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Pharmacological studies on the neural substrates of social behavior in health and disease states.

PhD Candidate

Sara Schiavi

Supervisor:

Prof.ssa Viviana Trezza, Roma Tre University, Rome

PhD Coordinator:

Prof. Paolo Visca, Roma Tre University, Rome

To Chiara, Roberta, Domiziana and Valeria.

Riassunto

Studi farmacologici sui substrati neurali del comportamento sociale in condizioni fisiologiche e patologiche.

Il comportamento sociale viene definito come quell'insieme di comportamenti che sono influenzati e che influenzano a loro volta membri della stessa specie, ed è essenziale per una serie di funzioni che consentono agli uomini e agli animali di sopravvivere. Comprendere i meccanismi neurali alla base del comportamento sociale risulta cruciale in quanto diversi studi suggeriscono che le esperienze sociali, soprattutto nelle prime fasi della vita, giocano un ruolo fondamentale nel determinare lo sviluppo neurale e comportamentale degli individui. Recenti studi clinici e preclinici hanno, infatti, evidenziato come la presenza di stimoli sociali adeguati durante le prime fasi della vita risulta critica per lo sviluppo di abilità sociali e cognitive appropriate. Al contrario, l'esposizione ad esperienze sociali negative viene associata ad un alterato sviluppo neuronale, ad alterazioni cognitive, sociali ed emozionali, e ad una maggiore suscettibilità allo sviluppo di patologie psichiatriche. Non a caso, le disfunzioni della sfera sociale sono un sintomo tipico di diverse patologie neuropsichiatriche, prima fra tutte l'autismo. La sindrome dello spettro autistico (*Autism Spectrum Disorder*, ASD) è infatti uno dei più gravi disturbi psichiatrici dello sviluppo, in termini di prevalenza ed esito, ed è caratterizzata da marcati deficit nella sfera sociale. Attualmente, non sono ancora disponibili trattamenti specifici per questa patologia. In questo contesto, la ricerca preclinica, grazie all'impiego di modelli animali, è essenziale sia per una migliore comprensione degli aspetti patofisiologici e neurobiologici della patologia, sia per validare nuovi target terapeutici e l'efficacia di nuovi potenziali farmaci. Fra i modelli animali impiegati per lo studio delle disfunzioni della sfera sociale, occupano una posizione fondamentale i roditori, e soprattutto i ratti (*Rattus Norvegicus*) che, per la loro innata socialità, sono modelli animali ideali per lo studio del comportamento sociale e delle sue alterazioni.

Alla luce di quanto descritto, lo scopo generale del mio dottorato di ricerca è stato quello di studiare forme tipiche e atipiche di comportamento sociale in modelli preclinici di ratto al fine di 1. studiare gli aspetti neurobiologici del comportamento sociale e far luce su possibili alterazioni neurochimiche che causano le disfunzioni sociali nelle patologie psichiatriche; 2. trovare nuove opportunità terapeutiche per curare le disfunzioni sociali che caratterizzano tali patologie.

Durante la prima fase del mio progetto di dottorato, ho incentrato i miei studi sul comportamento di gioco sociale poiché esso risulta essere il comportamento sociale più caratteristico mostrato dai giovani mammiferi ed è profondamente compromesso in diversi disturbi psichiatrici tipici dello sviluppo, incluso l'autismo. In particolare, ho studiato come il sistema endocannabinoide e il sistema oppioide endogeno interagiscono nella modulazione del comportamento di gioco sociale,

eseguendo esperimenti comportamentali e biochimici (Capitolo 3). I risultati di questo studio hanno mostrato che esiste una tolleranza crociata unidirezionale tra la neurotrasmissione oppioide ed endocannabinoide nella modulazione del comportamento di gioco sociale. Per chiarire invece il coinvolgimento del sistema dopaminergico e del sistema noradrenergico nel comportamento di gioco sociale del ratto, ho studiato come il Metilenedioxypyrovalerone (MDPV), un composto stimolante psicoattivo appartenente ai catinoni sintetici, interagisce farmacologicamente con questi due sistemi nella modulazione di tale comportamento (Capitolo 4). I risultati dei miei esperimenti hanno mostrato che l'MDPV riduce il comportamento sociale attraverso la stimolazione simultanea dei recettori adrenergici α-2 e dei recettori dopaminergici.

Durante la seconda fase del mio progetto di dottorato ho studiato comportamenti sociali atipici nel ratto allo scopo di identificare nuovi target terapeutici per le disfunzioni sociali che caratterizzano molte patologie psichiatriche. Innanzi tutto, ho validato nel nostro laboratorio il modello preclinico di autismo basato sull'esposizione prenatale ad acido Valproico (VPA) nel ratto, studiando sia la progenie di sesso maschile che quella di sesso femminile. Successivamente, ho studiato il coinvolgimento del sistema endocannabinoide nei deficit socio-emozionali, cognitivi e nei comportamenti ripetitivi mostrati da ratti prenatalmente esposti a VPA (Capitoli 5, 6). Questo studio ha evidenziato che le femmine di ratto sono in qualche modo meno vulnerabili agli effetti deleteri che l'esposizione prenatale a VPA ha sulla comunicazione sociale, sulla reattività emozionale e sulle prestazioni cognitive rispetto ai ratti maschi. Al contrario, le femmine di ratto esposte a VPA mostrano deficit selettivi nel comportamento di gioco sociale e nelle stereotipie. Inoltre, le analisi biochimiche effettuate in questo studio hanno evidenziato che l'esposizione prenatale a VPA altera la fosforilazione dei recettori cannabinoidi CB1 in un modo specifico a seconda del sesso e dell'età della prole, e a seconda del tessuto cerebrale analizzato. Sulla base di questi risultati, ho testato la capacità dell'inibitore selettivo dell'idrolisi dell'anandamide URB597 di migliorare i comportamenti sociali atipici mostrati dai ratti maschi e femmine esposti a VPA nel corso dello sviluppo. I risultati ottenuti mostrano che l'aumento della concentrazione dell'anandamide, indotta dall'inibizione farmacologica della sua degradazione, è in grado di migliorare i deficit comportamentali esibiti da animali esposti a VPA di entrambi i sessi (Capitolo 6). L'alterato comportamento sociale mostrato dai ratti esposti a VPA potrebbe essere dovuto sia a un deficit nell'elaborazione delle proprietà gratificanti degli stimoli sociali, che alla loro incapacità di comprendere e rispondere adeguatamente agli stimoli sociali. Per rispondere a tale quesito, ho fatto ulteriori esperimenti comportamentali, biochimici ed elettrofisiologici, per determinare se i deficit sociali mostrati dai ratti esposti a VPA fossero associati a cambiamenti nella risposta a diversi stimoli gratificanti, di natura sociale e non sociale (Capitolo 7). I risultati di questo studio hanno evidenziato che i ratti

esposti a VPA mostrano un'espressione alterata dei recettori della dopamina insieme ad ipereccitabilità intrinseca dei *Medium Spiny Neurons* (MSN) nel nucleus accumbens. Tuttavia, i ratti esposti a VPA si comportano in maniera analoga ai ratti di controllo quando vengono testati in test comportamentali finalizzati all'analisi delle proprietà gratificanti di stimoli sociali e non sociali. Alla luce di questo studio, è ragionevole pensare che le disfunzioni sociali presentate da ratti esposti a VPA siano più probabilmente causate da alterazioni degli aspetti cognitivi dell'interazione sociale, come l'interpretazione e la capacità di reciprocare lo stimolo sociale e / o la capacità di adattare il comportamento sociale alle circostanze ambientali, piuttosto che all'incapacità di elaborare gli aspetti gratificanti dell'interazione sociale.

Nell'ultima parte del mio progetto di dottorato, ho usato un modello di esposizione prenatale a cannabinoidi nel ratto per studiare come cambiamenti precoci nel sistema endocannabinoide possano interferire con il comportamento sociale in fasi più avanzate della vita (Capitolo 8). È noto infatti che le alterazioni della funzionalità del sistema endocannabinoide contribuiscono alla patogenesi di numerosi disturbi psichiatrici e neurologici. Difatti, gli endocannabinoidi sono modulatori chiave della plasticità neurale e dello sviluppo del cervello e una varietà di patologie implica la dis-regolazione delle loro funzioni di segnalazione. In questo studio, ho valutato gli effetti dell'esposizione prenatale all'agonista del recettore dei cannabinoidi WIN55,212-2, sulla reattività emozionale e sulle prestazioni cognitive di ratti maschi e femmina dall'infanzia fino all'età adulta, analizzando il ruolo del recettore mGlu5 negli effetti osservati. Da questo studio è emerso come ratti maschi prenatalmente esposti a WIN55,212-2 mostrino alterazioni della comunicazione sociale in infanzia ed un aumento dell'attività locomotoria, rispetto alla loro controparte femminile. Questi effetti sono stati normalizzati quando gli animali maschi sono stati trattati con il modulatore allosterico positivo del recettore mGlu5, CDGPB.

In conclusione, la ricerca svolta durante il mio progetto di dottorato ha aumentato le nostre conoscenze sui sistemi di neurotrasmissione e sulle aree cerebrali coinvolte nella regolazione del comportamento sociale, sia in condizioni tipiche che atipiche. Comprendere le fondamenta neurali del comportamento sociale, e in particolare del gioco sociale, in condizioni fisiologiche mi ha permesso di studiare le basi dei comportamenti sociali atipici osservati nel modello preclinico di ASD basato sull'esposizione prenatale a VPA e nel modello di esposizione prenatale ai cannabinoidi. Questi studi mi hanno portato a identificare nuovi potenziali target farmacologici per il trattamento delle disfunzioni sociali che caratterizzano molte patologie psichiatriche. Tra questi, il sistema endocannabinoide è emerso come un target farmacologico promettente nell'autismo, dato il suo ruolo fondamentale in alcuni comportamenti che risultano tipicamente alterati in questa patologia e la sua capacità di regolare la plasticità sinaptica e lo sviluppo del cervello.

Abstract

Pharmacological studies on the neural substrates of social behavior in health and disease states.

The terms “social behavior” refer to all the behaviors that influence, or are influenced by other members of the same species and are essential for a series of function that allows animals and humans to survive. For instance, it is instrumental to successfully interact with members of the same species, to obtain food, mates and avoid predation. A better understanding of the neural mechanisms underlying social behavior is crucial, as several studies suggest that adequate social stimuli during early life are critical for developing appropriate socio-emotional and cognitive skills, while adverse social experiences negatively affect the proper development of brain and behavior, increasing, for instance, the susceptibility to develop psychiatric conditions. Indeed, social dysfunctions are a key symptom of several neuropsychiatric disorders, including Autism Spectrum Disorder (ASD). ASD is a one of the most severe paediatric psychiatric condition, in terms of prevalence and outcome, and it is characterized by marked deficits in the social domain. To date, no specific treatments for ASD are available yet. In this context, preclinical research has a crucial role in the understanding of the pathophysiological and neurobiological aspects of ASD. Indeed, animal models allow to mimic specific symptoms, and to study the role of genetic and environmental factors, as well as their potential interaction, at the base of the onset of ASD. Besides, animal models are essential to validate new therapeutic targets and the efficacy of potential new drugs.

The general aim of my PhD was to study typical and atypical forms of social behavior in animal models, in order to: (1) study neurobiological aspects of social behavior and shed light on possible neurochemical alterations causing social dysfunctions in psychiatric diseases; (2) find new therapeutic opportunities to treat the social dysfunctions that characterize several psychiatric disorders. To address these aims, I used rodent models displaying either normal or atypical patterns of social behavior.

In the first part of my PhD project, I studied the neural mechanisms underlying social play behavior, that is the most characteristic form of social behavior displayed by young mammals and it is profoundly impaired in several psychiatric disorders. In particular, I performed behavioral and biochemical experiments to investigate how the endocannabinoid and the endogenous opioid systems interact in the modulation of social play (Chapter 3). The results of this study showed that a unidirectional cross-tolerance between opioid and endocannabinoid neurotransmission in the modulation of social play behavior exists. Then, to clarify the involvement of dopaminergic and noradrenergic neurotransmission in social play behavior, I studied how Methylenedioxypyrovalerone (MDPV), a psychoactive stimulant compound belonging to the

synthetic cathinones, pharmacologically interact with these two neurotransmitter systems to modulate social play behavior in rats (Chapter 4). This study showed that MDPV reduces social behavior in rats through the simultaneous stimulation of α-2 adrenoceptors and dopamine receptors.

During the second part of my PhD project, I studied atypical patterns of social behavior in rodents to find hints for new therapeutic opportunities to treat social dysfunctions. First, I set up and validated the well-characterized animal model of autism based on prenatal exposure to valproic acid (VPA) (Chapter 5). I also investigated the involvement of the endocannabinoid system in the sex-specific ASD-like socio-emotional, cognitive and repetitive symptoms displayed by rats prenatally exposed to VPA (Chapters 6). Thanks to this study, I found that female rats are somehow less vulnerable to the deleterious effects of prenatal VPA exposure on social communication, emotional reactivity and cognitive performance than male rats. I also found that prenatal VPA exposure alters the phosphorylation of CB1 cannabinoid receptors in a sex-, age- and tissue-specific manner. On these bases, I tested the ability of the selective anandamide hydrolysis inhibitor URB597 to rescue the atypical social behaviors displayed by male and female VPA-exposed rats in the course of development. I found that enhancing anandamide signaling reversed the behavioral deficits displayed by VPA-exposed animals of both sexes. As the altered social behavior displayed by VPA-exposed rats could be due to either a deficit in social reward processing or to a more general inability to properly understand and respond to social signals, I investigated whether the social deficits displayed by VPA-exposed rats are associated with changes in more specific reward-related behaviors, including social, drug and food rewards. Thus, I performed behavioral, electrophysiological and neurochemical experiments to test the involvement of the brain reward system in the social dysfunctions displayed by rats prenatally exposed to VPA (Chapter 7). I found that VPA-exposed rats show altered expression of dopamine receptors together with inherent hyperexcitability of medium spiny neurons (MSNs) in the NAc. However, when tested for tasks aimed at analyzing reward-related behavior such as socially-induced conditioned place preference, locomotor response to amphetamine and sucrose preference, control and VPA-exposed rats performed similarly, indicating normal responses to social, drug and food rewards. Thus, it is possible that social dysfunctions displayed by VPA-exposed rats are more likely caused by alterations in cognitive aspects of the social interaction, such as the interpretation and reciprocation of social stimuli and/or the ability to adjust the social behavior of the individual to the changing circumstances in the social and physical environment, rather than to inability to enjoy the pleasurable aspects of the social interaction.

In the last part of my PhD project, I focused on a model of prenatal exposure to cannabinoids in rats to study how early changes in the endocannabinoid system could interfere with social behavior later

in life (Chapter 8). It is known that alterations of the endocannabinoid functionality contribute to the pathogenesis of several psychiatric and neurological disorders. As endocannabinoids are key modulators of neural plasticity and brain development, a variety of pathologies are thought to involve dysregulation of their signaling functions. In this study, I studied the effects of prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 on the emotional reactivity and cognitive performance of male and female rat offspring from infancy through adolescence and tested the role of mGlu5 receptor signaling in the observed effects. We found that prenatally WIN-exposed male infant pups emitted less ultrasonic vocalizations compared with male control pups when separated from the dam and siblings and showed increased locomotor activity, while females were spared. These effects were normalized when male pups were treated with the positive allosteric modulator of the mGlu5 receptor CDPPB.

In conclusion, the research performed during my three-year PhD project increased our knowledge about the neurotransmitter systems and brain areas involved in the regulation of social behavior, both in health and disease states. Understanding the neural underpinning of social behavior, and particularly social play, in physiological conditions allowed me to study the foundation of the atypical social behaviors observed in both the preclinical model of ASD based on prenatal VPA exposure, and in the model of prenatal exposure to cannabinoids. These studies led me to identify some of the principal neurotransmitter systems involved in social behavior as new potential targets for the treatment of social dysfunctions. Among the others, the endocannabinoid system emerged as a promising pharmacological target for ASD, given its pivotal role in many functions that are disrupted in the disease, and its capability to mediate and participate in neuronal plasticity and brain development.

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CHAPTER 1

"Man is by nature a social animal; an individual who is unsocial naturally and not accidentally is either beneath our notice or more than human. Society is something that precedes the individual. Anyone who either cannot lead the common life or is so self-sufficient as not to need to, and therefore does not partake of society, is either a beast or a god."

- Aristotle, Politics

1. Introduction.

We are highly social animals. Our success in adapting to the natural world through the course of evolution is largely the result of our ability to form social networks. More than any other species, we depend on each other for company and survival. Consequently, we cannot develop normally on our own. Children have an innate predisposition to interpret the world they will meet as adults but the fundamental skills they will need, such as language, can only be learned from other people. For this reason, early social or sensory deprivation can compromise brain structure. Similarly, we need social interactions to keep the brain in good shape in old age (Kandel, 2018).

1.1 Social behavior in mammals.

Social behavior can be defined as all the behaviors that influence, or are influenced by other members of the same species. Thus, the term covers sexual and reproductive activities and behaviors that tend to bring individuals together as well as all forms of aggressive behavior (Grant, 1963). Social behavior holds a fundamental position among the behaviors expressed by mammals, including humans, being essential for the survival of individuals, groups and entire species. In humans, social behavior is an integral part of life, that emerges since early childhood (Kovacs, Teglas et al. 2010), and it is crucial throughout life (Slaughter, Imuta et al. 2015). According to the "social brain hypothesis", the bigger size of the brain of primates, compared to other vertebrates, could be probably due to an effort to handle complex social systems (Dunbar 2009). Besides humans, several species tend to form social organizations, ranging from small aggregations to permanent social groups. During the course of life, social behavior meets numerous changes. Indeed, social re-orientation is critical for development, as it occurs in a particular time window in which brain undergoes substantial functional and structural changes (Spear 2000, Nelson, Leibenluft et al. 2005, Blakemore 2008, Counotte, Goriounova et al. 2011). Some of the most prominent changes seem to occur during youth, both in terms of complexity of behaviors and in terms of the ability to interact with peers (Spear 2000, Nelson, Leibenluft et al. 2005). In mammals, the first forms of social behavior are directed toward parental figures; later in life, during adolescence, the interaction with peers becomes particularly important, both in animals and humans (Larson and Richards 1991, Spear 2000). The most prominent forms of social behavior displayed by adult mammals, such as affiliative, sexual, parental and aggressive behaviors, are probably the result of refinement of social behavioral components displayed during childhood and adolescence (Vanderschuren, Niesink et al. 1997). Indeed, social activities early in life shape brain development and adult behavior (Champagne and Curley 2005). Noteworthy, the social environment is fundamental for species that rely on parental care and social needs beyond the individuals (Miczek, Yap et al. 2008, Cacioppo and Hawkley 2009).

1.2 Functions of social behavior: the importance of being social.

Social behavior is essential for a series of function that allows animals and humans to survive. For instance, it is instrumental to successfully interact with members of the same species, to obtain food and mates and avoid predation. As it is essential for reproduction, the neural and hormonal processes sub-serving these categories of behaviors are likely to be highly conserved. Social behavior is not a unitary behavior with a unitary neurological basis. Rather, different aspects of social behavior have different neural and endocrine bases, while behavioral changes may depend on the situation (Whishaw and Kolb, 2006). Interestingly, abnormal patterns of social behavior have a key role in the development of psychiatric disorders such as schizophrenia and autism (Lord, Cook et al. 2000). Several studies suggest that adequate social stimuli during the early stages of postnatal life are crucial for developing appropriate socio-emotional and cognitive skills, while adverse social experiences such as prolonged isolation negatively affect proper development of brain and behavior, increasing, for instance, the susceptibility to develop psychiatric conditions such as alcoholism, drug addiction and eating disorders (Chatterjee, Chatterjee-Chakraborty et al. 2007, Zucker, Donovan et al. 2008, Cirulli, Berry et al. 2010, Hassel, McKinnon et al. 2011, Marco, Valero et al. 2013, Branchi and Cirulli 2014, Sale, Berardi et al. 2014, Turecki, Ota et al. 2014, Vartanian, Smyth et al. 2014). Indeed, evidence that social isolation is a serious risk factor for medical disorders exist, and it may challenge the well-known traditional risk factors. Social deprivation early in life leads to a variety of psychiatric disorders, such as disruptive behavior disorders, autism, early-onset schizophrenia and attention deficit hyperactivity disorder (Alessandri 1992, Moller and Husby 2000, Jordan 2003, Skodol 2012). Antisocial personality traits are a predisposing factor for alcohol and drug addiction, while in children and adolescents, antisocial behavior is a defining characteristic of disruptive behavior disorders (Kofoed and MacMillan 1986, Helzer and Pryzbeck 1988, Regier, Farmer et al. 1990, Alterman and Cacciola 1991, Hawkins, Catalano et al. 1992, Wilens and Biederman 1993, Harpur and Hare 1994, Kessler, Nelson et al. 1996, Bonomo, Bowes et al. 2004, Hesselbrock and Hesselbrock 2006). Thus, social species seems to be dramatically affected by social isolation during development, as it results in a variety of social deficits (van den Berg, Hol et al. 1999, Von Frijtag, Schot et al. 2002, Lukkes, Summers et al. 2009), cognitive impairments (Fone and Porkess 2008, Cacioppo and Hawkley 2009), and increased vulnerability for psychiatric disorders (Jones, Marsden et al. 1990, Jones, Hernandez et al. 1992, Wilkinson, Killcross et al. 1994, Hall, Huang et al. 1998, Howes, Dalley et al. 2000, Leussis and Andersen 2008). Consequently, the opportunity to engage in social behaviors plays a pivotal role in determining the development trajectories of individuals. Indeed, the way in which individuals learn to interact successfully with others includes a complex interaction between neural, behavioral, and environmental elements. These have a role in the achievement of positive

developmental outcomes, including peer acceptance and mental health (Soto-Icaza, Aboitiz et al. 2015).

1.3 Motivational, cognitive and rewarding aspects of social behavior.

1.3.1 The theory of social motivation.

According to the theory of social Motivation of Chevallier and colleagues (2012), social motivation is "a set of psychological dispositions and biological mechanisms biasing the individual to preferentially orient to the social world (social orienting), to seek and take pleasure in social interactions (social reward), and to work to foster and maintain social bonds (social maintaining)". In social orienting, social stimuli seem to be essential; human faces rapidly capture attention, and this preference is expressed since the early life, with infants preferentially attracted to face-like stimuli rather than to scrambled or inverted faces. Social orienting helps individuals to acquire face-related tasks, such as gender discrimination or encoding of identities (Fletcher-Watson, Findlay et al. 2008, Senju and Johnson 2009, Rosa Salva, Farroni et al. 2011). Social motivation does not only orient the social attention of individuals but allows them to find rewarding social stimuli. For instance, when given the choice to access a reward collaboratively or individually, toddlers strongly prefer to collaborate (Rekers, Haun et al. 2011). Moreover, social motivation includes also the desire to engage with others in prolonged periods. To do so, individuals adopt strategies to understand behaviors by which people may establish, maintain and enhance their relationships. Indeed, people try to be likable rather than unlikeable, as competent rather than incompetent, as more rather than less physically attractive, etc. (Lery and Allen, 2010). Also these behaviors emerge early in development, with pre-schoolers spontaneously engaging in positive self-presentation and prosocial lies.

1.3.2 Social cognition and the Theory of Mind.

Social cognition involves all the abilities that support us to understand social stimuli and to interact with them. In this process, it is crucial to be able to predict the behavior of others, by detecting, analyzing, and interpreting their intentions. To do so, individuals need to develop social skills, a varied group of ability that emerges from the appropriate execution of social cognition processing. The experience of adequate social performances allows individuals to interact and communicate with others. Thus, social-cognitive processes in humans describe the ways by which individuals became able to predict and understand other people's intentions, feelings, emotions, and behaviors (Soto-Icaza, Aboitiz et al. 2015). Among this field, lies the Theory of Mind, firstly postulated in 1978 by David Premack and Guy Woodruff and defined as the ability to impute mental states to oneself and to others (Premack and Woodruff, 1978). Although it was originally thought that the Theory of Mind

was a peculiar ability of humans, the literature on the Theory of Mind in nonhuman animals more recently suggested that some species share fundamental social cognitive mechanisms with humans (Krupenye and Call 2019). Noteworthy, the Theory of Mind was then applied to autistic subject by Baron-Cohen and co-workers in 1985 (Baron-Cohen, Leslie et al. 1985). Autism is a pervasive neurodevelopmental psychiatric disease characterized by large broad of heterogeneous impairments, in which deficit in social and communicative ability is prominent. According to the work of Baron-Choen, the heterogeneity of symptoms found in autistic children may be explained by the presence of coexisting cognitive difficulties in the area of Theory of Mind. While an individual with an intact Theory of Mind is able to understand the content of his/her own and others' minds, autistic children seem to experience difficulties in understanding other minds, a core cognitive feature of autism that is believed to be universal among such individuals.

1.3.3 The rewarding value of social behavior.

As social behavior is essential to survive, is not surprising that it owns rewarding proprieties. Indeed, reward helps us to select those actions that will lead to the most and best rewards and motivates us to carry out those actions. Thus, humans use social information to form evaluations and expectations of other people and to decide whether to trust or cooperate with others (Bhanji and Delgado 2014). Primary rewards have been extensively studied and more light has been shed on the neural systems underpinning reward processing. Recent research efforts highlight commonalities between neural systems underlying social evaluation and decision making and more well-characterized neural systems of reward processing (Delgado 2007, Behrens, Hunt et al. 2008, Izuma, Saito et al. 2008). It is nowadays generally accepted that certain forms of social behavior (i.e., social play, sexual behavior, parental care) are highly rewarding (Trezza, Campolongo et al. 2011).

1.4 Rodent models as useful tools to study the neural substrates of social behavior.

Studies on the neurobiological mechanisms underlying normal social behavior in animal models may provide insights into the neuropathology of psychiatric disorders characterized by aberrant social traits. Animal models have been defined for the first time by McKinney in 1984 as: "experimental preparations developed in one species for the purpose of studying phenomena occurring in another species" (McKinney 1984). In order to evaluate the concrete utility of animal models, the word "validity" has been introduced in the preclinical field as a key principle used to assess the real application of a specific animal as a model of human disease. Thus, many of the information that we have today on the neural substrates of social behavior has been provided not only by observational studies but also by animal models of psychiatric diseases. In this field, rodents have emerged as

election models. Indeed, differently from animal species more similar to humans such as primates, handling rodents has been proved to be less difficult given the rapid development of the nervous system and the possibility to generate transgenic animals (Belzung e al., 2005). Moreover, rodents are highly social animals (Insel, Cuthbert et al. 2010). Mice and rats have a strong tendency to group together in laboratory conditions and display good-natured behaviors. There is evidence that both species can recognize conspecific individuals and, depending on partner's identity, choose different social behaviors (Kondrakiewicz, Kostecki et al. 2019).

1.4.1 Rats supremacy in the social behavioral field.

Rats are usually the election model for the study of sociability or most of the psychiatric conditions linked to social behavior. Indeed, laboratory rats usually coexist in a peaceful manner. Apart from specific situations, they show mainly pro-social behavior. The most basic behavior reflecting a tendency to form groups, huddling, is related to thermoregulation and appears at birth and persists in a weaker form throughout adulthood (Alberts 2007). Importantly, single-housing or even reducing the number of animals kept in one cage can be a major stressor for the rats (Kask, Nguyen et al. 2001, Djordjevic, Djordjevic et al. 2012). Moreover, after variable periods of isolation rats display increased motivation to engage in social behaviors (Varlinskaya, Spear et al. 1999). Because of this innate characteristic, most of the experimental works on social behavior in mammals has been performed in the laboratory rat (*Rattus Norvegicus*), whose social behavioral repertoire allows both quantitative and qualitative analysis (Panksepp, Siviy et al. 1984, Vanderschuren, Niesink et al. 1997, Trezza, Baarendse et al. 2010). Indeed, since the first studies, rats exhibited a complex and well-organized repertoire of social behavior, compared to mice. Factors such as the age, sex, familiarity, and social rank and the nature of the setting in which social interactions occur, influence the social behaviors exhibited by the animals (Meaney, and Stewart, 1981). Noteworthy, rats are able to perform social play behavior; the first form of non-mother-directed social behavior displayed by most developing mammals which is thought to be important for the development of social, cognitive and emotional processes and their neural underpinnings, and it is disrupted in pediatric psychiatric disorders (Vanderschuren, Achterberg et al. 2016).

2. Neural substrates underlying social behavior.

Social interaction requires a network of interconnected brain regions that process social information. In 1990 Brothers proposed that there is a circumscribed set of brain regions dedicated to social cognition, and then sociability (Brothers, 1990). Although it is not possible to assert that social functions are specific to a precise group of brain regions, it is nowadays accepted that some brain areas are more involved than others. Thus, areas such as the prefrontal cortex, ventral striatum, hippocampus, and amygdala seem to be election brain areas able to process social information. Moreover, these regions are considered key parts of the 'social brain,' based on imaging and network studies (Wei, Allsop et al. 2017). Each area empowers the varied set of functions that allow mammals to interact with each other. These functions include proper processing of the rewarding properties of social behavior, the cognitive ability needed to perform it and also, the emotional setting arising during the social interaction. In these brain regions, several neurotransmitter systems work together to transmit and modulate social information and social behavior.

2.1 Major neurotransmitter systems involved in social behavior.

2.1.1 Endogenous opioid system.

The endogenous opioid system has been widely implicated in reward mechanisms, such as the positive emotional properties of food, sex and drugs of abuse (Le Merrer, Becker et al. 2009, Berridge and Kringelbach 2015). It consists of three prototypes of endogenous ligands (endorphins, enkephalins, and dynorphins) and three receptor types (mu, delta, and kappa) (Le Merrer, Becker et al. 2009). Based on striking similarities between social distress, physical pain and opiate withdrawal, mu receptors have been proposed to play a critical role in modulating social behavior in humans and animals. Indeed, in 1978, Panksepp and colleagues formulated their 'Brain Opioid Theory of Social Attachment', in which social and affiliative behaviors were proposed to depend on endogenous opioid peptides (Panksepp, Siviy et al. 1984). According to this theory, social deprivation-induced social distress and contact seeking are due to insufficient opioid tone (opioid withdrawal). Social contact would relieve this negative effect by triggering opioid (endorphin) release. Since then, experimental evidence has accumulated showing that mu receptors are primarily involved in the pro-social effects of opioids, which are not only exclusively observed under social distress conditions such as social deprivation or defeat, but also in a more neutral or positive social situation such as social comfort (Pellissier, Gandia et al. 2018). Indeed, moderate mu receptor activation facilitates social behavior. In rats, mu receptor agonists increase social and sexual behaviors, social play and enhance long-term social memory (Vanderschuren, Stein et al. 1995, Trezza and Vanderschuren 2008). In contrast,

blockade of mu receptor activity inhibits various social behaviors when administered under neutral social context in animals, including social interaction and social play (Trezza, Campolongo et al. 2011, Chaijale, Curtis et al. 2013). In humans, a low dose of mu receptor agonist increases female face attractiveness and opioids partial agonists improve memory for happy faces, increase pleasantness ratings of neutral or social images and decrease perception of social rejection (Gospic, Gunnarsson et al. 2008, Chelnokova, Laeng et al. 2014, Syal, Ipser et al. 2015, Bershad, Seiden et al. 2016, Pellissier, Gandia et al. 2018).

2.1.2 Endocannabinoid system.

Endocannabinoids are lipid signalling messengers. The most studied endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized following neuronal depolarization (Piomelli 2003, Mechoulam, Hanus et al. 2014). Once released from postsynaptic neurons, they bind to presynaptic G-protein coupled cannabinoid receptors (CB1 and CB2). Finally, their actions are terminated by uptake via one or more endocannabinoid membrane transporters, followed by degradation by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), hydrolytic enzymes that provide the primary clearance routes for anandamide and 2-AG, respectively (Piomelli 2003, Mechoulam, Hanus et al. 2014). The brain distribution of the molecular components of the endocannabinoid system is consistent with their role in social behavior (Wei, Allsop et al. 2017). Indeed, cannabinoid receptors are highly expressed in brain regions that support human social-emotional functioning such as the frontal cortex and subcortical structures (Glass, Dragunow et al. 1997, Stanley and Adolphs 2013). It has been proved that, in rats, a novel encounter of a social stimulus elevates anandamide levels in the striatum, compared to encounters with familiar or non-social animals (Marco, Rapino et al. 2011). Mutant mice in which genetic removal of the hydrolytic enzyme fatty acid amide hydrolase (FAAH) caused elevated levels of anandamide exhibit increased direct social interactions (Cassano, Gaetani et al. 2011). Furthermore, it has been found that social play behavior is associated with increased anandamide mobilization in the nucleus accumbens and amygdala (Trezza, Damsteegt et al. 2012). Also, 2-AG signaling is important in social play behavior and may interact with opioid or dopaminergic signaling in the nucleus accumbens (Manduca, Lassalle et al. 2016).

However, the prosocial effect induced by pharmacological potentiation of anandamide signalling is in contrast with the effect obtained using direct-acting cannabinoid receptor agonists, which have been proven to decrease social play (Trezza, Campolongo et al. 2008). For instance, administration of Delta-9-tetrahydrocannabinol (THC) during the pubertal period reduced social play (Trezza and Vanderschuren 2008, Trezza, Baarendse et al. 2014).

2.1.3 Dopaminergic system.

The study of the brain areas involved in reward processing started with the seminal discovery that animals are willing to work to obtain electrical stimulation to mesolimbic brain regions (Olds and Milner 1954). A key component of the reward circuit is the striatum, a brain area in which converge affective, cognitive and motor information (Alexander, DeLong et al. 1986, Cardinal, Parkinson et al. 2002, Bhanji and Delgado 2014). Signals in the striatum are related to several social functions, including the evaluation of social rewards, the modulation of reward experiences and behavior by social relationships and interactions (Bhanji and Delgado 2014). Here, dopaminergic neurons, producing the neurotransmitter dopamine (DA), have been found to have a central role. DA is produced in the ventral tegmental area (VTA) and the terminal region with the densest VTA dopaminergic projections in the ventral striatum, or nucleus accumbens (NAc), which is thought to encode reward-related signals from the VTA. The NAc comprises primarily the inhibitory projection neurons called medium spiny neurons (MSNs) that can be differentiated by the type of DA receptor they express: D1 or D2. These two subpopulations of NAc MSNs are thought to bi-directionally control reward and have been pharmacologically implicated in affiliative behaviors (Gunaydin and Deisseroth 2014). The NAc also receives inputs from other regions implicated in social behavior, such as the dorsal raphe, hypothalamus, and prefrontal cortex, as well as sensory inputs, and is believed to control the integration of socially relevant information into behavioral output. In humans, genetic studies showed that there are genes involved in the dopamine pathway able to modulate social behavior. Indeed, an increased dopaminergic tone was associated with stronger social approach tendency in an implicit social approach-avoidance task (Enter, Colzato et al. 2012). Moreover, the administration of L-DOPA, a DA precursor, improved the ability of individuals assumed to have lower endogenous striatal DA, to learn about a partner's prosocial preferences (Eisenegger, Pedroni et al. 2013). In rats, treatment with non-selective dopamine receptor antagonists, dopamine D1 receptor antagonist, and D2 receptor antagonist seems to inhibit social play (Beatty, Costello et al. 1984, Holloway and Thor 1985, Siviy, Fleischhauer et al. 1996, Trezza and Vanderschuren 2009). However, the effects of dopamine receptor agonist treatment on social play have been reported to be variable. For example, treatment with the non-selective dopamine receptor agonist apomorphine was found to increase (Beatty et al., 1984; Vanderschuren et al., 2008) as well as decrease social play (Niesink and Van Ree, 1989).

2.1.4 Noradrenergic system.

Noradrenergic neurotransmission has been implicated in a variety of cognitive processes, including learning, attention, and flexibility (Aston-Jones and Cohen 2005, Robbins and Arnsten 2009,

Roozendaal and McGaugh 2011, Berridge and Kringelbach 2015). Besides, it plays a role in the generation and perception of emotions, whereby there is an emerging body of work to indicate that noradrenaline is involved in reward processes (Ventura, Alcaro et al. 2005, Bouret and Richmond 2015). Noradrenaline (NA), belongs to the chemical class of catecholamines and is synthesized from the amino acid precursor phenylalanine and tyrosine. In the brainstem, noradrenergic neurons populate the medulla oblongata and the dorsal vagal nucleus with projections to the spinal cord. A high density of noradrenergic cells bodies can also be found in the locus coeruleus which innervates the thalamus, dorsal hypothalamus, hippocampus, and cortex. The ventral noradrenergic bundle, caudal to the locus coeruleus, is connected to subcortical limbic regions. Peripherally, noradrenaline is part of the sympathetic nervous system, mediating physiological responses to stress and acute anxiety (Terbeck, Savulescu et al. 2016). Recently it has been determined that changes in basic emotions, such as fear and anger, in which NA is implicated, might have a significant role in social and moral cognition. Indeed, the role of noradrenaline in higher-order social cognition suggests that the noradrenergic system may be important in social attitudes and moral judgments by mediating affective changes (Terbeck, Savulescu et al. 2016). Apart from this, several preclinical studies highlighted the role of the noradrenergic system in social behavior. For instance, noradrenaline is important for the proper execution of social play behavior. Treatment with noradrenaline reuptake inhibitors has been demonstrated to inhibit social play, which was prevented by pre-treatment with the α2-noradrenaline receptor antagonist RX821002 (Vanderschuren, Trezza et al. 2008). Interestingly, treatment with α2-noradrenaline receptor antagonists produced differential effects on social play (Normansell and Panksepp 1985, Vanderschuren, Trezza et al. 2008). Moreover, it has been reported a role of α1-noradrenaline receptors and β-noradrenaline receptors in the modulation of social play behavior, (Vanderschuren, Achterberg et al. 2016).

3. Aberrant social behavior in psychiatric disorders: focus on Autism Spectrum Disorder.

3.1 Autism Spectrum Disorder (ASD).

According to the Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), Autism Spectrum Disorder (ASD) includes pervasive developmental psychiatric disorders emerging in the early life characterized by impairments in social interaction, verbal and non-verbal communication and the presence of stereotyped and repetitive behaviors (American Psychiatric Association, 2013). Comorbid features, such as social and generalized anxiety or aberrant sensitivity to sensory stimulation and cognitive disability are often related to the above-mentioned characteristics (Lai, Lombardo et al. 2014). ASD is highly heterogeneous in its presentation and its etiology remains unclear considering both the environmental and genetic factors involved in it (Kim and Leventhal 2015, Karimi, Kamali et al. 2017). In terms of prevalence and outcome, ASD is considered, among others, some of the most severe developmental psychiatric disorder, but still, no specific treatments are currently available (Mohiuddin and Ghaziuddin 2013, Lai, Lombardo et al. 2014). During the last 30 years, the prevalence of ASD seems to be increased of about 1% (Lai, Lombardo et al. 2014). That could be due to a more precise definition of the diagnostic criteria and increasing epidemiological studies. As early as infancy, children with ASD demonstrate symptoms such as a weak response to their parents' voice, they not use their voice to attract attention to themselves, express emotions, or establish contact, and they present a lack of attempts at nonverbal communication. Furthermore, they may be unresponsive to social stimuli or may focus intently on one item, refusing to interact with others for long periods of time. As the children get older, withdrawal from social interactions, indifference to social activities and deficits in social communication become more evident, and several components of the social repertoire and language appear highly disrupted (Lai, Lombardo et al. 2014). At adulthood, the impairments in social and communicative behaviors become more evident, the IQ scores tend to remain stable or to decrease and language remains impaired (Magiati, Tay et al. 2014). Indeed, ASD patients have been found to be less able to orient toward social stimuli and to initiate social interactions with same-aged peers and adults compared to patients with other developmental disabilities.

3.1.1 Gender issues in ASD.

One of the most important findings in ASD research is the higher rate of ASD diagnosis in males than in females. Indeed, sex bias was already noted in the initial descriptions of autism, with eight of eleven cases described by Kanner (1943) being males (Kanner, 1943). Thus, gender bias in ASD was described with a ratio of about 1 female for every 4 males diagnosed, while more recently a 3:1 male-to-female ratio was suggested (Fombonne 2003, Werling and Geschwind 2013, Loomes, Hull et al.

2017). The clinical presentation of ASD symptoms is often dissimilar in male and female patients and this gender difference might lead to missed or delayed diagnosis in females (Halladay, Bishop et al. 2015, Lai and Baron-Cohen 2015, Bargiela, Steward et al. 2016). Even when females with ASD are identified, they receive their diagnosis later than males (Giarelli, Wiggins et al. 2010). Moreover, gender differences in ASD may manifest with regard to symptom domains, width and severity (Rivet et Matson, 2011a, 2011b). To date, clinical findings regarding gender differences in the core triad of impairments seen in ASD (communication, social interactions, and stereotyped and repetitive behaviors) are dissimilar. Compared to females, males with ASD demonstrate more externalizing behavioral issues than females, such as aggressiveness, hyperactivity, reduced pro-sociality, and increased repetitive/restricted behaviors and interests. In opposition, females with ASD are more likely to experience internalizing problems such as depression, anxiety and other emotional issues (Werling and Geschwind 2013). Moreover, females with ASD are able to "camouflage" the autistic core deficits with better language and social mimicry, masking their ASD-related behaviors to a greater extent than males (Lehnhardt, Falter et al. 2016, Hull, Mandy et al. 2017). Nowadays, clinical research is focused on the characterization of females with autism to determine whether males and females with ASD display similar behavioral and cognitive profiles and to revise the diagnostic criteria in order to prevent a delayed or missing ASD diagnosis in female patients. However, studies exploring sex differences in behavioral manifestations of autism found inconsistent and sometimes conflicting results.

3.2 Rodents models of ASD: from genetic to environmental models.

3.2.1 Validation of rodent models of ASD.

The concept of validity was originally postulated by McKinney and Bunney in 1969 assessing that animal models should be able to reproduce the etiology, biochemistry, symptomatology, and treatment of a certain disease (McKinney and Bunney, 1969). Following the McKinney and Bunney criteria, the concept of face, construct and predictive validity of animal models was postulated. In particular, similarities should be observed between the patients' symptoms and the behaviors displayed by the animal model (face validity) and the neurobiological mechanisms underlying the pathology in patients and the aberrant behaviors in the animal model should also be similar (construct validity). Moreover, the animal model should be able to predict successful or unsuccessful interventions in the clinical setting (predictive validity)(Blanchard, Summers et al. 2013, Servadio, Vanderschuren et al. 2015). Since ASD is defined principally by behavioral characteristics, and the clinical diagnosis is based on behavioral features, a useful approach in ASD research is to focus on

animal behaviors that are relevant to the core diagnostic symptoms of the disease (Crawley 2007, Servadio, Vanderschuren et al. 2015). Rodents seem to be the most attractive candidates to reproduce core and comorbid features of ASD. Thus, mice and rats have been extensively used to mimic the ASD phenotype, giving the possibility to create several models reproducing, fully or in part, the characteristic of human ASD. Over the years, different rodent models of ASD have been generated: models of mutant animals, models mimicking epigenetic factors that increase the risk for autism in humans, models of human genetic diseases associated with autism, models based on prenatal exposure to environmental factors, and models obtained after neonatal lesions of brain areas which are abnormal in autistic patients (Belzung et al., 2005). Interesting, in the behavioral field rats emerged as a better proxy than mice, revealing in a more accurate way behavioral and cognitive symptoms experienced by autistic patients. Moreover, recent advances in the ability to manipulate the rat genome offer unique opportunities, especially in the study of central nervous system diseases in which mice have long been the favourite species to model genetic disorders. However, despite the presence of different models mimicking the core and secondary symptoms of ASD, still, there is no animal model able to capture at once all the molecular, cellular and behavioral characteristic of ASD. As psychiatric disorders in general, ASD is indeed difficult to model in animals. Since the exact etiology of ASD is largely unknown, generating an animal model with face and construct validity is far than simple. Indeed, despite the presence of a large variety of rodent models, none of them is universally recognized as valuable or as invalid (Pietropaolo, Crusio et al. 2017). Moreover, it is difficult to choose the most appropriate behavioral markers to assess, and the complexity of ASD itself, including a highly heterogeneous group of disorders sometimes with markedly different symptoms, complicates the situation (Pietropaolo, Crusio et al. 2017).

3.2.2 The animal model of autism based on prenatal exposure to Valproic Acid in rats.

Valproic acid (VPA) is a medication used for epilepsy and mood disorders but it is also used off label for pathological states such as migraine (Tartaglione, Schiavi et al. 2019). The use of VPA during childbearing years, and during early pregnancy, is related to several minor and major malformations in the offspring. Indeed, the risk to develop major defects when exposed to VPA *in utero* is so high and characteristic that the term 'fetal valproate syndrome' (FVS) was created to group them all. For years VPA has been associated with numerous teratogenic effects and to a higher incidence of neural tube defects, developmental delay and ASD at the point that it is now considered the most teratogenic antiepileptic drug available (Kozma 2001, Werler, Ahrens et al. 2011, Meador, Baker et al. 2012, Tartaglione, Schiavi et al. 2019). On the basis of the clinical studies reporting an association between maternal exposure to VPA and an increased risk to develop ASD in the offspring (Kini, Adab et al.

2006, Christensen, Gronborg et al. 2013, Veroniki, Rios et al. 2017), prenatal VPA exposure in rodents has been validated as a preclinical model of ASD. Numerous studies have successfully reported that the VPA animal model is a useful tool to study ASD pathology, having both high face and construct validity (Roullet, Lai et al. 2013). Studies in both rats and mice confirm that prenatal VPA exposure leads to autistic-like behaviors in the offspring, including social abnormalities, repetitive behaviors and disrupted communication (Roullet, Lai et al. 2013). Indeed, VPA-exposed animals exhibit impairments in social interaction, repetitive and stereotyped behaviors, increased anxiety, alteration in fear memory and compromised communicative abilities (Tartaglione, Schiavi et al. 2019). In particular, VPA-exposed animals show impaired social behavior when tested in the social play behavior (Schneider, Turczak et al. 2006, Chomiak, Hung et al. 2014), three-chamber (Kim, Kim et al. 2011, Kerr, Downey et al. 2013, Baronio, Castro et al. 2015), resident-intruder (Felix-Ortiz & Febo, 2012), and social interaction tests (Schneider and Przewlocki 2005, Markram, Rinaldi et al. 2008, Felix-Ortiz and Febo 2012). Furthermore, several studies have shown alterations in the number of ultrasonic vocalizations (USVs) emitted by pups prenatally exposed to VPA when isolated from their nests (Schneider and Przewlocki 2005, Dufour-Rainfray, Vourc'h et al. 2010, Gandal, Edgar et al. 2010). Together with impairments in social behavior and communication, repetitive and stereotyped behaviors have also been found in VPA-exposed animals, such as increased digging behavior and repetitive self-grooming (Kim, Lee et al. 2014, Baronio, Castro et al. 2015, Servadio, Melancia et al. 2016, Melancia, Schiavi et al. 2018). The behavioral abnormalities displayed by animals exposed to VPA during pregnancy are often accompanied by neural impairments. In particular, the offspring prenatally exposed to VPA show cranial nerve abnormalities (Rodier, Ingram et al. 1996), a rearrangement of the dendritic morphology in several limbic and cortical regions (Snow, Hartle et al. 2008, Bringas, Carvajal-Flores et al. 2013), hyperreactivity of pyramidal neurons after electrical stimulation and increased synaptic plasticity in the amygdala (Markram, Rinaldi et al. 2008), decreased excitability with a reduction in putative synaptic contacts in pyramidal neurons (Rinaldi, Perrodin et al. 2008), and a reduced number of Purkinje cells in the posterior lobes of the cerebellum (Ingram, Peckham et al. 2000). Altogether, these findings suggest that the VPA rodent model of ASD has face and construct validity.

3.3 ASD relevant social behavioral phenotypes in the laboratory setting.

While a rodent model cannot fully replicate the human presentation of ASD, fundamental symptoms can be reproduced in the laboratory animal to test theories about the biochemical, genetic and environmental causes of the human condition (Crawley et al., 2007). Thus, ASD models should be based on behavioral impairments recapitulating ASD in humans. To date, the ASD definition lies on

two core symptoms: (a) persistent social interaction and communication deficits and (b) restricted, repetitive patterns of behaviors, interests or activities, along with other comorbid features, most of all anxiety (American Psychiatric Association, 2013). All these features can be easily assessed in the laboratory setting, as outlined below.

3.3.1 Assessing social communication in rodent models of ASD.

The analysis of ultrasonic vocalization (USV) emission has been proven to be an effective tool to study social communication in rodents. Rodents are able to emit USVs in different social context across their lifespan to transmit different types of information, such as pleasure during mating or playful interactions, distress due to a predator attack, food location and maternal retrieval in infancy (Servadio, Vanderschuren et al. 2015). Usually, low frequency (around 22-kHz) USVs are associated with negative social experiences (e.g., exposure to predator odor, inter-male fighting), while high frequency (around 50-kHz) USVs are associated to contexts involving potential reward (e.g., sexual approach, play fighting) (Burgdorf, Panksepp et al. 2011, Servadio, Vanderschuren et al. 2015). In particular, USVs emitted at frequencies between 30 and 90 kHz are fundamental in mother-offspring interaction and thus crucial for pups survival, since they elicit retrieval and caregiving behavior in the dam (Branchi, Santucci et al. 2001, Trezza, Campolongo et al. 2011). Since communicative deficits in ASD appear already in infancy (Dawson and Bernier 2013), analyzing the USVs emitted by the pups when separated from the mother and siblings could provide information on the possible presence of social-communicative deficit. Thus, an alteration in the frequency and duration of USVs emission could reveal an autistic-like behavior in rodents as it has been already proven in literature (i.e (Scattoni, Gandhy et al. 2008, Dawson and Bernier 2013, Wohr 2014, Servadio, Melancia et al. 2016, Melancia, Schiavi et al. 2018).

3.3.2 Assessing social interactions in rodent models of ASD.

Impairments in social interaction are the most investigated behavioral trait in animal models of ASD, since social interaction is widely recognized as the core defining feature of ASD (Wohr and Scattoni 2013, Tartaglione, Schiavi et al. 2019). Since rodents are highly social species, is it possible to assess different kind of social behaviors, such as social approach, reciprocal social interactions, sexual interactions, parental behaviors, and aggressive encounters by using several behavioral tasks (Panksepp, Siviy et al. 1984, Wohr and Scattoni 2013). Unusual and inappropriate social approach, lack of social reciprocity and of spontaneous seeking of interactions among other conspecifics have been selected to model the social abnormalities found in autism (Seltzer, Krauss et al. 2003). One of the most characteristic social behavior that is altered in ASD is social play behavior. Social play has

a key role in the identification and diagnosis of ASD (Jordan 2003, Young, Brewer et al. 2003). Indeed, children with ASD engage passive, stereotyped and rigid play patterns that employ fewer opportunities to join and share play scenarios. As a result, the play of children with ASD is less likely to involve the interest of other children resulting in the failure to engage in play with peers (Jordan 2003, Servadio, Vanderschuren et al. 2015). In mammals, social play behavior is the first form of non-mother directed social behavior; it is crucial for neural growth and proper development, it drives the development of communicative skills, cognitive and social competence, helping behavioral and mental flexibility (Vanderschuren, Achterberg et al. 2016).

In adult rodents, reciprocal social interactions can be easily studied by assessing the behaviors of pairs of animals placed together in a neutral arena, and optimizing the experimental conditions according to the specific aims of the experiment (File 1980). In mice, one of the most used behavioral paradigm engaged to assess sociability is the three-chamber task presented by Moy and coworkers (Moy et al 2004). This specific paradigm is based on a three-chambered apparatus with openings between the lateral chambers and the central compartment. The experimental animal, placed in the central chamber, is allowed to explore a novel object (empty wire cage) and a stimulus animal inside a wire cage placed in two lateral chambers respectively. Typically, rodents presenting normal sociability prefer to spend time with stimulus animal rather than alone. Conversely, rodents showing social dysfunctions tend to enter less and spend less or equal time with the stimulus animal and the empty cage (Servadio, Vanderschuren et al. 2015). Similarly, individuals with autism tend to engage themselves in non-social activities (Wohr and Scattoni 2013). This specific paradigm provides a simple measure of general sociability, since mice present very rudimentary forms of social play and this behavioral paradigm, compared to dyadic social interaction, prevents direct physical contact between the tested animal and the stimulus animal. Through the three-chamber paradigm, researcher are able to assess social approach task and detect unusual levels of mouse sociability that may be analogous to the deficits inappropriate social interactions seen in a patient affected by ASD (Wohr and Scattoni 2013, Servadio, Vanderschuren et al. 2015).

4. Role of the endocannabinoid system in the development of psychiatric disorders.

Alterations of the ECS functionality contribute to the pathogenesis of several psychiatric and neurological disorders (Zamberletti, Gabaglio et al. 2017). As endocannabinoids are key modulators of neural plasticity (Hallmayer, Cleveland et al. 2011) and brain development (Sandin, Lichtenstein et al. 2014) a variety of pathologies are thought to involve dysregulation of endocannabinoid signaling functions. Recently, several studies have documented an impaired endocannabinoid signaling in animal models of neuropsychiatric diseases where social impairment is a core feature, including

schizophrenia, ASD and developmental cannabinoid exposure (Wei, Allsop et al. 2017). Notably, the main active principle of cannabis, Δ9-tetrahydrocannabinol (THC), enters maternal circulation and readily crosses the placenta (Hutchings et al., 1989). Thus, prenatal cannabis exposure might exert deleterious effects on the fetus. In rats, CB1 cannabinoid receptors, already functional around gestational days (GD) 11-14, are involved in embryonal implantation, neural development, and control of synaptic communication (Berghuis, Rajnicek et al. 2007, Harkany, Guzman et al. 2007). Pioneering animal studies have demonstrated specific deficits in prenatally cannabis-exposed rodent offspring at different developmental periods (Trezza, Damsteegt et al. 2012, Richardson, Hester et al. 2016). In humans, several studies have proved the detrimental effects of prenatal cannabis exposure on the offspring from the neonatal period through early adulthood (Huizink 2014, Crume, Juhl et al. 2018, El Marroun, Brown et al. 2018, Ryan, Ammerman et al. 2018), revealing increased tremors, startles and altered sleep patterns at birth (Calvigioni, Hurd et al. 2014, Volkow, Compton et al. 2017) and significant impairment of higher cognitive functions beyond infancy (Leech, Richardson et al. 1999, Fried 2002, Smith, Longo et al. 2010, Huizink 2014, Passey, Sanson-Fisher et al. 2014, Grant, Campbell et al. 2018). Moreover, in rats cannabinoid exposure during pregnancy and/or lactation alters isolation-induced USVs and social play inducing anxiety-like behaviors (Trezza, Campolongo et al. 2008).

In the last decade, evidences for an involvement of the ECS in ASD emerged from the observation that this system is strongly implicated in the regulation of social and emotional reactivity as well as in the modulation of behaviors that are often altered in ASD (Trezza, Campolongo et al. 2008, Trezza and Vanderschuren 2009, Marco, Valero et al. 2013). Furthermore, human neuroimaging studies revealed associations between polymorphisms in the gene encoding for CB1 receptor, CNR1, and social reward responsivity (Chakrabarti, Kent et al. 2006, Chakrabarti and Baron-Cohen 2011), suggesting that alterations of CB1 receptors might contribute to deficits in social reward processing associated with ASD. Consistent with this, reduced CB1 receptor expression was found in post-mortem brains of individuals with autism (Purcell, Jeon et al. 2001, Baron-Cohen 2004). At the preclinical level, both genetic- and environmental-based models have been exploited to study the involvement and/or the therapeutic potential of the ECS in the context of ASD revealing that enhancing AEA signaling through inhibition of its degradation exerts prosocial effects in different animal models of ASD (Servadio, Melancia et al. 2016, Melancia, Schiavi et al. 2018). Moreover, CB1 receptor blockade seems to have ameliorate cognitive deficits in mouse models of FXS (Busquets-Garcia, Gomis-Gonzalez et al. 2013, Gomis-Gonzalez, Busquets-Garcia et al. 2016). A better understanding of the possible role of the ECS in the onset and/or progression of ASD symptoms

would allow for the evaluation of specific pharmacological interventions that may eventually aid the development of successful drug therapies (Zamberletti, Gabaglio et al. 2017).

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CHAPTER 2

1. Aims

The general aim of my PhD project was to study typical and atypical forms of social behavior in animal models, in order to 1. study neurobiological aspects of social behavior and shed light on possible neurochemical alterations causing social dysfunctions in psychiatric diseases; 2. find new therapeutic opportunities to treat the social dysfunctions that characterize several psychiatric disorders. To address these aims, I used rodent models displaying either normal or atypical patterns of social behavior.

To study the neural underpinnings of social behavior in healthy states, I mainly focused on the endogenous regulation of social play behavior in rats. Social play is the most characteristic social activity displayed by young mammals, it has a crucial role in the social and cognitive development of mammals, and it is particularly compromised in several developmental neuropsychiatric diseases, such as ASD and schizophrenia. In the first phase of my PhD project, I focused on the study of four specific neurotransmitter systems involved in the regulation of social play behavior: the endogenous opioid system, the endocannabinoid system, the dopaminergic system and the noradrenergic system. In particular, I first studied the interaction between the endocannabinoid and the endogenous opioid systems in social play (see Chapter 3). Then, to clarify the involvement of dopaminergic and noradrenergic neurotransmission in social play behavior, I studied the neural mechanisms underlying the effects of Methylenedioxypyrovalerone (MDPV) on social play (see Chapter 4). Indeed, MDPV is a psychoactive stimulant compound belonging to the synthetic cathinones that is thought to pharmacologically interact with dopaminergic and noradrenergic neurotransmission. During the second phase of my PhD project, I set up and validated in our lab the well-characterized animal model of autism based on prenatal exposure to VPA (see Chapter 5) and a model of prenatal exposure to cannabinoids in rats (see Chapter 8), in order to study: (1) social impairments in ASD and (2) the brain mechanisms underlying the social deficits induced at early life by prolonged cannabinoid exposure, putting particular attention on sex-related behavioral differences in the two models. First, I studied the neural underpinnings of the atypical social behaviors that characterize developmental psychiatric diseases such as ASD, and I mainly investigated the functionality of the brain reward system and the endocannabinoid system in different brain areas of rats prenatally exposed to VPA (see Chapters 6 and 7). Then, I tested the ability of the selective anandamide hydrolysis inhibitor URB597 to rescue the atypical social behaviors displayed by male and female VPA-exposed rats in the course of development (see Chapter 6). Last, in the third phase of my PhD project, as endocannabinoids are key modulators of neural plasticity, brain development and a variety of

pathologies are thought to involve dysregulation of their signaling functions, I studied the effects of prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 on the emotional reactivity and cognitive performance of male and female rat offspring from infancy through adolescence and tested the role of mGlu5 receptor signaling in the observed effects (see Chapter 8).

CHAPTER 3

Unidirectional opioid-cannabinoid cross-tolerance in the modulation of social play behavior in rats

Sara Schiavi[1]*, Antonia Manduca*[1], Marco Segatto[1], Patrizia Campolongo[2], Valentina Pallottini[1], Louk J.M.J. Vanderschuren[3], Viviana Trezza[1#]

*equal contribution

[1]Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Rome, Italy;

[2]Department of Physiology and Pharmacology "V. Erspamer", Sapienza University of Rome, Rome, Italy.

[3]Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

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Unidirectional opioid-cannabinoid cross-tolerance in the modulation of social play behavior in rats.

The first aim of my PhD project was to study neurobiological aspects of social behavior and shed light on possible neurochemical alterations causing social dysfunctions in psychiatric diseases. I initially clarified the role of different neurotransmitter systems that are thought to be normally involved in the regulation of typical forms of social behavior. First, I focused on the endocannabinoid and the endogenous opioid systems, given their well-known role in the modulation of socio-emotional behavior. In this study, I analyzed the role and the interaction of these neurotransmitter systems in social play behavior in adolescent rats, demonstrating that a dynamic opioid-cannabinoid interaction in the modulation of social play behavior exists, with limbic brain areas strongly involved. A better understanding of opioid-cannabinoid interactions in social play can contribute to clarify neurobiological aspects of social behavior at a young age, which may provide new therapeutic targets for social dysfunctions.

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ORIGINAL INVESTIGATION

Unidirectional opioid-cannabinoid cross-tolerance in the modulation of social play behavior in rats

Sara Schiavi[1] · Antonia Manduca[1] · Marco Segatto[1] · Patrizia Campolongo[2] · Valentina Pallottini[1] · Louk J. M. J. Vanderschuren[3] · Viviana Trezza[1]

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Abstract

Rationale The endocannabinoid and the endogenous opioid systems interact in the modulation of social play behavior, a highly rewarding social activity abundantly expressed in young mammals. Prolonged exposure to opioid or cannabinoid receptor agonists induces cross-tolerance or cross-sensitization to their acute behavioral effects.

Objectives and methods Behavioral and biochemical experiments were performed to investigate whether cross-tolerance or cross-sensitization occurs to the play-enhancing effects of cannabinoid and opioid drugs on social play behavior, and the possible brain substrate involved.

Results The play-enhancing effects induced by systemic administration of JZL184, which inhibits the hydrolysis of the endocannabinoid 2-AG, were suppressed in animals repeatedly pretreated with the opioid receptor agonist morphine. Conversely, acute morphine administration increased social play in rats pretreated with vehicle or with either JZL184 or the cannabinoid agonist WIN55,212-2. Acute administration of JZL184 increased the activation of both CB1 receptors and their effector Akt in the nucleus accumbens and prefrontal cortex, brain regions important for the expression of social play. These effects were absent in animals pretreated with morphine. Furthermore, only animals repeatedly treated with morphine and acutely administered with JZL184 showed reduced activation of CB1 receptors and Akt in the amygdala.

Conclusions The present study demonstrates a dynamic opioid–cannabinoid interaction in the modulation of social play behavior, occurring in limbic brain areas strongly implicated in social play behavior. A better understanding of opioid–cannabinoid interactions in social play contributes to clarify neurobiological aspects of social behavior at young age, which may provide new therapeutic targets for social dysfunctions.

Keywords Cannabinoid · Opioid · Social play behavior · Rats · 2-AG · Morphine

Introduction

In humans as in animals, positive social interactions are important for well-being, healthy development, and establishment and maintenance of adequate social structures. One of the most pleasurable social activities displayed in youth by mammals is social play behavior. Social play is important for the development of social, cognitive, and emotional competences, and it is disrupted in pediatric psychiatric disorders (Vanderschuren et al. 2016). In rodents, social play behavior peaks after weaning (postnatal day (PND) 21) until mid-adolescence (PND 35–46), equivalent to childhood to early/mid adolescence in humans (McCutcheon and Marinelli 2009; Panksepp 1981; Spear 2000).

Operant and classical conditioning studies in rodents have shown that social play is a highly rewarding activity

Sara Schiavi and Antonia Manduca contributed equally to this work.

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✉ Viviana Trezza
viviana.trezza@uniroma3.it

[1] Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Viale G. Marconi 446, 00146 Rome, Italy

[2] Department of Physiology and Pharmacology "V. Erspamer", Sapienza University of Rome, Rome, Italy

[3] Department of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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(Vanderschuren et al. 2016). Accordingly, pharmacological experiments have demonstrated that social play behavior is modulated by neurotransmitters involved in reward and motivation, such as endocannabinoids and endogenous opioids (Trezza et al. 2010, 2011b; Vanderschuren et al. 2016).

The endocannabinoid and the endogenous opioid systems have functional similarities in the sense that they share neuroanatomical, neurochemical, and pharmacological characteristics (Befort 2015; Parolaro et al. 2010; Wenzel and Cheer 2018).

The endocannabinoid system is abundant in brain regions involved in reward processes. It consists of lipid signaling messengers (endocannabinoids, mainly anandamide and 2-arachidonoylglycerol (2-AG)) which are synthesized following neuronal depolarization (Mechoulam et al. 2014; Piomelli 2003). Endocannabinoids are mostly synthesized postsynaptically and act as retrograde messengers regulating the release of a variety of neurotransmitters at the presynaptic level (Di Marzo 2006; Di Marzo et al. 2004; Piomelli 2003). When released from the postsynaptic neuron, endocannabinoids bind to Gi/Go-coupled cannabinoid receptors (CB1 and CB2, mainly expressed in the brain and periphery, respectively). Their actions end with the degradation by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), hydrolytic enzymes for anandamide and 2-AG, respectively (Mechoulam et al. 2014; Piomelli 2003).

Similarly to endocannabinoids, endogenous opioid peptides such as enkephalins, endorphins, and dynorphins activate Gi/Go protein-coupled metabotropic receptors (mu, delta, and kappa) (Filizola and Devi 2013; Kieffer 1995) that are highly expressed in brain areas that mediate reward and motivation (Erbs et al. 2015; Koob and Volkow 2010; Le Merrer et al. 2009; Vanderschuren et al. 2016).

Although different neurotransmitter systems are involved in social play behavior (Siviy and Panksepp 2011; Vanderschuren et al. 1997), the endocannabinoid and endogenous opioid systems play a key role in the modulation of the rewarding properties of social play, and they have been shown to closely interact in the regulation of this behavior (Vanderschuren et al. 2016). Endocannabinoids are released during social play in the nucleus accumbens (NAc) and amygdala (Trezza et al. 2012), and drugs that increase the levels of the endocannabinoids anandamide and 2-AG by inhibiting their hydrolysis enhance social play acting within these brain areas (Manduca et al. 2016; Trezza et al. 2012). The prefrontal cortex also has a role in cannabinoid modulation of social play behavior (Schneider and Koch 2005). Likewise, changes in brain opioid activity occur during social play in different brain areas, including the NAc (Vanderschuren et al. 1995a) and both systemic (Vanderschuren et al. 1995b) and intra-NAc (Trezza et al. 2011a) administration of opioid receptor agonists and antagonists increase and suppress social play, respectively, through interaction with mu-opioid receptors. Noteworthy, reciprocal interactions between the endocannabinoid and the opioid systems are involved not only in drug (Befort 2015; Fattore et al. 2005) and food (Solinas and Goldberg 2005) rewards but also in social play reward (Manduca et al. 2016; Trezza and Vanderschuren 2008; Vanderschuren et al. 2016).

Previous studies have demonstrated that prolonged exposure to either opioid or cannabinoid receptor agonists results in cross-tolerance or cross-sensitization to most of their acute behavioral effects (Maldonado 2002; Robledo et al. 2008). Given the previously reported opioid–cannabinoid interaction in the modulation of social play behavior (Manduca et al. 2016; Trezza et al. 2010; Vanderschuren et al. 2016), the aim of the present study was to investigate whether cross-tolerance or cross-sensitization would occur to the play-enhancing effects of cannabinoid and opioid drugs. To address this aim, we used two play-enhancing drugs whose effects on social play have been previously well characterized: the 2-AG hydrolysis inhibitor JZL184 (Manduca et al. 2016) and the opioid receptor agonist morphine (Vanderschuren et al. 1995a, b). JZL184 is a high selective 2-AG hydrolysis inhibitor that increases brain 2-AG level when administered systemically (Fowler 2012; Long et al. 2009). We have recently shown that the increase in social play induced by systemic administration of JZL184 requires the activation of both opioid and cannabinoid receptors (Manduca et al. 2016). However, since JZL184 is an indirect cannabinoid agonist (i.e., it increases 2-AG levels, but it does not directly bind cannabinoid receptors), we performed also an additional experiment in which we tested whether the play-enhancing effects of morphine were maintained, or not, when rats were repeatedly pretreated with the CB1 cannabinoid receptor agonist WIN 55,212-2.

There is experimental evidence that CB1 receptor activation by endogenous and/or exogenous cannabinoids leads to the induction of signaling cascades culminating in Akt protein activation (Bouaboula et al. 1995; Gomez del Pulgar et al. 2000; Ozaita et al. 2007; Wartmann et al. 1995). Thus, the evaluation of Akt phosphorylation is a reliable readout to assess CB1 cannabinoid receptor activation. For this reason, we measured the expression of both phosphorylated and total CB1 receptor protein and its effector Akt in the amygdala, NAc and prefrontal cortex, key brain regions implicated in social play behavior (Vanderschuren et al. 2016).

Experimental procedures

Animals

Male Wistar rats (Charles River Laboratories, Italy) arrived in the animal facility at 21 days of age, and they were housed in groups of five in Macrolon cages (43 × 26 × 20 cm) under

controlled conditions (temperature 21 ± 1 °C, 60 ± 10% relative humidity and 12/12 h light cycle with lights on at 07:00 a.m.). Food and water were available ad libitum. Animals were experimentally naive and were used only once. Sample size (*n*) is indicated in the figure legends. The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) (Kilkenny et al. 2010) guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

Drugs

The monoacylglycerol lipase (MAGL) inhibitor JZL184 [4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester] (National Institute of Mental Health's (NIMH) Chemical Synthesis and Drug Supply Program, USA) and the CB1 cannabinoid receptor agonist WIN 55,212-2 (WIN) (TOCRIS, UK) were dissolved in 5% Tween 80/5% polyethylene glycol/saline. The opioid receptor agonist morphine (SALARS, Italy) was dissolved in saline. JZL184 (1 mg/kg) and WIN (0.3 mg/kg) were given intraperitoneally (i.p.), while morphine (1 mg/kg) was administered subcutaneously (s.c.). These doses of JZL184 and morphine are known to increase social play behavior in rats following acute systemic administration (Manduca et al. 2016; Trezza and Vanderschuren 2008). As vehicle for JZL184 and WIN (VEH), we used a mixture of 5% Tween 80, 5% polyethylene glycol and 90% saline, while the vehicle for morphine was saline solution (SAL). Solutions were freshly prepared on the day of the experiment and were administered in a volume of 2 ml/kg.

Behavioral experiments

Social play behavior

Social play was assessed as previously described (Manduca et al. 2016). The experiments were performed in a sound attenuated chamber under dim light conditions. The testing arena consisted of a Plexiglas cage (40 × 40 × 60 cm) with approximately 2 cm of wood shavings covering the floor.

At 26–28 days of age, rats were individually habituated to the test cage for 10 min on 2 days prior to testing. On the test day, the animals were socially isolated for 3.5 h before testing. This isolation period has been shown to induce a half-maximal increase in the amount of social play behavior (Niesink and van Ree 1982). At the appropriate time before testing, pairs of animals were treated with drugs or vehicle. In all experiments, both animals of a pair received the same drug treatment. The test consisted of placing two animals into the test cage for 15 min. The animals of each pair did not differ more than 10 g in body weight and had no known previous common social experience (i.e., they were not cage mates).

Behavior was assessed per pair of animals using the Observer 3.0 software (Noldus Information Technology BV, Wageningen, The Netherlands). In rats, a bout of social play behavior starts with one rat soliciting ("pouncing") another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways. If the animal that is pounced upon fully rotates to its dorsal surface, pinning is the result, i.e., one animal lying with its dorsal surface on the floor with the other animal standing over it. From this position, the supine animal can initiate another play bout, by trying to gain access to the other animal's neck. Thus, during social play, pouncing is considered an index of play solicitation, while pinning can be regarded as the terminal component of a single play bout as well as a releaser of a prolonged play bout (Pellis and Pellis 1987). Pinning and pouncing events can be easily quantified, and they are considered to be the most characteristic parameters of social play behavior in rats (Panksepp and Beatty, 1980). During the social encounter, animals may also display social behaviors not directly associated with play, such as sniffing or grooming the partner's body. A pair of rats was considered as one experimental unit. The following parameters were therefore scored per pair of animals:

Social behaviors directly related to play:

- Number of pinning events during the 15-min test session.
- Number of pouncing events during the 15-min test session.

Social behaviors unrelated to play:

- Time spent in social exploration: the total amount of time (s) spent in non-playful forms of social interaction (i.e., one animal sniffing or grooming any part of the partner's body).

First, we investigated whether cross-tolerance would occur to the effects of JZL184 and morphine on social play after repeated treatment with either compounds. To this aim, three experiments were performed (Fig. 1). In experiment 1, animals were pretreated with either morphine (MOR, 1.0 mg/kg, s.c.) or saline solution (SAL) for five consecutive days (postnatal days 23–27). On day 28, 1 day after the last pretreatment injection, animals were isolated for 3.5 h. Next, half of both pretreatment groups (MOR or SAL) was treated with either JZL184 (1 mg/kg, i.p., 2 h before testing) or its vehicle (VEH), and tested for social play behavior as described above. In experiment 2, animals were pretreated with JZL184 (JZL, 1.0 mg/kg, i.p.) or its vehicle (VEH) for five consecutive days (postnatal days 23–27). On day 28, animals were isolated for 3.5 h. Next, half of both pretreatment groups (JZL or VEH)

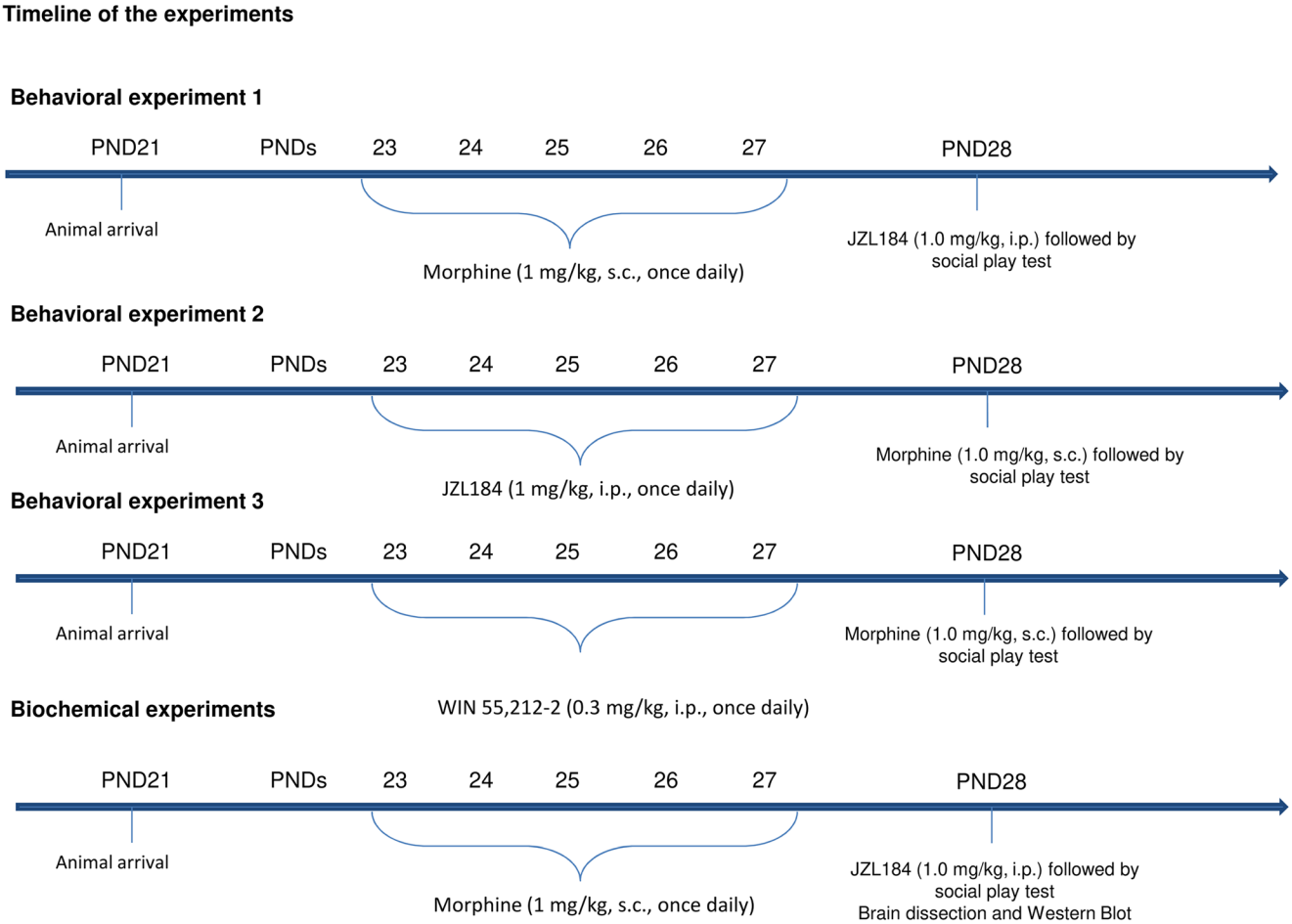

Fig. 1 Timeline of the experiments

was treated with either morphine (MOR, 1 mg/kg, s.c., 1 h before testing) or saline (SAL), and tested for social play behavior as described above. In experiment 3, animals were pretreated with WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) or its vehicle (VEH) for five consecutive days (postnatal days 23–27). On day 28, animals were isolated for 3.5 h. Next, half of both pretreatment groups (WIN55,212-2 or VEH) was treated with either morphine (MOR, 1 mg/kg, s.c., 1 h before testing) or saline (SAL), and tested for social play behavior as described above.

Western blot analysis of phosphorylated and total CB1 cannabinoid receptor

Lysate preparation from brain tissue

Immediately after testing for social play behavior, rats repeatedly treated with morphine (or saline solution) and acutely treated with JZL184 (or its vehicle) were rapidly decapitated and their brains quickly removed and rinsed in ice-cold distilled water for 10 s. The brains were then cut into coronal slices on a cold plate, and the amygdala, NAc, and prefrontal cortex were dissected by hand under microscopic control within 2 min (Gray et al. 2015; Hill et al. 2010). Tissues were stored at − 80 °C until use. Lysates from brain regions were prepared accordingly to the previously used protocol (Segatto et al. 2014). Briefly, brain regions were lysed by sonication in a sample buffer (0.125 M TrisHCL pH 6.8, 10% SDS, Protease and Phosphatase Inhibitor Cocktails, Sigma, Italy). The lysate was then centrifuged at 13.000 rpm for 10 min to remove cell/tissue debris. Protein concentration was estimated by the method of Lowry et al. (1951). Samples were subsequently boiled for 3 min before loading to the SDS-PAGE for Western blotting analysis.

Western blotting analysis

Western blot experiments were performed as previously described (Segatto et al. 2014) Briefly, proteins (30 μg) from amygdala, prefrontal cortex, and NAc lysates were resolved by 10% (for p-Akt, Santa Cruz sc-7985-R; t-Akt, Santa Cruz sc-8312; p-CB1 Santa Cruz (ser-316) sc-17555; t-CB1 (k-15) sc-10068 Santa Cruz) SDS-PAGE

at 40 mA (constant current) for 60 min. Proteins were then transferred onto nitrocellulose membrane by using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Milan, Italy). The nitrocellulose membrane was blocked at room temperature with 5% fat-free milk in Tris-buffered saline (0.138 M NaCl, 0.027 M KCl, 0.025 M Tris-HCl, and 0.05% Tween-20, pH 6.8), and probed at 4 °C overnight with primary antibodies followed by incubation for 1 h with horseradish peroxidase-conjugated secondary IgG antibodies (Bio-Rad Laboratories, Milan, Italy). Subsequently, the nitrocellulose membrane was incubated with anti-tubulin (α-tubulin DM-1A; Sigma, Italy) antibody. Bound antibodies to proteins onto nitrocellulose were visualized by using enhanced chemoluminescence detection (GE Healthcare) and exposure to Amersham Hyperfilm ECL (GE Healthcare). Western blotting images were analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA) software for Windows. The visualization of the housekeeping protein α-tubulin served as loading control. Thus, each reported value was obtained from the ratio between arbitrary units derived by the protein band and the respective α-tubulin.

Statistical analysis

Data are expressed as mean ± SEM, and statistical significance was set at $p < 0.05$. To assess the effects of treatments on behavioral parameters, data were analyzed using two-way ANOVA (with pretreatment and acute treatment as independent factors), followed by Student–Newman–Keuls post hoc tests where appropriate. To assess the effects of treatments on biochemical parameters, data were analyzed using the non-parametric Kruskall–Wallis test followed by Mann–Whitney *U* test. To check the normality of the behavioral data, the Shapiro–Wilk test was performed (Shapiro and Wilk 1965).

Results

Behavioral experiments

Repeated administration of the opioid receptor agonist morphine causes cross-tolerance to the play-enhancing effects of the 2-AG hydrolysis inhibitor JZL184

To evaluate whether cross-tolerance or sensitization occurs to the play-enhancing effects of JZL184 on social play behavior after repeated treatment with morphine, animals were treated with morphine (1 mg/kg, s.c.) or saline once daily for five consecutive days. On the sixth day, the animals were tested after treatment with either a dose of the MAGL inhibitor JZL184 (1.0 mg/kg, i.p.) that is known to increase social play behavior (Manduca et al. 2016) or its vehicle.

Systemic administration of JZL184 increased social play in saline-pretreated rats but not in morphine-pretreated rats (pinning: $F_{(acute)1,33} = 4.160$, $p = 0.049$; $F_{(repeated)1,33} = 3.272$, $p = 0.080$; $F_{(acute \times repeated)1,33} = 2.170$, $p = 0.150$; pouncing: $F_{(acute)1,33} = 6.039$, $p = 0.019$; $F_{(repeated)1,33} = 2.040$, $p = 0.163$; $F_{(acute \times repeated)1,33}$ = n.s., $p = 0.328$). Post hoc analyses revealed that JZL184 increased the number of pinning (Fig. 2a) and pouncing (Fig. 2b) in animals pretreated with saline but not with morphine, indicating that tolerance to the effect of JZL184 had occurred after repeated stimulation of mu-opioid receptors. Social exploration was not affected by either treatment ($F_{(acute)1,33}$ = n.s., $p = 0.801$; $F_{(repeated)1,33} = 1.716$, $p = 0.199$; $F_{(acute \times repeated)1,33}$ = n.s., $p = 0.637$, Fig. 2c).

Repeated administration of either the 2-AG hydrolysis inhibitor JZL184 or the CB1 cannabinoid receptor agonist WIN does not interfere with the play-enhancing effects of morphine

To evaluate whether cross-tolerance or sensitization occurs to the play-enhancing effects of morphine after repeated

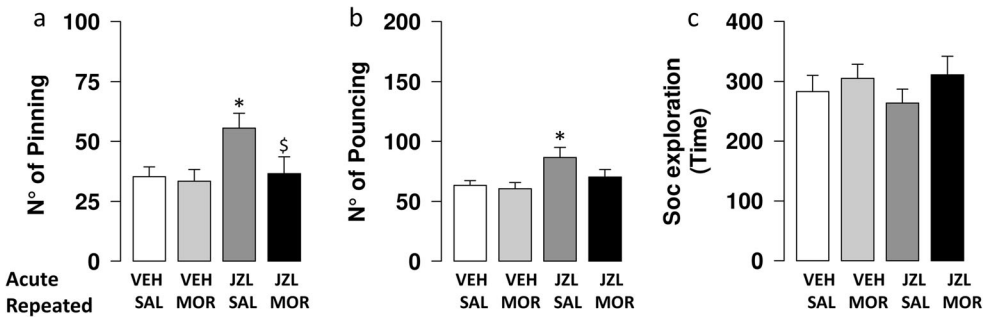

Fig. 2 Repeated administration of the opioid receptor agonist morphine causes cross-tolerance to the play-enhancing effects of the 2-AG hydrolysis inhibitor JZL184. Systemic administration of the 2-AG hydrolysis inhibitor JZL184 (JZL; 1 mg/kg, i.p.) increased the number of pinning (**a**) and pouncing (**b**) in animals repeatedly treated with saline (SAL) but not in animals pretreated with morphine (MOR; 1.0 mg/kg, s.c.). Social exploration was not affected by either treatment (**c**) (n = VEH-SAL 10, n = VEH-MOR 8, n = JZL-SAL 11, n = JZL-MOR 8). Data represent mean values ± SEM; *$p < 0.05$ vs VEH-SAL group, $$p < 0.05$ vs JZL-SAL group (Student–Newman–Keuls post hoc test)

treatment with the 2-AG hydrolysis inhibitor JZL184, animals were treated with JZL184 (1 mg/kg, i.p.) or its vehicle once daily for five consecutive days. On the sixth day, the animals were tested after treatment with a dose of the opioid receptor agonist morphine (1.0 mg/kg, s.c.) that is known to increase social play (Trezza and Vanderschuren 2008).

Acute administration of morphine markedly increased social play in both rats repeatedly pretreated with vehicle or with JZL184 (pinning, $F_{(acute)1,24} = 23.87$, $p < 0.001$; $F_{(repeated)1,24}$ = n.s., $p = 0.857$; $F_{(acute \times repeated)1,24}$ = n.s., $p = 0.983$; pouncing, $F_{(acute)1,24} = 26.50$, $p < 0.001$; $F_{(repeated)1,24}$ = n.s., $p = 0.765$; $F_{(acute \times repeated)1,24}$ = n.s., $p = 0.333$). Post hoc analysis revealed that morphine increased the number of pinning (Fig. 3a) and pouncing (Fig. 3b) regardless of whether the animals were repeatedly pretreated with vehicle or JZL184, indicating that cross-tolerance to the play-enhancing effects of morphine after repeated administration of the 2-AG hydrolysis inhibitor JZL184 had not occurred. Social exploration was not affected in any treatment group ($F_{(acute)1,24} = 2.044$, $p = 0.166$; $F_{(repeated)1,24} = 1.883$, $p = 0.183$; $F_{(acute \times repeated)1,24}$ = n.s., $p = 0.369$, Fig. 3c).

To exclude the possibility that JZL184 failed to induce cross-tolerance (or sensitization) to the play-enhancing effects of acute morphine administration due to its pharmacological profile (i.e., JZL184 is a 2-AG hydrolysis inhibitor rather than a direct CB1 receptor agonist), an additional experiment was performed: animals were repeatedly treated with the CB1 cannabinoid receptor agonist WIN55,212-2 (WIN, 0.3 mg/kg, i.p.) or its vehicle once daily for five consecutive days. On the sixth day, the animals were tested after treatment with a dose of the opioid receptor agonist morphine (1.0 mg/kg, s.c.) that is known to increase social play (Trezza and Vanderschuren 2008).

Acute administration of morphine markedly increased social play in rats repeatedly pretreated with vehicle or with WIN (pinning, $F_{(acute)1,28} = 80.74$, $p < 0.001$; $F_{(repeated)1,28}$ = n.s., $p = 0.755$; $F_{(acute \times repeated)1,28}$ = n.s., $p = 0.734$; pouncing, $F_{(acute)1,28} = 90.31$, $p < 0.001$; $F_{(repeated)1,28}$ = n.s., $p = 0.66$; $F_{(acute \times repeated)1,28} = 1.054$, $p = 0.313$). Post hoc analysis revealed that morphine increased the number of pinning (Fig. 4a) and pouncing (Fig. 4b) regardless of whether the animals were repeatedly pretreated with vehicle or WIN, indicating that cross-tolerance to the play-enhancing effects of morphine after repeated administration of the CB1 cannabinoid receptor agonist WIN had not occurred. Social exploration was not affected in any treatment group ($F_{(acute)1,28}$ = n.s., $p = 0.731$; $F_{(repeated)1,28}$ = n.s., $p = 0.375$; $F_{(acute \times repeated)1,28}$ = n.s., $p = 0.472$, Fig. 4c). These results confirm that unidirectional opioid–cannabinoid cross-tolerance exists in the modulation of social play behavior in rats.

Biochemical experiments

Since the behavioral experiments showed that repeated administration of the opioid receptor agonist morphine caused cross-tolerance to the play-enhancing effects induced by acute administration of the 2-AG hydrolysis inhibitor JZL184, we measured CB1 cannabinoid receptor and p-Akt phosphorylation in the amygdala, NAc, and prefrontal cortex of rats acutely treated with JZL184 after repeated treatment with morphine, since these brain regions have a key role in the modulation of social play behavior (Vanderschuren et al. 2016).

CB1 cannabinoid receptor and p-Akt phosphorylation in the amygdala of rats acutely treated with JZL184 after repeated treatment with morphine

Animals treated with morphine for 5 days, followed by acute treatment with JZL184 before testing showed a reduction in the ratio between phosphorylated and total CB1 receptor protein compared to all the other experimental groups ($p < 0.01$, Mann–Whitney U test for JZL-MOR vs VEH-SAL, VEH-MOR, and JZL-SAL, Fig. 5a). Likewise, in animals injected for 5 days with morphine and treated acutely with JZL184 before testing a reduction in the ratio between phosphorylated and total Akt protein when compared to all the other

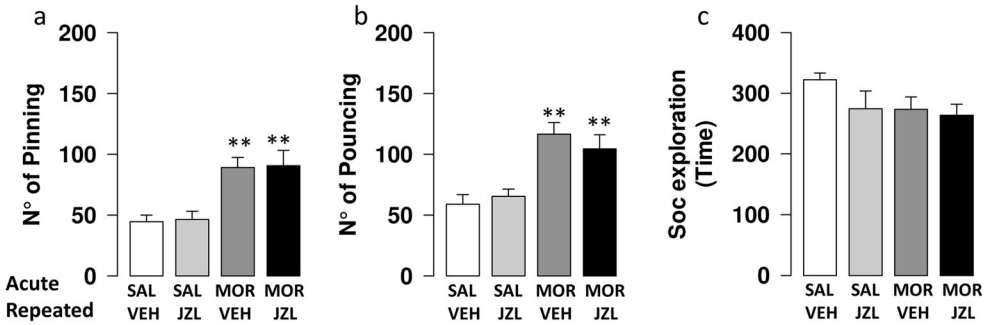

Fig. 3 Repeated administration of the 2-AG hydrolysis inhibitor JZL184 does not cause cross-tolerance to the play-enhancing effects of morphine. Systemic administration of morphine (MOR; 1 mg/kg s.c.) before testing increased the number of pinning (**a**) and pouncing (**b**) regardless the animals were repeatedly treated with vehicle (VEH) or JZL184 (JZL; 1 mg/kg, i.p.). Social exploration was not affected by either treatment (**c**) (n = SAL-VEH 7, n = SAL-JZL 7, n = MOR-VEH 6, n = MOR-JZL 8). Data represent mean values ± SEM; $^{**}p < 0.01$ vs SAL-VEH group (Student–Newman–Keuls post hoc test))

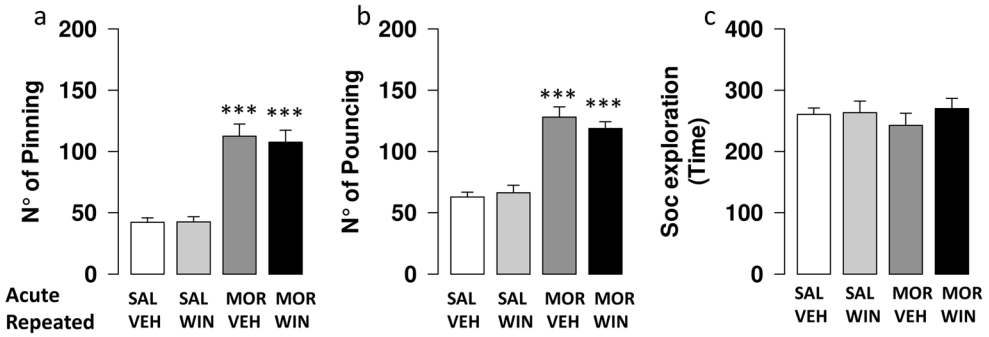

Fig. 4 Repeated administration of the CB1 cannabinoid receptor agonist WIN 55,212-2 does not cause cross-tolerance to the play-enhancing effects of morphine. Systemic administration of morphine (MOR; 1 mg/kg s.c.) before testing increased the number of pinning (**a**) and pouncing (**b**) regardless the animals were repeatedly treated with WIN 55,212-2 (WIN; 0.3 mg/kg, i.p.) or its vehicle (VEH). Social exploration was not affected by either treatment (**c**) (n = SAL-VEH 8, n = SAL-JZL 8, n = MOR-VEH 8, n = MOR-JZL 8). Data represent mean values ± SEM; ***$p < 0.001$ vs SAL-VEH group (Student–Newman–Keuls post hoc test)

experimental groups was found ($p < 0.05$, Mann–Whitney U test for JZL-MOR vs VEH-SAL, VEH-MOR, and JZL-SAL, Fig. 5b). These results indicate that the 2-AG hydrolysis inhibitor JZL184 reduced the activation of amygdala CB1 receptors only in animals pretreated with morphine.

CB1 cannabinoid receptor and p-Akt phosphorylation in the NAc of rats acutely treated with JZL184 after repeated pretreatment with morphine

The acute administration of JZL184 increased the ratio between phosphorylated and total CB1 receptors in the NAc, but this effect was absent in rats pretreated with morphine ($p < 0.05$, Mann–Whitney U test for JZL-MOR vs JZL-SAL and JZL-SAL vs VEH-SAL, Fig. 6a). Likewise, the ratio between phosphorylated and total Akt in the NAc was increased only in rats repeatedly treated with saline followed by acute treatment with JZL184 ($p < 0.05$, Mann–Whitney U test for JZL-MOR vs JZL-SAL and $p < 0.01$, Mann–Whitney U test for JZL-SAL vs VEH-SAL, Fig. 6b).

CB1 cannabinoid receptor and p-Akt phosphorylation in the prefrontal cortex of rats acutely treated with JZL184 after repeated pretreatment with morphine

Treatment with JZL184 increased the ratio between phosphorylated and total CB1 receptors in the PFC of saline- but not morphine-pretreated rats ($p < 0.05$, Mann–Whitney U test for

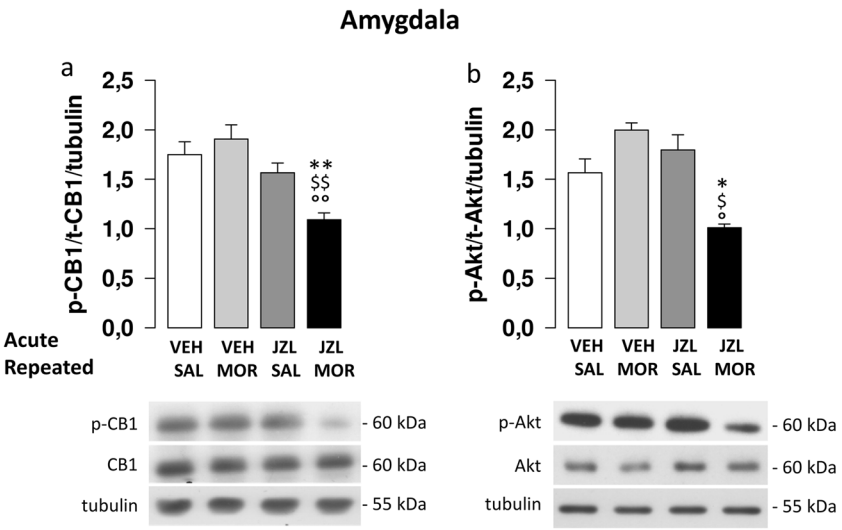

Fig. 5 CB1 cannabinoid receptor and p-Akt phosphorylation in the amygdala of rats repeatedly treated with morphine and acutely treated with JZL184. Animals injected for 5 days with morphine (MOR) and treated acutely with JZL184 (JZL) before testing showed a reduction in the ratio between phosphorylated and total CB1 receptor protein in the amygdala compared to all the other experimental groups (**a**). Similarly, in the amygdala of repeatedly morphine-exposed rats treated with JZL184 before testing, the ratio between phosphorylated and total Akt protein decreased compared to the other experimental groups (**b**) (n = VEH-SAL 5, n = VEH-MOR 5, n = JZL-SAL 6, n = JZL-MOR 5). Data represent mean values ± SEM; *$p < 0.05$, **$p < 0.01$ vs VEH-SAL group, °$p < 0.05$, °°$p < 0.01$ vs VEH-MOR group, $$p < 0.05$, $$$p < 0.01$ vs JZL-SAL group (Mann–Whitney U test)

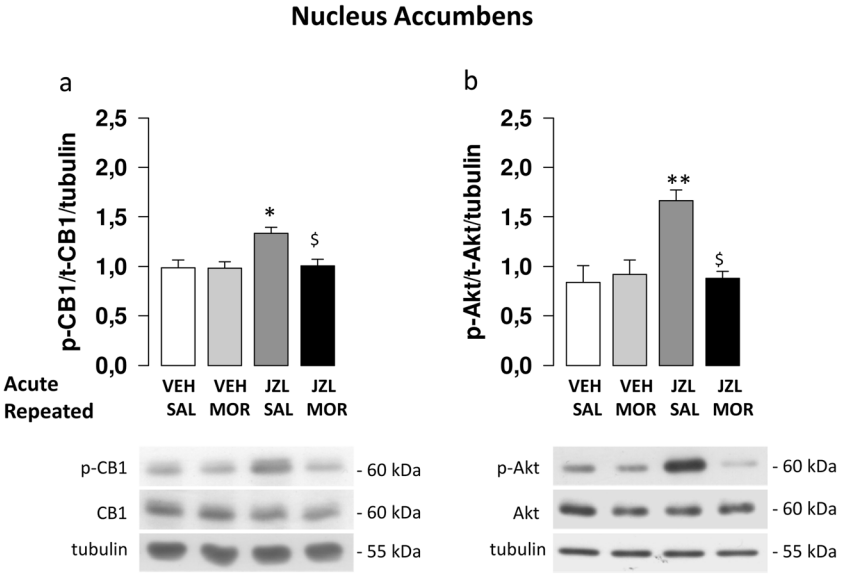

Fig. 6 CB1 cannabinoid receptor and p-Akt phosphorylation in the NAc of rats repeatedly treated with morphine and acutely treated with JZL184. Acute administration of JZL184 (JZL) increased the ratio between phosphorylated and total CB1 receptors in the NAc, but this effect disappeared when rats acutely treated with JZL184 were repeatedly pretreated with morphine (MOR) (**a**). Similarly, the ratio between phosphorylated and total Akt protein increased in the NAc of saline- (VEH) but not morphine-pretreated rats (**b**) (n = VEH-SAL 4, n = VEH-MOR 4, n = JZL-SAL 4, n = JZL-MOR 4). Data represent mean values ± SEM; *$p < 0.05$, **$p < 0.01$ vs VEH-SAL group, $$p < 0.05$ vs JZL-SAL group (Mann–Whitney U test)

JZL-MOR vs JZL-SAL and JZL-SAL vs VEH-SAL Fig. 7a). Similarly, the acute administration of JZL184 increased the ratio between phosphorylated and total Akt protein, but this effect disappeared when rats acutely treated with JZL184 were repeatedly pretreated with morphine ($p < 0.05$, Mann–Whitney U test for JZL-MOR vs JZL-SAL and JZL-SAL vs VEH-SAL Fig. 7b).

Discussion

In this study, we provide the first evidence for unidirectional cross-tolerance between opioid and endocannabinoid neurotransmission in the modulation of social play behavior. In particular, we found that cross-tolerance to the play-enhancing effects of the 2-AG hydrolysis inhibitor JZL184

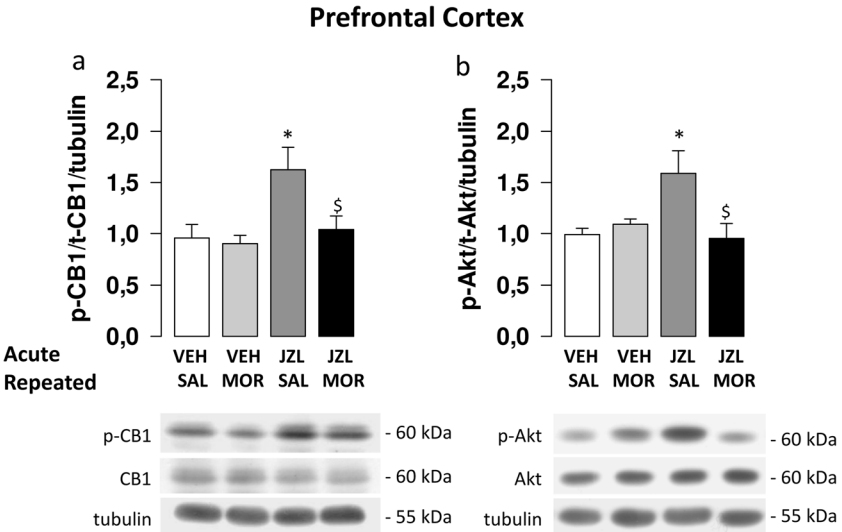

Fig. 7 CB1 cannabinoid receptor and p-Akt phosphorylation in the prefrontal cortex of rats repeatedly exposed to morphine and acutely treated with JZL184. Acute administration of JZL184 (JZL) increased the ratio between phosphorylated and total CB1 receptors in the PFC of saline- (VEH) but not morphine- (MOR) pretreated rats (**a**). Similarly, the ratio between phosphorylated and total Akt protein increased in the NAc of saline- but not morphine-pretreated rats (**b**) (n = VEH-SAL 5, n = VEH-MOR 5, n = JZL-SAL 5, n = JZL-MOR 5). Data represent mean values ± SEM; *$p < 0.05$ vs VEH-SAL group, $$p < 0.05$, vs JZL-SAL (Mann–Whitney U test)

occurred after repeated administration of morphine, accompanied by changes in the phosphorylation, and therefore the activation, of both CB1 receptors and their effector Akt in the NAc, amygdala and prefrontal cortex.

The endocannabinoid and endogenous opioid systems share similar characteristics. Thus, both opioid and cannabinoid receptors belong to the super-family of seven-transmembrane G protein-coupled receptors, they activate Gi/Go GTP-binding proteins, modulate similar intracellular systems, co-localize in several brain regions and their activation results in similar behavioral outcomes (Fattore et al. 2005, 2010; Gerrits et al. 2003; Maldonado et al. 2011; Manzanares et al. 1999; Parolaro et al. 2010; Pellissier et al. 2018; Volkow et al. 2017; Wenzel and Cheer 2018). A large body of evidence indicates that endocannabinoid and opioid neurotransmission interact to mediate reward processes, including social play reward (Fattore et al. 2005; Manzanares et al. 1999; Parolaro et al. 2007; Trezza et al. 2010; Wei et al. 2017; Wei et al. 2016). For example, it has been shown that the play-enhancing effects of drugs that magnify endocannabinoid activity by interfering with either anandamide or 2-AG degradation are blocked by pretreatment with either cannabinoid or opioid receptor antagonists (Manduca et al. 2016; Solinas and Goldberg 2005; Trezza and Vanderschuren 2008, 2009). This functional interaction is bidirectional, since the play-enhancing effects of opioid receptor agonists are antagonized not only by opioid but also by cannabinoid receptor antagonists (Trezza and Vanderschuren 2008, 2009). More recently, we have shown that endocannabinoid-opioid interaction in social play occurs via the NAc, since the play-enhancing effects induced by systemic administration of the 2-AG hydrolysis inhibitor JZL184 were counteracted by blockade of NAc μ-opioid receptors. Conversely, the increase in social play behavior induced by systemic administration of the opioid receptor agonist morphine was counteracted by intra-NAc administration of the cannabinoid receptor antagonist SR141716A (Manduca et al. 2016).

Studies in laboratory animals have also shown that repeated administration of cannabinoid or opioid drugs induces cross-tolerance to their acute behavioral and physiological effects (Maldonado 2002; Robledo et al. 2008). For example, tolerance to the analgesic and hypothermic effects of morphine developed in laboratory animals pre-exposed to cannabinoid agonists, and vice versa (Massi et al. 2001; Smith et al. 1994; Thorat and Bhargava 1994). However, cross-sensitization has also been reported. Thus, rats pre-exposed to cannabinoids showed a heightened locomotor response to opioids (Cadoni et al. 2001; Lamarque et al. 2001; Pontieri et al. 2001a) and vice versa (Pontieri et al. 2001b). Furthermore, morphine-dependent rats showed sensitization to the acute antinociceptive effect of cannabinoids (Rubino et al. 1997; Vigano et al. 2005a), and repeated cannabinoid exposure has been shown to increase opiate self-administration in rats (Biscaia et al. 2008; Ellgren et al. 2007; Norwood et al. 2003).

Here, we extend this scenario by showing that opioid–cannabinoid cross-tolerance exists in social play behavior. Indeed, the play-enhancing effects induced by systemic administration of the 2-AG hydrolysis inhibitor JZL184 were suppressed in animals repeatedly pretreated with morphine, indicating that cross-tolerance to the effects of JZL184 had occurred after repeated administration of morphine.

However, this cross-tolerance was unidirectional. That is, we found that acute administration of morphine before testing markedly increased social play in rats pretreated with both JZL184 and vehicle, indicating that tolerance to the play-enhancing effects of morphine had not occurred after repeated treatment with the indirect cannabinoid agonist. A possible explanation for this negative finding is that JZL184 is not a cannabinoid receptor agonist. Rather, it acts as indirect agonist by enhancing local 2-AG signaling through the inhibition of its hydrolysis (Long et al. 2009). Since endocannabinoids are released on demand (Alger and Kim 2011; Di Marzo 2006; Piomelli 2003), i.e., only when appropriate stimuli mobilize them, it is possible that a direct, impulse-independent activation of CB1 receptors is needed to induce cross-tolerance to the play-enhancing effects of morphine. In support of this possibility, it has been shown that the development of tolerance to the effects of endocannabinoids involves different mechanisms than those implicated in tolerance to direct cannabinoid agonists. For instance, anandamide-tolerant mice did not show cross-tolerance to the antinociceptive responses induced by mu-, delta-, or kappa-opioid agonists, while THC-tolerant mice exhibited cross-tolerance to opioid agonists (Welch 1997). However, it is still unknown whether the same holds true for 2-AG, or not. To address this issue, we performed an additional experiment in which we tested whether the play-enhancing effects of morphine were maintained, or not, when rats were repeatedly pretreated with the CB1 cannabinoid receptor agonist WIN55,212-2. We found that acute administration of morphine before testing markedly increased social play in rats pretreated with either WIN or its vehicle, indicating that regardless how the endocannabinoid system is repeatedly activated (i.e., indirectly by inhibiting the hydrolysis of 2-AG or directly by WIN binding to cannabinoid receptors), the opioid-cannabinoid cross-tolerance in the modulation of social play behavior remains unidirectional.

As for the mechanisms underlying the development of morphine-induced tolerance to the play-enhancing effects of JZL184, it has been reported that chronic exposure to opiates results in alterations of cannabinoid receptor density and/or signal transduction (Gonzalez et al. 2002; Romero et al. 1998; Rubino et al. 1997; Shapira et al. 1998). To test the possibility that the development of tolerance to the play-enhancing effects of JZL184 in animals pretreated with morphine is due to opioid-induced changes in cannabinoid receptor expression or activity, we measured the phosphorylation of the CB1 receptor and its effector Akt in the amygdala, NAc

and prefrontal cortex, key brain regions implicated in social play behavior (Manduca et al. 2016; Trezza et al. 2012; van Kerkhof et al. 2013; Vanderschuren et al. 2016). Since CB1 receptor activation by endogenous and/or exogenous cannabinoids leads to the induction of signaling cascades culminating in Akt activation, the evaluation of Akt phosphorylation is useful to assess CB1 receptor activation (Gomez del Pulgar et al. 2000). It is generally accepted that the phosphorylation of the carboxy terminus of the CB1 cannabinoid receptor induces the internalization of the agonist-activated full-length receptor (Daigle et al. 2008; Garcia et al. 1998; Martin et al. 2000; Nogueras-Ortiz and Yudowski 2016; Rubino et al. 2005). For this reason, increased CB1 receptor phosphorylation reflects the compensatory response to reduce CB1 receptor-mediated signaling after endocannabinoid system activation, and can be used as an indirect parameter to evaluate CB1 receptor activation, as already described (Servadio et al. 2016; Trezza et al. 2012). We found that acute administration of JZL184 increased the phosphorylation, and therefore the activation, of both CB1 receptors and their effector Akt in the NAc and prefrontal cortex. This is in line with previous studies showing that 2-AG signaling in the NAc (Manduca et al. 2016; Wei et al. 2016) and prefrontal cortex (Gould et al. 2012; Robinson et al. 2010) is involved in social (play) behavior. Interestingly, however, we found that the JZL-induced increase in CB1 and Akt phosphorylation in both the NAc and prefrontal cortex disappeared when JZL-treated rats were pretreated with morphine. Furthermore, we found that only animals repeatedly treated with morphine and acutely administered with JZL184 before testing showed a reduced phosphorylation of both CB1 receptors and Akt in the amygdala.

Several mechanisms have been hypothesized to explain the existence of opioid/cannabinoid cross-tolerance, ranging from modifications in receptor expression to alterations in receptor signaling. In our experimental conditions, it seems unlikely that pretreatment with morphine altered cannabinoid receptor expression, since the total amount of CB1 receptor did not differ among the experimental groups. On the other hand, the endogenous opioid system may interfere with endocannabinoid neurotransmission by affecting its signaling pathway. For instance, chronic morphine exposure may impact CB1 activity by decreasing the level of G protein activation (Gonzalez et al. 2002; Welch 1997). Additionally, long-term exposure to opioid receptor agonists induces CB1 desensitization through inhibition of cAMP production, since both opioid and endocannabinoid signaling pathways converge at the level of adenylyl cyclase (Vigano et al. 2005a, b). Another explanation is that the functional interaction between opioid and cannabinoid drugs is due to alterations of their endogenous tone. To support this possibility, it has been shown that, in addition to alterations in CB1 receptor function in the NAc, chronic morphine exposure strongly lowered 2-AG content in several brain regions, including the NAc and amygdala, without significant changes in anandamide levels (Vigano et al. 2003). On this basis, it is still possible that the play-enhancing effects of JZL184 disappear as a consequence of reduced 2-AG levels induced by repeated treatment with morphine.

In conclusion, we provide new evidence that the endogenous opioid and the endocannabinoid systems interact in the modulation of social play. In particular, unidirectional heterologous cross-tolerance exists to the effects of the 2-AG hydrolysis inhibitor JZL184 on social play in animals repeatedly pretreated with the opioid agonist morphine. Our results indicate that the mechanism involved in such interactions could take place at the cannabinoid receptor level, although the changes in the downstream events associated or a common release of several neurotransmitters cannot be excluded. A better understanding of opioid–cannabinoid interactions in social play can contribute to clarify neurobiological aspects of social behavior at young age and may provide new therapeutic targets for social dysfunctions.

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Compliance with ethical standards

Conflict of interest The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 5 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CHAPTER 4

Detrimental effects of the abused 'bath salt' Methylenedioxypyrovalerone (MDPV)

on social play behavior in adolescent rats

Running title: MDPV and social behavior

Sara Schiavi[1], Antonia Manduca[1], Francesca Melancia[1], Emilia Carbone[1], Patricia Jiménez Peinado[1], Antonello Mai[2,3], Patrizia Campolongo[4], Louk L.J.M. Vanderschuren[5], Viviana Trezza[1]

[1]Dept. of Science, University "Roma Tre", Rome, Italy;

[2]Dept. of Drug Chemistry & Technologies, Sapienza University of Rome, Rome, Italy; [3]Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy

[4]Dept. of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy

[5]Dept. of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;

Ready for submission.

Detrimental effects of the abused 'bath salt' Methylenedioxypyrovalerone (MDPV) on social play behavior in adolescent rats.

In the present paper I studied the involvement of dopaminergic and noradrenergic neurotransmission in social behavior by analyzing their role in the effects of Methylenedioxypyrovalerone (MDPV) in social play behavior in adolescent rats. Indeed, MDPV is a psychoactive designer drug belonging to synthetic cathinones able to act as a selective uptake blocker at catecholamine transporters, such as dopamine and noradrenaline transporters (DAT and NET, respectively). This study has a twofold implication: 1. it revealed for the first time the effects of MDPV social behavior, which is important given the widespread use of synthetic cathinones among adolescents; 2. It clarified the involvement of the dopaminergic and noradrenergic systems in social play behavior.

Detrimental effects of the abused 'bath salt' Methylenedioxypyrovalerone (MDPV) on social play behavior in adolescent rats

Running title: MDPV and social behavior

Sara Schiavi[1], Antonia Manduca[1], Francesca Melancia[1], Emilia Carbone[1], Valeria Buzzelli[1], Patricia Jiménez Peinado[1], Antonello Mai[2,3], Patrizia Campolongo[4], Louk L.J.M. Vanderschuren[5], Viviana Trezza[1]

[1]Dept. of Science, University "Roma Tre", Rome, Italy;
[2]Dept. of Drug Chemistry & Technologies, Sapienza University of Rome, Rome, Italy; [3]Istituto Pasteur- Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy
[4]Dept. of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy
[5]Dept. of Animals in Science and Society, Division of Behavioural Neuroscience, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;

Corresponding author:
Viviana Trezza, PhD
Department of Science, Section of Biomedical Sciences and Technologies
University "Roma Tre"
Viale G. Marconi 446
00146 Rome, Italy
tel: +39-0657336343
fax: +39-0657336365
email: viviana.trezza@uniroma3.it

Abstract

Methylenedioxypyrovalerone (MDPV) is the most popular synthetic cathinone found in products marketed as 'bath salts', available online and widespread among teenagers and young adults. Synthetic cathinones have pharmacological effects resembling those of psychostimulants, which are known to disrupt a variety of social behaviors. However, despite the popular use of MDPV by young people in social contexts, scarce information is available about its effects on social behavior. Social play behavior is the most characteristic social behavior displayed by young mammals and it is crucial for neurobehavioral development. Here, we investigated the effects of MDPV on social play behavior in adolescent rats, and the neurobehavioral mechanisms underlying its effects. MDPV suppressed both the initiation to play and the responsivity to play initiation, and its play-suppressant effect was subjected to tolerance but not sensitization. As the behavioral effects of MDPV have been ascribed to dopaminergic and noradrenergic neurotransmission, and given the role of these neurotransmitters in social play, we investigated whether the play-suppressant effects of MDPV depend on activation of dopaminergic and/or noradrenergic receptors. The effects of MDPV on social play were blocked by either the α-2 adrenoceptor antagonist RX821002 or the dopamine receptor antagonist flupenthixol, given alone or together at sub-effective doses. Thus, MDPV selectively suppresses the most vigorous social behavior of adolescent rats through both noradrenergic and dopaminergic mechanisms. This study provides the first preclinical evidence of the deleterious effects of MDPV on social behavior, and increases our understanding of the neural correlates of the behavioural effects induced by this popular cathinone.

Introduction

Methylenedioxypyrovalerone (MDPV) is a designer drug belonging to synthetic cathinones, a group of psychoactive substances emerged in the mid-2000s as legal alternatives to psycostimulants such as amphetamine, ecstasy, MDMA or cocaine (Karila *et al*, 2018; Valente *et al*, 2014). Currently, MDPV is the most popular cathinone found in products marketed as 'bath salts', which are easily available online and widespread consumed among teenagers and young adults, particularly in social contexts, such as festivals and rave parties (Ashrafioun *et al*, 2016; German *et al*, 2014; Karila *et al*, 2018; Karila *et al*, 2015; Valente *et al*, 2014; Zawilska and Wojcieszak, 2013). Due to their high abuse potential, their constantly growing market and the lack of any accepted medicinal use, starting from 2011 many of the most common cathinones, including MDPV, have been classified as Schedule I controlled substances in the United States (Drug Enforcement Administration, 2011) and are monitored by the European Monitoring Center for Drugs and Drugs Addiction (EMCDDA) (EMCDDA, 2018).

Despite the use of synthetic cathinones is a growing public health concern, their mechanism of action and their impact on brain and behavior is still a matter of debate. Users report MDPV-related positive psychoactive effects resembling those of cocaine, such as euphoria, talkativeness, sexual arousal, alertness, motor excitation, increased concentration, and also increased sociability, productivity, motivation and libido (Karila *et al*, 2017, EMCDDA, 2018). However, several other psychiatric adverse effects such as hallucinations, psychosis, delirium, depressed mood, anxiety, agitation, disorganized thoughts and cognitive alteration have also been reported following acute intoxication (Froberg *et al*, 2015; Karila *et al*, 2018; Weaver *et al*, 2015).

Preclinical findings in rodents have shown that MDPV induces dose-dependent stereotyped behaviors and abuse potential, together with alterations in thermoregulation (Aarde *et al*, 2013; Atehortua-Martinez *et al*, 2019; Baumann *et al*, 2013; Glennon and Young, 2016). Furthermore, it has been recently reported that synthetic cathinones are β-keto analogs of amphetamine with pharmacological effects resembling those of cocaine and amphetamines (Angoa-Perez *et al*, 2017).

Psychostimulants are known to disrupt a variety of social behaviors (Achterberg *et al*, 2014; Miczek *et al*, 1989; Moro *et al*, 1997; Schiorring, 1979; Simmler *et al*, 2013). Surprisingly, however, despite the popular use of synthetic cathinones by young people in social contexts, scarce information is currently available about their effects on social behavior.

One of the most characteristic social behaviors displayed by the young of all mammalian species, including humans, is social play behavior. This form of social behavior is highly conserved throughout evolution and it is essential to develop behavioral and mental flexibility and to acquire cognitive and social competence (Vallersnes *et al*, 2016). Accordingly, abnormalities in social play are observed in childhood psychiatric disorders such as autism, early-onset schizophrenia and attention deficit/hyperactivity disorder (Alessandri, 1992; Helgeland and Torgersen, 2005; Jones *et al*, 1994; Jordan, 2003; Moller and Husby, 2000). Therefore, because of the scarce information about the effects of MDPV on social behavior and the importance of social play for proper behavioral development, we investigated the effects of MDPV on social play in adolescent rats, and the neurobehavioral mechanisms underlying its effects.

Experimental procedures

Experimental design

Six different experiments were performed. To assess the effects of MDPV on social play behavior, in *experiment 1* animals were treated with two doses of MDPV (0.1 or 0.5 mg/kg) or saline (control group) given intraperitoneally (i.p.) 30 min before testing. To investigate whether the effects of MDPV on social play depended on the behavior of the test partner, in *experiment 2* none, one, or both members of a test pair were treated with MDPV (0.5 mg/kg i.p.), and behavior of both test partners was scored separately. In *experiment 3*, we investigated whether tolerance or sensitization would occur to the effect of MDPV on social play behavior after repeated treatment. To this aim, animals were pretreated with either MDPV (0.5 mg/kg, i.p.) or saline for 5 consecutive days (postnatal days 25-29). On day 30 (i.e. one day after the last pretreatment injection), animals were isolated for 3.5 h. Next, half of both pretreatment groups (MDPV or saline) was treated 30 min before testing with either saline or MDPV, given at the effective dose of 0.5 mg/kg (i.p.) to assess the presence of tolerance, or at the sub-effective dose of 0.1 mg/kg (i.p.) to assess the occurrence of sensitization.

The behavioral effects of MDPV have been mainly ascribed to dopaminergic and noradrenergic neurotransmission, as it acts in the brain as a potent uptake inhibitor at plasma membrane transporters for dopamine (DAT) and noradrenaline (NET) (Baumann et al., 2013). Furthermore, both dopaminergic and noradrenergic neurotransmission are involved in social play (Trezza *et al*, 2010). Thus, to clarify whether MDPV exerts its effects on social play through dopaminergic (*experiment 4*) or noradrenergic (*experiment 5*) neurotransmission, animals were treated with either the α-2 adrenoceptor antagonist RX821002 (0.2 mg/kg, i.p.) or the dopamine receptor antagonist flupenthixol (0.125 mg/kg, i.p.) 15 min before MDPV (0.5 mg/kg i.p.). Last, in *experiment 6* we investigated whether dopaminergic and noradrenergic neurotransmission are simultaneously involved in the effect of MDPV on social play, by treating the animals with either sub-effective

doses of both RX821002 (0.1 mg/kg i.p.) and flupenthixol (0.06 mg/kg i.p.), or with saline (control group) 15 min before treatment with MDPV (0.5 mg/kg i.p.).

Animals

Male Wistar rats (Charles River Laboratories, Italy) arrived in the animal facility at 21 days of age and were housed in groups of five in Macrolon cages (43 × 26 × 20 cm) under controlled conditions (temperature 21±1 °C, 60±10% relative humidity and 12/12 h light cycle with lights on at 07:00 a.m). Food and water were available *ad libitum*. Animals were experimentally naive and were used only once. Sample size (n) is indicated in the figure legends. The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) (Kilkinney et al., 2010) guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

Drugs

3,4-Methylenedioxypyrovalerone (MDPV) (0.1-0.5 mg/kg,), flupenthixol-dihydrochloride (0.125-0.06 mg/kg) and RX821002-hydrochloride (0.2-0.1 mg/kg), were dissolved in saline and given intraperitoneally (i.p.). Solutions were freshly prepared on the day of the experiment and were administered in a volume of 2 ml/kg. MDPV was administered 30 min before testing while both RX821002 and flupenthixol were administered 15 min prior to MDPV administration. Saline solution was used as control treatment for all the drugs used.

Social Play Behavior

All the experiments were performed in a sound-attenuated chamber under dim light conditions. The testing arena consisted of a Plexiglas cage (40×40×60 cm) with approximately 2 cm of wood shavings covering the floor. Social play behavior was assessed as previously described (Manduca et

al., 2016). Rats were individually habituated to the test cage for 10 min on 2 days prior to testing. On the test day, the animals were socially isolated for 3.5 h before testing. This isolation period has been shown to induce a half-maximal increase in the amount of social play behavior (Niesink and van Ree, 1982). At the appropriate time before testing, pairs of animals were treated with drugs or vehicle. In all experiments except for *experiment 2*, both animals of a pair received the same drug treatment. The test consisted of placing two animals into the test cage for 15 min. The animals of each pair did not differ more than 10 g in body weight and had no previous common social experience. Behavior was assessed per pair of animals in *experiment 1, 3* and *4*, while in *experiment 2* behavior was assessed per single animal. The Observer 3.0 software (Noldus Information Technology BV, Wageningen, The Netherlands) was used to score behaviors related to play. In rats, a bout of social play behavior starts with one rat soliciting ('pouncing') another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways. If the animal that is pounced upon fully rotates to its dorsal surface, 'pinning' is the result, i.e., one animal lying with its dorsal surface on the floor with the other animal standing over it. From this position, the supine animal can initiate another play bout, by trying to gain access to the other animal′s neck. Thus, during social play, pouncing is considered an index of play solicitation, while pinning can be regarded as the terminal component of a single play bout as well as a releaser of a prolonged play bout (Pellis and Pellis, 1987). Pinning and pouncing frequencies can be easily quantified and they are considered to be the most characteristic parameters of social play behavior in rats (Trezza *et al*, 2010). During the social encounter, animals may also display social behaviors not directly associated with play, such as sniffing or grooming the partner′s body. A pair of rats was considered as one experimental unit and the behavioral parameters were therefore scored per pair of animals, except for *experiment 2*, in which the behavioral parameters were scored per each individual animal of a test pair.

The following parameters were scored:

Social behaviors directly related to play:

- Frequency of pinning.
- Frequency of pouncing.
- Play responsiveness: the percentage of response to play solicitation, calculated as the probability of an animal of being pinned in response to play solicitation (pouncing) by the stimulus partner.

Social behaviors unrelated to play:

- Time spent in social exploration: the total amount of time (s) spent in non-playful forms of social interaction (i.e., one animal sniffing or grooming any part of the partner′s body).

Locomotor activity during the social encounter:

- Crossing: a grid, dividing the arena into equally sized squares, was projected over the recordings, and the number of line crossings made by the animal was recorded.

Statistical analysis

Data are expressed as mean ± SEM and statistical significance was set at $p < 0.05$. To assess the effects of single or combined treatments on social play behavior, data were analyzed using one-way or two-way analysis of variance, respectively, followed by Student-Newman-Keuls *post hoc* tests where appropriate.

Results

Experiment 1: MDPV suppresses social play behavior.

Systemic administration of MDPV (0.5 mg/Kg) suppressed social play behavior. A one-way ANOVA analysis performed on the parameters measured during testing gave the following results: pinning: $F_{2,22}$ =7.72, p=0.003; pouncing: $F_{2,22}$ =5.93, p=0.010; play responsiveness: $F_{2,22}$ =11.72, p<0.001. Post-hoc analyses showed that MDPV, administered at the dose of 0.5 mg/kg, suppressed pinning (p<0.05, Figure 1a) and pouncing (p<0.05, Figure 1b) and reduced play responsiveness (p<0.01 Figure 1c), whereas the dose of 0.1 mg/kg had no effect. General social exploration (i.e., sniffing and grooming any part of the body of the test partner, including the anogenital area) was not affected by MDPV treatment ($F_{2,22}$ =1.34, p=n.s., Figure 1d). Interestingly, the effects of MDPV on social play were not secondary to changes in locomotor activity: indeed, at the dose that decreased social play behavior (0.5 mg/kg), MDPV did not affect the number of crossings ($F_{2,22}$ =0.04, p=n.s Figure 1d).

Experiment 2: MDPV reduces social play regardless of the treatment received by the test partner.

To investigate whether the effects of MDPV on social play depend on the behavior exhibited by the test partner, we performed an experiment in which none, one, or both members of a test pair were treated with MDPV (0.5 mg/kg), and behavior of both test partners was scored separately. A two-way ANOVA analysis gave the following results (pinning: $F_{(subject)1,54}$ =9.06, p=0.004; $F_{(partner)1,54}$ =4.14, p=0.05; $F_{(subject\ x\ partner)1,54}$ =5.19, p=0.03; pouncing: $F_{(subject)1,54}$ =2.28, p=0.03; $F_{(partner)1,54}$ =4.02, p=0.05; $F_{(subject\ x\ partner)1,54}$ =1.72, p=0.19; % of play response: $F_{(subject)1,54}$ =5.00, p=0.03; $F_{(partner)1,54}$ =4.23, p=0.04; $F_{(subject\ x\ partner)1,54}$ =0.10, p=n.s). Consistent with the previous experiments, post-hoc analyses revealed that pinning (p<0.05), pouncing (p<0.05) and the percentage of response

to play solicitation ($p<0.05$) were reduced when both members of a test pair were treated with MDPV (Figure 2 a,b,c). When only the scored subject of a test pair was treated with MDPV, there was also a decrease of pinning ($p<0.05$) and pouncing ($p<0.05$) events, indicating that the MDPV-treated rats did not pin and pounce their partner, regardless the partner was treated with saline or MDPV (Figure 3a,b, c). Social exploration was not affected by MDPV ($F_{(subject)1,54}$ =0.00, p=n.s; $F_{(partner)1,54}$=0.83, p=n.s; $F_{(subject\ x\ partner)1,54}$ =0.16, p=n.s, *data not shown*).

Experiment 3: The effects of MDPV on social play are subjected to tolerance but not sensitization.

To evaluate whether tolerance occurs to the effects of MDPV on social play behavior after repeated treatment, animals were treated with the dose of MDPV that reduced social play in the previous experiments (0.5 mg/kg, i.p.) or saline (control group) once daily for 5 consecutive days. On the sixth day, half of both pretreatment groups were tested after acute treatment with MDPV (0.5 mg/kg, i.p.), or saline (control group). A two-way ANOVA analysis performed on the parameters measured during testing test gave the following results: pinning: $F_{(repeated)1,26}$ =0.45, p=n.s; $F_{(acute)1,26}$ =9.20, p=0.005; $F_{(repeated\ x\ acute)1,26}$ =1.32, p=n.s; pouncing: $F_{(repeated)1,26}$ =0.01, p=n.s; $F_{(acute)1,26}$ =9.90, p=0.004; $F_{(repeated\ x\ acute)1,26}$ =2.17, p=n.s). Post-hoc analyses revealed that acute administration of MDPV (0.5 mg/kg, i.p.) decreased pinning ($p<0.01$, Figure 3a) and pouncing ($p<0.01$, Figure 3b) in animals repeatedly treated with saline but not in animals repeatedly pretreated with MDPV (0.5 mg/kg, i.p.), indicating that tolerance to the effect of MDPV on social play occurs. Social exploration was not affected by either the repeated or acute treatment ($F_{(repeated)1,26}$ =0.80, p=n.s; $F_{(acute)1,26}$ =0.91, p=n.s; $F_{(repeated\ x\ acute)1,26}$ =0.072, p=n.s, Figure 3c).

Next, to evaluate whether sensitization occurs to the effects of MDPV on social play behavior after repeated treatment, animals were treated with MDPV (0.5 mg/kg, i.p.) or saline once daily for 5 consecutive days. On the sixth day, half of both pretreatment groups were treated with a dose of MDPV that does not affect social play by itself (0.1 mg/kg, i.p.), or saline. Acute administration of

MDPV (0.1 mg/kg, i.p.) did not affect social play in rats repeatedly treated with either saline or MDPV (0.5 mg/kg, i.p), indicating that sensitization to the effect of MDPV on social play behavior had not occurred after repeated treatment (pinning: $F_{(repeated)1,26}$ =0.14, p=n.s; $F_{(acute)1,26}$ =1.51, p=n.s; $F_{(repeated\ x\ acute)1,26}$ =0.68, p=n.s, Figure 4a; pouncing: $F_{(repeated)1,26}$ =0.01, p=n.s; $F_{(acute)1,26}$ =1.16, p=n.s; $F_{(repeated\ x\ acute)1,26}$ =1.47, p=n.s, Figure 4b). Social exploration was not affected by either the repeated or acute treatment ($F_{(repeated)1,26}$ =0.77, p=n.s; $F_{(acute)1,26}$ =1.55, p=n.s; $F_{(repeated\ x\ acute)1,26}$ =1.05, p=n.s, Figure 4c).

Experiment 4: MDPV reduces social play through dopaminergic receptors.

It has recently been shown that certain behavioral effects induced by MDPV are mediated by activation of dopaminergic receptors (Atehortua-Martinez *et al*., 2019; Bernstein *et al*., 2019; Cameron *et al*., 2013). To investigate whether the effects of MDPV on social play also depend on dopaminergic neurotransmission, we administered the dopamine receptor antagonist cis-(Z)-flupenthixol, at a dose (0.125 mg/kg i.p.) that does not affect social play by itself (Vanderschuren *et al*., 2018), before administration of MDPV (0.5 mg/kg i.p.). Systemic administration of flupenthixol blocked the effects of MDPV on social play (pinning: $F_{(pretreat)1,26}$ =0.025, p=n.s; $F_{(treat)1,26}$ =4.57, p=0.042; $F_{(pretreat\ x\ treat)1,26}$ =0.95, p=n.s, Figure 5a; pouncing: $F_{(pretreat)1,26}$ =0.33, p=n.s; $F_{(treat)1,26}$ =5.11, p=0.032; $F_{(pretreat\ x\ treat)1,26}$ =0.62 p=n.s, Figure 5b). Indeed, post-hoc analysis revealed that MDPV reduced both pinning ($p<0.05$) and pouncing ($p<0.05$) in animals pretreated with saline but not in animals pretreated with flupenthixol (Figure 6a, b). No differences were found in the time spent in social exploration ($F_{(pretreat)1,26}$ =1.03, p=n.s; $F_{(treat)1,26}$ =1.01, p=n.s; $F_{(pretreat\ x\ treat)1,26}$ =1.15, p=n.s, Figure 6 c).

Experiment 5: MDPV reduces social play through alpha-2 noradrenergic receptors.

We have previously shown that psychostimulants like amphetamine and methylphenidate exert their

play-suppressant effects through alpha-2 noradrenergic receptors (Vanderschuren *et al*., 2008; Achterberg *et al*., 2014; Achterberg *et al*., 2015). To investigate whether these receptors are also involved in the reduction of social play induced by MDPV, animals were treated with the α-2 adrenoceptor antagonist RX821002 (0.2 mg/kg, i.p.) before administration of MDPV (0.5 mg/kg, i.p.) (*experiment 5*). Systemic administration of the α-2 adrenoceptor antagonist RX821002 (0.2 mg/kg, i.p.) 15 min before MDPV administration counteracted the effects of MDPV on social play behavior (pinning: $F_{(pretreat)1,20}$ =7.35, p=0.013; $F_{(treat)1,20}$ =4.25, p=n.s; $F_{(pretreat\ x\ treat)1,20}$ =4.98, p=0.04,; pouncing: $F_{(pretreat)1,20}$ =13.2, p=0.002; $F_{(treat)1,20}$ =6.71, p=0.02; $F_{(pretreat\ x\ treat)1,20}$ =4.68, p=0.04). Post-hoc analysis revealed that MDPV reduced both pinning (p<0.01) and pouncing (p<0.01) in animals pretreated with saline but not in animals pretreated with RX821002 (Figure 5d, e). No differences were found in social exploration ($F_{(pretreat)1,20}$ =0.21, p=n.s; $F_{(treat)1,20}$ =0.56, p=n.s; $F_{(pretreat\ x\ treat)1,20}$ =0.16, p=n.s, Figure e f).

Experiment 6: MDPV reduces social play through simultaneous activation of dopaminergic and alpha-2 noradrenergic receptors

The combined administration of sub-effective doses of the alpha-2 noradrenergic receptor antagonist RX821002 (0.1 mg/kg i.p.) and the dopamine receptor antagonist flupenthixol (0.06 mg/kg i.p.) 15 minute before MDPV administration antagonized the play-suppressant effects of MDPV (pinning: $F_{(pretreat)1,26}$ =1.22, p=n.s; $F_{(treat)1,26}$ =0.93, p=n.s; $F_{(pretreat\ x\ treat)1,26}$ =4.61, p=0.04, Figure 5g; pouncing: $F_{(pretreat)1,26}$ =1.27, p=n.s; $F_{(treat)1,26}$ =0.64, p=n.s; $F_{(pretreat\ x\ treat)1,26}$ =14.2, p<0.001, Figure 5h). Post-hoc analyses revealed that MDPV reduced both pinning (p<0.05) and pouncing (p<0.01) in animals pretreated with saline but not in animals pretreated with sub-effective doses of both RX821002 and flupenthixol (Figure 6 g, h). No differences were found in social exploration ($F_{(pretreat)1,26}$ =0.63, p=n.s; $F_{(treat)1,26}$ =0.95, p=n.s; $F_{(pretreat\ x\ treat)1,26}$ =0.016, p=n.s, Figure 5 i).

Discussion

Synthetic cathinones are widely abused by young people, often in a social setting (Karila *et al*, 2018). However, scarce information is available about their effect on social behavior, and the mechanisms by which they affect brain and behavior are still poorly understood. Here, we provide the first evidence that the most popular abused cathinone, MDPV, suppresses social play, that is the most characteristic and rewarding form of social interaction displayed by young mammals (Panksepp *et al*, 1984; Pellis and Pellis, 2009; Trezza *et al*, 2010). This effect was behaviorally specific: MDPV did not alter social exploratory behavior or locomotor activity during social interaction, demonstrating that changes in general sociability or locomotion did not underlie the effects of MDPV on social play.

Social play behavior is influenced by the level of social activity of the partner (Pellis and McKenna, 1995; Pellis and McKenna, 1992; Trezza and Vanderschuren, 2008; Varlinskaya *et al*, 1999). Therefore, to investigate whether the effects of MDPV on social play depend on the behavior of the test partner, we performed an experiment in which none, one, or both members of a test pair were treated with MDPV, and the behavior of both test partners was scored separately. We found that MDPV reduced social play behavior regardless of the treatment received by the social partner. That is, pinning and pouncing were decreased in all MDPV- treated rats, regardless they were interacting with vehicle- or MDPV-treated partners.

In humans, the development of craving, tolerance, dependence and withdrawal has been reported after the frequent use of high doses of MDPV (Andrabi *et al*, 2015). Here, we show that the effects of MDPV on social play behavior are subjected to tolerance but not sensitization. Indeed, the reduction in social play induced by systemic administration of MDPV was suppressed following repeated treatment, indicating that tolerance to the effects of MDPV in social play had occurred. Conversely, social play was unaffected when animals were pretreated with MDPV for 5 consecutive days and treated with a sub-effective dose of MDPV on the test day, showing that sensitization to the effects of MDPV on social play behavior had not occurred.

In the brain, MDPV acts as a potent uptake inhibitor at plasma membrane transporters for both dopamine (DAT) and noradrenaline (NET), but it is not considered a substrate releaser (Baumann *et al*, 2017; Simmler *et al*, 2013). In particular, in vitro studies reported that MDPV is 50-fold more potent at DAT and 10-fold more potent at NET compared to cocaine (Baumann *et al*, 2013; Karila *et al*, 2018). Thus, it is not surprising that MDPV has abuse potential, as demonstrated at the preclinical level with both drug self-administration and intracranial self-stimulation (ICSS) experiments (for an extensive review see (Glennon *et al*, 2016)). Furthermore, similarly to other psychostimulants, MDPV induces different types of stereotyped movements (Marusich *et al*, 2012) and hyperlocomotion (Aarde *et al*, 2013; Atehortua-Martinez *et al*, 2019), the latter reduced by pretreatment with the dopamine D1 receptor antagonist SCH23390 (Marusich *et al*, 2014). More recently, it has been shown that MDPV has anxiolytic-like effects in rats tested in the elevated plus maze test , and it reinforces memory consolidation without altering spatial orientation based on the two-trial recognition task test (Atehortua-Martinez *et al*, 2019). These effects have been related to activation of dopaminergic neurotransmission by MDPV (Atehortua-Martinez *et al*, 2019).

A recent study compared the electroencephalogram (EEG) response to MDPV versus the hallucinogenic drugs MK-801 and ketamine in rats. The effects of MDPV on EEG synchronization were blocked by the D1 receptor antagonist SCH23990 and D2 receptor antagonist sulpiride, suggesting that the hallucinogen effect of the drugs was ascribed to dopaminergic neurotransmission (Shokry *et al*, 2019).

Similarly to cocaine, MDPV was able to evoke 50-kHz USVs in rats (Simmons *et al*., 2018), which have been proposed as an index of positive affect; interestingly, cocaine modulation of 50-kHz USVs has been associated with activation of adrenergic α1- and β-receptor (Burgdorf *et al*, 2001; Simmons *et al*, 2018; Wright *et al*, 2012).

Both dopaminergic and noradrenergic neurotransmission are involved in social play behavior, although in a different manner. Dopamine plays a role in the motivational properties of social play (Manduca *et al*, 2016), whereas noradrenaline seems to modulate more cognitive components of

social play (Achterberg *et al*, 2015; Vanderschuren *et al*, 2008). Furthermore, we have previously shown that noradrenergic but not dopaminergic neurotransmission underlie the play-suppressant effects of psychostimulants like amphetamine and methylphenidate (Achterberg *et al*, 2015; Trezza *et al*, 2009; Vanderschuren *et al*, 2008). Indeed, both amphethamine and methylphenidate exert their play-suppressant effects through alpha-2 noradrenergic receptors but not through dopamine receptors (Achterberg *et al*, 2014; Achterberg *et al*, 2015; Vanderschuren *et al*, 2008). Based on these findings, we investigated whether MDPV exerts its effects on social play through dopaminergic or noradrenergic neurotransmission, by treating rats with the α-2 adrenoceptor antagonist RX821002 or the dopamine receptor antagonist flupenthixol prior to MDPV. We found that both the α-2 adrenoceptor antagonists RX821002 and the dopamine receptor antagonist flupenthixol, given alone, counteracted the effects of MDPV on social play. Furthermore, combined administration of sub-effective doses of both RX821002 and flupenthixol also antagonized the play-suppressant effects of MDPV, indicating that the effects of MDPV on social play are mediated by activation of both α-2 adrenoceptors and dopamine receptors. α-2 adrenoceptors usually act as presynaptic autoreceptors (Starke *et al*, 1989), thus we would expect that blocking these receptors would enhance, rather than inhibit, the effects of MDPV. However, α-2 adrenoceptors are also located postsynaptically (Arnsten, 2006; Arnsten and Dudley, 2005; Aron *et al*, 2004), and it has been showed that these postsynaptic receptors mediate the play-suppressant effects induced by methylphenidate (Vanderschuren *et al*, 2008) and amphetamine (Achterberg *et al*, 2014; Achterberg *et al*, 2015). On the basis of these results, similarities and differences exist between the effects induced by MDPV and other psychostimulants on social play. Thus, compared to amphetamine and methylphenidate that exert their play-suppressant effects through alpha-2 noradrenergic receptors but not through dopaminergic neurotransmission (Achterberg *et al*, 2014; Achterberg *et al*, 2015; Vanderschuren *et al*, 2008), and to cocaine that reduces social play by simultaneous increases in dopamine, noradrenaline and serotonin neurotransmission (Achterberg *et al*, 2014), MDPV suppresses social play acting through both noradrenergic and dopaminergic neurotransmission. Two

possible explanations can account for the present findings. Since social play is a highly rewarding social activity (Trezza *et al*, 2010; Vanderschuren *et al*, 2016), and given the role of limbic dopamine in the rewarding aspects of social play (Manduca et al., 2016), it is possible that MDPV reduces social play by interfering with its pleasurable properties through dopaminergic neurotransmission. The second explanation is related to the impact of MDPV on noradrenergic neurotransmission. It is known that noradrenergic neurotransmission is involved in cognitive processes, such as learning, attention and flexibility (Berridge, 2007). Social play requires a complex interplay between cognitive and emotional processes, in which noradrenaline plays an important role (Vanderschuren *et al*, 2016). Thus, MDPV may reduce social play by altering the ability of the animals to interpret and adequately reciprocate social stimuli and to adjust their social behaviour to the changing circumstances in the social and physical environment. However, since the combined administration of sub-effective doses of both RX821002 and flupenthixol also antagonized the play-suppressant effects of MDPV, our data suggest that both the cognitive ability needed to process the social stimuli, and the pleasurable aspects of social play are simultaneously disrupted by MDPV.

Collectively, our data are the first preclinical evidence of the deleterious effects of MDPV on social behavior and shed light increases on the neural correlates of the behavioural effects induced by this popular cathinone. Since social play behavior is critical for proper neurobehavioral development, constitutive suppression of this behavior may lead to enduring behavioral deficits (Vanderschuren *et al*, 2016). Since MDPV can induce a wide range of behavioural alterations in humans (Karila *et al*, 2017, EMCDDA, 2018), more research into the persistent behavioral effects of chronic exposure to this synthetic cathinone is warranted.

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Figure legends

Figure 1. Acute administration of MDPV at the dose of 0.5 mg/kg suppressed social play and do not alter the locomotor activity of adolescent rats. MDPV (0.5 mg/kg) suppressed the number of pinning (a) and pouncing (b) and reduced the percentage of play response (c) in adolescent rats. Social exploration was not affected by either treatment (d) (n=SAL 8, n= MD0.1 8, n=MD0.5 7). Both MD0.1- and MD0.5-treated animals do not shown alterations in crossing frequency compared to SAL-treated animals (e) (n=SAL 8, n= MD0.1 8, n=MD0.5 7). Data represent mean values ± SEM; *p<0.05, **p<0.01 vs SAL group (Student–Newman–Keuls post hoc test).

Figure 2. MDPV reduced social play behavior in rats regardless of whether both animals in a test pair were treated with MDPV or SAL and MDPV. In the figure 'Subject' represents the treatment of the animal whose behavior was scored; 'partner' represents the treatment of its test partner. When either one or both animals in a pair were treated with MDPV, pinning and pouncing behavior was decreased (a, b). Whereas the percentage of play response was decreased only in MDPV-treated animals interacting with MDPV-treated partner (c) (n=SAL-SAL 16 , n=SAL-MD 12, n=MD-SAL 12 n=MD-MD 18). Data represent mean values ± SEM; *p<0.05, vs SAL-SAL group (Student–Newman–Keuls post hoc test).

Figure 3. MDPV caused tolerance to its effects in rats social play behavior. Acute administration of MDPV (0.5 mg/kg, i.p.) decreased the number of pinning (a) and pouncing (b) in animals pretreated with saline (SAL-MD0.5 group) but not with MDPV (MD0.5-MD0.5 group) indicating that tolerance to the effect of MDPV had occurred. Social exploration was not affected by either the repeated and acute treatment (c) (n=SAL-SAL 7, n=SAL-MD0.5 8, n=MD0.5-SAL 7 n=MD0.5-MD0.5 8). Data represent mean values ± SEM; **p<0.01, vs SAL-SAL group (Student–Newman–Keuls post hoc test).

Figure 4. MDPV does not cause sensitization to its effects on social play behavior. Acute administration of MDPV (0.1 mg/kg, i.p.) did not decrease pinning (a) and pouncing (b) in saline pretreated and MDPV (0.5 mg/kg, i.p.) pretreated rats revealing that sensitization to the effect of MDPV on social play behavior had not occurred. Social exploration was not affected by either the repeated and acute treatment (c) (n=SAL-SAL 7, n=SAL-MD0.5 8, n=MD0.5-SAL 7 n=MD0.5-MD0.5 8). Data represent mean values ± SEM.

Figure 5. Acute administration of RX821002 and Flupenthixol reverted the effects of MDPV on social play. The systemic administration of the dopamine receptor antagonist flupenthixol (0.125 mg/kg i.p.) 15 min. before MDPV administration blocked the MDPV effects on pinning (a) and pouncing (b) (n=SAL-SAL 9, n=SAL-MD 8, n=FLU-SAL 7, n=FLU-MD 6). Similarly, the systemic administration of the α-2 adrenoceptor antagonist RX821002 (0.2 mg/kg i.p.) 15 min. before MDPV administration was able to block the MDPV effects on pinning (d) and pouncing (e) (n=SAL-SAL 6, n=SAL-MD 7, n=RX-SAL 5, n=RX-MD 6). Moreover, the combined administration of RX821002 (0.1 mg/kg i.p.) and flupenthixol (0.06 mg/kg i.p.) at lower doses 15 minute before MDPV administration was able to block the MDPV effects on pinning (g) and pouncing (h) (n=SAL-SAL 6, n=SAL-MD 8, n=FLU/RX-SAL 8, n=FLU/RX-MD 8). Data represent mean values ± SEM; *$p<0.05$,**$p<0.01$, vs SAL-SAL group (Student–Newman–Keuls post hoc test).

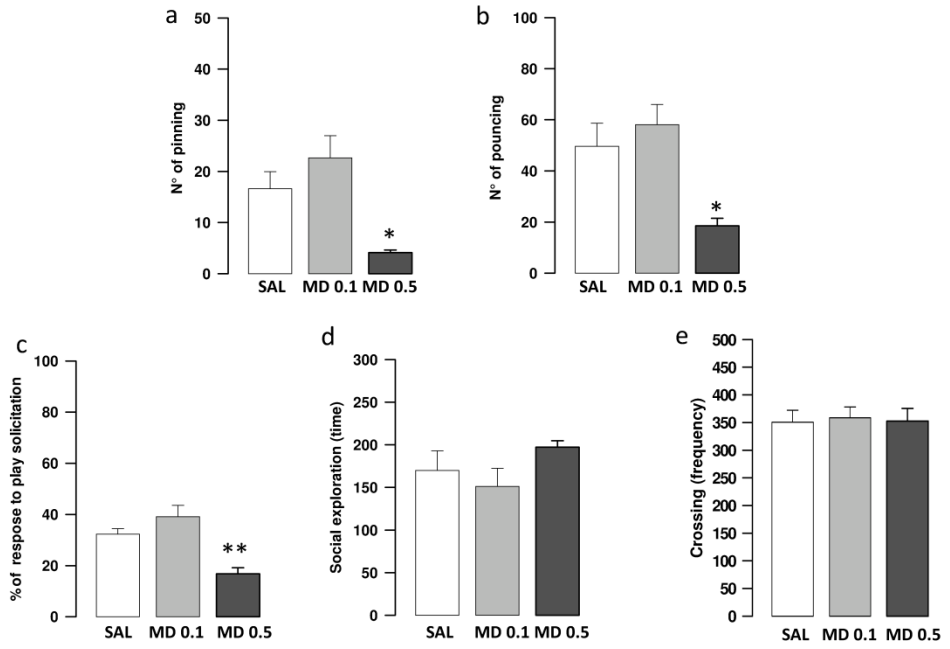

Figure 1

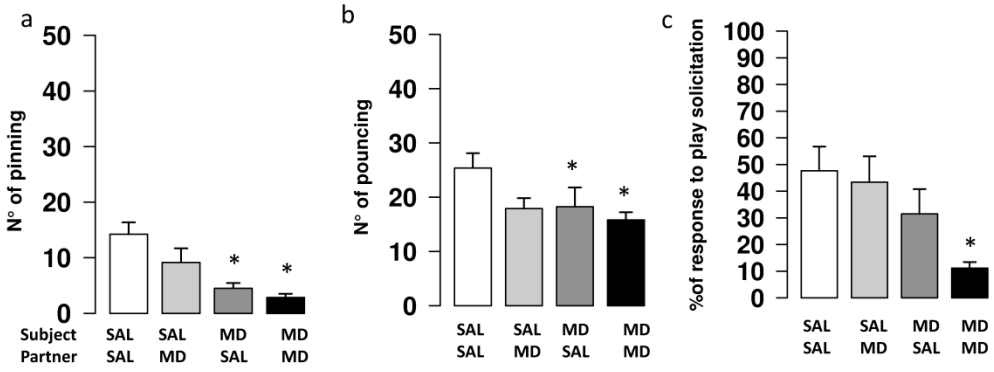

Figure 2

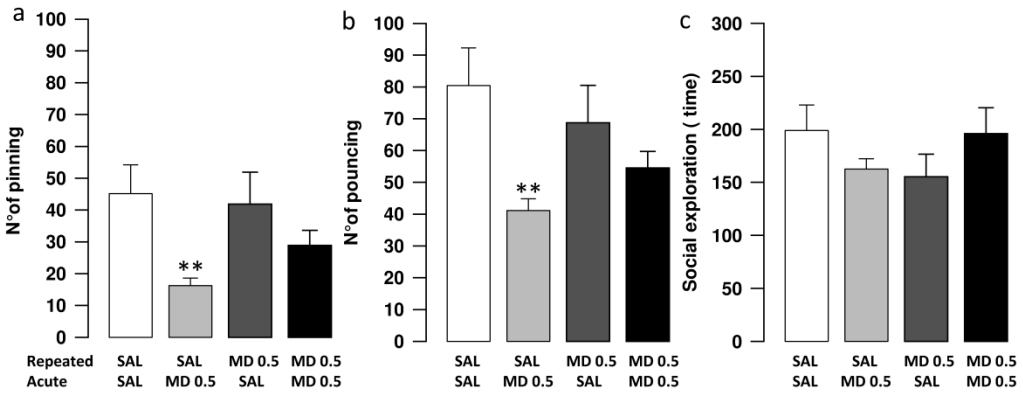

Figure 3

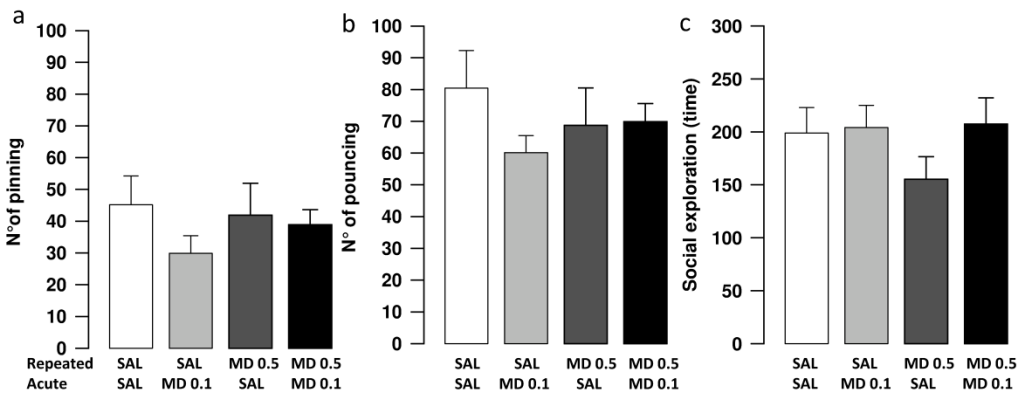

Figure 4

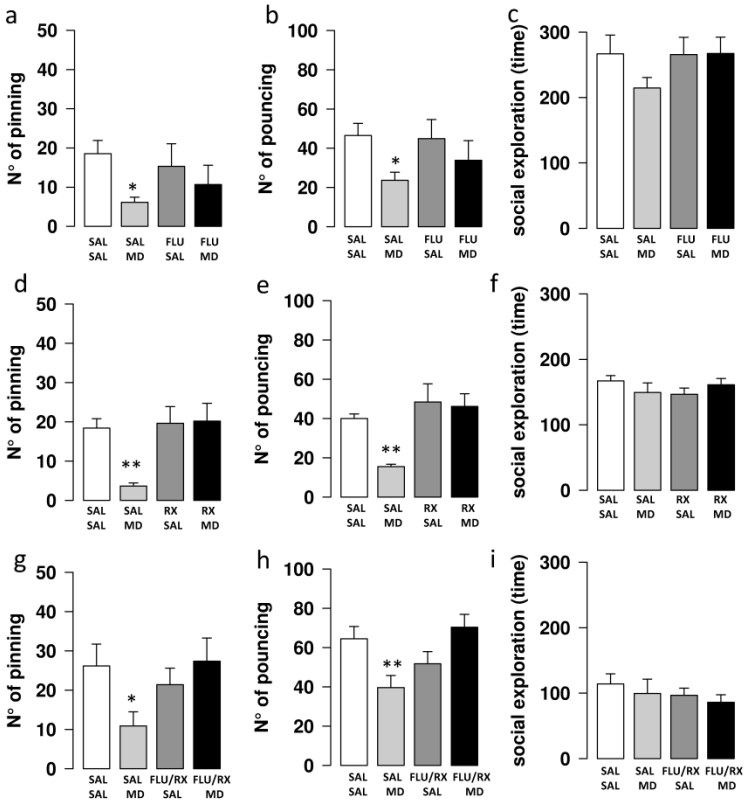
a
b
c
d
e
f
g
h
i
N° of pinning
N° of pouncing
social exploration (time)
SAL SAL
SAL MD
FLU SAL
FLU MD
RX SAL
RX MD
FLU/RX SAL
FLU/RX MD
*
**

Figure 5

CHAPTER 5

Prenatal valproate in rodents as a tool to understand the neural underpinnings of social dysfunctions in Autism Spectrum Disorder

Anna Maria Tartaglione[1], Sara Schiavi[2], Gemma Calamandrei[1*], Viviana Trezza[2*]

[1]Centre for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy;

[2]Department of Science, Roma Tre University, Rome, Italy

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Prenatal valproate in rodents as a tool to understand the neural underpinnings of social dysfunctions in Autism Spectrum Disorder.

The second aim of my PhD project was to study neural substrates of atypical social behavior in rodents to find new therapeutic opportunities to treat social dysfunctions in psychiatric disorders. To this aim, I focused on the atypical social behavior observed in animal models of Autism spectrum disorder (ASD), a group of neurodevelopmental psychiatric disorders whose core symptoms include impaired communication and social interaction. The present review paper provides an overview on one of the most used environmental preclinical model of ASD, i.e., prenatal exposure to Valproic Acid (VPA) in rodents.

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Invited review

Prenatal valproate in rodents as a tool to understand the neural underpinnings of social dysfunctions in autism spectrum disorder

Anna Maria Tartaglione[a], Sara Schiavi[b], Gemma Calamandrei[a,1,**], Viviana Trezza[b,*,1]

[a] Centre for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Rome, Italy
[b] Department of Science, Roma Tre University, Rome, Italy

HIGHLIGHTS

- Prenatal VPA exposure is a risk factor for autism spectrum disorder (ASD).
- Prenatal VPA exposure is a preclinical model of ASD.
- VPA-exposed rodents show ASD-like social and communication deficits.
- The VPA model is a valuable tool to identify new pharmacological targets for ASD.

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ABSTRACT

Impairments in social interaction and verbal and non verbal communication are among the main features of Autism Spectrum Disorder (ASD). The causes of ASD are still unknown but the research efforts of the last decade have identified a number of factors (rare gene mutations, gene variations and adverse environmental events) that, interacting in complex ways, affect early brain development. The clinical evidence that prenatal exposure to the antiepileptic drug valproate (VPA) is associated with increased risk of neurodevelopmental delay, cognitive deficits and autism in children, has drawn the attention of scientists on VPA as a tool to unravel the environment contribution to ASD risk in children. In agreement with the clinical evidence, rodents prenatally exposed to VPA display behavioral anomalies resembling ASD symptoms. The mechanisms by which administration of VPA in pregnancy increases the risk of autism are still far to be clear as are still undetermined the specific targets of VPA in the developing brain both in humans and rodents. However, the robustness of the behavioral alterations, mainly in the social domain, and the neural/molecular changes revealed so far support the VPA model as a reliable instrument to investigate the neural underpinnings of social impairment.

Here we provide an update of preclinical studies on prenatal exposure to VPA in rodents with a focus on the social and communication deficits induced by VPA, discussing potential pitfalls and future directions in this research field and corroborating the potential of the VPA model to identify new pharmacological targets for ASD.

This article is part of the Special Issue entitled 'The neuropharmacology of social behavior: from bench to bedside'.

1. Use of valproate during pregnancy: fetal valproate syndrome and beyond

1.1. Public awareness of the teratogen potential of VPA

Valproate (valproic acid, VPA) is a widely prescribed and effective medication for both epilepsy and bipolar disorder and it is also used *off label* for other pathological states including migraine (Moller and Nasrallah, 2003; Nevitt et al., 2017; Wieck and Jones, 2018). Nowadays VPA is considered the most teratogenic of all the antiepileptic drugs (AEDs) currently available (Meador, 2008; Werler et al., 2011), with a teratogenic risk of 6.3% compared to other medications (Atturu and Odelola, 2015; Goodwin and Consensus Group of the British Association for, 2009; Ornoy, 2006).

* Corresponding author. Dept. Science, Roma Tre University, Viale Marconi 446, 00146 Rome, Italy.
** Corresponding author. Centre for Behavioral Sciences and Mental Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy.
E-mail addresses: gemma.calamandrei@iss.it (G. Calamandrei), viviana.trezza@uniroma3.it (V. Trezza).
[1] Equal contribution.

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Despite the longstanding evidence of its teratogenicity (Robert and Guibaud, 1982), the public awareness of the deleterious consequences induced by prenatal VPA exposure in the offspring is still limited. Studies conducted in the UK and other European Countries indicate that a substantial proportion of women of childbearing age taking VPA has not been informed by health professionals about the risks related to VPA use during pregnancy, or is not aware of them (Friedrich et al., 2018; Langan et al., 2013; Wieck et al., 2007). The scenario is similar outside Europe. Thus, a study published in 2011 involving a cohort of epileptic pregnant women identified within the Quebec Pregnancy Registry revealed that VPA was still the second most common AED taken (Kulaga et al., 2011). In the US, a recent retrospective study showed that only 13.2% of all the reproductive-aged female patients who were prescribed VPA as treatment for their psychiatric illness at a major medical center in the Midwest was documented about the possible teratogenicity of the drug (Gotlib et al., 2016). Similarly, the analysis of data contained in the Australian Register of Antiepileptic Drugs revealed that 17.9% of monotherapy pregnant patients received VPA (Vajda et al., 2012).

In line with this scenario, in March 2016, the French Inspection Générale des Affaires Sociales (IGAS) reported at least 450 cases of malformations in children born between 2006 and 2014 from mothers who had taken VPA during pregnancy (IGAS, 2016). These reports demonstrate that a considerable number of patients across many Countries continues to take VPA during pregnancy. As a result, both national and international Medicines Agencies have recently strengthened warnings on the use of VPA medicines in women of childbearing age. For instance, in May 2016, the Italian Medicines Agency (AIFA) published an informative note directed to medical doctors and pharmacists about the risks associated with the exposure to drugs containing VPA during pregnancy (Valproato_NII_25052016). In February 2018, the Pharmacovigilance Risk Assessment Committee (PRAC) of the European Medicines Agency (EMA) recommended that VPA should not be used during pregnancy unless the woman has a form of epilepsy that is not responsive to other AEDs, and that the drug should not be prescribed to women of childbearing age who are not enrolled in a pregnancy prevention program (EMA/67672/2018). Thus, doctors in the EU are advised not to prescribe VPA for epilepsy or bipolar disorder to pregnant women, or to women of childbearing age, unless other treatments are ineffective or not tolerated. When VPA is the only therapeutic option, women should be advised on the use of effective contraception and treatment should be started and supervised by a doctor experienced in treating these conditions (EMA/145600/2018).

However, the management of bipolar disorder and epilepsy during pregnancy continues to be a complex challenge for both clinicians and patients, who may well be faced with situations in which they must weigh up the benefits and risk of VPA use (Macfarlane and Greenhalgh, 2018). For example, among the AEDs that could be offered to a woman of childbearing age, VPA remains one of the most effective, it is still used in emergency settings and it is effective where other drugs are not (Macfarlane and Greenhalgh, 2018). At the same time, in bipolar disorder, VPA may become necessary when patients suffering of acute mania either do not respond to lithium, or do not tolerate it (Miura et al., 2014; Pope et al., 1991). Furthermore, in women with unstable forms of bipolar disorder, the high risk of relapse associated with rapid withdrawal of mood stabilizers may balance the potential risk to the fetus (Macfarlane and Greenhalgh, 2018). Similarly, it has been shown that withdrawal of VPA for the treatment of epilepsy in the first trimester was associated with a significantly higher rate of generalized tonic clonic seizures compared to when it was continued. More strikingly, the rate of seizures was also elevated when VPA was switched to another anti-epileptic medication (Tomson et al., 2016). Thus, women may decide that the benefits of VPA to their mood and quality of life still outweigh the risks. Particularly if they do not plan to carry a pregnancy to term and are aware of the other health risks, they could decide that VPA is the best option for them personally (Gotlib et al., 2017).

However, since a safe dose of VPA during pregnancy has not been identified (Wieck and Jones, 2018), in these particular cases a pregnancy prevention program must be followed. This includes an assessment of the women taking VPA, pregnancy tests before and during treatment, effective communication about the risks of VPA to the fetus and about the importance of contraception throughout treatment. Every year, a specialist evaluation must be provided to patients and a complete a risk acknowledgement form should be filled in. At each VPA prescription, patients should receive a warning card explaining the pregnancy risks. Furthermore, the packaging should carry a visual warning and pharmacists dispensing VPA to women of childbearing age should make them aware of the risks (Wieck and Jones, 2018) (EMA/145600/2018).

1.2. Effects of in utero VPA exposure: fetal valproate syndrome and autism spectrum disorder (ASD)

VPA is known to cross the human placenta and its clearance is increased during pregnancy (Albani et al., 1984; Nau et al., 1982). Its levels in cord serum are often higher than in the mother, suggesting a better binding in the fetal than in the maternal compartment (Ornoy, 2009). Moreover, VPA is excreted into human milk in low concentrations (von Unruh et al., 1984), and it has been estimated that infants may ingest about 5% of the weight-adjusted maternal daily dose (Albani et al., 1984).

The effects of *in utero* VPA exposure change according to the administered dose and the time window of the exposure. The VPA dose treatment usually ranges from 200 mg to 3600 mg per day and higher doses are associated with greater risks to the fetus (Roullet et al., 2013). During the first trimester of gestation, VPA exposure results in major organ malformations (Harden et al., 2009), while exposure later in pregnancy increases the risk of more subtle neurodevelopmental effects (Barrett and Richens, 2003). Neural tube defects are the most frequent malformation induced by VPA exposure at embryonic stages, with spina bifida being10–20 times more frequent in VPA-exposed fetuses than in the general population (Ornoy, 2009). One of the first studies that linked *in utero* VPA exposure to neural tube defects was published in 1982 and documented a 20-fold higher risk for the occurrence of spina bifida after exposure to VPA during pregnancy (Robert and Guibaud, 1982). Nowadays, the association between maternal use of VPA and the development of neural tube defects in the offspring is well-established. This suggests a specific association with caudal defects involving an aberrant neuronal differentiation, even if it is still unclear how VPA initiates the molecular and biochemical events that trigger these outcomes (Alsdorf and Wyszynski, 2005). Besides neural tube defects, *in utero* VPA exposure has been associated to congenital malformations affecting the gastrointestinal, cardiovascular, dermatologic, genital, urinary, pulmonary, and musculoskeletal systems (Meador, 2008). Indeed, the risk to develop major defects when exposed to *in utero* VPA is high and characteristic to the point that the term 'fetal valproate syndrome' (FVS) was created to group them all. The first characterization of the FVS was provided by Di Liberti and coworkers in 1984, who described a specific set of facial dysmorphic features in seven infants exposed *in utero* to VPA (DiLiberti et al., 1984). Since then, an increasing amount of studies reported similar facial features together with other congenital anomalies, developmental delay and neurological impairment in children exposed *in utero* to VPA (Ardinger et al., 1988; Kini et al., 2006; Mawer et al., 2002; Moore et al., 2000; Winter et al., 1987), including also fetal or neonatal distress (Alsdorf and Wyszynski, 2005) and withdrawal symptoms in the neonatal period (Clayton-Smith and Donnai, 1995). Nowadays, it is generally accepted that FVS includes neural tube defects, trigonocephaly, radial ray defects, pulmonary abnormalities, coloboma of iris/optic disc, low verbal IQ and features of ASD (Kini et al., 2006).

Studies of neurodevelopmental outcomes caused by *in utero*

exposure to VPA have expanded in last years, and children exposed to VPA have been reported to be intellectually less able (Bromley et al., 2014) and to have developmental delay and ASD (Christensen et al., 2013; Veroniki et al., 2017). In particular, children exposed to VPA *in utero* display cognitive deficit such as delayed verbal reasoning abilities and language skills (Adab et al., 2004; Bromley et al., 2014; Meador et al., 2013; Nadebaum et al., 2011) together with poorer levels of attention and working memory (Kantola-Sorsa et al., 2007; Vinten et al., 2005). Some of the studies published in the 90's on the association between maternal VPA exposure and ASD were originally based on the increased frequency of autistic symptoms in children diagnosed with FVS (Christianson et al., 1994; Williams et al., 2001; Williams and Hersh, 1997). One of the first case studies was published by Christianson and coworkers, who described four children exposed to VPA during pregnancy that showed developmental delay and one of them had ASD (Christianson et al., 1994). Other studies confirmed this association, reporting that VPA was used during pregnancy by mothers of children with ASD and Asperger syndrome (Moore et al., 2000; Williams et al., 2001). Since the 90's, several studies proved that, among all the anticonvulsants used during pregnancy, VPA was the drug most commonly associated with ASD (Rasalam et al., 2005). Recently, a large Danish population-based study reported an increased risk of ASD in children exposed to VPA during pregnancy (Christensen et al., 2013). Case, population, retrospective and observational studies reported increased rates of ASD in VPA exposed subjects (Bromley et al., 2008, 2013; Rasalam et al., 2005; Wood et al., 2015). The neurodevelopmental outcomes of children exposed to VPA consistently demonstrate an association with dose, showing that higher doses are linked to poorer global cognitive abilities (e.g. DQ or IQ scores) (Adab et al., 2004; Baker et al., 2015; Bromley et al., 2013; Meador et al., 2013). Most commonly, doses of 800–1000 mg daily are associated with higher risk. Additionally, a relationship with dose has been reported for verbal/language abilities (Baker et al., 2015; Meador et al., 2013; Nadebaum et al., 2011). However, although a significant proportion of children exposed to VPA *in utero* exhibit the typical phenotypic characteristics of ASD, to date it remains difficult to identify specific behavioral traits that associate a certain type of ASD manifestation with prenatal exposure to VPA.

2. Prenatal exposure to VPA in rodents: a suitable model of autism?

Based on the robust clinical evidence, it is not surprising that prenatal exposure to VPA is being increasingly studied in rodents as a possible drug-induced model of autism. An animal model of human disease is robust when it mimics, at least in part, pathogenic mechanisms (construct validity) and disease phenotypes (face validity) that occur in patients; therefore, positive preclinical studies performed with these animal models may predict positive outcomes in clinical trials (predictive validity). However, such categorization is difficulty applicable to animal models of ASD. The pathogenic mechanisms of ASD are far to be understood, and complex gene × environment interactions have been called upon, involving rare gene variations/mutations and adverse environmental factors in early developmental stages. Consequently, the rodent models established so far attempted to identify molecular mechanisms associated with a given factor by replicating some features of the syndrome, either the gene variants found in the clinical studies or the behavioral symptoms described in affected individuals (Risch et al., 2014). Among these models, the prenatal VPA model might represent cases of idiopathic ASD with a possible environmental cause. The mechanisms by which administration of VPA in pregnancy increases the risk of ASD are still far to be clear as are still undetermined the targets of VPA in the developing brain both in humans and rodents. Thus, the use of this animal model is based on the cluster of symptoms resembling the human pathology, most of them belonging to the social domain, which are induced even by a single administration of VPA during gestation. In this section, we will present preclinical studies on prenatal exposure to VPA in rodents that corroborate the strength of the VPA model mainly from the behavioral perspective. The discovery that agents - mostly off-label drugs - acting on different molecular targets and systems protect or ameliorate from the adverse effects of prenatal VPA on social function (see par. 2.2) supports the use of this rodent model to uncover the environmental etiology of ASD and to identify new pharmacological targets for therapy.

2.1. Modeling core behavioral features of ASD in the VPA model

At present, ASD is defined by two core symptoms: (a) persistent social communication and interaction deficits and (b) restricted, repetitive patterns of behaviors, interests or activities (2013), along with other comorbid signs, of which anxiety is one of the most common (Gillott et al., 2001; Leyfer et al., 2006; Vasa and Mazurek, 2015).

Thus, face validity of the VPA model is based upon detection of behavioral impairments recapitulating ASD in humans (Nicolini and Fahnestock, 2018; Roullet et al., 2013). In the present review we will focus on the social and communication deficits induced in rodents by prenatal VPA exposure.

Dose and timing of exposure to VPA are key variables for ASD-like behavioral outcome (Roullet et al., 2013). At variance with prenatal exposure occurring in humans, since VPA treatment is usually scheduled for the whole duration of pregnancy, the rodent model mainly implies a single injection of VPA at doses ranging from 300 to 800 mg/kg between gestational day (GD) 9–12.5. Very few experimental studies assessed the effects of repeated VPA treatment at lower dosages during gestation (Bertelsen et al., 2017; Fujimura et al., 2016; Juliandi et al., 2015; Sabers et al., 2014; Wellmann et al., 2014). As for the timing of exposure, mice exposed to VPA on GD 12.5 but not on GD 9 and 14.5 have been shown to exhibit autism-like behavioral alterations (Kataoka et al., 2013). Similarly, rats exposed to VPA on day 12.5 of gestation but not on GD 7, 9.5 and 15 display the most significant changes in social behaviors (Kim et al., 2011).

As for the dose, Servadio et al. (2018) compared different VPA doses (350, 400, 500 mg/kg) administered at GD 12.5: VPA induced dose-dependent deficits in social communication and social discrimination in the rat offspring. As already mentioned, the large body of literature produced on the VPA model shows that VPA exposure at doses from 300 to 800 mg/kg leads to autism-like phenotype in both rats and mice of different strains. However, the 500 mg/kg dose of VPA appears as the most effective in inducing core and associated autism-like symptoms in the exposed offspring, in the absence of overt toxicological effects in the pregnant females (Hara et al., 2016, 2017a, 2017b; Servadio et al., 2016, 2018).

2.1.1. Social communication and interaction deficits

According to the DSM-5 criteria for diagnosing ASD, social behavior and communication have been grouped together under a single umbrella of the behavioral domain of socialization. One of the most consistent social deficit in children who develop ASD is the lack of non-verbal social gestures such as pointing, showing, and giving. Consequently, social play behavior is significantly altered in ASD children (Hobson et al., 2013; Jordan, 2003; Rutherford et al., 2007).

Given that impaired social interaction is widely recognized as the defining feature of ASD, we would expect a valid animal model to exhibit deficits in this behavioral domain. Rodents are highly social species exhibiting complex patterns of social behavior such as parental care, social play behavior and sexual behavior (Panksepp et al., 1984; Ricceri et al., 2007). Social play, also known as “rough-and-tumble” play, is the first form of non-mother directed social behavior displayed by most mammals at young age (Panksepp et al., 1984; Vanderschuren et al., 1997, 2016). For both animals and humans, social play is crucial

for neural growth and proper development: it helps to develop communicative skills, to acquire cognitive and social competence, and it contributes to the development of behavioral and mental flexibility (Vanderschuren et al., 2016; Vanderschuren and Trezza, 2014).

The laboratory rat is an ideal species to study social play behavior, which can be objectively quantified by measuring the frequency and duration of specific behaviors (Panksepp et al., 1984; Vanderschuren and Trezza, 2014). In rats, an episode of social play behavior usually starts with a rat soliciting a conspecific, by attempting to nose or rub the nape of its neck, hence the term 'pouncing' (Vanderschuren et al., 2016). The most characteristic response to this play initiation is when the recipient rat rolls onto its dorsal surface, commonly known as 'pinning'. Other components of the social repertoire of adolescent rats, such as social investigation (face and ano-genital sniffing) and social grooming are considered social behaviors not primarily related to play, and can also be easily assessed. For methodological details about the experimental paradigms most commonly used to study social play behavior in rodents, see (Ricceri et al., 2007) and (Trezza et al., 2010).

Several studies indicate that social play behavior is profoundly impaired in the VPA model (Chomiak et al., 2010; Raza et al., 2015). Notably, VPA-exposed adolescent rats showed a decrease in the frequency of pinning during social play, pointing to social deficits observed also in adulthood in terms of increased latency to engage in social behavior and decreased number of social explorations (Schneider and Przewlocki, 2005; Schneider et al., 2008). Further support for VPA effects on social play behavior comes from studies (Markram et al., 2008) showing that VPA-exposed rats exhibited decreased play behavior, reduced exploration of a conspecific and active interaction avoidance. Furthermore, rats prenatally exposed to VPA showed reduced responsiveness to play solicitation compared to control rats, with no changes in general social exploration (Melancia et al., 2018; Servadio et al., 2016, 2018).

In mice treated with VPA prenatally, only few studies have evaluated sociability in an open interaction session measuring the number of contacts with unfamiliar mice. Exposure to VPA reduced the duration of face sniffing to an age- and sex-matched conspecific (Hara et al., 2016, 2017a, 2017b; Kataoka et al., 2013; Moldrich et al., 2013).

Beyond the traditional assays to evaluate social interaction, a standardized test for assessing sociability both in mice and rats is the three-chamber test proposed by Crawley et al. (Crawley, 2004; Moy et al., 2004, 2008; Yang et al., 2011). Specifically, in a three-chamber apparatus, the mouse or the rat, placed in the central chamber, is allowed to explore a novel object (empty wire cage) and a stimulus animal inside a wire cage placed in two lateral chambers, respectively. The time spent in each chamber and time sniffing- or nose-pokes towards the wire cage are measured. This behavioral paradigm, compared to dyadic social interaction, prevents direct physical contact between the tested animal and the stimulus animal.

Rats and mice prenatally exposed to VPA showed significantly reduced preference for the social stimulus relative to controls, suggesting lower interest in social cues (Campolongo et al., 2018; Cartocci et al., 2018; Gandal et al., 2010; Kerr et al., 2013; Kim et al., 2011, 2014a, 2014b; Lucchina and Depino, 2014; Melancia et al., 2018; Moldrich et al., 2013; Roullet et al., 2010). Recently, a study by Hirsch and coworkers (Hirsch et al., 2018) found impaired social transmission of food preference (STFP) in rats prenatally exposed to VPA. This task implies that after an interaction period between the demonstrator animal, previously provided to eat a flavored food, and the observer animal, this latter should prefer the food presented by the demonstrator rat with respect to an alternative food (for methodological aspects, see (Bessieres et al., 2017)). Whereas the reciprocal social interaction test primarily focuses on the social behavior of the test animal with an unfamiliar conspecific (Schneider and Przewlocki, 2005), the STFP test investigates long term consequences of social deficits on establishment of food preferences (Bessieres et al., 2017; Hirsch et al., 2018), thus providing additional information on the rewarding properties of social contact, specifically compromised in ASD (Greene et al., 2018; Supekar et al., 2018).

While the social competencies can be easily described in laboratory rodents, the communicative impairment is more difficult to assess. Although rodents do not use language, they emit ultrasonic vocalizations (USVs) that entail different meanings depending on the context where they are emitted. As an example, USVs are used by males in courtship behavior to elicit the female's responsiveness; females emit USVs when encountering an unfamiliar conspecific possibly to promote affiliation (Egnor and Seagraves, 2016). The communicative value of USVs is evident since the very early developmental phases, as new born rats and mice emit USVs when separated from the mother and the nest (Blumberg and Alberts, 1991; Scattoni et al., 2009). The most widely used protocol for the study of isolation-induced USVs in infant rodents is to separate the pup from its mother and littermates and place it alone in a soundproof arena for a few minutes. The USVs emitted by the pup are detected by an ultrasonic microphone fixed above the arena, connected to an ultrasound detector, and are analyzed qualitatively and quantitatively using specific software (for a comprehensive methodological review, see (Branchi et al., 2006). Studies measuring USVs in mice and rats prenatally exposed to VPA when separated from the dam and siblings, revealed a reduction in the number of calls (Cartocci et al., 2018; Gandal et al., 2010; Melancia et al., 2018; Servadio et al., 2018). Interestingly, Moldrich et al. (2013) observed also a decrease of complex call types in mouse pups prenatally exposed to VPA.

Furthermore, in rodents, USVs frequencies vary depending on age, emotional state and environmental factors. It has been largely demonstrated that adult rats emit low frequency (22-kHz) USVs when they detect danger or aversion and high frequency (50-kHz) USVs for positive or non-aversive conditions (Brudzynski, 2015). Few studies investigated USVs in adult rodents prenatally exposed to VPA. So far, reduced adult 70-kHz premating vocalizations in VPA-exposed mice (Gandal et al., 2010) and a decrease of complex 50 kHz USVs during social interaction in VPA-exposed rats have been documented (Wellmann et al., 2014).

In the context of early communicative impairment, it is worth considering that there are other early indicators of developmental delay in rodent models. Specifically, failure to recognize familiar olfactory cues (i.e. nest odor) may be prodromic of impaired adult social recognition and interaction (Melo et al., 2006; Terry and Johanson, 1996). Early deficits in recognizing familiar olfactory cues can be detected through the homing behavior test. The experimental procedure includes a brief isolation of the pup before testing. Next, the pup is placed for a few minutes in a cage with two parts of the floor covered with clean sawdust and one part with sawdust from its own nest. The latency to reach the familiar bedding and the total time spent by the pup in the familiar bedding are scored (Scattoni et al., 2009). Homing deficits have been described in VPA-exposed rats and mice by a large body of studies that reported increased latency to find home bedding during a nest-seeking response in VPA-exposed pups (Melancia et al., 2018; Moldrich et al., 2013; Roullet et al., 2010; Schneider and Przewlocki, 2005; Servadio et al., 2016, 2018; Tartaglione et al., 2018).

2.1.2. Repetitive behavior

As described in the DSM-5, the second core feature of ASD includes repetitive/stereotypic behaviors such as unusual and/or repetitious vocalizations or actions with one's own body or with objects (2013). Several studies assessing repetitive behaviors in the VPA model found increased self-grooming (Gandal et al., 2010; Mehta et al., 2011; Moldrich et al., 2013), digging (Kim et al., 2014a; Mehta et al., 2011; Moldrich et al., 2013) and dipping behavior (Melancia et al., 2018). Perseverative behavior in VPA-exposed rats has also been reported by Markram et al. (2008), who found increased re-entry of the same previously explored arm in a Y-maze, and by Schneider and coworkers (Schneider and Przewlocki, 2005; Schneider et al., 2008) who reported increased locomotion and stereotyped and repetitive behaviors to the

detriment of exploratory activity.

2.1.3. Comorbid traits

The most frequent associated symptoms in ASD include anxiety, epileptic seizures and cognitive impairments (2013). Comorbidities are prevalently seen in individuals with ASD, whereas studies in animal models are rather focused on the actual core symptoms of the disease (Argyropoulos et al., 2013). However, several studies revealed increased anxiety in rodents prenatally exposed to VPA, as indicated by decreased time spent and lower number of entries in the open arms of the elevated plus maze (Cartocci et al., 2018; Kataoka et al., 2013; Markram et al., 2008; Melancia et al., 2018; Schneider et al., 2006, 2007, 2008; Servadio et al., 2018) and by reduced exploration of the central area in open-field tests (Gandal et al., 2010; Kataoka et al., 2013). Some of these studies found also lower sensitivity to pain and higher tactile sensitivity, suggesting sensory system deficits (Kerr et al., 2013; Schneider and Przewlocki, 2005; Schneider et al., 2006, 2008) and reduced prepulse inhibition pointing to deficits in sensorimotor integration and a lack of appropriate inhibition that could lead to increased repetitive and stereotypic behaviors (Gandal et al., 2010; Schneider and Przewlocki, 2005; Schneider et al., 2006). Among comorbid traits, cognitive impairment is often found in association with social deficits, in particular in more severe autism. VPA-exposed rodents have been largely tested in learning and memory tasks (Gao et al., 2016a, 2016b; Hara et al., 2016, 2017b; Markram et al., 2008; Melancia et al., 2018; Mychasiuk et al., 2012; Schneider et al., 2007). Rats prenatally exposed to VPA showed impaired emotional memory in the inhibitory avoidance task [83] and altered spatial memory in the spatial water maze task (Gao et al., 2016a, 2016b). Markram et al. (2008) reported enhanced and resistant conditioned fear memories in rats prenatally exposed to VPA that might be caused by lateral amygdala hyperactivity and hyperplasticity. Enhanced anxiety and abnormal fear processing observed in the VPA model could amplify aversion to environmental fear-evoking stimuli. As for the ability of prenatal VPA exposure to affect novel object recognition, VPA-exposed rats showed intact object recognition abilities when tested either 3 (Schneider et al., 2007) or 30 (Melancia et al., 2018) minutes after the training trial; however, when the interval between the training and test trials was longer, both VPA-exposed mice (Hara et al., 2016, 2017b) and rats (Mychasiuk et al., 2012) showed impaired object recognition abilities, thus suggesting that prenatal VPA exposure may have a more deleterious impact on long-term rather than on short-term object recognition. Altogether, the deficits displayed by VPA-exposed animals in different cognitive domains may result in a cognitive state that is incompatible with the adequate execution of complex social acts, thus contributing to the impaired social interactions displayed by VPA-exposed animals.

In Table 1 we report in detail the experimental studies described in this section.

2.2. Unraveling the neurobiological bases of social impairment in the VPA model: an avenue for treatment development?

While ASD is by now considered as a multifactorial disease, finding its exact causes and mechanisms is still a huge challenge yet to overcome. The VPA model effectively mimics one of the environmental components of ASD etiology, and experimental studies so far suggest that the neurodevelopmental effects of VPA exposure can be attributed to a complex set of intersecting pathways. Of note, some of these pathways are related to fundamental brain developmental processes, such as the early imbalance between gamma-aminobutyric acid (GABA) and glutamate system for the most part responsible for brain network connectivity (Bozzi et al., 2018). Other pathways, including the serotonergic, dopaminergic, oxytocinergic and endocannabinoid systems, are also pivotal in the modulation of social behaviors in mammals (Dolen, 2015; Narita et al., 2002; Vanderschuren et al., 2016; Wei et al., 2017).

In Table 2 we report in detail the effects of the drugs described in this section on the social domain of VPA-exposed rodents.

2.2.1. Excitatory and inhibitory (E/I) imbalance

The excitatory and inhibitory (E/I) imbalance has been largely implicated in ASD etiology and symptoms (Dani et al., 2005; Rubenstein and Merzenich, 2003). Attenuated GABAergic and enhanced glutamatergic signals have been reported, which might be responsible for derailed neural network connectivity and ASD-like behaviors including impaired social interaction (Mehta et al., 2011; Rinaldi et al., 2007, 2008a, 2008b; Silva et al., 2009). Prenatally VPA-exposed male rats showed E/I imbalance, similar to ASD patients (Kim et al., 2013, 2014b, 2016). Specifically, studies reported macrocephaly, increased neuronal number and increased protein markers such as postsynaptic density protein 95 (PSD95), α-Ca (2+)/calmodulin-dependent protein kinase II (α-CaMKII), N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (NMDAR and AMPAR) in postnatal VPA rat brain, pathways related to the increased glutamatergic neuronal density of the rat brain (Gandal et al., 2010; Go et al., 2012; Kim et al., 2013, 2014b, 2016; Mehta et al., 2011; Nagode et al., 2017).

Studies on the VPA rat model revealed hyper-connected microcircuits at the cellular level in different brain areas implicated in ASD, such as the prefrontal cortex and the somatosensory cortex, which was reflected on the molecular level in enhanced expression and function of the NMDA receptor protein (Rinaldi et al., 2007, 2008a, 2008b; Silva et al., 2009). Thus, pharmacological compounds targeting this receptor system have been investigated. Two studies reported that both acute and chronic administration of the NMDA receptor antagonist memantine restored sociability and reduced repetitive behaviors induced by prenatal VPA exposure (Kang and Kim, 2015; Kumar and Sharma, 2016a).

Treatment with D-cycloserine (DCS), a partial agonist of the glutamate and glycine sites of glutamatergic receptors, was able to ameliorate the social impairment induced by prenatal VPA. In males, a single dose of DCS ameliorated the VPA-induced increase in play fighting, and in females, DCS increased social motivation (Wellmann et al., 2014). Kim et al. (2017) tested the therapeutic potential of agmatine, an endogenous NMDAR antagonist, to rescue the behavioral alterations induced by prenatal VPA. Agmatine restored behavioral deficits and the overly activated extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in the prefrontal cortex and hippocampus of VPA-exposed rats, possibly by modulating over-excitability due to enhanced excitatory neural circuit.

Treatment with the metabotropic glutamate receptor 5 (mGluR5) antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP), which reduces NMDAR function through inhibition of mGluR5 (Alagarsamy et al., 1999), reversed E/I imbalance, sensorimotor integration deficits and stereotyped/perseverative behaviors (self-grooming and digging) induced by VPA (Gandal et al., 2010; Mehta et al., 2011). Recently, the study by Kim et al. (2018) suggested that modulation of AMPAR can be a potential target for the treatment of social behavior deficits associated with ASD. Specifically, the administration of CP465022, an AMPAR antagonist, ameliorated only social deficits, without affecting repetitive behaviors induced by prenatal VPA espoure.

Remarkably, these data are consistent with the hypothesis that E/I imbalance might contribute to autism-like behavioral deficits in VPA-exposed rodents.

2.2.2. Serotonin

Among the neurotransmitter systems involved in ASD, the role of serotonin (5-HT) in early neural development has been also investigated (Lam et al., 2006). Starting from the evidence of hyperserotonemia (elevated plasma 5HT levels) occurring in about one-third of the ASD population, the serotonergic system has been studied in VPA rodent models (Miyazaki et al., 2005; Narita et al., 2002; Tsujino et al.,

Table 1
Behavioral outcome following prenatal VPA exposure in rodent models.

Species	Day of exposure	Dose/Route	Age of assessment	Behavioral alterations	Reference
Rat	GD 12.5	600 mg/kg i.p.	PND 7-90	↓ maturation and motor development; ↓ olfactory discrimination at PND9; ↓ prepulse inhibition; ↑ locomotor and repetitive/stereotyped activity; ↓ exploratory activity; ↓ social play behavior; ↓ adult social interaction; ↑sensitivity to nonpainful stimuli; ↓ sensitivity to pain; ↑ anxiety; ↓place aversion to naxolone; N.E. novel object recognition;	Schneider and Przewlocki (2005); Schneider et al., 2006, 2007, 2008
Rat	GD 12.5	500 mg/kg i.p.	Adulthood	↓ social interaction; ↑ repetitive/stereotyped behavior; ↓ sensitivity to pain; ↓ prepulse inhibition; ↑ anxiety; ↑ fear- memories and –generalization; ↓ fear extinction; N.E. spatial learning and memory	Markram et al. (2008)
Mouse	GD 13	600 mg/kg s.c.	PND 2-75 PND 42-71	↓ pup distress calls; ↓ adult 70-kHz premating vocalizations; ↓ social preference; ↑ repetitive/stereotyped behavior; ↓ prepulse inhibition; N.E. motor coordination (PND 63) and olfactory function (PND 75) ↑ anxiety; ↑ repetitive/stereotyped behaviors; N.E. locomotor activity	Gandal et al., (2010); Mehta et al. (2011)
Mouse	GD 11	800 mg/kg orally (mixed in 1.5 g peanut butter)	PND 9 -25	↓ maturation; ↓ olfactory discrimination at PND9; ↓ sociability	Roullet et al. (2010)
Rat	GD 7 GD 9.5 GD 12 GD 15	400 mg/kg s.c.	4 weeks	N.E. N.E. ↓ sociability and preference for social novelty; ↑ locomotion N.E.	Kim K C et al. 2011; Kim K C et al. 2014
Rat	GD 12	800 mg/kg orally (mixed in 1.5 g peanut butter)	PND 65-105	↓ novel object recognition; ↓ anxiety	Mychasiuk et al. (2012)
Mouse	GD 9 GD 12.5 GD 14.5	500 mg/kg i.p.	4 and 8 weeks	N.E. ↓ social interaction; ↓ locomotor and exploratory activity; ↑ anxiety N.E.	Kataoka et al. (2013)
Rat	GD 12.5	600 mg/kg s.c.	PND 33-40	↓ sociability; ↓ sensitivity to pain; ↓ locomotor activity; NE anxiety	Kerr et al. (2013)
Mouse	GD 12.5	600 mg/kg i.p.	PND 8-38	↓ pup distress calls; ↓ olfactory discrimination; ↓ adult complex calls; ↓ sociability; ↓ social interaction; repetitive/stereotyped behaviors; NE locomotor activity; NE olfactory dishabituation	Moldrich et al. (2013)
Rat	GD 10	300 mg/kg s.c.	PND 21-40	↓ sociability and preference for social novelty; ↑ locomotor activity; ↑ repetitive/stereotyped behaviors; ↓ anxiety; ↓ novel object recognition; ↓ nest building	Kim J W et al. 2014
Mouse	GD 12.5	400 mg/kg s.c. 600 mg/kg s.c.	8–10 weeks	N.E. ↓ sociability; ↑ anxiety; NE depressive behavior	Lucchina and Depino (2014)
Rat	GD 12.5	800 mg/kg orally (mixed in 1.5 g peanut butter)	PND 29-34	↓ social play behavior	Raza et al. (2015)
Rat	GD 12.5	600 mg/kg i.p.	PND 35-40	↓ spatial learning and memory; ↓ sociability	Gao et al., (2016a), 2016b
Mouse	GD 12.5	500 mg/kg i.p.	8 weeks	↓ social interaction; ↓ novel object recognition	Hara et al., (2016); 2017a, 2017b
Rat	GD 12.5	500 mg/kg i.p. 350 mg/kg i.p 400 mg/kg i.p. 500 mg/kg i.p.	PND 9-90	↓ pup distress calls; ↓ olfactory discrimination; ↓ social play behavior; ↓ sociability; ↑ repetitive/stereotyped behavior; ↑ anxiety ↓ inhibitory avoidance; N.E. novel object recognition N.E. ↓ pup distress calls ↓ pup distress calls; ↓ olfactory discrimination; ↓ social play behavior; ↓ sociability; ↑ anxiety	Servadio et al., (2016); Cartocci et al. (2018); Melancia et al., (2018) Servadio et al. (2018)
Rat	GD 12.5	600 mg/kg i.p.	PND 46-64	↓ social interaction; ↓ social transmission of food preference; ↑ repetitive/stereotyped behavior	Hirsch et al. (2018)

N.E. no effect.

Table 2
Effects of pharmacological manipulations on the social impairment induced by prenatal VPA exposure in rodents.

VPA model			Drug			Social outcome			Reference
Species	Day of exposure	Dose/Route	Compound	Time of treatment*	Dose/Route	Age of assessment	Behavioral test	Outcome	
Mouse	GD 13.5	600 mg/kg s.c.	Memantine	30 min before	10 mg/kg i.p.	8–16 weeks	Three chamber	↑	Kang &Kim 2015
Rat	GD 12.5	500 mg/kg s.c.	Memantine	PND 21–50 once daily	10 or 20 mg/kg orally	7 weeks	Three chamber	↑	Kumar & Sharma 2016a,b
Rat	GD 12, 12.5, 13	200 mg/kg i.p.	D-cycloserine	1 h -; one daily for 4 days - before	32 or 64 mg/kg s.c.	4 weeks	Social interaction	↑	Wellmann et al. (2014)
Rat	GD 12	400 mg/kg s.c.	Agmatine	30 min before	25, 50 or 100 mg/kg i.p.	4–8 weeks	Three chamber	↑ (50 and 100 mg/kg)	Kim et al. (2017)
Mouse	GD 10	300 mg/kg s.c.	CP465022	30 min before-	0.25, 0.5 or 1 mg/kg i.p.	4 weeks 5 weeks	Social play Three chamber	↑ ↑ (0.5 and 1 mg/kg)	Kim et al., 2018
Rat	GD 12.5	500 mg/kg i.p.	8-OH-DPAT	ILPFC infusion	10 μg/μL	- -	Three chamber	↑	Wu et al. (2018)
Mouse	GD 12.5	500 mg/kg i.p.	Methylphenidate Atomoxetine	one daily for 2 weeks before	3 mg/kg i.p. 1 mg/kg i.p.	8 weeks	Social interaction	↑	Hara et al., 2016
Mouse	GD 12.5	500 mg/kg i.p.	Risperidone Aripiprazole Haloperidol	24 h -; once daily for 2 weeks before	0.2 mg/kg i.p. 3 mg/kg i.p. 0.1 mg/kg i.p.	8 weeks	Social interaction	↑ ↑ -(chronic)	Hara et al. 2017a,b
Mouse	GD 12.5	500 mg/kg i.p.	Oxytocin	24 h -; once daily for 2 weeks before	50, 100 or 200 μg/kg; 100 μg/kg intranasal	8 weeks	Social interaction	↑ (100 and 200 mg/kg) ↑	Hara et al.2017a,b
Species	Day of exposure	Dose/Route	Compound	Time of treatment*	Dose/Route	Age	Behavioral test	Outcome	
Mouse	GD 10	300 mg/kg s.c.	Donepezil	P14-P40 once daily before-	0.3 mg/kg i.p.	4 weeks	Three chamber	↑	Kim et al. 2014a,b
Mouse	GD 12.5	500 mg/kg i.p.	DL77 Donepezil	once daily for 21 days-	5, 10 or 15 mg/kg i.p. 1 mg/kg i.p.	8 weeks	Three chamber	↑ (10 and 15 mg/kg) ↑	Eissa et al., 2018
Rat	GD 12.5	500 mg/kg i.p.	URB597	2 h -	2 ml kg^{-1} (PND35) i.p. 1 ml kg^{-1} (PND90) i.p.	5 weeks 5–12 weeks	Social play Three chamber	↑ ↑	Servadio et al. (2016)
Rat	GD 12.5	600 mg/kg i.p.	Resveratrol	GD 6.5–18.5once daily	3.6 mg/kg s.c.	5–7 weeks	Three chamber	↑	Bambini-Junior et al., 2014
Rat	GD 12.5	500 mg/kg i.p.	Hesperitin	GD 0 – end lactation	10 mg/kg orally	5 weeks	Three chamber	↑	Khalajet al. 2018
Mouse	GD 12.5	600 mg/kg i.p.	Astaxanthin	PND 26-55	2 mg/kg orally	PND 56	Three chamber	↑	Al-Amin et al., 2015
Rat	GD 12.5	500 mg/kg s.c.	Mynocycline	PND 21–50 once daily before-	25 or 50 mg/kg orally	PND 44-45	Three chamber	↑	Kumar & Sharma 2016a,b
Rat	GD 12.5	400 mg/kg i.p.	Zinc	1 h after VPA	2 mg/kg s.c.	5 weeks	Social play	↑	Cezar et al. (2018)

*Time of treatment: before behavioral assessment.

2007).

In pregnant rats exposed to VPA on GD9, serotonergic neurons abnormally developed in the dorsal raphe nucleus of the offspring at postnatal day 50 (Miyazaki et al., 2005; Narita et al., 2002; Tsujino et al., 2007). Moreover, prenatal VPA administration increased 5-HT levels in the blood as well as in the frontal cortex, hippocampus, and cerebellum (Narita et al., 2002; Tsujino et al., 2007). A recent study (Wu et al., 2018) demonstrated that both deep brain stimulation (DBS) in the infralimbic PFC (ILPFC) and administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT into the same brain region improve sociability, anxiety and hyperlocomotion via modulation of the 5-HT system in rats prenatally exposed to VPA. Moreover, one-day ILPFC DBS, alone ineffective, combined with 8-OH-DPAT treatment significantly reversed the impaired sociability. These behavioral changes were associated with decreased expressions of the NR2B subunit of NMDAR and the β3 subunit of the GABAR type A in the PFC region.

2.2.3. Dopamine

The dopaminergic system affects a wide range of behaviors and functions, including cognition, attention, motor function, reward mechanisms, eating, drinking, sexual behaviors and neuroendocrine regulation. Interest in the role of dopamine (DA) in ASD began with the evidence that some DA blockers (i.e., antipsychotics) are effective in treating some behavioral impairment in ASD children (Anderson et al., 2007). Thus, several drugs targeting the dopaminergic system have been tested also in the VPA model. In particular, Hara et al. (2017b) tested the ability of the atypical antipsychotics risperidone and aripiprazole and the typical antipsychotic haloperidol to counteract the social interaction deficits, the recognition memory impairment and the reduction in dendritic spine density observed in the VPA mouse model of ASD. Chronic treatment with risperidone and aripiprazole, but not with haloperidol, improved the social interaction deficits, the recognition memory impairment and the reduction in prefrontal dendritic spine density in VPA-exposed mice. However, neurotransmitter systems other than dopamine may contribute to the effects induced by risperidone and aripiprazole in VPA-exposed mice. Indeed, besides having antidopaminergic (D2-like) activity, risperidone has prominent antiserotonergic (5-HT2A), antiadrenergic (α1) and antihistaminic (H1) activity, while aripiprazole has partial-agonist activity at D2 and 5-HT1A receptors, as well as antagonistic activity at 5-HT2A receptors. The effects of risperidone and aripiprazole in ASD have been also tested in the clinical setting. Both risperidone (McCracken et al., 2002; Shea et al., 2004) and aripiprazole (Marcus et al., 2009; Owen et al., 2009) were found effective in the treatment of the irritability displayed by ASD patients. However, unlike what observed in the VPA model of ASD (Hara et al., 2017b), risperidone (McCracken et al., 2002; Shea et al., 2004) and aripiprazole (Marcus et al., 2009; Owen et al., 2009) had no effects on social withdrawal in ASD patients.

It has been shown that chronic treatments with the ADHD drugs methylphenidate and atomoxetine ameliorate the deficit in social interaction and novel object recognition displayed by VPA-exposed mice and suppress the reduction in dendritic spine density induced by VPA in the prefrontal cortex. Interestingly, the effects of methylphenidate and atomoxetine on behaviors and dendritic spine morphology were antagonized by concomitant treatment with the dopamine-D1 receptor antagonist SCH39166 or the dopamine-D2 receptor antagonist raclopride, but not by the α2-adrenoceptor antagonist idazoxan, thus suggesting that these effects are mediated by activation of prefrontal DA-D1 and/or DA-D2 receptors (Hara et al., 2016).

Taken together, the present findings suggest that changes in dopaminergic neurotransmission may contribute to the behavioral deficits displayed by VPA-exposed mice. However, since the drugs used in the studies outlined above act not only at DA receptors, the contribution of other neurotransmitter systems in the observed effects cannot be excluded.

2.2.4. Oxytocin

Given that social impairment is a primary symptom of autistic disorder, the oxytocin (OXT) system has been investigated in ASD due its role in social and affiliative behaviors (Husarova et al., 2016; Insel, 2010; Insel et al., 1999). In accordance with evidence showing the pivotal role of OXT in social behaviors in rodent models (Ferguson et al., 2001; Pobbe et al., 2012), a reduction in OXT receptor binding in the amygdala has been also observed in the VPA model (Bertelsen et al., 2017). Furthermore, the study by Hara et al. (2017a) found that a single intranasal administration of OXT restored social interaction deficits for up to 2 h in mice prenatally exposed to VPA, but there was no effect on recognition memory impairments. Additionally, administration of OXT across 2 weeks improved VPA-induced social interaction deficits for at least 24 h. Moreover, intranasal administration of OXT increased c-Fos expression in the paraventricular nuclei, PFC, and somatosensory cortex, but not in the hippocampal CA1 and CA3 regions of VPA-exposed mice, suggesting the former regions contribute to the OXT effects. These findings suggest that OXT attenuates social interaction deficits through the activation of higher cortical areas and the PVN.

2.2.5. Acetycholine

The cholinergic system has been investigated in ASD mainly in relation to the cognitive deficits associated to the core ASD symptoms (Buckley et al., 2011; Handen et al., 2011). Rats and mice prenatally exposed to VPA show up-regulation of acetylcholinesterase (AChE) in the PFC, known as an important area for social recognition and social behavior (Vanderschuren et al., 2016; Yizhar, 2012). Subchronic treatment with the AChE inhibitor donepezil ameliorated social deficits and decreased repetitive digging behavior in VPA-exposed mice (Eissa et al., 2018; Kim et al., 2014a).

2.2.6. Histamine

There has been a growing interest in the study of the histaminergic system as a pharmacological target for the treatment of brain disorders, including ASD (Linday, 1997; Rossi et al., 1999). Notably, recent studies evaluated the effects of histamine receptor 3 (H3R) antagonists on the autistic-like behavioral deficits induce by VPA in mice. Baronio et al. (2015) demonstrated the efficacy of the acute administration of the H3R antagonist ciproxifan (CPX) in attenuating the impaired social behavior and stereotypies induced by prenatal VPA. Eissa et al. (2018) showed that subchronic administration of the novel potent and selective H3R antagonist DL77 ameliorated social interaction deficits and stereotypies in VPA-exposed mice. Importantly, the sociability- and social novelty-enhancing effect induced by DL77 was dose-dependent. These results provide evidence that modulation of brain histaminergic neurotransmission may serve as an effective therapeutic target to rescue ASD-like behaviors in VPA-exposed animals.

2.2.6. Endocannabinoid system

The endocannabinoid system is known to regulate key behaviors that are altered in ASD, such as social communication, social play and anxiety-like behaviors (Campolongo and Trezza, 2012; Kathuria et al., 2003; Trezza and Vanderschuren, 2008).

Increasing evidence suggests that endocannabinoid dysfunction may underlie the behavioral abnormalities observed in the VPA rat model (Kerr et al., 2013; Melancia et al., 2018; Servadio et al., 2016). Servadio et al. (2016) found changes in brain anandamide synthesis and metabolism in rats prenatally exposed to VPA; also, pharmacological modulation of anandamide metabolism through inhibition of its hydrolysis mitigated the social dysfunctions, the altered communicative abilities, the cognitive deficits, the stereotypies and the altered emotional reactivity displayed by VPA-exposed rats (Kerr et al., 2016; Servadio et al., 2016).

2.2.7. Oxidative stress/inflammation

Accumulating evidence has suggested a role for oxidative stress and

neuroinflammation in the embriotoxicity induced by VPA (Cipriani et al., 2018; Deckmann et al., 2018; Hegazy et al., 2015). It has been demonstrated that antioxidant pretreatment protects against VPA-induced teratogenesis in mice (Al Deeb et al., 2000; Zhang et al., 2010). Furthermore, VPA exposure resulted in increased expression of apoptotic markers that was attenuated by catalase supplementation (Tung and Winn, 2010).

Specifically, two studies investigated the preventive effects of prenatal resveratrol (RSV), a naturally occurring polyphenolic compound found in grapes, peanuts and red wine (Vang et al., 2011), on ASD-like phenotype induced by maternal challenge with VPA. Prenatal treatment with RSV ameliorated the alterations in social behavior (Bambini-Junior et al., 2014) and sensory integration (Fontes-Dutra et al., 2018) in VPA-exposed offspring. The mechanisms by which prenatal RSV counteracts the effects of VPA on postnatal behavior are unknown, as well as the potential effects of postnatal treatment with RSV on social impairment induced by prenatal VPA exposure. Among antioxidant treatments, hesperetin (Hst), a natural compound belonging to the flavanone class of flavonoids (Cho, 2006), has been also investigated. Hst administration, from conception through gestation and lactation, ameliorated the behavioral deficits and reduced the oxidative stress and inflammation induced by prenatal VPA (Khalaj et al., 2018). The administration of astaxanthin (AST), known for its antioxidant and neuroprotective effects in ischemia (Dose et al., 2016), improved the deficits in pain sensation, the altered locomotor activity, the impaired social behavior and the increased anxiety displayed by VPA-exposed mice (Al-Amin et al., 2015). Furthermore, VPA-treated mice showed an increased level of oxidative stress, that was counteracted by AST administration (Al-Amin et al., 2015).

Minocycline, a tetracycline antibiotic active against both gram-positive and gram-negative bacteria, has a variety of biological actions that are independent of its anti-microbial activity, including antioxidant, anti-inflammatory and neuroprotective effects (Garrido-Mesa et al., 2013). For this reason, it has been suggested that minocycline may have therapeutic potential in different psychiatric conditions (Dean et al., 2012). It has been shown that minocycline, given once daily for 30 days to VPA-exposed rats, improved their ASD-like behavior, including their reduced social interaction. Minocycline also attenuated the altered 5-HT levels and PFC mitochondrial complex activity exhibited by VPA-exposed animals. Moreover, minocycline decreased brain oxidative and nitrosative stress, inflammation, calcium and blood brain barrier permeability induced by prenatal VPA exposure (Kumar and Sharma, 2016b).

Increasing evidence also supports the design of dietary patterns and supplementation with antioxidants to protect brain function under adverse environmental challenges.

Zinc is an essential constituent of over 200 metalloenzymes participating in carbohydrate and protein metabolism and in nucleic acid synthesis, and it has antioxidant functions (Mistry and Williams, 2011). Cezar et al. (2018) investigated the potential therapeutic role of Zinc supplementation during pregnancy in the VPA model. Zinc treatment was able to alleviate the deficits in cognitive flexibility and the impairments in social play displayed by rats prenatally exposed to VPA. In addition, the altered vocalization pattern of these animals was attenuated.

The ketogenic diet, a high-fat low carbohydrate diet that improves mitochondrial function and decreases oxidative stress (Pinto et al., 2018), has been found to reverse the social deficits and mitochondrial dysfunction in rats prenatally exposed to VPA (Ahn et al., 2014).

Increasing evidence suggests that the vast repertoire of commensal bacteria within the gut plays a key role in CNS functioning and its alteration may contribute to neuropsychiatric disease risk. Several functions have been attributed to the gut microbiota, including degradation of non-digestible carbohydrates, protection against pathogens, activation of anti-inflammatory and antioxidant pathways, and stimulation of the immune system (Eshraghi et al., 2018; Rogers et al., 2016). The gut microbiota has been the subject of investigation as a contributing factor to ASD symptoms because there is evidence suggesting that alterations in the intestinal microflora are correlated with gastrointestinal and ASD symptom severity (Codagnone et al., 2019; Kelly et al., 2017; Li et al., 2017). It has been proposed that the neurobiological mechanisms underlying ASD may be caused and/or sustained by alterations in the composition of the gut resident commensal communities. Indeed, the disruption of the gut microbiota can alter the systemic antioxidant homeostasis and can trigger epigenetic changes, thus contributing to the pathogenesis of ASD (Eshraghi et al., 2018).

So far, only two studies (de Theije et al., 2014; Lim et al., 2017) investigated the relation between gut microbiota and ASD-like behavior in the VPA model, finding altered microbial colonization and activity in VPA-exposed animals, with preponderance in the male offspring (de Theije et al., 2014).

Altogether, the studies highlighted in this section indicate that antioxidant and anti-inflammatory agents of various sources mitigate the consequences induced by prenatal VPA exposure in rodents. Whether the same holds true for autistic children born from VPA-treated mothers remains to be determined.

2.3. The VPA rodent model to study the epigenetic contribution to ASD

Epigenetic processes play a key role in the etiology of neurodevelopmental disorders. They are responsible for the biological encoding of environmental influences, representing the meeting point of genes and environment (Dall'Aglio et al., 2018; LaSalle, 2013). Epigenetic processes can regulate gene expression via a number of mechanisms including DNA methylation, histone modifications and ATP-dependent chromatin remodeling, without affecting the underlying DNA sequences. Recently, epigenetic factors have been proposed to have a key role in the pathogenesis of ASD (LaSalle, 2013; Loke et al., 2015), and increasing evidence has been provided that diverse environmental factors might alter the typical brain maturation trajectory by interfering with DNA expression. For this reason, the role of VPA in epigenetic events has started to be the subject of intense investigation (Andrews et al., 2017). Further to the direct interaction of VPA exposure with several brain systems/pathways, it is worth remembering that VPA is also a non-selective inhibitor of histone deacetylase of class I and II (HDAC1 and HDAC2) expressed in the brain (Fukuchi et al., 2009). HDAC reduces the acetylation of histones, by inducing chromatin changes and thereby contributing to gene transcription regulation (Bannister and Kouzarides, 2011). Transient hyperacetylation of H3 and H4 histones induced by VPA during a critical window of embryonic mouse brain might play a key role in autism pathogenesis (Kataoka et al., 2013; Moldrich et al., 2013). Of note, exposure to valpromide, a VPA analog lacking HDAC inhibitory activity, neither affects behavior nor induces a transient increase in the levels of acetylated histones (Kataoka et al., 2013).

Moreover, VPA treatment changes the expression of various genes (Barrett et al., 2017; Cipriani et al., 2018; Kim et al., 2014b, 2016; Konopko et al., 2017; Stodgell et al., 2006; Wiltse, 2005), including the Hox genes that are critical to the early patterning of the embryo and the methyl-CpG-binding protein 2 (MECP2) gene, implicated in CNS maturation. More in detail, Hoxa1 is important in the development of the brainstem, as higher or lower Hoxa1 protein is teratogenic (Stodgell et al., 2006; Zhang et al., 1994). MeCP2 is the gene (located on X chromosome) responsible for Rett syndrome, an X-linked neurodevelopmental disorder, which shows commonalities with ASD-like behavioral phenotype (Amir et al., 1999). The potential for VPA of acting through epigenetic mechanisms is also supported by recent rodent studies indicating that the autism-like neurobehavioral phenotype shows transgenerational epigenetic inheritance (Choi et al., 2016; Tartaglione et al., 2018).

2.4. The VPA model: a tool to study sex differences in the social brain

Based on the reported higher incidence in males than in females, ASD is considered a prototypical sex-biased neurodevelopmental disorder. Early population prevalence studies of ASD estimated a 4:1 male-to-female ratio (Fombonne, 2002). More recently, it has been suggested that the true male-to-female ratio in ASD is lower than previously assumed, being closer to 3:1 (Loomes et al., 2017). This discrepancy is due to the fact that the existing assessment tools and diagnostic criteria may contain a sex/gender bias, meaning that girls who meet criteria for ASD are at disproportionate risk of not receiving a clinical diagnosis (Evans et al., 2018; Mandy et al., 2018).

Interestingly, the prevalence of ASD in children exposed to VPA during pregnancy is characterized by an even (1:1) male to female ratio (Rasalam et al., 2005; Schneider et al., 2008). In spite of this evidence, most of the preclinical studies that used the VPA model have focused on the male offspring; the few rodent studies considering the two sexes have shown that prenatal exposure to VPA induces ASD-like symptoms in both sexes. However, the effects of VPA in females are either different or milder than those observed in males (Anshu et al., 2017; Kazlauskas et al., 2016; Kim et al., 2013, 2016; Melancia et al., 2018; Schneider et al., 2008). Concerning the social domain, some authors reported that only male rats prenatally exposed to VPA show impairments in social behavior, while female rats exhibit normal levels on social interaction (Kim et al., 2013; Schneider et al., 2008). A similar result was obtained in mice (Kataoka et al., 2013).

It has been also suggested that VPA-exposed female rats may display age-dependent social deficits: thus, at adolescence, both VPA-exposed male and female rats showed atypical patterns of social play behavior. However, only the male but not the female VPA-exposed offspring showed enduring social deficits when tested at adulthood in the three-chamber test (Melancia et al., 2018). It should be noted, however, that other authors reported that both adult male and female VPA-exposed rats showed impaired sociability in the three-chamber apparatus (Anshu et al., 2017). Differences in the rat strain used (Wistar in the study by Melancia and coworkers and Sprague-Dawley in the study by Anshu and coworkers) and in the VPA dose administered during pregnancy [500 (Melancia et al., 2018) vs 450 (Anshu et al., 2017) mg/kg] may account for these controversial results.

The sexually dimorphic behavioral effects of prenatal VPA exposure may involve sex-specific differences in neurotransmission and in neuroendocrine and immune functions (Hara et al., 2015; Kim et al., 2013, 2016; Melancia et al., 2018; Schneider et al., 2008).

Specifically, Kim and coworkers (Kim et al., 2013) found perturbed synaptic maturation due to altered glutamatergic neuronal differentiation in VPA exposed male but not female rats. Interestingly, male, but not female rats prenatally exposed to VPA had significantly lower MeCP2 expression compared to control levels (Kim et al., 2016). Changes in MeCP2 levels may be responsible for the time-specific increase in postsynaptic density as well as in excitatory neuronal markers within four weeks of age in the VPA male offspring (Kim et al., 2016).

Moreover, prenatal VPA exposure has been found to decrease the expression of DA (both D1 and D2) receptors in the PFC of the male offspring, while the female offspring was unaffected (Hara et al., 2015). As previously described, DA neurotransmission is involved in motivational processes and incentive salience (Berridge, 2007; Salamone and Correa, 2012; Trezza et al., 2010) and the reduced DA activity could have a contributing role in the social deficits well documented in this model.

In line with these data, Melancia et al. (2018) reported that male rats prenatally exposed to VPA display altered phosphorylation of CB1 receptors in the dorsal striatum and hippocampus both at adolescence and adulthood and in the amygdala at adulthood only. Conversely, VPA-exposed female rats display altered activation of CB1 receptors only in the PFC at adolescence. The alterations observed in male rats prenatally exposed to VPA may contribute to their socio-emotional and cognitive deficits, given the well-known role of endocannabinoid neurotransmission within these brain areas in the control of emotional and cognitive states (Campolongo and Trezza, 2012; Freund et al., 2003; Trezza and Vanderschuren, 2008).

Another promising perspective to explain the sex differences in the effects induced by prenatal VPA exposure comes from recent molecular data supporting sex-dependent differences of epigenetic regulation in early ontogeny; the findings by Konopko and coworkers (Konopko et al., 2017) indicated that the interference of gestational VPA with brain-derived neurotrophic factor (BDNF) gene transcription is different in the female and male fetal brain. Furthermore, Perez-Pouchoulen et al. (2016) found that the expression levels of androgen receptor in the developing cerebellum are altered by VPA more in neonate females than in males. In line with these data, Tartaglione et al. (2018) reported sex differences in early sensorimotor development in VPA exposed offspring as well in the transcriptional activity of endogenous retroviruses, these latter found increased in ASD subjects (Balestrieri et al., 2012, 2014, 2016). All this considered, the VPA model lends itself to investigate the sex dimorphisms of the social brain, including sex hormone signaling in the brain regions and neurotransmitter systems implicated in social interaction and reward. This may ultimately shed light on sex differences and sex-dependent vulnerabilities in ASD.

3. Concluding remarks and future perspectives

Collectively, the preclinical studies described in the present review indicate that a single prenatal injection of VPA in rodents results in behavioral impairments resembling the core signs of ASD, supporting the high face validity for prenatal VPA as an animal model of autism. In agreement with the clinical data, rodents exposed to VPA in pregnancy show birth defects, deficits in neurodevelopment and cognitive/social anomalies of varying degree depending on dose and time of administration (see (Roullet et al., 2013)). Of course, in every VPA animal model, the replication of the core symptoms of ASD may be followed by detailed molecular investigations of how VPA induces ASD-like phenotypes. Indeed, as discussed in this review, several pathogenic mechanisms are thought to contribute to the increased ASD risk induced by prenatal VPA exposure (see Fig. 1).

Nonetheless, given the complex etiology of ASD, the VPA model explains only some aspects of ASD etiology, namely those related to a selected environmental perturbation in a critical developmental phase. However, the pathogenic cascade induced by VPA may share pathways with other etiological factors implicated in the development of the disease, including genetic, epigenetic and environmental (i.e. environmental chemicals, endocrine disruptors, maternal infections and obstetric complications) factors. Although we do not expect any rodent model to recapitulate the entire spectrum of brain and behavioral changes characteristic of ASD, prenatal administration of VPA in rodents has demonstrated to be a reliable tool to understand which processes are most vulnerable to environmental stressors during early neurodevelopment. The advantage of this model resides in its feasibility, as it is possible to identify critical windows for adverse effects on different levels/systems/pathways by simply varying the timing of prenatal VPA exposure.

Importantly, from a neurotoxicological perspective, the VPA model may help us to understand which pregnancies are most vulnerable to prenatal VPA, which gestational time points are most sensitive, how to safely manage (or replace) the VPA treatment during pregnancy to prevent deleterious effects on fetal brain development.

Furthermore, the behavioral and genetic alterations observed support the use of prenatal VPA exposure as an effective tool for the study of pathways underlying social dysfunction relevant to ASD in the search for earlier diagnosis and identification of biomarkers/potential therapeutic targets in human neurodevelopmental disorders. Last but not least, this model may be important to evaluate the possible roles of epigenetic changes associated with brain and behavior dysfunctions

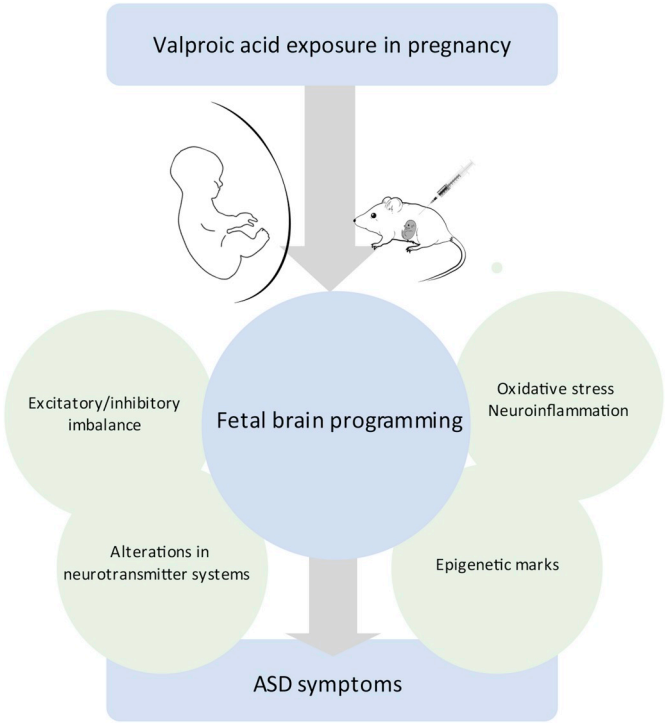

Fig. 1. Possible pathogenic mechanisms contributing to ASD risk by prenatal VPA exposure. Experimental studies on VPA rodent models suggest that VPA might affect brain programming by a complex set of intersecting pathways (i.e. excitatory/inhibitory imbalance, alterations in neurotransmitter systems involved in the modulation of emotional/social/cognitive behaviors in mammals, oxidative stress and neuroinflammation, epigenetic marks). Interference with one or more of these pathways may explain the increased risk of ASD in children exposed *in utero* to VPA.

caused by exposure to chemicals, specifically during embryogenesis.

Conflicts of interest

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past five years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CHAPTER 6

Sex specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signaling

Running title: Sex specific valproate-induced autism and anandamide

Francesca Melancia[1*], Sara Schiavi[1*], Michela Servadio[1], Veronica Cartocci[1], Patrizia Campolongo[2], Maura Palmery[2], Valentina Pallottini[1], Viviana Trezza[1#]

[1]Department of Science, Section of Biomedical Sciences and Technologies, University 'Roma Tre', Rome, Italy,

[2]Department of Physiology and Pharmacology 'V. Erspamer', Sapienza University of Rome, Rome, Italy

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Sex specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signaling.

The present paper is the result of the second aim of my PhD project: find new therapeutic opportunities to treat social dysfunctions in neuropsychiatric disorders, and in particular in ASD. Given the well-known role of endocannabinoids in the modulation of socio-emotional behavior, in the present paper, I investigated the involvement of the endocannabinoid system in the ASD-like socio-emotional, cognitive and repetitive symptoms displayed by rats prenatally exposed to VPA, with special emphasis on sex-specific differences. I first validated the ASD-like symptoms observed in this preclinical model, and then I analyzed the functionality of the endocannabinoid system in different brain areas of rats of both sexes prenatally exposed to VPA. Last, I tested the ability of the selective anandamide hydrolysis inhibitor URB597 to rescue the atypical behaviors displayed by both male and female rats prenatally exposed to VPA in the course of development.

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RESEARCH PAPER

Sex-specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signalling

Correspondence Viviana Trezza, PhD, Department of Science, Section of Biomedical Sciences and Technologies, University 'Roma Tre', Viale G. Marconi 446, Rome 00146, Italy. E-mail: viviana.trezza@uniroma3.it

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Francesca Melancia[1,*], Sara Schiavi[1,*], Michela Servadio[1], Veronica Cartocci[1], Patrizia Campolongo[2], Maura Palmery[2], Valentina Pallottini[1] and Viviana Trezza[1]

[1]Department of Science, Section of Biomedical Sciences and Technologies, University 'Roma Tre', Rome, Italy, and [2]Department of Physiology and Pharmacology 'V. Erspamer', Sapienza University of Rome, Rome, Italy

*Francesca Melancia and Sara Schiavi made equal contributions to this study.

BACKGROUND AND PURPOSE

Autism spectrum disorder (ASD) is more commonly diagnosed in males than in females. Prenatal exposure to the antiepileptic drug valproic acid (VPA) is an environmental risk factor of ASD. Male rats prenatally exposed to VPA show socio-emotional autistic-like dysfunctions that have been related to changes in the activity of the endocannabinoid anandamide. Here, we have investigated if prenatal VPA induced sex-specific autistic endophenotypes involving anandamide signalling.

EXPERIMENTAL APPROACH

We studied sex-specific differences in the ASD-like socio-emotional, cognitive and repetitive symptoms displayed during development of Wistar rats of both sexes, prenatally exposed to VPA. The involvement of anandamide was followed by Western blotting of cannabinoid CB_1 receptors and by inhibiting its metabolism.

KEY RESULTS

Female rats were less vulnerable to the deleterious effects of prenatal VPA exposure on social communication, emotional reactivity and cognitive performance than male rats. Conversely, as observed in male rats, prenatal VPA exposure induced selective deficits in social play behaviour and stereotypies in the female rat offspring. At the neurochemical level, prenatal VPA exposure altered phosphorylation of CB_1 receptors in a sex-specific, age-specific and tissue-specific manner. Enhancing anandamide signalling through inhibition of its degradation reversed the behavioural deficits displayed by VPA-exposed animals of both sexes.

CONCLUSIONS AND IMPLICATIONS

These findings highlight sexually dimorphic consequences of prenatal VPA exposure that may be related to sex-specific effects of VPA on endocannabinoid neurotransmission in the course of development and introduce a new therapeutic target for reversing autistic-like symptoms in both sexes.

Abbreviations

% OE, percentage of open-arm entries; % TO, percentage of time spent in the open arms; ASDs, autism spectrum disorders; GD, gestational day; PND, postnatal day; USVs, isolation-induced ultrasonic vocalizations; VPA, valproic acid

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Introduction

The term autism spectrum disorder (ASD) refers to a group of pervasive developmental psychiatric disorders, emerging in early life and characterized by impairments in social interaction, restricted communication abilities and stereotyped/repetitive behaviours (American Psychiatric Association, 2013). Common co-morbid features include anxiety and intellectual disability (Lai *et al*., 2014). The aetiology of ASD is still controversial, involving both environmental and genetic factors (Kim and Leventhal, 2015; Karimi *et al*., 2017).

One of the most striking but consistent findings in ASD epidemiology is the higher rate of diagnosis in males than in females, with a recently suggested 3:1 male-to-female ratio (Loomes *et al*., 2017). Sex differences in the phenotypic presentation of the disease might lead to missed or delayed diagnosis in females (Rivet and Matson, 2011a,b; Lai and Baron-Cohen, 2015; Bargiela *et al*., 2016). Compared with females, males with ASD show more aggression, hyperactivity, reduced prosocial behaviour and increased repetitive/restricted behaviours (Werling and Geschwind, 2013). Conversely, females with ASD often camouflage the autistic core deficits with better language and social competences (Lehnhardt *et al*., 2016; Hull *et al*., 2017), and they often have lower average intellectual abilities than males (Banach *et al*., 2009).

As the male predominance in ASD has been long documented, most clinical and epidemiological studies have been conducted on the male population (Rivet and Matson, 2011a, b). The lack of research focused on gender differences in ASD is mirrored at the preclinical level, where animal models of ASD have been mainly developed and validated in males (Beery and Zucker, 2011; Kokras and Dalla, 2014). Nevertheless, it is essential that preclinical research is performed on animals of both sexes, to enhance the validity of animal models and contribute to gender-oriented prevention, diagnosis and treatment of psychiatric disorders (Hughes, 2007).

Valproic acid (**VPA**) is a widely used and effective antiepileptic and mood stabilizer drug. However, VPA is also a known teratogen and, when given during pregnancy, it can induce various congenital malformations (Kozma, 2001; Kini, 2006), including autistic-like features in the exposed children, such as impaired communication, reduced sociability and stereotyped behaviours. For this reason, prenatal VPA exposure is a recognized environmental risk factor for ASD (Williams and Hersh, 1997; Williams *et al*., 2001).

Based on thsee clinical observations, prenatal VPA exposure in rodents is a widely used environmental preclinical model of ASD with face and construct validity (Williams *et al*., 2001; Schneider and Przewlocki, 2005; Wagner *et al*., 2006; Roullet *et al*., 2013; Sabers *et al*., 2014; Servadio *et al*., 2015, 2016). This model, however, has been mainly validated and used in male rodents.

Here, we have performed a longitudinal study from birth to adulthood to evaluate potential sexually dimorphic effects induced in rodents by prenatal VPA exposure on core and comorbid behavioural traits that are frequently impaired, often in a sex-specific manner, in ASD patients: (i) social communication, (ii) social behaviour, (iii) social discrimination, (iv) stereotypies, (v) anxiety and (vi) cognitive performance. Furthermore, because the endocannabinoid system plays a key role in brain development (Maccarrone *et al*., 2014), it has been recently involved in ASD (Siniscalco *et al*., 2013; Chakrabarti *et al*., 2015; Zamberletti *et al*., 2017) and it is modulated by sex hormones during critical developmental ages (Viveros *et al*., 2011). Here, we have investigated its role in the sexually dimorphic behavioural consequences of prenatal VPA exposure.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU), and were approved by the Italian Ministry of Health (Rome, Italy). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al*., 2010; McGrath and Lilley, 2015).

Primiparous female Wistar rats (Charles River, Calco (Lecco), Italy), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day (GD) 1. Pregnant rats were singly housed in Macrolon cages (40 × 26 × 20 cm; $l \times w \times h$), under controlled conditions (temperature 20–21°C, 55–65% relative humidity, 12/12 h light cycle with lights on at 07:00 h, standard bedding (Charles River), enriched environmental conditions [wooden toys and irradiated certified diamond twists (Envigo, Italy)] and food and water *ad libitum*). On GD 12.5, dams received a single i.p. injection of either sodium valproate (VPA, n = 54) or saline (SAL, n = 53). On postnatal day (PND) 1, litters were culled to four males and four females. On PND 21, pups were weaned and housed in groups of three. The experiments were carried out on the male and female offspring during infancy (PNDs 5, 9 and 13), adolescence (PND 35) and adulthood (PND 90) (Figure 1). One pup per litter from different litters per treatment group (SAL or VPA) was randomly used in each experiment. Sample size (n) was based on our previous experiments and power analysis with the software GPower. Potential outliers within each data set were calculated using the GraphPad software. Sample size is indicated in the figure legends. All behavioural tests were assessed by a trained observer who was unaware of the treatments.

To collect brain samples for the Western blot experiments, at PNDs 35 and 90, rats were rapidly decapitated, as it is known that other killing methods such as anaesthetic overdose or CO_2 inhalation can alter brain neurochemistry (Woodbury *et al*., 1958; Karmarkar *et al*., 2010; Pierozan *et al*., 2017), thus affecting the results of the biochemical experiments described here. At the end of the behavioural experiments, rats were killed by CO_2 inhalation.

Drug administration

VPA was dissolved in saline and administered at a dose (500 mg·kg^{-1} on GD 12.5) that induces autistic-like behavioural changes in male rat offspring (Servadio *et al*., 2016). The anandamide hydrolysis inhibitor **URB597** was dissolved in 5% Tween 80/5% polyethylene glycol/saline and administered i.p. at a dose of 0.050 mg·kg^{-1}: URB597 was administered 2 h before each behavioural test (Servadio *et al*., 2016), as *in vivo* experiments have shown that, 2 h after URB597

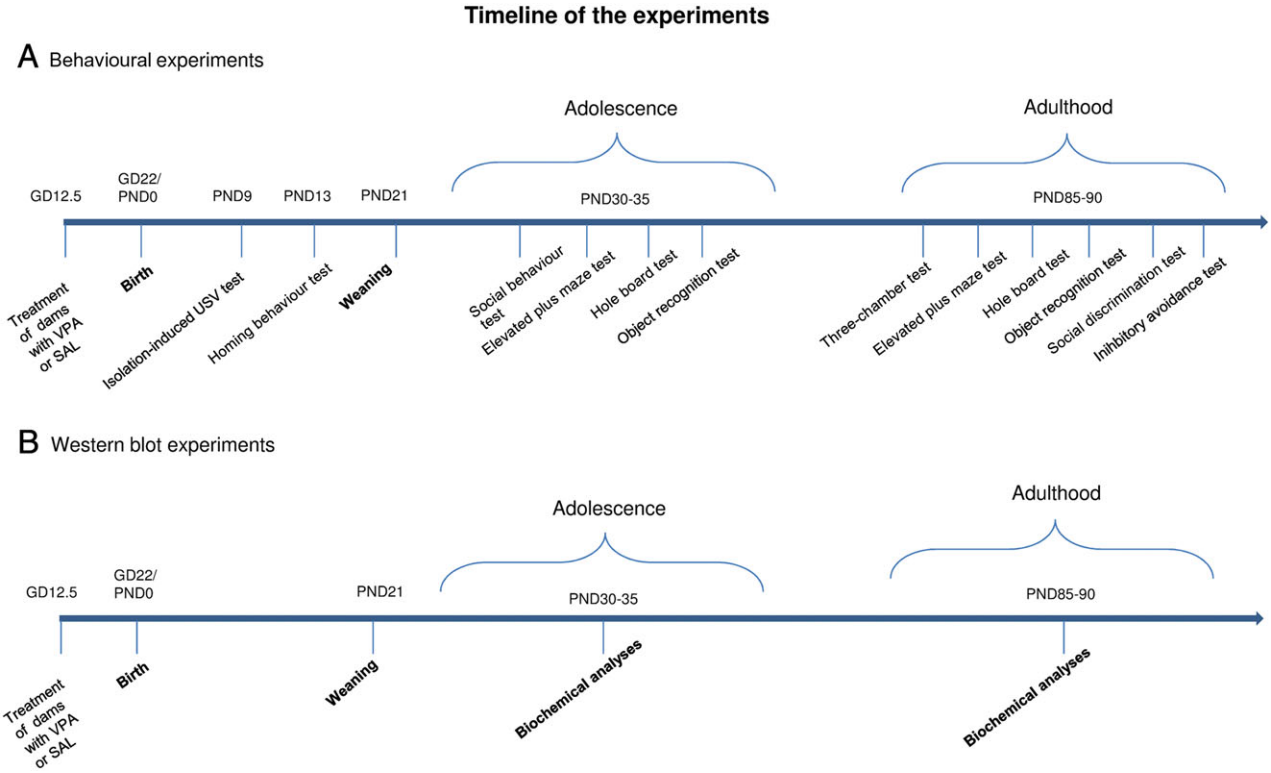

Figure 1
Timeline of the behavioural (A) and biochemical (B) experiments.

injection, the enzyme hydrolysing anandamide, **fatty acid amide hydrolase**, is maximally inhibited and **anandamide** levels are significantly increased (Gobbi *et al.*, 2005; Kathuria *et al.*, 2003). In the inhibitory avoidance test only, URB597 was administered immediately after the acquisition trial, in order to exclude any drug-induced variability in the training phase (e.g. pain sensitivity, motivation and locomotion). Solutions were administered in a volume of 2 mL·kg^{-1} at adolescence and 1 mL·kg^{-1} at adulthood.

Determination of the oestrous cycle

To rule out the possibility that any change observed in the female offspring could be due to cycle fluctuations, we monitored the oestrous cycle in both VPA-exposed and control females, to ensure that they would always be tested at the same phase of the oestrous cycle.

Western blot analysis of phosphorylated and total CB_1 receptors

Rats were rapidly decapitated, and their brains were removed and cut into coronal slices on a cold plate. The prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus, amygdala and cerebellum were dissected by hand under microscopic control within 2 min. Tissues were stored at −80°C until use. Lysates and protein separation were performed as previously described (Servadio *et al.*, 2016). Immunoblots were incubated with primary antibodies against CB_1 receptors and against phosphorylated CB_1 receptors (1:500) (Santa Cruz Biotechnology, Dallas (Texas), USA), followed by secondary peroxidase-conjugated antibodies (1:3000) (Santa Cruz Biotechnology). Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare, UK). Sample loading was normalized with anti-tubulin (Sigma-Aldrich, Milano, Italy) antibody. Bound antibodies to proteins on nitrocellulose were visualized by using enhanced chemoluminescence detection (GE Healthcare) and exposure to ChemiDoc Imaging System (Bio-Rad, Milano, Italy). Images were analysed with ImageJ (National Institutes of Health, MD, USA). Western blot experiments were performed in duplicate.

Behavioural tests

Isolation-induced ultrasonic vocalizations. On PND 9, the isolation-induced ultrasonic vocalizations (USVs) emitted by each pup removed from the nest and placed into a Plexiglas arena were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Germany) sensitive to frequencies between 10 and 200 kHz. The USVs were analysed quantitatively using Avisoft Recorder software (version 5.1).

Homing behaviour. On PND 13, following 30 min of isolation, each pup was placed for 4 min into a box whose floor was covered for 1/3 with bedding from the pup's home cage and for 2/3 with clean bedding. The following parameters were scored using the Observer 3.0 software (Noldus, The Netherlands): latency (s) to reach the home cage bedding and total time (s) spent in the nest bedding area (Scattoni *et al.*, 2008).

Social play behaviour. The test was performed in a sound-attenuated chamber under dim light conditions, as previously described (Trezza and Vanderschuren, 2008; Trezza and Vanderschuren, 2009). The 35-day-old rats were individually habituated to the test cage for 10 min on the two days before testing. On the test day, the animals were isolated for 3 h before testing. The test consisted of placing VPA-exposed or SAL-exposed rats together with an untreated animal for 15 min.

The following parameters were scored for each animal of a pair using the Observer 3.0 software (Noldus) (Trezza *et al.*, 2010):

- Pinning: the most characteristic posture of social play in rats that occurs when one animal is solicited to play by its test partner and rotates to its dorsal surface with the other animal standing over it.
- Pouncing: one animal is soliciting the other to play, by attempting to nose or rub the nape of the neck of the partner.
- Partial rotation: upon nape contact, the recipient animal rotates along its longitudinal axis but then stops and keeps one or both hind feet firmly planted on the ground.
- Evasion: upon solicitation, the recipient animal avoids nape contact by leaping, running or turning away from the partner.
- Social exploration: sniffing any part of the body of the test partner.
- Play responsiveness: the percentage of response to play solicitation, calculated as the probability of an animal of being pinned in response to play solicitation (pouncing) by the stimulus partner.

Three-chamber test. The test was performed as previously described (Servadio *et al.*, 2016). The apparatus was a rectangular three-chamber box, with two lateral chambers (30 × 35 × 35 cm; $l \times w \times h$) connected to a central chamber (15 × 35 × 35 cm; $l \times w \times h$). Each lateral chamber contained a small Plexiglas cylindrical cage. At PND 90, each experimental rat was individually allowed to explore a three-chamber apparatus for 10 min and then confined in the central compartment. An unfamiliar stimulus animal was confined in a cage located in one chamber of the apparatus, while the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to explore the apparatus for 10 min. The per cent of time spent in social approach (sniffing the stimulus animal) and the percentage of time spent exploring the empty chamber were scored using the Observer 3.0 software (Noldus).

Hole board test. The test was performed in a sound-attenuated chamber under dim light conditions, as previously described (Makanjuola *et al.*, 1977; Servadio *et al.*, 2016). The apparatus consisted of a grey square metal table (40 × 40 × 10 cm; $l \times w \times h$) with 16 evenly spaced holes (4 cm in diameter), inserted in a Plexiglas arena (40 × 40 × 60 cm; $l \times w \times h$). Each rat was individually placed in the apparatus for 5 min. Each session was recorded with a camera positioned above the apparatus for subsequent behavioural analysis performed using the Observer 3.0 software (Noldus). Dipping behaviour was scored by the number of times an animal inserted its head into a hole at least up to the eye level.

Elevated plus maze. The elevated plus maze apparatus comprised two open (50 × 10 × 40 cm^3; $l \times w \times h$) and two closed arms (50 × 10 × 40 cm^3; $l \times w \times h$) that extended from a common central platform (10 × 10 cm^2). Rats were individually placed on the central platform for 5 min and allowed to explore the apparatus. The following parameters were scored using the Observer 3.0 software (Noldus):

- % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100;
- % open arm entries (% OE): (number of entries into the open arms of the maze/number of entries into open + closed arms) × 100.

Social discrimination. The test was performed at PND 90. Briefly, animals were isolated for 7 days before testing. The test consisted of a learning trial and a retrieval trial, which were separated by a 30 min intertrial interval. During the learning trial, a juvenile (30 days old), unfamiliar rat was introduced into the home cage of the experimental rat for 5 min. The time spent by the experimental rat investigating (sniffing, allogrooming and following) the juvenile was measured. Thirty minutes after, the juvenile used in the learning trial was returned to the same adult's cage together with a novel juvenile. The time spent by the adult exploring the novel and the familiar juveniles was monitored for 5 min. The discrimination index was calculated as the difference in time exploring the novel and the familiar animal, expressed as the percentage ratio of the total time spent exploring both animals (Campolongo *et al.*, 2007).

Novel object recognition. On the training trial, each rat was individually placed into an open-field arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 5 min. Thirty minutes later, one copy of the familiar object (A3) and a new object (B) were placed in the same location as during the training trial. Each rat was placed in the apparatus for 5 min, and the time spent exploring each object was recorded. The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects (Campolongo *et al.*, 2013).

Inhibitory avoidance. On the first day, 90-day-old rats were individually placed in the illuminated compartment of an inhibitory avoidance apparatus (Ugo Basile, Gemonio (Varese), Italy). After 10 s, the sliding door was opened, and the time taken by the animal to enter into the dark compartment was measured (latency). Once the animal entered the dark compartment, the sliding door was closed, and a mild shock (0.6 mA) was delivered through the floor for 2 s. Twenty-four hours later, the animal was placed in the lit compartment, and the latency to re-enter (retention

latency) the dark compartment was recorded (Campolongo *et al.*, 2007).

Locomotor activity. To determine whether the behavioural effects induced by prenatal VPA exposure were secondary to any change in locomotor activity, at PND 35 and PND 90, rats from both experimental groups were individually placed into an open-field arena, and their locomotor activity was scored for 5 min as follows: a grid, dividing the arena into equally sized squares, was projected over the recordings, and the number of line crossings made by the animal was recorded using the Observer 3.0 software (Noldus).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). Data are expressed as mean ± SEM. To assess the effects of the prenatal treatments (VPA or SAL) in the male and female offspring, the behavioural and biochemical data were analysed by two-way ANOVA, with treatment and sex as factors. Two-way ANOVA was also used to assess the effects of prenatal (VPA or SAL) and postnatal (URB597 or vehicle) treatments.

The accepted value for significance was $P < 0.05$. If main or interaction effects were significant, the Student–Newman–Keuls *post hoc* test was used for individual group comparisons. The software Sigma Plot (13.0; Systat Software, Inc, USA) was used for statistical analysis of the data.

Materials

The compounds used in these studies were supplied as follows: VPA was supplied by Cayman (Michigan, USA) and URB597 by Sigma.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

Reproduction data

No differences in body weight gains were observed between VPA-treated and SAL-treated dams. Prenatal VPA exposure did not affect pregnancy length, litter size at birth, male/female ratio, pup weight gain and postnatal vitality (Supporting Information Table S1).

Locomotor activity

Although there was a significant effect of sex on locomotor activity (two-way ANOVA), no differences between VPA-exposed and control animals of both sexes were found in locomotor activity both at adolescence and adulthood (data not shown).

Sex-specific effects of prenatal VPA exposure on social communication and social discrimination in the infant rat offspring

Prenatal VPA exposure differentially affected the USVs emitted by male and female pups separated from the nest on PND 9, with no effect of sex or of prenatal treatment but a significant interaction of (sex × prenatal treatment). Indeed, VPA-exposed male but not female pups vocalized significantly less (Figure 2A) compared with SAL-exposed pups. As

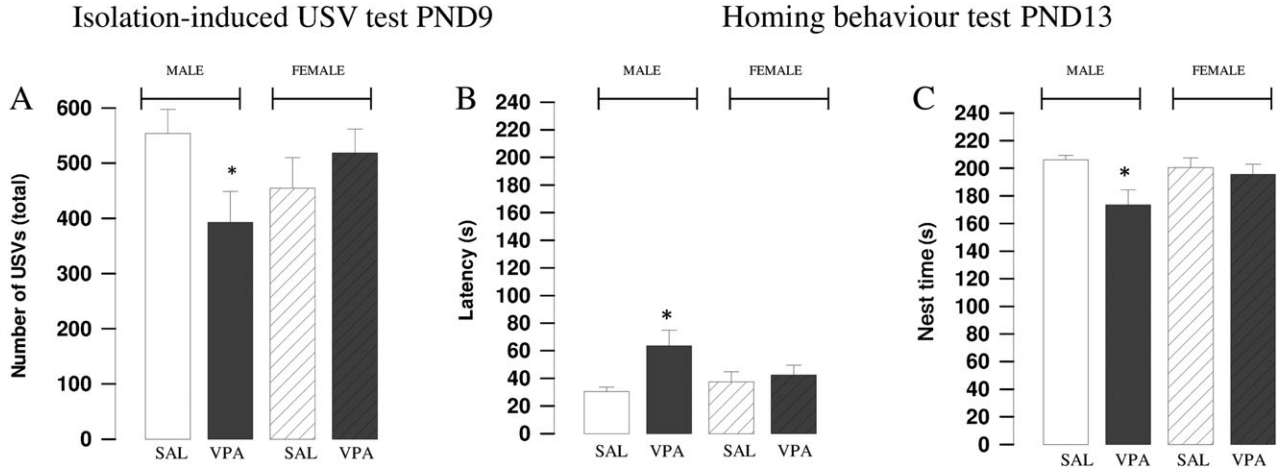

Figure 2

Sex-specific effects of prenatal VPA exposure on social communication and social discrimination in the infant rat offspring. (A) VPA-exposed male but not female pups vocalized significantly less compared with SAL-exposed pups (male: SAL, $n = 13$ and VPA, $n = 17$; female: SAL, $n = 13$ and VPA, $n = 16$). When tested in the homing behaviour test, the male but not the female offspring prenatally exposed to VPA displayed a lower latency to reach the home cage bedding (B) and spent less time in the nest area (C) compared with SAL-exposed male animals (male: SAL, $n = 24$ and VPA, $n = 26$; female: SAL, $n = 10$ and VPA, $n = 14$). Data are means ± SEM. *$P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

for the parameters measured in the homing behaviour test, analysis with two-way ANOVA showed significant effects only for prenatal treatment. *Post hoc* analysis revealed that VPA-exposed male but not female pups showed longer latency to reach the home cage bedding (Figure 2B) and spent less time in the nest area (Figure 2C) compared with SAL-exposed pups.

Sex-specific effects of prenatal VPA exposure on core and secondary autistic-like features in the adolescent rat offspring

A two-way ANOVA analysis performed on the parameters measured in the social play behaviour test gave the following results. For frequency of pinning (male: SAL = 15.6 ± 1.9, VPA = 13.7 ± 1.9; female: SAL = 3.8 ± 1.9, VPA = 2.7 ± 1.8) and for frequency of pouncing (male: SAL = 38.1 ± 3.4, VPA = 44.5 ± 3.4; female: SAL = 15.8 ± 3.4, VPA = 21.2 ± 3.1), there were significant effects of sex only. For the frequency of partial rotation there were significant effects of sex and prenatal treatment, for frequency of evasion there were significant effects of prenatal treatment and for play responsiveness (male: SAL = 49.9 ± 4.8, VPA = 32.8 ± 4.6; female: SAL 23.0 ± 4.3, VPA 9.4 ± 4.3) there were significant effects of sex and prenatal treatment. *Post hoc* analysis revealed that, when solicited to play by the test partner, VPA-exposed male and female rats responded with an increased frequency of evasion (Figure 3A) and partial rotation (Figure 3B) than SAL-exposed rats. However, general social exploration, although showing significant effects of sex differing between sexes, was not affected by prenatal exposure to VPA in both males and females (data not shown).

Prenatal VPA exposure induced sex-specific effects in the offspring tested in the hole board test with head dipping being affected significantly by sex, prenatal treatment and the interaction. In the elevated plus maze test, values for %

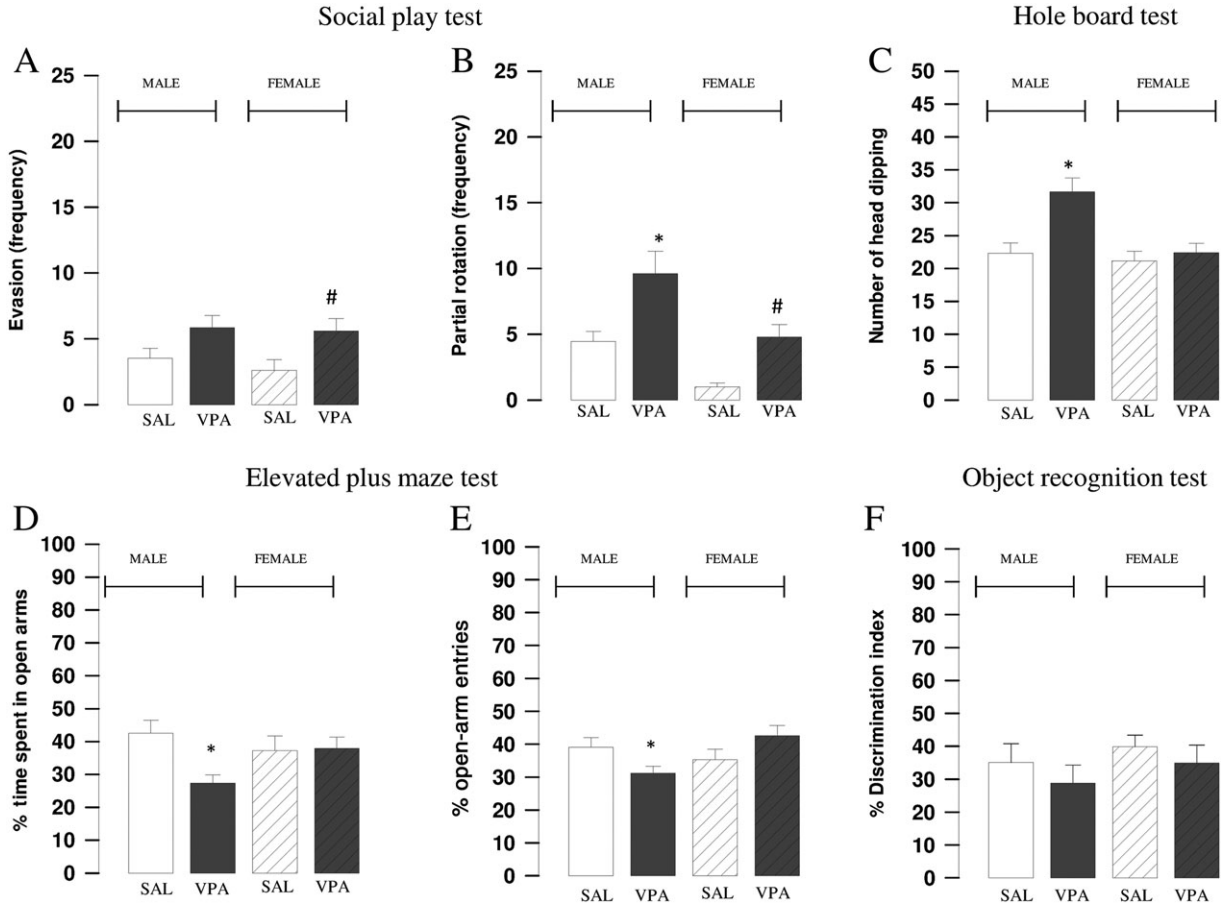

Figure 3

Effects of prenatal VPA exposure on core and secondary autistic-like features in the male and female rat offspring at PND 35. VPA-exposed male and female rats responded to play solicitation with an increased frequency of evasion (A) and partial rotation (B) compared with SAL-exposed animals (male: SAL, $n = 13$ and VPA, $n = 13$; female: SAL, $n = 13$ and VPA, $n = 15$). VPA-exposed males but not females showed stereotypic behaviours in the hole board test (C) compared with SAL-exposed male animals (male: SAL, $n = 12$ and VPA, $n = 12$; female: SAL, $n = 15$ and VPA, $n = 15$). VPA-exposed males but not females spent less time in the open arms (D) and made less open entries (E) in the elevated plus maze test compared with SAL-exposed male animals (male: SAL, $n = 23$ and VPA, $n = 21$; female: SAL, $n = 13$ and VPA, $n = 13$). No differences among groups were found in the object recognition test (F) (male: SAL, $n = 7$ and VPA, $n = 11$; female: SAL, $n = 10$ and VPA, $n = 11$). Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

TO were affected only by the interaction, as were the values for % OE, measured at PND 35. Indeed, VPA-exposed males but not females showed increased head-dipping behaviour in the hole board test (Figure 3C), spent less time in the open arms of the elevated plus maze (Figure 3D) and made less open-arm entries (Figure 3E) compared with SAL-exposed animals. VPA-exposed male and female animals did not show impaired novel object recognition at PND 35 (Figure 3F).

Sex-specific effects of prenatal VPA exposure on core and secondary autistic-like features in the adult rat offspring

A two-way ANOVA analysis performed on the percentage of time spent by the experimental rat sniffing the stimulus animal in the three-chamber test showed there were significant effects of sex only. *Post hoc* analysis revealed that 90-day-old male, but not female, rats prenatally exposed to VPA showed decreased sociability, as they spent less time sniffing the stimulus animal (Figure 4A) compared with SAL-exposed rats. No difference between VPA-exposed and SAL-exposed animals of both sexes was found in the percentage of time spent exploring the empty chamber.

Prenatal VPA exposure induced stereotypic behaviour in the adult offspring of both sexes with frequency of head dipping showing there were significant effects of sex and of prenatal treatment. Compared with SAL-exposed animals, both VPA-exposed adult males (Figure 4B) and females (Figure 4B) showed an increased number of head dippings.

Prenatal VPA induced sex-specific effects in the adult offspring tested in the elevated plus maze test with % TO values showing there were significant effects of sex and the interaction (sex x prenatal treatment), whereas for the % OE values there were significant effects of sex, prenatal treatment and the interaction. Indeed, VPA-exposed males but not females spent less time in the open arms (Figure 4C) and made less open-arm entries (Figure 4D) compared with SAL-exposed animals.

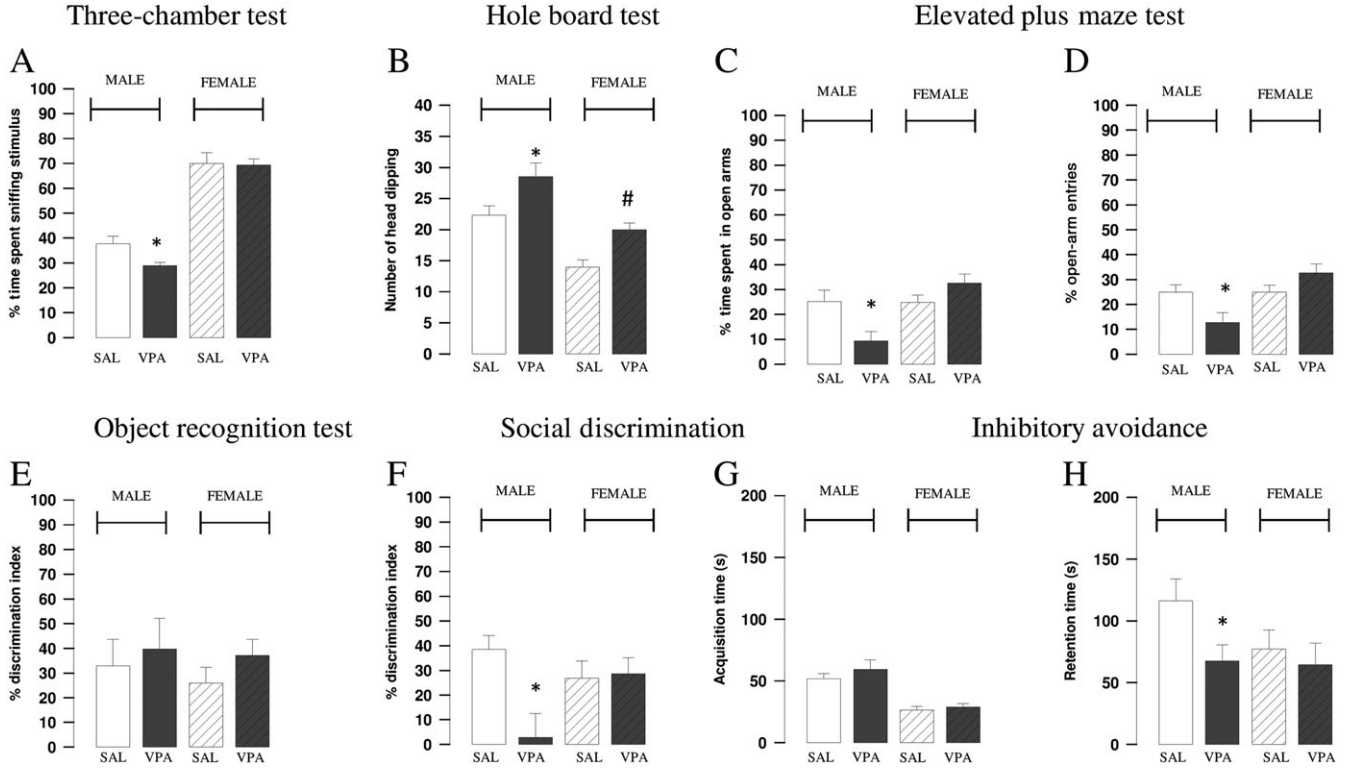

Figure 4

Effects of prenatal VPA exposure on core and secondary autistic-like features in the male and female rat offspring at PND 90. Prenatal VPA exposure reduced sociability of male but not female rats in the three-chamber test (A) (male: SAL, $n = 9$ and VPA, $n = 9$; female: SAL, $n = 8$ and VPA, $n = 7$) while it induced stereotypic behaviour in the hole board test both in male and female animals (B) (male: SAL, $n = 12$ and VPA, $n = 12$; female: SAL, $n = 15$ and VPA, $n = 15$). VPA-exposed male but not female rats spent less time in the open arms (C) and made less open-arm entries (D) in the elevated plus maze test (male: SAL, $n = 13$ and VPA, $n = 10$; female: SAL, $n = 16$ and VPA, $n = 16$). No differences among groups were found in the object recognition test (E) (male: SAL, $n = 12$ and VPA, $n = 10$; female: SAL, $n = 15$ and VPA, $n = 16$). Male but not female rats prenatally exposed to VPA showed impaired social discrimination (F) (male: SAL, $n = 11$ and VPA, $n = 11$; female: SAL, $n = 10$ and VPA, $n = 10$). No differences among groups were found in the acquisition trial of the inhibitory avoidance test (G). However, male but not female rats prenatally exposed to VPA showed impaired memory consolidation during the retention session (H) (male: SAL, $n = 15$ and VPA, $n = 15$; female: SAL, $n = 12$ and VPA, $n = 14$). Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

Similar to the test results obtained on PND 35, VPA-exposed male and female animals did not show impaired novel object recognition at PND 90 (Figure 3E). However, VPA exposure did induce sex-specific deficits in social discrimination, by prenatal treatment and for the interaction. VPA-exposed adult males but not females showed a lower discrimination index (Figure 4F) compared with SAL-exposed animals.

Approach latencies during the first trial of the inhibitory avoidance test were similar among groups, although there were significant effects of sex (Figure 4G). However, after 24 h, prenatal VPA exposure induced sex-specific effects on memory retention . Males, but not females, prenatally exposed to VPA had retention latencies significantly shorter than SAL-exposed rats (Figure 4H), indicating a decreased ability of VPA-exposed males to consolidate aversive memories.

Sex-specific, age-specific and tissue-specific changes in phosphorylation of CB_1 receptors

Phosphorylated CB_1 receptors, which may reflect the activation of this receptor (Garcia *et al.*, 1998; Daigle *et al.*, 2008), are abundant in brain areas such as the nucleus accumbens and the amygdala (Orio *et al.*, 2009), which are also involved in anandamide modulation of social reward (Trezza *et al.*, 2012). To investigate whether VPA prenatal exposure induced changes in the activity of male and female brain CB receptors, we measured phosphorylated and total CB_1 receptor protein expression in prefrontal cortex, dorsal striatum, hippocampus, amygdala, nucleus accumbens and cerebellum of male and female rats at PNDs 35 and 90. The results of the two-way ANOVA analyses performed for each brain region at both ages are shown in Supporting Information Table S2 (ratio between phosphorylated and total CB_1 receptors and Supporting Information Table S3 (total content of CB_1 receptors), while the original Western blots for each data set are shown in Figure 5. *Post hoc* analyses revealed that, compared with SAL-exposed animals, VPA-exposed males but not females displayed increased phosphorylation of CB_1 receptors at PNDs 35 and 90 in the dorsal striatum (Figure 5A, E), reduced phosphorylation of CB_1 receptors in the hippocampus at both ages (Figure 5C, G) and only at PND 90 in the amygdala (Figure 5H). Conversely, VPA-exposed females displayed increased phosphorylation of CB_1 receptors in the prefrontal cortex only at PND 35 (Figure 5B).

Pharmacological blockade of anandamide hydrolysis corrects the partial ASD-like symptoms displayed by VPA-exposed female rats

Systemic administration of the anandamide hydrolysis inhibitor URB597 has been found to rescue the communicative deficits, the socio-emotional alterations and the stereotypies displayed by VPA-exposed male rats (Servadio *et al.*, 2016). Here, we showed that URB597 also mitigated the altered social play patterns displayed by VPA-exposed female rats at PND 35. For frequency of partial rotation, there were significant effects of prenatal treatment (VPA), of treatment (URB597) and of the interaction (prenatal treatment x treatment). URB597 was also able to mitigate the stereotypic behaviour displayed by female rats at PND 90, as significant effects of prenatal treatment and the interaction were found. Indeed, *post hoc* analyses showed that at PND 35, VPA-exposed females responded to play solicitation with a higher frequency of partial rotation (Figure 6A) and evasion (Figure 6B) compared with SAL-exposed rats. However, when they were treated with URB597, the frequency of partial rotation was normalized to the level of SAL-exposed rats, while the frequency of evasion was attenuated. Furthermore, VPA-exposed females displayed stereotypies at PND 90 (Figure 6C). However, when they were treated with URB597, this parameter was normalized to the level of SAL-exposed rats.

Pharmacological blockade of anandamide hydrolysis corrects the cognitive deficits displayed by VPA-exposed male rats

Systemic administration of URB597 counteracted the deficits displayed by VPA-exposed adult males in social discrimination (significant effects of treatment and the interaction). For the inhibitory avoidance task, the acquisition time showed no significant effects and retention time was significantly affected only by the interaction (prenatal treatment x treatment). *Post hoc* analysis revealed that, compared with SAL-exposed rats, VPA-exposed animals showed impaired social discrimination (Figure 6D) and memory retention (Figure 6F), which were normalized when VPA-exposed animals were treated with URB597.

Discussion

We found that prenatal exposure to VPA, an environmental risk factor for ASD, induced sex-specific autistic-like features in the rat offspring. While VPA induced a wide range of socio-emotional and cognitive deficits in the male offspring in the course of development, the female offspring was only partly affected. Despite these sex-specific endophenotypes, we identified a pharmacological target to correct the behavioural deficits displayed by VPA-exposed rats of both sexes.

Persistent deficits in social communication and social interaction are key features of ASD, and it has been suggested that the delayed or missed diagnosis of ASD in girls may be related to marked sex differences in the phenotypic presentation of these symptoms (Rivet and Matson, 2011b; Lai and Baron-Cohen, 2015; Bargiela *et al.*, 2016). Consistent with this possibility, at infancy, male but not female pups prenatally exposed to VPA showed communicative deficits, since they emitted less USVs than control pups when isolated from the dam and siblings. In rodents, these USVs play a fundamental role in mother–offspring interactions, as they induce maternal retrieval and elicit care-giving behaviours in the dam (Servadio *et al.*, 2015).

Because olfaction and, in particular, the learned association between maternal odours and maternal stimulation, is essential for the proper development of social behaviour and social recognition (Melo *et al.*, 2006), we next analysed the ability of male and female pups to discriminate between a neutral odour and their own nest odour in the homing behaviour test. While male pups prenatally exposed to VPA showed early deficits in social discrimination in this test, VPA-exposed female pups showed intact discriminative

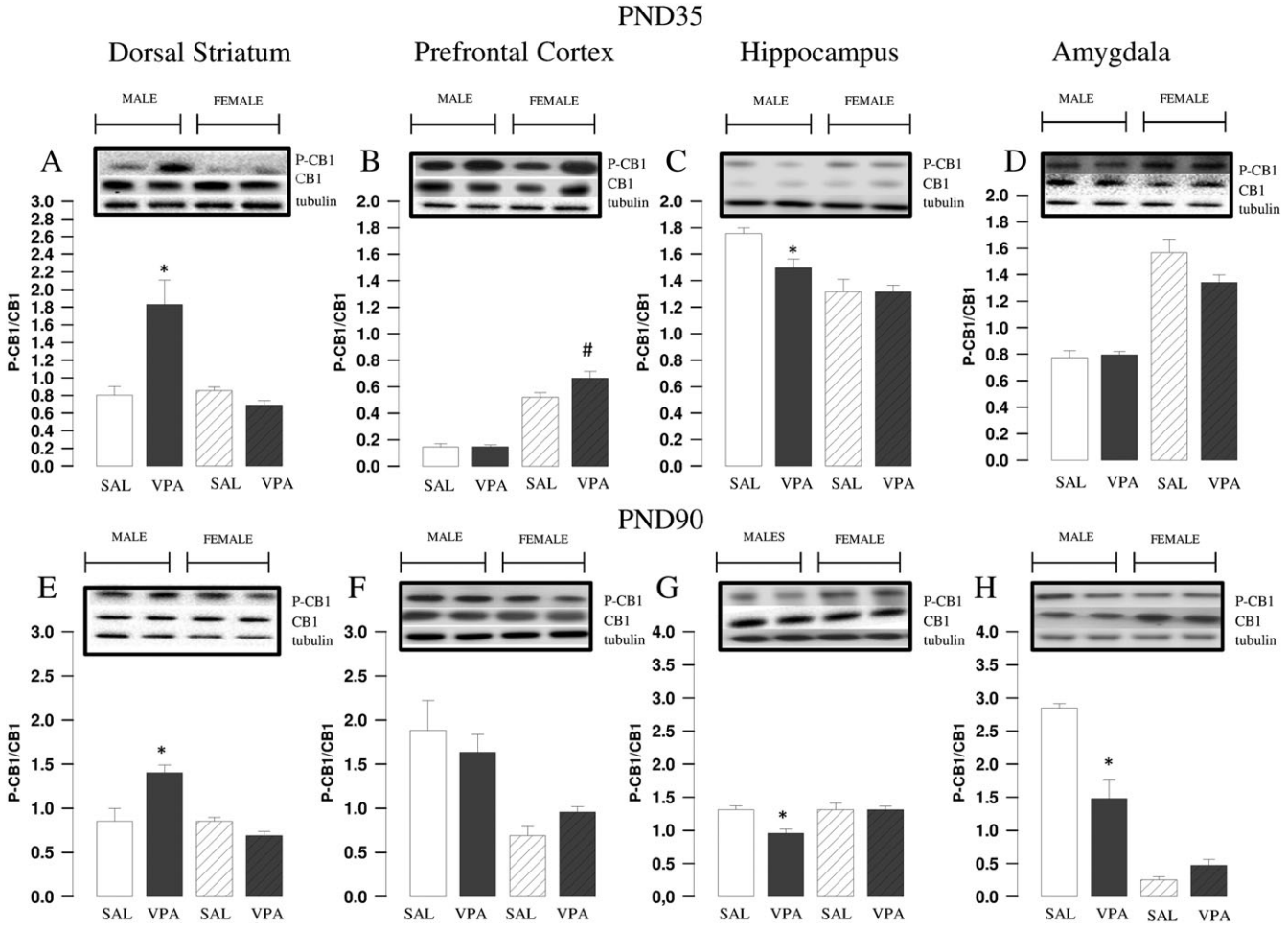

Figure 5

Sex-specific, age-specific and tissue-specific changes in phosphorylation of CB_1 receptors induced by prenatal VPA exposure. VPA-exposed male but not female rats displayed altered phosphorylation of CB_1 receptors in dorsal striatum [PND 35 (A), male: SAL, $n = 5$ and VPA, $n = 5$; female: SAL, $n = 5$ and VPA, $n = 6$; PND 90 (E), male: SAL, $n = 5$ and VPA, $n = 5$; female: SAL, $n = 5$ and VPA, $n = 6$] and hippocampus [PND 35 (C), male: SAL, $n = 10$ and VPA, $n = 8$; female: SAL, $n = 5$ and VPA, $n = 6$; PND 90 (G), male: SAL, $n = 8$ and VPA, $n = 9$; female: SAL, $n = 5$ and VPA, $n = 6$]. VPA-exposed male but not female rats displayed reduced phosphorylation of CB_1 receptors at PND 90 in the amygdala (H) (male: SAL, $n = 6$ and VPA, $n = 10$; female: SAL, $n = 5$ and VPA, $n = 6$). Conversely, VPA-exposed female rats displayed increased phosphorylation of CB_1 receptors in the prefrontal cortex only at PND 35 (B) (male: SAL, $n = 6$ and VPA, $n = 6$; female: SAL, $n = 4$ and VPA, $n = 5$). Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

abilities. The sex-related differences observed in the homing behaviour test may be due to a sex-specific alteration of the olfactory system in the course of development. Furthermore, given the role of the endocannabinoid system in olfactory processes (Soria-Gomez *et al.*, 2014), changes in endocannabinoid activity may also be involved.

Together, these results show that, at infancy, female rat pups are less vulnerable than males to the deleterious effects induced by prenatal VPA exposure on social communication and social discrimination.

In rats, play behaviour peaks during adolescence, and it is considered the first form of non-maternal-oriented social behaviour, whose practice is crucial for social, cognitive, emotional and sensorimotor development (Vanderschuren *et al.*, 2016). At adolescence, both VPA-exposed male and female rats showed atypical patterns of social play behaviour. They responded to play solicitation mainly by partial rotation and evasion, rather than reciprocating the playful interaction. However, only the male but not the female VPA-exposed offspring showed enduring social deficits when tested at adulthood in the three-chamber test. A possible explanation of this finding is that prenatal VPA exposure induces in male rats a wide range of social impairments in the course of development, ranging from social play deficits at adolescence to altered sociability at adulthood. Conversely, the social deficits displayed by female rats prenatally exposed to VPA may be restricted to play-related behaviours at adolescence. To support this possibility, 4-week-old male but not female rats prenatally exposed to VPA showed altered sociability in the three-chamber test (Kerr *et al.*, 2016).

These findings are in line with the observation that female children, adolescents and adults with ASD demonstrate fewer socio-communicative symptoms compared with their male counterparts (Lai *et al.*, 2011; Dworzynski *et al.*, 2012;

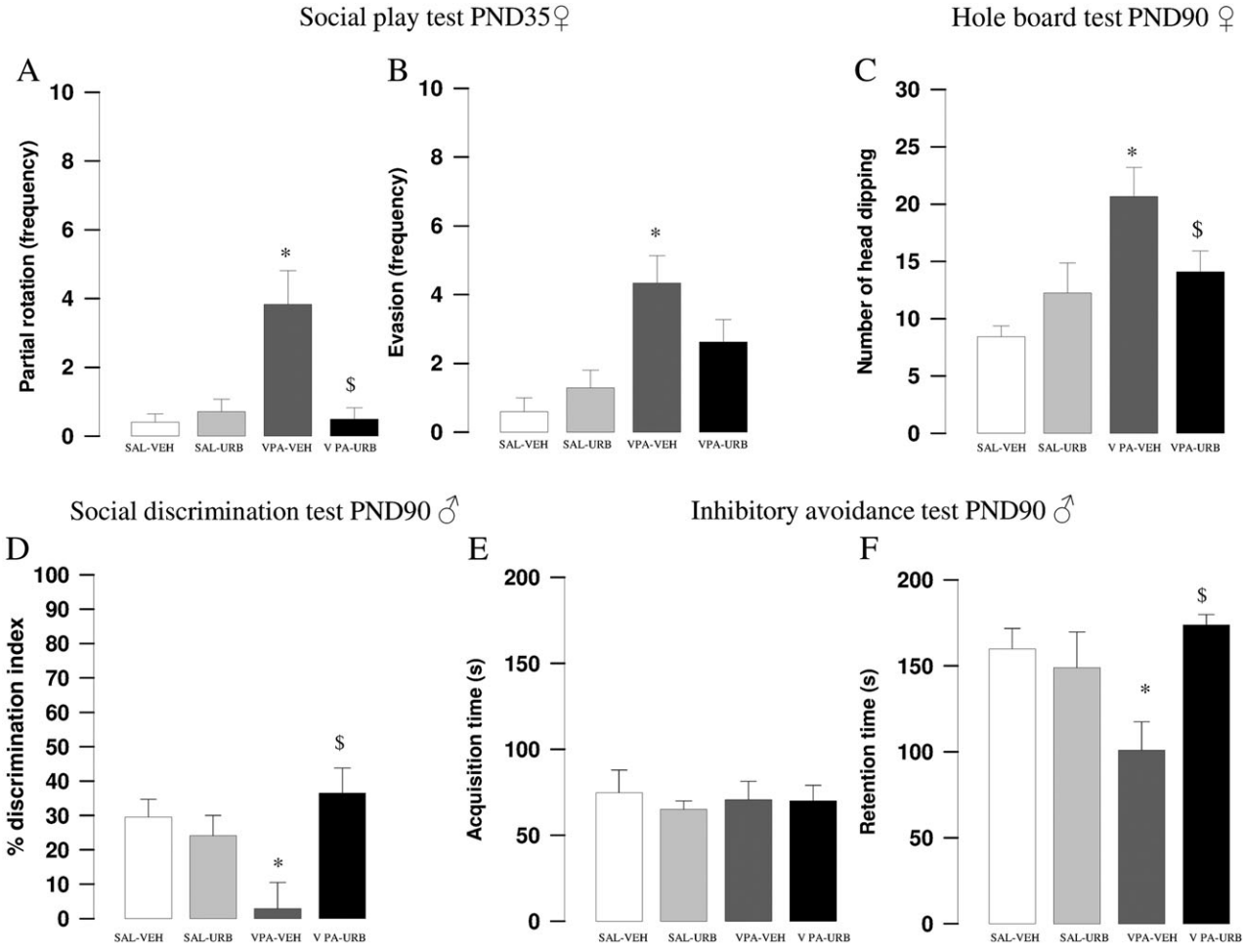

Figure 6

Pharmacological interference with anandamide hydrolysis corrects the partial behavioural alterations displayed by VPA-exposed female rats and the cognitive deficit found in VPA-exposed male rats. The administration of URB597 normalized the altered pattern of social play behaviour (A, B) displayed by VPA-exposed female rats at PND 35 [SAL-vehicle (VEH), $n = 5$; SAL-URB, $n = 7$; VPA-VEH, $n = 6$; and VPA-URB, $n = 8$] and their stereotypic behaviour at PND 90 (C) (SAL-VEH, $n = 7$; SAL-URB, $n = 8$; VPA-VEH, $n = 9$; and VPA-URB, $n = 10$). URB597 also reversed the deficits displayed by VPA-exposed male rats in the social discrimination (D) (SAL-VEH, $n = 8$; VPA-VEH, $n = 9$; SAL-URB, $n = 8$; and VPA-URB, $n = 8$) and inhibitory avoidance (E, F) (SAL-VEH, $n = 14$; VPA-VEH, $n = 12$; SAL-URB, $n = 8$; and VPA-URB, $n = 7$) tests. Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL-VEH group; $^{\$}P < 0.05$, significantly different from VPA-VEH group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

Head *et al.*, 2014), making it difficult to recognize ASD in female subjects on the basis of their social and communicative competences.

Stereotypies and/or restricted interests are the second core symptoms of ASD and seem to be more evident in boys rather than in girls with ASD (Hartley and Sikora, 2009; Supekar and Menon, 2015). Accordingly, VPA-exposed male rats showed marked stereotypies both at adolescence and adulthood. Conversely, as previously reported (Schneider *et al.*, 2008), VPA-exposed female rats showed stereotypic behaviours only at adulthood. Interestingly, behavioural differences were found between male and female rats in baseline condition, with control females spending more time sniffing the stimulus cage of the three-chamber apparatus and making less head dips in the hole board test compared with control males. These sex-specific differences may be due to a more explorative behaviour found in females rather than males, as previously reported (Lynn and Brown, 2009).

Anxiety is a common co-morbid feature displayed by autistic patients (Lai *et al.*, 2014). We found that VPA-exposed male, but not female, rats showed an anxious-like phenotype both in adolescence, as previously reported (Schneider *et al.*, 2008), and in adulthood.

ASD patients frequently display atypical cognitive performance, such as impaired social cognition (Lai *et al.*, 2014), while object recognition is often intact (Dawson *et al.*, 2002). Interestingly, female patients demonstrate better access to emotionally salient memories than males (Goddard *et al.*, 2014). Consistent with these findings, we found that male, but not female, rats prenatally exposed to VPA showed deficits in social discrimination from infancy till adulthood. Furthermore, VPA-exposed male but not female adult rats showed impaired emotional memory in the inhibitory avoidance task. Both VPA-exposed male and female rats, however, showed intact object recognition, as previously reported in male animals only (Schneider *et al.*, 2007; Banerjee *et al.*, 2014).

The attenuated ASD-like phenotype found in VPA-exposed female rats mirrors the sex differences in the symptoms displayed by ASD patients. It is unlikely that these sex differences are due to interference by sex hormones, with the teratogenic potential of VPA. Indeed, equal morphological changes are observed in the brain of male and female rats exposed to the same dose of VPA used in the present study (Favre *et al.*, 2013). Moreover, oestrogen receptors are not detected in the rat brain before GD 16 (Miranda and Toran-Allerand, 1992). It is more likely that the ASD-like attenuated phenotype found in VPA-exposed female rats arise in the course of development. Indeed, sexual dimorphism exists in the expression of genes related to ASD, with males being more susceptible than females to perturbations in genes involved in synaptic plasticity. Furthermore, sexually dimorphic neural pathways are involved in synaptic structure, function and plasticity. Thus, a higher male-to-female ratio in autism may arise because males have a lower threshold than females for aberrant changes in synaptic dynamics during development following a genetic or environmental insult (Mottron *et al.*, 2015). In line with this possibility, a perturbed synaptic maturation due to an altered glutamatergic neuronal differentiation was found in VPA-exposed male but not female rats (Kim *et al.*, 2013).

Endocannabinoids play a key role in brain development and synaptic plasticity (Maccarrone *et al.*, 2014), and they have been recently involved in ASD. Indeed, a consistent number of studies indicated that endocannabinoids modulate several behaviours and processes that are compromised in ASD. Furthermore, an impaired endocannabinoid activity has been observed in preclinical models of ASD (Kerr *et al.*, 2013; Zamberletti *et al.*, 2017) and in ASD patients (Siniscalco *et al.*, 2013). Sex hormones have been found to modulate brain endocannabinoid activity, thus influencing cannabinoid-mediated physiopathological processes (Fattore and Fratta, 2010; Viveros *et al.*, 2011). Thus, an alternative explanation to the sexually dimorphic behavioural consequences of prenatal VPA exposure may involve a sex-specific effect on endocannabinoid neurotransmission in the course of development.

In line with this possibility, we here report that male rats prenatally exposed to VPA display altered phosphorylation of CB_1 receptors in the dorsal striatum and hippocampus both at adolescence and adulthood and in the amygdala at adulthood only. Conversely, VPA-exposed female rats display altered activation of CB_1 receptors only in the prefrontal cortex at adolescence. The altered activation of CB_1 receptors found in the dorsal striatum, hippocampus and amygdala of male rats prenatally exposed to VPA may underlie their profound behavioural deficits in the socio-emotional and cognitive domains, given the well-known role of endocannabinoid neurotransmission within these brain areas in the control of emotional and cognitive states (Freund *et al.*, 2003).

Enhancement of anandamide activity by inhibiting its degradation ameliorates the socio-emotional and communicative deficits and the stereotypies displayed by VPA-exposed male rats (Kerr *et al.*, 2016; Servadio *et al.*, 2016). Here, we have extended these findings by showing that pharmacological interference with anandamide metabolism ameliorated (i) the deficits in social discrimination and emotional memory displayed by VPA-exposed adult male rat and (ii) the atypical social phenotype and the stereotypies displayed by VPA-exposed female rats.

Overall, two main conclusions can be drawn from our results. First, sex-specific changes in endocannabinoid neurotransmission may underlie the deleterious effects of environmental risk factors on ASD-relevant behaviours. Second, although more studies are needed to test the consequences of chronic inhibition of anandamide hydrolysis, our study points to an important role of this endocannabinoid in the autistic-like traits displayed by male and female VPA-exposed rats. Thus, the endocannabinoid system may be a therapeutic target for the core and associated symptoms displayed by autistic patients of both sexes.

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Author contributions

S.S., F.M. and M.S. performed, analysed and contributed to the design of the behavioural experiments. P.C., V.P. and M.P. contributed to the design of the experiments and edited the manuscript. V.C. performed, analysed and contributed to the design of the biochemical experiments. S.S. and F.M. wrote the manuscript. V.T. supervised the project, designed the experiments and wrote the manuscript.

Conflict of interest

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 5 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table S1 Reproduction parameters.
Table S2 Two-way ANOVA analyses performed on the Western blot data (ratio between phosphorylated and total CB1 cannabinoid receptor) for each brain region at PNDs 35 and 90.
Table S3 Two-way ANOVA analyses performed on the Western blot data (total CB1 cannabinoid receptor) for each brain region at PNDs 35 and 90.

CHAPTER 7

Reward-related behavioral, neurochemical and electrophysiological changes in a rat model of autism based on prenatal exposure to valproic acid

Sara Schiavi[1*], Daniela Iezzi[2*], Antonia Manduca[1], Stefano Leone[1], Francesca Melancia[1], Carmen Carbone[2], Michele Petrella[3], Guido Mannaioni[2], Alessio Masi[2,3*#] and Viviana Trezza[1*#]

[*] equal contribution

[1]Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Rome, Italy;

[2]Department of Neuroscience, Psycology, Drug Research and Child Health -NEUROFARBA-, Section of Pharmacology and Toxicology.University of Florence, Florence, Italy.

[3]School of Pharmacy, University of Camerino, Via Madonna delle Carceri. Cap 62032 Camerino, (MC), Italy.

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Reward-related behavioral, neurochemical and electrophysiological changes in a rat model of autism based on prenatal exposure to valproic acid.

In order to pursuit the second aim of my PhD project and find new therapeutic opportunities to treat social dysfunctions in ASD, in the present paper I investigated the involvement of the brain reward system in social abnormalities displayed by rats prenatally exposed to VPA. In particular, I aimed to clarify whether the altered social behavior displayed by VPA-exposed rats may be due to either a deficit in social reward processing or to a more general inability to properly understand and respond to social signals. I addressed these issues by behavioral, electrophysiological and neurochemical experiments.

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Reward-Related Behavioral, Neurochemical and Electrophysiological Changes in a Rat Model of Autism Based on Prenatal Exposure to Valproic Acid

Sara Schiavi[1†], Daniela Iezzi[2†], Antonia Manduca[1], Stefano Leone[1], Francesca Melancia[1], Carmen Carbone[2], Michele Petrella[3], Guido Mannaioni[2], Alessio Masi[2,3†] and Viviana Trezza[1*†]*

[1]Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Rome, Italy, [2]Department of Neuroscience, Psychology, Drug Research and Child Health -NEUROFARBA-, Section of Pharmacology and Toxicology, School of Psychology, University of Florence, Florence, Italy, [3]School of Pharmacy, University of Camerino, Camerino, Italy

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Edited by:
João Peça,
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Rong Zhang,
Neuroscience Research Institute, Peking University, China
Victorio Bambini-Junior,
University of Central Lancashire, United Kingdom

***Correspondence:**
Alessio Masi
alessio.masi@unifi.it
Viviana Trezza
viviana.trezza@uniroma3.it

[†]These authors have contributed equally to this work

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Prenatal exposure to the antiepileptic drug valproic acid (VPA) induces autism spectrum disorder (ASD) in humans and autistic-like behaviors in rodents, which makes it a good model to study the neural underpinnings of ASD. Rats prenatally exposed to VPA show profound deficits in the social domain. The altered social behavior displayed by VPA-exposed rats may be due to either a deficit in social reward processing or to a more general inability to properly understand and respond to social signals. To address this issue, we performed behavioral, electrophysiological and neurochemical experiments and tested the involvement of the brain reward system in the social dysfunctions displayed by rats prenatally exposed to VPA (500 mg/kg). We found that, compared to control animals, VPA-exposed rats showed reduced play responsiveness together with impaired sociability in the three-chamber test and altered social discrimination abilities. In addition, VPA-exposed rats showed altered expression of dopamine receptors together with inherent hyperexcitability of medium spiny neurons (MSNs) in the nucleus accumbens (NAc). However, when tested for socially-induced conditioned place preference, locomotor response to amphetamine and sucrose preference, control and VPA-exposed rats performed similarly, indicating normal responses to social, drug and food rewards. On the basis of the results obtained, we hypothesize that social dysfunctions displayed by VPA-exposed rats are more likely caused by alterations in cognitive aspects of the social interaction, such as the interpretation and reciprocation of social stimuli and/or the ability to adjust the social behavior of the individual to the changing circumstances in the social and physical environment, rather than to inability to enjoy the pleasurable aspects of the social interaction. The observed neurochemical and electrophysiological alterations in the NAc may contribute to the inability of VPA-exposed rats to process and respond to social cues, or, alternatively, represent a compensatory mechanism towards VPA-induced neurodevelopmental insults.

Keywords: autism, valproate, social play behavior, dopamine, electrophysiology

INTRODUCTION

Although the precise causes of autism spectrum disorder (ASD) are still the subject of significant debate, a number of factors (rare gene mutations, gene variations and adverse environmental events) have been identified that, interacting in complex ways, affect early brain development contributing to the risk of ASD. Among the environmental factors involved in the pathogenesis of ASD, it has been well documented that prenatal exposure to the antiepileptic drug valproic acid (VPA) is associated with increased risk of neurodevelopmental delay and autistic symptoms in the offspring. Indeed, when given during gestation, VPA not only increases the risk for various congenital malformations (Kozma, 2001; Kini et al., 2006), but also induces core autistic symptoms in the offspring, i.e., impaired communication, reduced sociability and stereotyped behaviors (Williams and Hersh, 1997; Williams et al., 2001). Based on this clinical evidence, prenatal exposure to VPA in rodents has been validated as a drug-induced preclinical model of ASD (Roullet et al., 2013; Nicolini and Fahnestock, 2018; Tartaglione et al., 2019). In agreement with the clinical data, rodents exposed to VPA during pregnancy show marked behavioral impairments resembling the core and secondary signs of ASD (Rodier et al., 1996; Narita et al., 2002; Miyazaki et al., 2005; Schneider and Przewłocki, 2005; Servadio et al., 2016; Melancia et al., 2018). Since impaired social interaction is a key feature of ASD, a valid animal model is expected to exhibit deficits in this behavioral domain. Rodents are highly social species that engage in complex patterns of social behavior such as parental care and social play behavior (Panksepp et al., 1984; Ricceri et al., 2007). Rats prenatally exposed to VPA show a wide range of deficits in the social domain. At infancy, they show deficits in social communication and social discrimination, i.e., they are unable to properly communicate with their mother and siblings when removed from the nest and cannot discriminate between a neutral scent and their own nest odor (Schneider and Przewłocki, 2005; Dufour-Rainfray et al., 2010; Favre et al., 2013; Servadio et al., 2016; Bronzuoli et al., 2018; Cartocci et al., 2018; Melancia et al., 2018). At adolescence, VPA-exposed rats display atypical patterns of social play behavior, that is the most characteristic and rewarding social activity displayed by young mammals: indeed, compared to control animals, rats prenatally exposed to VPA respond to play solicitation mainly by partial rotation and evasion, rather than reciprocating the playful interaction (Servadio et al., 2016; Melancia et al., 2018). The social deficits displayed by VPA-exposed rats are long lasting, since they also persist at adulthood (Schneider and Przewłocki, 2005; Schneider et al., 2006, 2008; Markram et al., 2008; Kim et al., 2011, 2014; Servadio et al., 2016, 2018; Hirsch et al., 2018; Melancia et al., 2018; Fontes-Dutra et al., 2019) and are evocative of the social disturbances displayed by autistic patients over the course of development.

The pervasive social deficits found in autistic patients have been initially explained in terms of cognitive impairments and inability to infer others' mental states. More recently, they have been related to blunted social reward processing, i.e., inability to enjoy and prolong reciprocal social interactions, which has been hypothesized to be the consequence of abnormal activity of the brain reward circuit in social contexts (Chevallier et al., 2012; Pellissier et al., 2018). Along this line, the social dysfunctions displayed by VPA-exposed rats may be caused by either their inability to properly understand and respond to social signals by the social partner or by a failure of their reward system to assign a positive value to the social experience. The aim of the present study was to address this issue by performing behavioral, neurochemical and electrophysiological experiments in rats prenatally exposed to VPA. In particular, we determined whether the social deficits displayed by VPA-exposed rats are associated with changes in more specific reward-related behaviors, including social, drug and food rewards. Furthermore, given the important role of corticolimbic dopamine in (social) reward processes (Gunaydin et al., 2014; Vanderschuren et al., 2016), we measured the expression of D1 and D2 dopamine receptors in the prefrontal cortex (PFC), dorsal striatum (DS), nucleus accumbens (NAc) and hippocampus (HIPP) of VPA-exposed rats, since these brain areas play an important role in the modulation of social behavior. Last, since activation of dopaminergic terminals in the NAc of rats during bouts of interaction with novel conspecifics has been reported (Robinson et al., 2002; Gunaydin et al., 2014) and given the important role of NAc dopamine in rewarding forms of social interaction such as social play (Manduca et al., 2016), we addressed the role of the NAc in the social impairment displayed by VPA-exposed rats by performing electrophysiological experiments in this brain area.

MATERIALS AND METHODS

Animals

Female Wistar rats (Charles River, Italy), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day 1. Pregnant rats were singly housed in Macrolon cages [40 (length) × 26 (width) × 20 (height) cm], under controlled conditions (temperature 20–21°C, 55%–65% relative humidity and 12/12 h light cycle with lights on at 07:00 h). Food and water were available *ad libitum*. On gestational day 12.5, females received a single intraperitoneal injection of either VPA or saline (SAL). Newborn litters found up to 17:00 h were considered to be born on that day [postnatal day (PND) 0]. On PND 1, the litters were culled to eight animals (six males and two females), to reduce the litter size-induced variability in the growth and development of pups during the postnatal period. On PND 21, the pups were weaned and housed in groups of three. The experiments were carried out on the male offspring during adolescence (PNDs 35–40) and adulthood (PNDs 90–95). One male pup per litter from different litters per treatment group was used in each experiment. For the flow cytometric experiments, we used brain samples from the VPA- and SAL-exposed rats tested in the social play behavior, the three-chamber and the social discrimination tests. Other cohorts of VPA- and SAL-exposed rats were used for the electrophysiology experiments and to investigate amphetamine-

induced hyperlocomotion, sucrose preference and socially-induced Conditioned Place Preference (sCPP). The exact sample size (n) for each experimental group/condition is indicated in the figure legends. The sample size was based on our previous experiments and power analysis performed with the software G power.

The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the Animals in Research: Reporting *in vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2010), with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU. In particular, the experimental protocol was approved by the Animal Care Committees of both Roma Tre and Florence Universities and by the Italian Ministry of Health (authorization numbers: 31-2019-PR and 955/2015-PR).

Drugs

VPA (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in saline at a concentration of 250 mg/ml and administered at a dose (500 mg/kg) and time (gestational day 12.5) that have been shown to induce autistic-like behavioral changes in the offspring (Servadio et al., 2016; Melancia et al., 2018). Amphetamine (AMPH, Research Biochemicals International) was dissolved in saline and administrated at the dose of 0.5 mg/kg 30 min before the open field test to both VPA- and SAL-exposed offspring. We used a dose of AMPH that is known to affect locomotor activity without inducing stereotyped behaviors (Fowler et al., 2003; Manduca et al., 2014).

Behavioral Tests

Social Play Test

The test was performed in a sound-attenuated chamber under dim light conditions, as previously described (Trezza and Vanderschuren, 2008, 2009). At PNDs 35–40, rats were individually habituated to the test cage for 10 min on the 2 days before testing. On the test day, the animals were isolated for 3 h before testing. The test consisted of placing each experimental rat together with an untreated animal for 15 min in the testing chamber. In rats, a bout of social play behavior starts with one rat soliciting ("pouncing") another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways: if the animal fully rotates to its dorsal surface, "pinning" is the result (one animal lying with its dorsal surface on the floor with the other animal standing over it), which is considered the most characteristic posture of social play behavior in rats. The following parameters were scored for each animal of a pair using the Observer 3.0 software (Noldus, The Netherlands): (1) number of pinning events; (2) number of pouncing events; (3) evasion (the animal that is pounced upon does not prolong the playful interaction but rather runs away); and (4) play responsiveness [the percentage of response to play solicitation, as the probability of an animal of being pinned in response to pouncing by the stimulus partner (Servadio et al., 2016)]. Time spent in social exploration (the total amount of time spent in non-playful forms of social interaction, like sniffing any part of the body of the test partner, including the anogenital area, or grooming any part of the partner body).

Three-Chambers Test

The test was performed as previously described (Servadio et al., 2016). The apparatus was a rectangular three-chamber box, with two lateral chambers (30 × 35 × 35 cm; l × w × h) connected to a central chamber (15 × 35 × 35 cm; l × w × h). Each lateral chamber contained a small Plexiglas cylindrical cage. At PND 90, each experimental rat was individually allowed to explore the three-chamber apparatus for 10 min and then confined in the central compartment. An unfamiliar stimulus animal was confined in a cage located in one chamber of the apparatus, while the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to explore the apparatus for 10 min. The discrimination index was scored using the Observer 3.0 software (Noldus, The Netherlands) and was calculated as the difference in time spent sniffing the stimulus animal and the time spent exploring the empty chamber, expressed as the percentage ratio of the total time spent exploring both the stimulus animal and the empty chamber.

Social Discrimination Test

The test was performed as previously described (Melancia et al., 2018). Briefly, animals were isolated for 7 days before testing. The test consisted of a learning trial and a retrieval trial, which were separated by a 30 min intertrial interval. During the learning trial, a juvenile (30 days old) unfamiliar rat was introduced into the home cage of the experimental rat for 5 min. The time spent by the experimental rat investigating (sniffing, allogrooming and following) the juvenile was measured. Thirty-minutes after, the juvenile used in the learning trial was returned to the same adult's cage together with a novel juvenile. The time spent by the adult exploring the novel and the familiar juveniles was monitored for 5 min. The discrimination index was calculated as the difference in time exploring the novel and the familiar animal, expressed as the percentage ratio of the total time spent exploring both animals (Campolongo et al., 2007).

Open Field Test

To assess whether adolescent and adult VPA- and SAL-exposed rats similarly responded to AMPH-induced hyperlocomotion, animals from both experimental groups were tested for horizontal locomotor activity in a squared box [40 (length) × 40 (width) × 60 (height) cm]. Each animal was placed in the central zone of the apparatus and allowed to explore for 30 min. Total locomotor activity (expressed as the frequency of crossings in the arena) was analyzed during the 30-min test session. After each session, the apparatus was cleaned with ethanol 70%.

Sucrose Preference Test

At both adolescence and adulthood, rats were tested for preference of a 2% sucrose solution, using a two-bottle choice procedure (Monteggia et al., 2007) with slight modifications. Subjects were housed singly for the 3 days of test. Rats were given two bottles, one of sucrose (2%) and one of tap water.

Every 24 h the amount of sucrose and water consumed was evaluated. To prevent potential location preference of drinking, the position of the bottles was changed every 24 h. The preference for the sucrose solution was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed.

Socially-Induced Conditioned Place Preference

The sCPP test was performed at adolescence and adulthood as previously described (Wei et al., 2015). Briefly, rats were placed in an acrylic box [75 (length) × 35 (width) × 35 (height) cm], divided into two chambers by a clear acrylic wall with a small opening. Each chamber contained different types of autoclaved, novel bedding (Sanyx Bio Ultra litter and Padovan Sandy Litter), which differed in texture and shade (white vs. dark-brown). A 30-min preconditioning test was used to establish any baseline preference for either of the two types of novel bedding. Individual rats with strong preference for either type of bedding were excluded (typically, those that spent more than 1.5× time on one bedding over the other). The next day, each experimental animal was assigned to a social cage with cage-mates to be conditioned to one type of novel bedding for 24 h (CS+). Then, the experimental rat was moved to an isolated cage with the other type of bedding for 24 h (CS−). Bedding assignments were counterbalanced for an unbiased design. Animals were then tested alone for 30 min in the two-chambered box and the time spent in each chamber was calculated to determine post-conditioning preference for either type of bedding. Fresh bedding was used at each step and chambers were thoroughly cleaned between sessions with ethanol 70% to avoid olfactory confounders.

Brain Samples Collection

Rats were rapidly decapitated, and their brains were removed and cut into coronal slices on a cold plate. The PFC, DS, NAc and hippocampus (HIPP) were dissected by hand under microscopic control within 2 min as previously described (Hill et al., 2010; Trezza et al., 2012; Gray et al., 2015).

Flow Cytometric Experiments

Brain samples for each brain area were transferred with PBS into 1.5 ml tube. After centrifugation, the pellet was digested with Trypsin (0.1%) for 30 min at 37°C on slight agitation. Cell suspensions were filtered with CellTrics (100 μm) and washed with 5 ml of PBS. A little sample of suspension was analyzed for forward and side scatter parameter for neuron population quality control (Cruz et al., 2013). Each sample was fixed with PFA (1%), permeabilized with ice-cold ethanol (70%) and incubated for at least 2 h at −20°C. Next, cells were centrifuged, rehydrated with 1 ml PBS/BSA (1%)/Triton (0.1%), aliquoted in 96 well tissue culture plates with conical bottom. Samples were incubated for 1 h RT with primary antibody anti-D1 (1:100 diluted, Novusbio AB81296) and D2 dopamine receptors (1:100 diluted, Santa Cruz SC-7523). The cells were washed and incubated with secondary antibody anti-rabbit and anti-goat Alexa 488 conjugated. All samples were counterstained with propidium iodide/RNase buffer for nuclei identification (with G0 cell cycle phase DNA content) and for singlet/doublets discrimination. Mean fluorescence intensity of 20,000 useful cellular events for each sample, was calculated.

Whole-Cell Patch Clamp Recordings in Acute Brain Slices

Preparation of acute brain slices was performed with established procedures (Carbone et al., 2017). In brief, 1-month old male Wistar rats were anesthetized with isoflurane and decapitated. The brain was quickly removed and glued to the bottom of a vibroslicer slicing chamber (Leica VT 1000S, Leica Microsystem, Wetzlar, Germany). Coronal brain slices (250 μm) containing the NAc were cut in a slicing solution composed of (in mM): N-methyl-D-glucamine (92), 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (20), glucose (25), $NaHCO_3$ (30), NaH_2PO_4 (1.25), KCl (2.5), $MgSO_4$ (10), $CaCl_2$ (0.5). During slicing, the solution was kept cold and infused with a 95% O_2 + 5% CO_2 gas mixture. Slices were transferred to a warm (34–35°C), carbo-oxygenated recovery bath containing artificial Cerebral Spinal Fluid (aCSF) of the following composition (in mM): NaCl (130), KCl (3.5), NaH_2PO_4 (1.25), $NaHCO_3$ (25), glucose (10), $CaCl_2$ (2) and $MgSO_4$ (1). Slices were allowed to recover for at least 30 min prior to experiments. During recordings, a single slice was kept in a flow chamber positioned under the microscope objective and continuously perfused with warm (34–35°C), carbo-oxygenated aCSF. Whole-cell pipettes were pulled from thin-walled borosilicate capillaries (Harvard Apparatus, London, UK) with a vertical puller (Narishige PP830, Narishige International Limited, London, UK) and back-filled with an intracellular solution containing (in mM): K+ methanesulfonate (120), KCl (15), HEPES (10), EGTA (0.1), $MgCl_2$ (2), Na_2Phosphocreatine (5), Na_2GTP (0.3) and MgATP (2), resulting in a bath resistance of 3–5 MΩ. Unless otherwise specified, all drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and bath-applied. Access resistance was monitored during voltage clamp recordings with 100-ms, −10-mV steps, throughout the experiment. Recordings undergoing a drift in access resistance ≥ 10% were discarded. No whole-cell compensations were used. Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA). NAc-MSNs were identified by their morphological and electrophysiological properties (Cepeda et al., 2008). To examine the effects of VPA prenatal exposure on medium spiny neuron (MSN) excitability, current clamp input-output curves were obtained by injecting 800 ms-current steps with amplitude ranging from −50 to +550 pA with 50 pA increments. Inwardly rectifying potassium currents (IKir) were obtained by imposing 500 ms-voltage steps with amplitude ranging from −150 to −60 mV with 10 mV increments. Potassium currents were recorded in the presence of tetrodotoxin (TTX, 1 μM, Tocris, Biosciences, Bristol, UK), a blocker of voltage-dependent sodium channels. IKir was isolated as the 1 mM CsCl-sensitive component. IKir current density was obtained by normalizing current amplitudes for membrane capacitance (derived from the area underlying current peaks elicited by −10 mV steps) and expressed as pA/pF.

Statistical Analysis

Behavioral and neurochemical data are expressed as mean ± SEM. To assess the effects of the prenatal treatment (VPA or SAL) on the parameters measured, data were analyzed with Student's *t*-tests. Two-way analysis of variance (ANOVA) was used to assess the effects of prenatal and postnatal treatments in the open field test, using prenatal (VPA or SAL) and postnatal (AMPH or vehicle) treatments as between-subjects factor. Two-way ANOVA was followed by Student's–Newman–Keuls *post hoc* test where appropriate. All behavioral tests were scored by a trained observer who was unaware of treatment condition to reduce performance bias. Random allocation of animals to treatment groups and to behavioral tasks and blinding of investigators assessing outcomes were adopted to reduce selection and detection bias. All behavioral data were tested for normality.

For electrophysiological experiments, data are presented as mean ± SEM of *n* cells obtained from N animals. Statistical significance was assessed with student's *t*-test for unpaired samples (Microcal Origin 9.1; Northampton, MA, USA). Significance at the $P < 0.05$, 0.01 and 0.001 level is indicated with *, ** and ***, respectively, in figures. Graphs and representative traces were generated with Microcal Origin 9.1. Example traces represent typical observations.

RESULTS

Social Play Test

VPA-exposed rats showed reduced play responsiveness compared with SAL-exposed animals ($t = 3.67$, $p = 0.002$, $df = 16$; **Figure 1A**). Indeed, while no differences were found between SAL- and VPA-exposed animals in the number of pinning ($t = 0.81$, p = n.s., $df = 16$; data not shown) and pouncing ($t = -0.86$, p = n.s., $df = 16$; data not shown), VPA-exposed rats displayed a higher frequency of partial rotation ($t = -2.81$, $p = 0.013$, $df = 16$; **Figure 1B**) compared to SAL-exposed animals. No differences in the total time spent in general social exploration were found between SAL- and VPA-exposed animals ($t = 1.43$, p = n.s., $df = 16$, data not shown).

Three-Chambers Test

VPA-exposed rats showed decreased sociability in the three-chamber test, as they spent less time sniffing the stimulus animal compared to SAL-exposed rats, showing a lower discrimination index ($t = 2.27$, $p = 0.039$, $df = 14$; **Figure 1C**).

Social Discrimination Test

VPA-exposed animals showed impaired social discriminative abilities as they showed a lower discrimination index in the social discrimination test compared with SAL-exposed animals ($t = 2.27$, $p = 0.044$, $df = 14$; **Figure 1D**).

Amphetamine-Induced Hyperlocomotion

AMPH increased locomotor activity of SAL- and VPA-exposed rats both at adolescence and adulthood. A two-way ANOVA analysis performed on the frequency of crossing after treatment with AMPH or its vehicle gave the following results: PNDs 35–40 ($F_{(\text{prenatal treat.})1,28} = 1.56$, p = n.s.; $F_{(\text{treat.})1,28} = 10.97$, $p < 0.01$; $F_{(\text{prenatal treat.} \times \text{treat.})1,28} = 0.1$; p = n.s.); PNDs 90–95 ($F_{(\text{prenatal treat.})1,27} = 1.40$, p = n.s.; $F_{(\text{treat.})1,27} = 33.05$, $p < 0.001$; $F_{(\text{prenatal treat.} \times \text{treat.})1,27} = 0.76$; p = n.s.). *Post hoc* analysis revealed that AMPH increased the frequency of crossing both in SAL- (PNDs 35–40: $p < 0.05$; PNDs 90–95: $p < 0.001$) and VPA-exposed rats (PNDs 35–40: $p < 0.05$; PNDs 90–95: $p = 0.002$; **Figures 2A–D**).

Sucrose Preference Test

Both SAL- and VPA-exposed rats preferred the sucrose over the water solution the in the sucrose preference test, at both PNDs 35–40 and 90–95 (PNDs 35–40: SAL: $t = -10.53$, $p < 0.001$, df = 16; VPA: $t = -17.65$, $p < 0.001$, $df = 12$, **Figure 2B**; PNDs 90–95: SAL: $t = -10.43$, $p < 0.001$, $df = 18$; VPA: $t = -11.76$, $p < 0.001$, $df = 18$; **Figure 2E**).

Socially-Induced Conditioned Place Preference

Both at adolescence and adulthood, VPA-exposed rats did not show deficits in the sCPP test. Indeed, during the test session animals of both experimental groups spent more time in the chamber containing the bedding used for the social conditioning (CS+; PNDs 35–40: Saline group: $t = -2.63$, $p < 0.05$, $df = 22$; VPA group: $t = -3.31$, $p < 0.01$, $df = 22$; **Figure 2C**; PNDs 90–95: Saline group: $t = -2.19$, $p < 0.05$, $df = 14$; VPA group: $t = -2.55$, $p < 0.05$, $df = 20$; **Figure 2F**).

Flow Cytometric Analysis of Dopamine Receptors

At adolescence, VPA-exposed rats shows a significative increase in D2 dopamine receptor expression in the NAc (SAL-exposed animals: MFI = 2,891; VPA-exposed animals: MFI = 5,272, $p < 0.05$; **Figures 3A,B**).

At adulthood, VPA-exposed rats showed a significative increase in D1 dopamine receptor expression in the NAc (SAL-exposed animals: MFI = 4,789; VPA-exposed animals: MFI = 6,690, $p = 0.025$; **Figure 3C**) and HIPP (SAL-exposed animals: MFI = 3,987; VPA group: MFI = 5,299, $p = 0.008$; **Figure 3C**), whereas D2 dopamine receptors were significative increased only in the NAc (SAL-exposed animals: MFI = 1,697; VPA-exposed animals: MFI = 2,152, $p = 0.022$; **Figure 3D**).

Electrophysiological Recordings of NAc MSNs in Acute Brain slices

Whole cell patch clamp recordings were obtained from NAc MSNs in acute coronal slices prepared from SAL- and VPA-exposed rats at PNDs 30–35 ($N = 9$ and 7, respectively, **Figures 4A,B**). While we found no differences in passive membrane properties between MSNs from SAL-exposed rats (SAL MSNs) and VPA-exposed rats (VPA MSNs; input resistance: SAL MSNs = 111.4 ± 8.81 MΩ, $n = 22$; VPA MSNs = 139.2 ± 12.78 MΩ, $n = 23$; membrane capacitance: SAL MSNs = 45.73 ± 3.07 pF, $n = 23$; VPA MSNs = 47.36 ± 4.49 pF $n = 26$; threshold: SAL MSNs = −39.43 ± 1.3 mV, $n = 35$; VPA MSNs = −39.96 ± 0.93 mV, $n = 33$; p = n.s.; all; **Figure 4C**), the latter group showed a significant depolarization

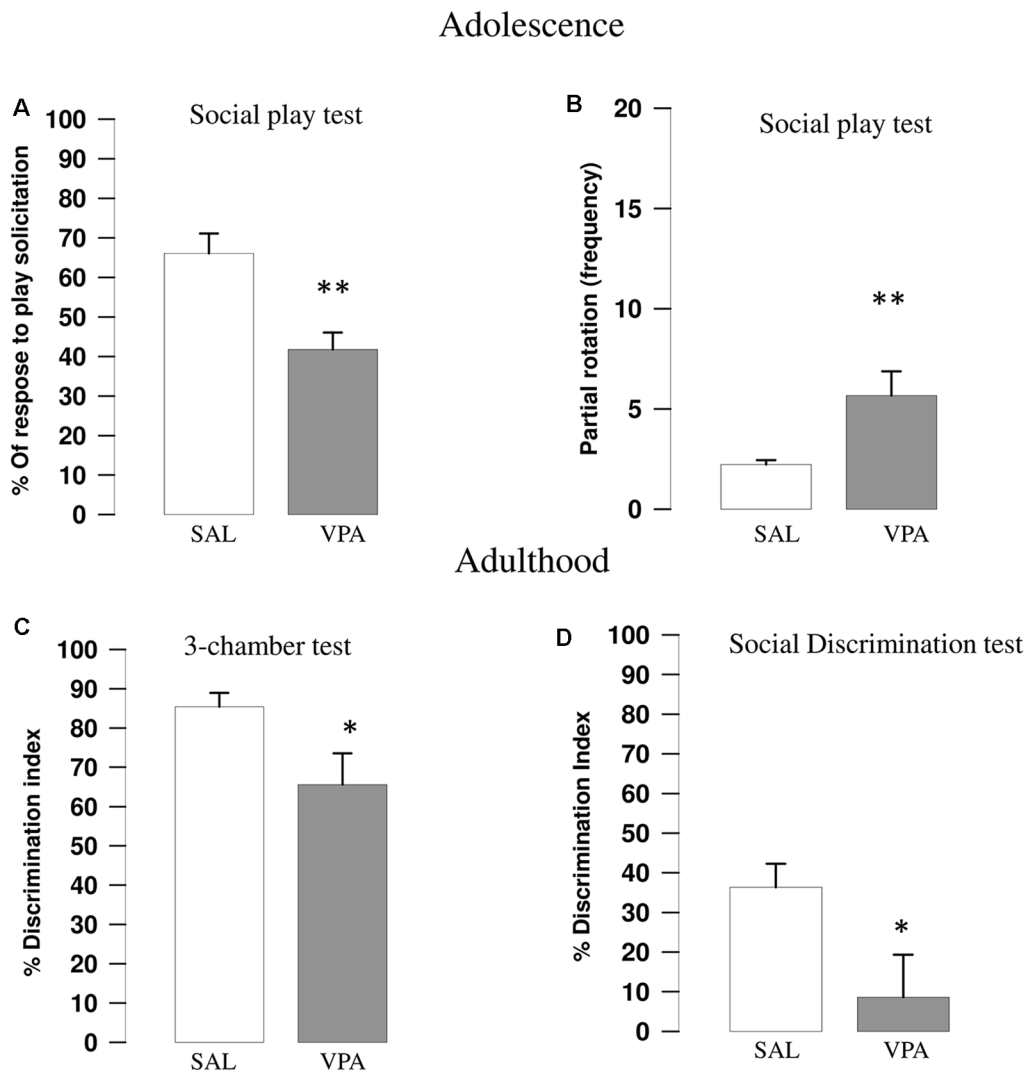

FIGURE 1 | Valproic acid (VPA)-exposed rats showed altered social play behavior, impaired sociability in the three-chamber test and reduced social discrimination abilities. VPA-exposed rats showed reduced response to play solicitation **(A)** and increased frequency of partial rotation (**B**; n = SAL 9, n = VPA 9). VPA-exposed rats also showed reduced sociability in the three-chamber test **(C)**, since they showed a lower discrimination index indicating (i.e., they spent less time sniffing the stimulus animal compared to SAL-exposed rats; n = SAL 8, n = VPA 8). VPA-exposed rats also showed impaired social discrimination as they displayed a lower discrimination index compared to SAL-exposed animals in the social discrimination task (**D**; n = SAL 8, n = VPA 8). Data represent mean values ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. SAL group (Student's t-test).

of the resting membrane potential as compared to SAL MSNs (SAL MSNs = −77.59 ± 0.83 mV, n = 35; VPA MSNs = −71.72 ± 0.98 mV, n = 37; p = 0.0008; **Figure 4C**). We then determined the intrinsic excitability of MSNs in acute slices containing the NAc by measuring the number of action potentials elicited by depolarizing current steps of increasing amplitude. **Figure 5A** shows a direct comparison of representative voltage responses obtained at each value of imposed current from SAL and VPA MSNs. The minimal value of current amplitude required to elicit an action potential in VPA MSNs was significantly lower compared to SAL MSNs (100 pA vs. 200 pA). However, as the amplitude of the depolarizing current increased (≥250 pA), while the number of APs fired by SAL MSNs increased linearly, VPA MSNs progressively lost the ability to fire action potentials. The plot in **Figure 5B** reports the mean number of APs ± SEM fired by SAL (black) and VPA (green) MSNs vs. imposed current (SAL MSNs, n = 35, vs. VPA MSNs n = 37; 150 pA; p = 0.01; 200 pA; p = 0.03; 350 pA; p = 0.02; ≥ 400 pA; $p < 0.0001$). Depolarized resting potential and altered AP discharge pattern are consistent with a change in the density of whole-cell K^+ currents. Based on the reported relevance of the inward-rectifying K^+ current (IKir) in setting resting potential in MSNs (Kreitzer, 2009), we measured whole-cell currents elicited at hyperpolarizing potentials in control aCSF and in the presence of 1 mM Cs^+, a IKir blocker (Cazorla et al., 2012).

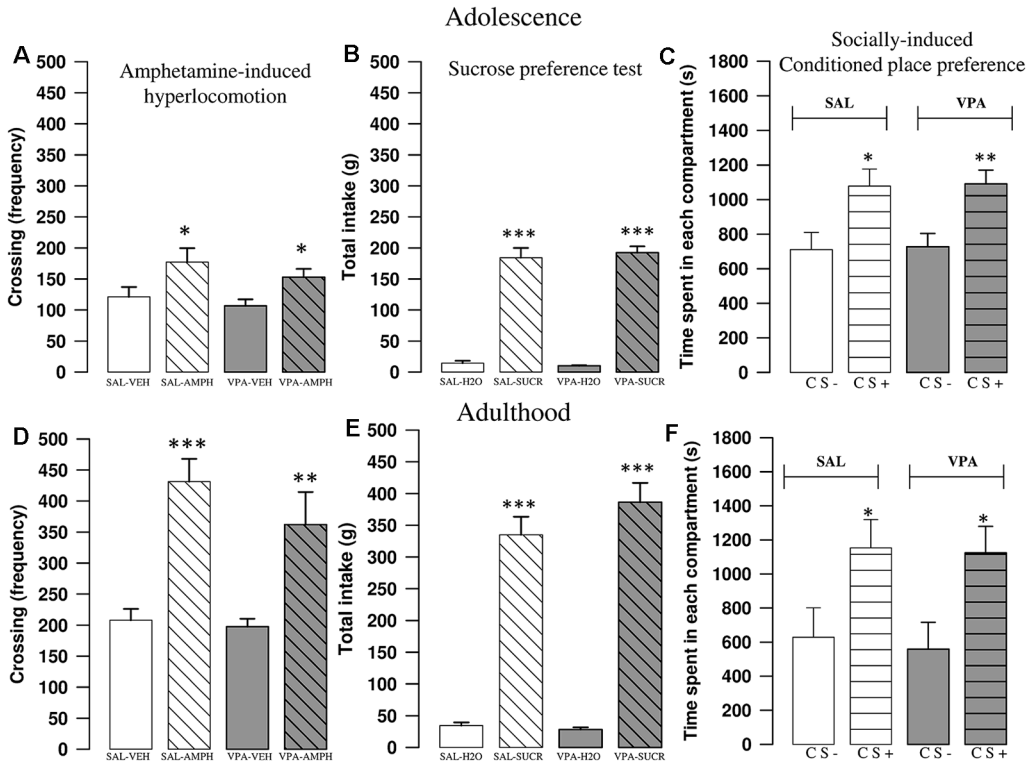

FIGURE 2 | VPA-exposed rats did not differ from control rats in AMPH-induced hyperlocomotion, sucrose preference and socially-induced Conditioned Place Preference (sCPP). In the open field test, AMPH increased the frequency of crossing both in SAL- and VPA-exposed rats at postnatal days (PNDs) 30–35 (**A**; n = SAL-VEH 7, n = SAL-AMPH 7, n = VPA-VEH 9, n = VPA-AMPH 9) and PNDs 90–95 (**D**; n = SAL-VEH 8, n = SAL-AMPH 7, n = VPA-VEH 8, n = VPA-AMPH 8). Data represent mean values ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SAL-VEH group (Student-Newman-Keuls *post hoc* test). No differences were found between SAL- and VPA-exposed rats in the sucrose preference test, both at PNDs 30–35 (**B**; n = SAL 10, n = VPA 10) and PNDs 90–95 (**E**; n = SAL 10, n = VPA 10). Data represent mean values ± SEM; *** $p < 0.001$ vs. SAL-H_2O and vs. VPA-H_2O groups (student's t-test). In the sCPP test, both SAL- and VPA-exposed rats spent more time in the chamber containing the bedding where social interaction previously occurred (CS+), both at PNDs 30–35 (**C**; n = SAL 12, n = VPA 12) and PNDs 90–95 (**F**; n = SAL 8, n = VPA 11). Data represent mean values ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. SAL-CS- and VPA-CS- groups (Student's t-test).

Figure 6A shows representative inward currents obtained in control solution (I_{aCSF}) and 1 mM Cs^+ (I_{Cs}). IKir is obtained by subtracting I_{Cs} from I_{aCSF}. Current-voltage relationship reveals a significant reduction of IKir current density in VPA MSNs (SAL MSNs, n = 7, VPA MSNs, n = 5; $p < 0.05$ where indicated; **Figure 6B**).

DISCUSSION

Clinical studies have repeatedly reported that maternal use of VPA during pregnancy can induce a wide range of abnormalities in the exposed children, ranging from structural malformations to more subtle autistic-like behaviors. For this reason, prenatal VPA exposure is nowadays considered an environmental risk factor involved in the pathogenesis of ASD (Christensen et al., 2013; Nicolini and Fahnestock, 2018). Based on the robust clinical evidence, prenatal exposure to VPA in rodents has been validated as a drug-induced preclinical model of ASD (Roullet et al., 2013; Ranger and Ellenbroek, 2016; Tartaglione et al., 2019).

In the present study, we show that prenatal exposure to VPA causes selective deficits in the social domain in the rat offspring at different developmental periods, together with changes in D1 and D2 dopamine receptor expression in the NAc and hyperexcitability in this same brain area, but without inducing changes in social and non-social reward-related behaviors.

According to the DSM-5 diagnostic criteria, persistent deficits in social communication and social interaction are key features of ASD. Social play behavior has a crucial role in the identification and diagnosis of ASD (Jordan, 2003; Jarrold et al., 2013) Social play is the first form of non-mother directed social behavior displayed by most mammals at young age (Panksepp et al., 1984; Vanderschuren et al., 1997, 2016). In ASD, play patterns are characterized not only by deficient cognitive complexity but also by a typical asocial dimension. Since the opportunity to engage in social play is crucial to acquire proper social and cognitive skills (Vanderschuren et al., 2016; Nijhof et al., 2018), the lack of social play in children with ASD has deleterious effects on their development, leading to long lasting deficits in self-awareness, social competence, problem solving and behavioral flexibility. In line with the social deficits reported in previous

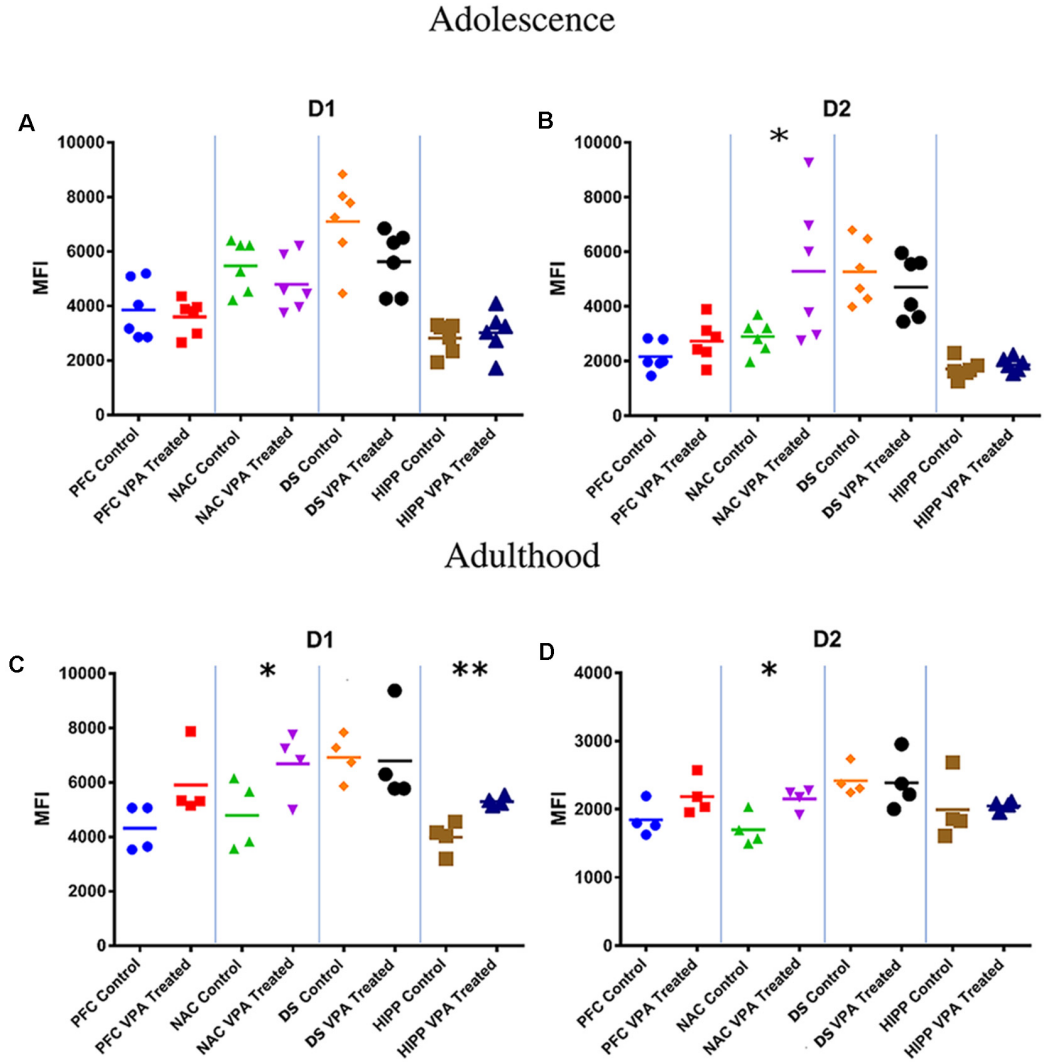

FIGURE 3 | Flow cytometric analysis of dopamine receptors. No differences between VPA-exposed and SAL-exposed rats were found in D1 dopamine receptor expression in prefrontal cortex, nucleus accumbens, dorsal striatum and hippocampus **(A)**. At PNDs 30–35, VPA-exposed rats showed a significant increase in D2 dopamine receptor expression in the nucleus accumbens (NAc) **(B)** compared to SAL-exposed rats (n = SAL 6, n = VPA 6). At PNDs 90–95, VPA-exposed rats showed increased expression of D1 dopamine receptor expression in NAc and hippocampus **(C)**, while D2 receptor expression was increased only in the NAc (**D**; n = SAL 5, n = VPA 5). Data represent mean values ± SEM; *$p < 0.05$, **$p < 0.01$ vs. SAL group (Student's t-test).

studies (Schneider and Przewłocki, 2005; Schneider et al., 2006; Felix-Ortiz and Febo, 2012; Servadio et al., 2016; Melancia et al., 2018), we confirm here that adolescent rats prenatally exposed to VPA show decreased responsiveness to play solicitation, since they respond to play solicitation mainly by partial rotation than reciprocating the playful interaction. Notably, these social deficits were long lasting. Indeed, VPA exposed rats showed social deficits also when tested at adulthood in the three-chamber and social discrimination tests. These data corroborate our previous findings showing that prenatal VPA exposure induces in the rat offspring a wide range of social impairments in the course of development, ranging from social play deficits to altered sociability and social discrimination (Servadio et al., 2016, 2018; Melancia et al., 2018) and are in line with clinical observation that children, adolescents and adults with ASD demonstrate marked socio-communicative deficits (Lai et al., 2011; Dworzynski et al., 2012; Head et al., 2014).

As it has been hypothesized for autistic patients (Chevallier et al., 2012), the wide range of social dysfunctions displayed by VPA-exposed rats may be due to either their inability to properly percept, understand and respond to socially relevant cues, or to a failure of their brain reward system to assign a positive value to the social experience (Pellissier et al., 2018). To address this issue, we performed neurochemical and electrophysiological experiments focusing on brain areas involved in reward processing, and performed additional behavioral experiments to investigate if VPA-exposed rats differ from control animals in responding to social and other (non-social) rewarding stimuli.

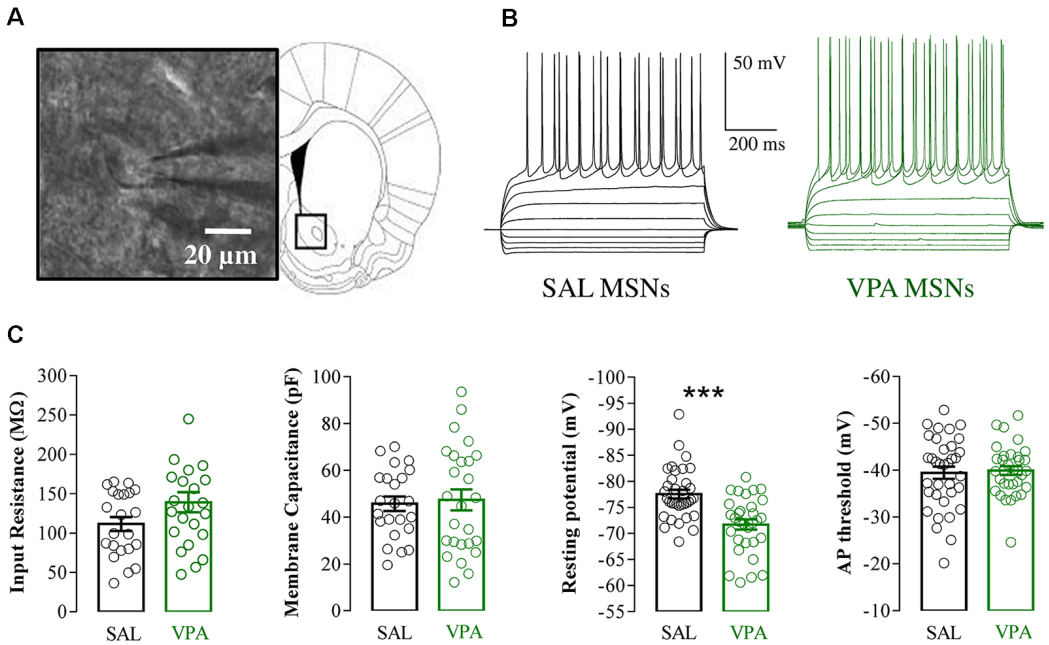

FIGURE 4 | Electrophysiological properties of SAL medium spiny neurons (MSNs) and VPA MSNs. **(A)** NAc-MSN visualized with infrared video microscopy (*left*) and schematic representation of a coronal brain slice at the level of the NAc (*right*). **(B)** Representative voltage traces of SAL MSNs and VPA MSNs evoked by injection of hyperpolarizing and depolarizing current pulses (800 ms from −200 pA to 250 pA in 50 pA steps). **(C)** Passive membrane properties of SAL MSNs and VPA MSNs; input resistance (SAL MSNs 111.4 ± 8.81 MΩ, $n = 22$; VPA MSNs 139.2 ± 12.78 MΩ, $n = 23$), membrane capacitance (SAL MSNs 45.73 ± 3.07 pF $n = 23$; VPA MSNs 47.36 ± 4.49 pF, $n = 26$); resting membrane potential (SAL MSNs −77.59 ± 0.83 mV, $n = 35$; VPA MSNs −71.72 ± 0.98 mV, $n = 37$) and AP threshold (SAL MSNs −39.43 ± 1.3 mV, $n = 35$; VPA MSNs −39.96 ± 0.93 mV, $n = 33$). Data represent mean values ± SEM; $^{***}p < 0.001$ vs. SAL group (Student's *t*-test).

Brain regions involved in the control of social behavior include corticolimbic structures and their altered functionality may represent one neural substrate contributing to the social impairments characteristic of ASD (Scott-Van Zeeland et al., 2010; Chevallier et al., 2012; Ameis and Catani, 2015; Supekar et al., 2018). Notably, these regions are subjected to modulation by dopaminergic neurons and it has been suggested that a dysfunction of dopaminergic neurotransmission in the mesocorticolimbic circuit leads to the social deficits observed in ASD (Paval et al., 2017): thus, it has been demonstrated that striatal MSNs show enriched expression of genes associated with ASD (Chang et al., 2015) and that ASD-associated mutations affect specific MSN subtypes (Portmann et al., 2014; Rothwell et al., 2014; for a review, see Rothwell, 2016).

In autistic subjects, dopamine imbalances, manifested as either hyperactivity or hypoactivity of midbrain dopaminergic pathways, have been detected, highlighting the heterogeneity of the disease (Paval et al., 2017).

Dopamine activation of D1 and D2 receptors in corticolimbic regions is also important for the expression of social behavior in rodents (Robinson et al., 2002; Gunaydin et al., 2014; Manduca et al., 2016; Kopec et al., 2018). Therefore, although the social impairments observed in VPA-exposed animals likely cannot be ascribed to the alteration of a single neurotransmitter system, we focused the biochemical and electrophysiological analyses on dopaminergic neurotransmission in corticolimbic brain areas. We measured the expression of D1 and D2 dopamine receptors in the PFC, DS, NAc and HIPP since these brain areas play not only an important role in the modulation of the rewarding properties of social interactions but have also a key role in cognitive aspects of the social repertoire (e.g., social cognition). While the DS and NAc have a well-recognized role in the modulation of several aspects of reward-related behaviors (for a review, see Bhanji and Delgado, 2014), the PFC and HIPP have also been found to be deeply involved in cognitive aspects of social behavior (Vanderschuren et al., 2016; Montagrin et al., 2018).

We found that, compared to control animals, adolescent rats prenatally exposed to VPA showed increased expression of D2 dopamine receptors in the NAc. At adulthood, they showed increased expression of D1 dopamine receptors in the NAc and hippocampus and of D2 receptors in the NAc. These results suggest that the social deficits displayed by VPA-exposed rats in the course of development may arise from dopaminergic dysfunctions in both these brain regions.

In line with our results, changes in hippocampal dopamine and D1 receptor levels associated with social deficits have been demonstrated in a genetic mouse model exhibiting autism-relevant behavioral abnormalities (Liu et al., 2017). Furthermore, mice knockout for genes strongly associated with ASD, such as Cntnap4 (Karayannis et al., 2014) and neuroligin-3 (Rothwell et al., 2014) mutant mice, show changes in NAc dopaminergic neurotransmission together

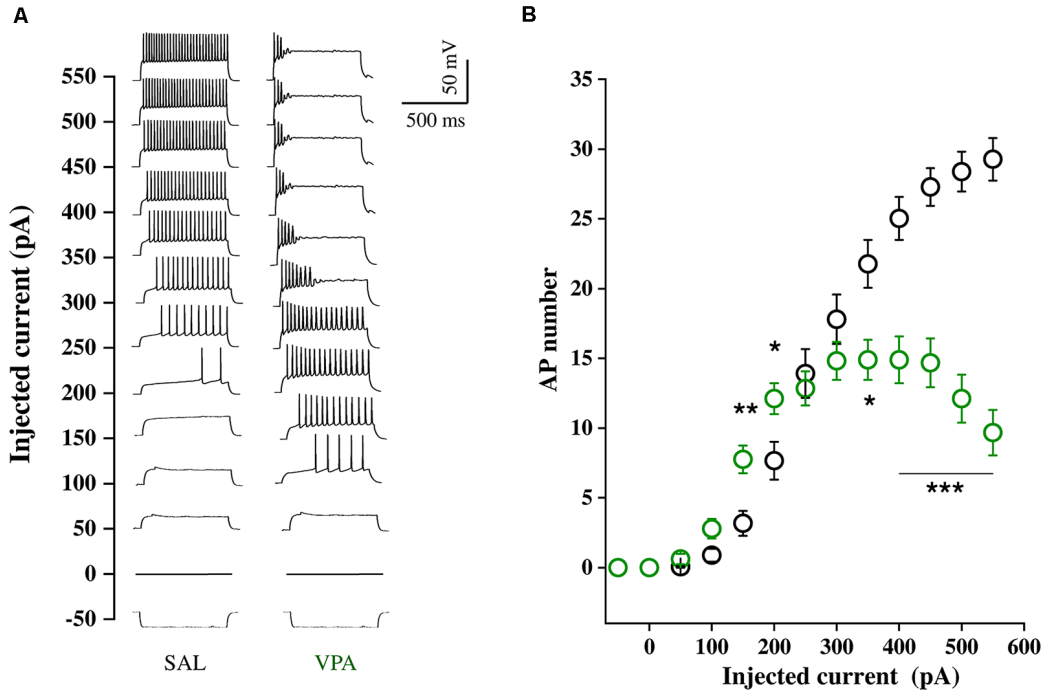

FIGURE 5 | Intrinsic excitability of SAL MSNs and VPA MSNs. **(A)** Representative voltage traces obtained in response to–50 to 550 pA current steps (800 ms duration, 50 pA amplitude) from resting potential, recorded from SAL and VPA MSNs. **(B)** Number of APs fired plotted against current steps of increasing value (SAL MSNs, $n = 35$; VPA MSNs, $n = 37$). Data represent mean values ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SAL group (Student's *t*-test).

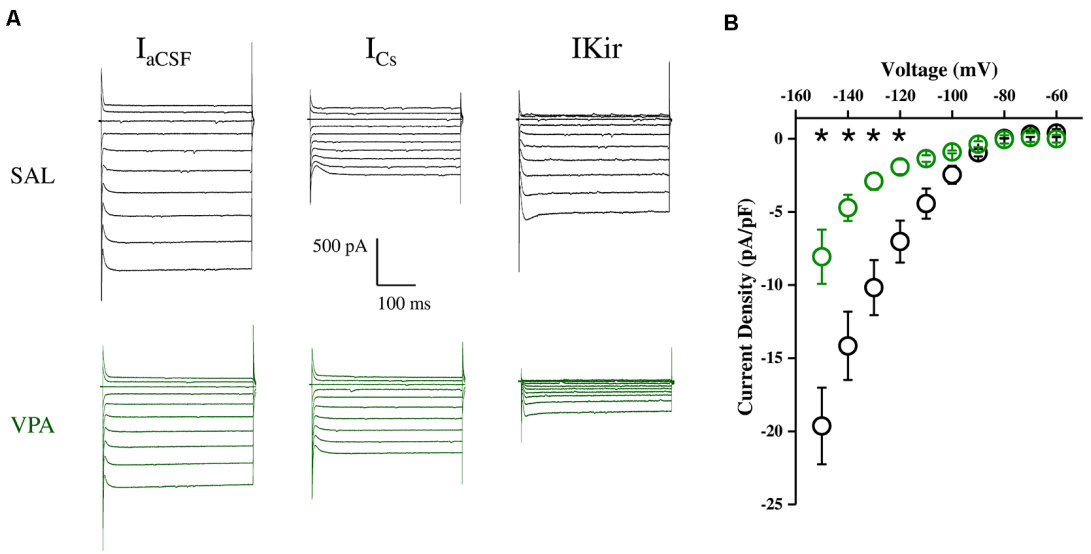

FIGURE 6 | Inwardly rectifying potassium current (IKir) density in SAL MSNs and VPA MSNs. **(A)** Representative IKir traces obtained at hyperpolarized membrane potentials (−60 to −150 mV in 10 mV steps) obtained from SAL and VPA MSNs. IKir is obtained by subtracting I_{Cs} from I_{aCSF}. Current-voltage relationship of IKir current density in SAL and VPA MSNs. **(B)** Current-voltage relationship of IKir current density in SAL and VPA MSNs (SAL MSNs, $n = 7$; VPA MSNs $n = 5$). Data represent mean values ± SEM; $^{*}p < 0.05$ vs. SAL group (Student's *t*-test).

with deficits in the core ASD behavioral domains. Recently, it has also been demonstrated that altered VTA dopamine neuron function represents a key mechanism by which insufficiency of SHANK3, encoding the synapse scaffolding protein SHANK3, generates impaired social preference in mice (Bariselli et al., 2016).

Building on this evidence, we studied the basic electrophysiological properties of NAc MSNs in acute striatal brain slices of VPA- and SAL-exposed offspring. We found that NAc MSNs of VPA-exposed animals show a significant depolarization of the resting membrane potential and increased excitability in the lower part of the excitability curve, indicating higher firing probability in conditions of normal synaptic excitation. These changes are likely caused by altered Kir current density, known to determine the hyperpolarized value of membrane potential in normal MSNs (∼−85 mV) and to affect their AP discharge pattern (Kreitzer, 2009; Cazorla et al., 2012). The NAc is a major node of the mesolimbic dopaminergic system and the overall impact of NAc output on behavior depends on the relative activity of D1- vs. D2-expressing MSNs. Increased excitability of MSNs of VPA-exposed animals, combined with increased expression of D2R-expressing neurons, is consistent with an imbalance in the direct and indirect pathway activity, in favor of the latter. Proper activation of these pathways underlies proper motor learning as well as the acquisition of reward-related behaviors (Yawata et al., 2012; Shin et al., 2018). Furthermore, extensive alterations in normal gene expression pattern have been found in multiple striatal neuronal populations in the VPA model of ASD (Lauber et al., 2016).

Based on the evidence of neurochemical and electrophysiological changes in the brain reward system of VPA-exposed rats, we tested their behavioral response to different (i.e., social and non-social) rewarding stimuli. First, we tested whether VPA- and SAL-exposed rats differently responded to a dose of amphetamine known to induce hyperlocomotion (Bolanos et al., 1998), a proxy for the ability of the dopaminergic system to respond to pharmacological activation. We found a robust amphetamine-induced increase in motor activity in the open field test in both adolescent and adult VPA- and SAL-exposed animals, showing a same susceptibility to amphetamine-induced hyperlocomotion in both experimental groups.

To examine whether the social impairment displayed by VPA-exposed rats was accompanied by a generalized anhedonic behavior, that is, a reduction in the interest for natural reward, we performed a sucrose preference test. In line with previous studies performed with other preclinical models of ASD (Jamain et al., 2008; Radyushkin et al., 2009) and with clinical studies reporting intact hedonic responses to sweet taste in autistic patients (Damiano et al., 2014), we found that both VPA-exposed and control animals showed preference for the sucrose over the water solution, either at adolescence or adulthood.

To assess whether the social deficits displayed by VPA-exposed animals may be due to aberrant social reward processing, we tested VPA- and SAL-exposed rats in a socially-induced place-conditioning task (sCPP), which involves the association between a social stimulus and a distinct set of contextual cues (Trezza and Vanderschuren, 2009; Dölen et al., 2013; Wei et al., 2015). Attenuated sCPP has been demonstrated in genetic models of ASD such as in fmr1 mutant mice, a model for Fragile X syndrome (Pacey et al., 2011), and BTBR T+tf/J (BTBR) mice (Pearson et al., 2012). However, we here failed to find any difference in sCPP: thus, both VPA- and SAL-exposed animals spent more time during testing in the chamber associated with a bedding where social interaction previously occurred. Thus, the altered pattern of social play behavior, the reduced sociability and the impaired social discrimination abilities displayed by VPA-exposed rats are not accompanied by altered social reward processing.

LIMITATIONS

From the behavioral point of view, the present study has some methodological limitations. Indeed, while we here found that VPA- and SAL-exposed rats similarly respond to amphetamine-induced hyperlocomotion, it still needs to be determined whether VPA- and SAL-exposed animals differ in behavioral set-ups specifically designed to assess drug intake and drug addiction. Similarly, we cannot exclude that VPA-exposed animals would show altered sucrose preference if different concentrations of sucrose solution were used, as found in the SHANK3 mouse model of ASD, where Shank3 mice preferred a sucrose solution at high but not at low concentrations (Bariselli et al., 2016). Last, it is still possible that VPA-exposed rats would show altered social motivation when tested in socially-driven operant conditioning paradigms (Achterberg et al., 2016). Concerning the electrophysiological experiments, the potential limitations in the validity of the information obtained with brain slice recordings is related to the nature of the methodology, which allows for in-depth, reliable interrogation of cell-autonomous electrical properties, but inevitably involves profound alteration of network connectivity, even within the area under examination. Aware of the intrinsic advantages and limitations of this approach, here we focused on neuronal properties that are better preserved in the acute brain slice preparation, such as intrinsic neuronal excitability.

CONCLUSION

Although more levels of analysis are needed to shed light on the mechanisms underlying the aberrant behavior found in VPA-exposed rats, on the basis of the results obtained here we suggest that the reduced play responsiveness, the impaired sociability in the three-chamber test and the reduced discrimination abilities in the social discrimination tasks displayed by VPA-exposed animals reported in the present and in previous studies (Kim et al., 2011, 2013, 2014; Servadio et al., 2016; Melancia et al., 2018) are more likely due to changes in the cognitive functions required for proper social interaction, such as to understand and predict the behaviors of other conspecifics or to adapt the social behaviors of the animal to the changing circumstances in its social and physical environment, or may be due to impairments in aspects of reward processing that could not be detected with the behavioral tasks used in the present work. In this picture, the alteration in the expression of striatal dopamine receptors and in the electrical

properties of MSNs in the NAc may be interpreted as a homeostatic mechanism deployed by reward-related brain areas to compensate for VPA-induced neurodevelopmental perturbations. Further behavioral, neurochemical and electrophysiological investigations are required to support this interpretation.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting *in vivo* Experiments; Kilkenny et al., 2010) guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU. In particular, the experimental protocol was approved by the Animal Care Committees of both Roma Tre and Florence Universities and by the Italian Ministry of Health (authorization numbers: 31-2019-PR and 955/2015-PR).

AUTHOR CONTRIBUTIONS

SS and FM performed, analyzed and contributed to the design of the behavioral experiments. SL performed, analyzed and designed the flow cytometric experiments. DI, CC and MP performed, analyzed and contributed to the design of the electrophysiology experiments. AMan and GM contributed to the design of the experiments and edited the manuscript. SS and DI wrote the manuscript. VT and AMas supervised the project, designed the experiments and wrote the manuscript.

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CHAPTER 8

Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 depend on mGlu5 receptor signaling

Running title: Sex specific cannabinoid-induced behavioral deficits and mGlu5R

Antonia Manduca[1,2,3], Michela Servadio[1], Francesca Melancia[1], Sara Schiavi[1], Olivier Manzoni[2,3], Viviana Trezza[1]

[1]Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Rome, Italy;

[2]Aix Marseille Université, INSERM, INMED, Marseille, France;

[3]Cannalab, Cannabinoids Neuroscience Research International Associated Laboratory. INSERM-Indiana University.

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Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 depend on mGlu5 receptor signaling.

In this last paper, I studied how prolonged prenatal exposure to cannabinoid drugs could impact on social behavior in order to understand the role of the endocannabinoid system in the development of socio-emotional dysfunction. The present study demonstrated that fetal exposure to cannabinoids causes sex-specific, mGlu5-related behavioral alterations in the progeny at early developmental periods. Interestingly, potentiating mGlu5R signaling reverted the early behavioral deficits displayed by WIN-exposed infant male rats.

Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 depend on mGlu5 receptor signaling

Running title: Sex specific cannabinoid-induced behavioral deficits and mGlu5R

Antonia Manduca[1,2,3#], Michela Servadio[1], Francesca Melancia[1], Sara Schiavi[1], Olivier Manzoni[2,3], Viviana Trezza[1]

[1]Department of Science, Section of Biomedical Sciences and Technologies, University "Roma Tre", Rome, Italy;
[2]Aix Marseille Université, INSERM, INMED, Marseille, France;
[3]Cannalab, Cannabinoids Neuroscience Research International Associated Laboratory. INSERM-Indiana University.

[#]Corresponding author:
Antonia Manduca, PhD
Department of Science, Section of Biomedical Sciences and Technologies
University "Roma Tre"
Viale G. Marconi 446
00146 Rome, Italy
tel: +39-0657336367
fax: +39-0657336365
email: antonia.manduca@uniroma3.it

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Abstract

Background and Purpose: Cannabis sativa is the illicit drug most commonly used among pregnant and breastfeeding women. Different studies reported long-term adverse effects induced by in utero exposure to the main component of Cannabis, Δ^9-tetrahydrocannabinol (THC), both in rodents and humans. However, little is known about any potential sex-dependent effects of cannabis consumption during pregnancy on newborns at early developmental ages.

Experimental Approach: We studied the effects of prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 (WIN; 0.5 mg/kg from GD5 to GD20) on the emotional reactivity and cognitive performance of male and female rat offspring from infancy through adolescence and tested the role of mGlu5 receptor signaling in the observed effects.

Key Results: Prenatally WIN-exposed male infant pups emitted less isolation-induced ultrasonic vocalizations (USVs) compared with male control pups when separated from the dam and siblings and showed increased locomotor activity, while females were spared. These effects were normalized when male pups were treated with the positive allosteric modulator of mGlu5 receptor CDPPB. When tested at the prepubertal and pubertal periods, WIN-prenatally exposed rats of both sexes did not show any difference in social play behavior, anxiety and temporal order memory.

Conclusion and Implications: We reveal a previously undisclosed sexual divergence in the consequences of fetal cannabinoids on newborns at early developmental ages, which is dependent on mGlu5 receptor signaling. These results provide new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both the male and female offspring.

Keywords: in utero cannabis, ultrasonic vocalizations, social play, elevated plus-maze, temporal order memory, mGlu5 receptors.

Abbreviations: WIN, the cannabinoid receptor agonist WIN 55,212-2; CTRL, control; GD, gestational day; PND postnatal day; USVs, Isolation-induced ultrasonic vocalizations; % TO, percentage of time spent in the open arms; % OE, percentage of open arm entries; SAP, number of stretched-attend postures; HDIPS, number of exploratory head dips; SEM, standard error of mean; VEH, vehicle; CDPPB, the positive allosteric modulator of mGlu5 receptors.

Bullet point summary:

What is already known?

- Cannabis sativa is the illicit drug most commonly used among pregnant and breastfeeding women and the long-term adverse effects induced by in utero cannabis have been described both in rodents and humans.
- However, the majority of these studies have been conducted exclusively in the male progeny

What this study adds?

- We demonstrate for the first time that fetal exposure to cannabinoids causes sex-specific, mGlu5-related behavioral alterations in the progeny at early developmental periods.
- Our results provide new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both the male and female offspring.

Clinical significance

- The dissemination of our results represents a means of preventative education for women using marijuana while pregnant.
- Our results provide new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabis exposure in both sexes.

Introduction

Cannabis sativa (marijuana) is the illicit drug most commonly used among pregnant and breastfeeding women (Brown, 2017; SAMHSA, 2013; Scheyer, 2019). The main active principle of cannabis, Δ^9-tetrahydrocannabinol (THC), enters maternal circulation and readily crosses the placenta (Hutchings *et al.*, 1989). Thus, prenatal cannabis exposure might exert deleterious effects on the fetus. Nowadays, the legalization of medical and recreational cannabis is increasing throughout the United States and many other Countries debate on its possible legalization. In this context, rigorous scientific research about the impact of cannabis use on health and well-being becomes more important than ever.

Human studies have provided invaluable information on the detrimental effects of prenatal cannabis exposure on the offspring from the neonatal period through early adulthood (Crume *et al.*, 2018; El Marroun *et al.*, 2018; Huizink, 2014; Ryan *et al.*, 2018), revealing increased tremors, startles and altered sleep patterns at birth (Calvigioni *et al.*, 2014; Volkow *et al.*, 2017) and significant impairment of higher cognitive functions beyond infancy (Fried, 2002; Fried *et al.*, 1998; Grant *et al.*, 2018; Huizink *et al.*, 2006; Leech *et al.*, 1999; Passey *et al.*, 2014; Smith *et al.*, 2006). However, one weakness of human studies is that they cannot control for environmental and genetic factors. Therefore, a wide array of animal studies has been performed to better evaluate the contribution of prenatal cannabis to adverse, even subtle neurodevelopmental consequences in the offspring (Grant *et al.*, 2018; Trezza *et al.*, 2012).

The endocannabinoid system plays a relevant role in a broad spectrum of neurodevelopmental processes: notably, CB1 cannabinoid receptors, already functional around gestational days (GD) 11-14 in rats, are involved in embryonal implantation, neural development and control of synaptic communication (Berghuis *et al.*, 2007; Harkany *et al.*, 2007). Pioneering animal studies have demonstrated specific deficits in prenatally cannabis-exposed rodent offspring at different developmental periods (Grant *et al.*, 2018; Richardson *et al.*, 2016; Trezza *et al.*, 2012). Interestingly, some of the behavioral deficits displayed by rodents prenatally exposed to cannabinoids have been related to changes in brain glutamatergic neurotransmission (Antonelli *et al.*, 2004; Antonelli *et al.*, 2005; Castaldo *et al.*, 2007; Mereu *et al.*, 2003).

Noteworthy, the majority of these studies were conducted exclusively in the male progeny. Therefore, an urgent need exists to understand the effects of prenatal cannabis exposure also in female progeny. Pioneering preclinical and clinical studies reported sexually-dimorphic responses to cannabinoids when administered during the gestational and/or early postnatal periods (Navarro *et al.*, 1995; Vela *et al.*, 1998; Wang *et al.*, 2006; Wang *et al.*, 2004).

Recently, our laboratories also revealed a previously undisclosed sexual divergence in the consequences of fetal cannabinoids at adulthood: prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 (WIN) reduced social interactions in adult male but not female rats and altered neuronal excitability and synaptic plasticity in the prefrontal cortex of male rats only. These deficits were paralleled by decreased levels of mGlu5R mRNA. Amplifying mGlu5R signaling with a positive allosteric modulator of mGlu5R normalized the social and synaptic deficits displayed by male WIN-exposed rats (Bara *et al.*, 2018).

Based on these findings, this study follows up our recent report showing sex-dependent effects of in utero cannabinoid exposure at adulthood and aimed to test the effects of prenatal exposure to WIN in the male and female infant and prepubertal and pubertal rat offspring, to evaluate possible sex-dependent effects induced by in utero WIN exposure on emotional reactivity and cognitive performance at developmental ages earlier than adulthood. The interaction between cannabinoid and mGlu5R has been extensively explored by using pharmacological, electrophysiological and anatomical approaches (Araque *et al.*, 2017; Jung *et al.*, 2012; Katona *et al.*, 2008; Lafourcade *et al.*, 2007; Liang *et al.*, 2014; Won *et al.*, 2012). Importantly, mGlu5R partecipate in the developmental regulation of the endocannabinoid system: indeed, the developmentally dependent increase in endocannabinoid mobilization (that occurs between the neonatal and juvenile stages) correlates with increases in the levels of protein expression of mGlu5R (Liang *et al.*, 2014). Based on this evidence and our recent findings on the interaction between cannabinoid and mGlu5R in modulating behavioral and synaptic states in the context of nutrition (Manduca *et al.*, 2017) and in utero cannabinoid exposure (Bara *et al.*, 2018), we here investigated whether the positive allosteric modulator of mGlu5R CDPPB normalized the behavioral deficits induced by in utero cannabinoid exposure.

Methods

Animals

Wistar (RGD_13508588) female rats (Charles River, Italy), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day 0 (GD0). Pregnant rats were singly housed in Macrolon cages (40 × 26 × 20 cm), under controlled conditions (temperature 20-21 °C, 55-65% relative humidity and 12/12 h light cycle with lights on at 07:00 a.m.). Food and water were available ad libitum. The synthetic cannabinoid receptor agonist WIN55,212-2 (WIN, 0.5 mg/kg) was administered to the dams subcutaneously (s.c.) daily from GD 5 to GD 20 (WIN group, n=27). Control dams (CTRL group, n=26) received a similar volume injection of the vehicle solution. Newborn litters found up to 05:00 p.m. were considered to be born on that day (postnatal day (PND) 0). On Postnatal day (PND) 1, litters were culled to four males and four females. On PND 21, pups were weaned and housed in groups of three. The experiments were carried out on the male and female offspring at three different developmental ages: 1. infancy (PNDs 10 and 13); 2. prepubertal period (PND 28-35 for males and PND 22-28 for females) and 3. puberty (PND 50-60 for males and PND 30-40 for females offspring). Puberty corresponds to vaginal opening and first ovulation (i.e. around 5 weeks) for female and preputial separation for male rats (Beckman *et al.*, 2003; Korenbrot *et al.*, 1977; Schneider, 2013).

To avoid the so called “litter effects”, one pup of both sexes per litter from different litters per treatment group was randomly used in each experiment (CTRL= 118 males and 89 females or WIN= 123 males and 87 females) as described in the “Handbook of Behavioral Teratology (CV Vorhees); Developmental and Reproductive Toxicology: A Practical Approach (RD Hood”. For power analysis, sample size (n) was based on our previous experiments and power analysis with the software GPower. Potential outliers within each data set were calculated using the GraphPad software. Sample size is indicated in the figure legends and represented in the figures as scatter dot plot. All behavioral tests were assessed by a trained observer who was unaware of treatment condition to reduce performance bias. Reproduction data including body weights of the dams (calculated from GD 1 to GD 21 and expressed as body weight gain in percentage) and the length of pregnancy, the litter size, weight gains of pups and postnatal viability (calculated as the number of live animals of both sexes at PND 21 (i.e. weaning) / the number of live animals of both sexes at PND 1 in percentage) were also measured.

The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the ARRIVE (Animals in Research: Reporting In Vivo

Experiments) (Kilkenny *et al.*, 2010) guidelines, with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

Drug treatment

WIN 55,212-2 mesylate ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone) (WIN, Sigma, Italy and National Institute of Mental Health, USA) was suspended in 5% polyethylene glycol, 5% Tween 80, and 90% saline and given subcutaneously (s.c.) at a volume of 1 ml/kg to the gestating dams. The dose of WIN used in this study (0.5 mg/kg) has been estimated to correspond to a moderate, or even to a low, exposure to cannabis in humans (Antonelli *et al.*, 2004; Compton *et al.*, 1992; French *et al.*, 1997), and it does not induce any sign of toxicity and/or gross malformations in the rat offspring (Mereu *et al.*, 2003). The positive allosteric modulator of mGlu5 receptors CDPPB (3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide) (National Institute of Mental Health, USA) was dissolved in 5% Tween 80/5% polyethylene glycol/saline and given intraperitoneally (i.p.) at the dose of 1.5 mg/kg 30 min before testing to offspring. Drug doses and pre-treatment intervals were based on previous work and pilot experiments. Solutions were freshly prepared on the day of the experiment and were administered in a volume of 2.5 ml/kg to offspring.

Behavioral tests

Isolation-induced ultrasonic vocalizations (USVs)

USVs are emitted by rodent pups when removed from the nest and play an important communicative role in mother-offspring interactions (Manduca *et al.*, 2012). On PND 10, the isolation-induced USVs emitted by CTRL- and WIN-exposed pups were recorded as previously described (Antonelli *et al.*, 2005; Melancia *et al.*, 2018). Briefly, pups were individually removed from the nest and placed into a black Plexiglas arena (30 cm × 30 cm), located inside a sound-attenuating and temperature-controlled chamber. Pup USVs were detected for 15 sec by an ultrasound microphone (Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies between 10 and 200 kHz and fixed at 15 cm above the arena and analyzed quantitatively (numbers of calls/15 sec).

Homing behavior

The homing behavior test exploits the tendency of immature rodent pups to maintain body contact with the dam and siblings and to discriminate their own home cage odor from a

neutral odor, which is an early indicator of social discrimination (Bignami, 1996). The homing behavior test was performed as previously described (Servadio *et al.*, 2018). Briefly, on PND 13 the litter was separated from the dam and kept for 30 min in a temperature-controlled holding cage. Then, each pup was placed into a Plexiglas box whose floor was covered for 1/3 with bedding from the pup's home cage, and for 2/3 with clean bedding. The pup was located at the side of the box covered by clean bedding, and its behavior was video recorded for 4 min for subsequent analysis. The following parameters were scored by an observer, unaware of animal treatment, using the Observer 3.0 software (Noldus, The Netherlands): latency (sec) to reach the home-cage bedding area; total time (sec) spent by the pup in the nest bedding area; total number of entries into the nest bedding area; locomotor activity, expressed as the total number of crossings in the test box.

Social play behavior

Social play behavior is one of the earliest forms of non-mother-directed social behavior very abundant during the juvenile phases of life in mammalian species, including rats (Vanderschuren *et al.*, 2016). The test was performed as previously described (Manduca *et al.*, 2016). Prepubertal and pubertal rats were individually habituated to the test cage for 10 min on each of the 2 days before testing. The test was performed between 9 a.m. and 2 p.m. under low light condition and consisted of placing the animal together with a similarly treated partner into the test cage for 15 min. Behavior was assessed per pair of animals and analyzed by a trained observer who was unaware of the treatment condition to reduce performance bias, using the Observer 3.0 software (Noldus Information Technology, The Netherlands). Both animals in a test pair had received the same treatment during gestation (CTRL- or WIN- in utero). Animals in a test pair did not differ by >10 g in body weight and had no previous common social experience (i.e., they were not cage mates).

In rats, a bout of social play behavior starts with one rat soliciting ('pouncing') another animal, by attempting to nose or rub the nape of its neck. If the animal that is pounced upon fully rotates to its dorsal surface, 'pinning' is the result (one animal lying with its dorsal surface on the floor with the other animal standing over it), which is considered the most characteristic posture of social play behavior in rats (Pellis *et al.*, 2009).

We determined: 1. frequency of pinning; 2. frequency of pouncing; 3. time spent in social exploration (i.e. the total amount of time spent in non-playful forms of social interaction, like sniffing any part of the body of the test partner, including the anogenital area, or grooming any part of the partner body).

Elevated plus-maze test

The elevated plus-maze apparatus comprised two open (50 × 10 × 40 cm3; l × w × h) and two closed arms (50 × 10 × 40 cm3; l × w × h) that extended from a common central platform (10 × 10 cm2). The test was performed as previously described (Manduca *et al.*, 2015; Trezza *et al.*, 2008). Rats were individually placed on the central platform of the maze for 5 min. Each 5-min session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis carried out an observer, unaware of animal treatment, to reduce performance bias, using the Observer 3.0 software (Noldus, The Netherlands).The following parameters were analyzed:

• % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100. Time on the open quadrants was timed from the moment all four paws of the rat were placed on an open section and ended when all four paws re-entered a closed quadrant;

• % open arm entries (% OE): (the number of entries into the open arms of the maze/number of entries into open + closed arms) × 100;

• Number of closed arm entries;

• Number of stretched-attend postures (SAP) made from the exit of a "closed" quadrant towards an "open" quadrant. This exploratory posture can be described as a forward elongation of the body, with static hind-quarters, followed by a retraction to the original position.

• Number of exploratory head dips (HDIPS) made over the edge of the platform, either from the exit of the "closed" quadrant, or whilst on the "open" quadrant;

Temporal order memory test

Animals were habituated to the experimental arena (40 × 40 cm) without objects for 10 min daily for 2 d before testing. This task consisted of two sample phases and one test trial (Barker *et al.*, 2007; Manduca *et al.*, 2017). In each sample phase, rats were allowed to explore two copies of an identical object for a total of 4 min. Different objects were used for sample Phases 1 and 2, with a delay between the sample phases of 1 h. After 3 h from sample Phase 2, rats performed the test trial (4 min duration) where a third copy of the objects from sample Phase 1 and a third copy of the objects from sample Phase 2 were used. The positions of the objects in the test and the objects used in sample Phase 1 and sample Phase 2 were counterbalanced between the rats. An intact temporal order memory requested the subjects to

spend more time exploring the object from Sample 1 (i.e., the object presented less recently) compared with the object from Sample 2 (i.e., the "new" object). The discrimination ratio was calculated as the difference in time spent by each animal exploring the object from sample Phase 1 compared with the object from sample Phase 2 divided by the total time spent exploring both objects in the test phase. Negative discrimination means that animals investigated more the object in Phase 2 than the object in Phase 1. Each 4-min session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis carried out an observer, unaware of animal treatment, to reduce performance bias, using the Observer 3.0 software (Noldus, The Netherlands).

Statistical analysis

Data are expressed as mean ± SEM and adhere to BJP guidelines. To assess the effects of the prenatal treatments (WIN or CTRL) in the male and female offspring, the behavioral data were analyzed by Two-way ANOVA, with treatment and sex as factors. Two-way ANOVA was also used to assess the effects of prenatal (WIN or CTRL) and postnatal (CDPPB or vehicle) treatments. Three-way ANOVA was also used to assess the effects of prenatal (WIN or CTRL) treatments in both male and female offspring depending on the different developmental ages (prepubertal and pubertal periods). To assess the effects on reproduction data, the data were analyzed by using Student's t-test (WIN or CTRL). Statistical significance was set at $p < 0.05$ with no further distinction made for $p < 0.01$ and $p< 0.001$. If main or interaction effects were significant, the Student–Newman–Keuls post hoc test was used for individual group comparisons. The software Sigma Plot (RRID:SCR_003210) and GraphPad Prism (RRID:SCR_002798) were used for statistical analysis of the data. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Random allocation of animals to treatment groups and to behavioral tasks and blinding of investigators assessing outcomes were adopted to reduce selection and detection bias in our trials.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Reproduction data

No differences in body weight gains were observed between WIN- and CTRL-treated dams. Prenatal WIN exposure did not affect pregnancy length, litter size at birth, postnatal viability and pup weight gain at different developmental ages (Table 1).

Prenatal exposure to the cannabinoid receptor agonist WIN caused sex-dependent deficits in social communication and locomotion in the infant rat offspring

Prenatally WIN-exposed male pups emitted less USVs at infancy (PND 10) when separated from the dam and siblings compared with male CTRL-pups (Figure 1: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$<0.05]). Interestingly, the deleterious effects of in utero WIN exposure on USVs were specific to the male offspring. Post hoc analysis revealed that prenatally-WIN exposed female pups showed no difference in the rate of USVs at PND 10 when compared to age-matched females from CTRL-group (Figure 1) suggesting that prenatal exposure to the cannabinoid WIN causes sex-dependent deficits in early social communication of the offspring.

When tested in the homing behavior test at PND13, male and female pups prenatally exposed to WIN did not differ from control animals in the latency to reach the nest arena (Figure 2A: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s]), in the total time spent in the nest zone (Figure 2B: [$p_{(sex)}$=n.s; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and in the number of entries in the nest zone (Figure 2C: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]). However, the frequency of crossing in the test arena was increased specifically in the male WIN-exposed offspring, while WIN-exposed females were spared (Figure 2D: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$<0.05]), suggesting a sex-dependent detrimental effects induced by prenatal cannabinoid exposure on early life locomotion.

Prenatal exposure to the cannabinoid receptor agonist WIN had no effect on social play, anxiety-like behaviors and temporal order memory in the prepubertal progeny

When tested at prepubertal period, WIN- and CTRL-prenatally exposed rats did not show any difference in social play behavior (Figure 3A-B). A detailed analysis of the various social play parameters revealed that pinning (Figure 3A: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and pouncing (Figure 3B: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) frequencies were similar between WIN- and CTRL- animals of both sexes. Moreover, the time spent in general social

exploration (including non-playful forms of social interaction, like sniffing) was unchanged during the 15-min session ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not showed).

To test whether prenatal cannabinoid exposure induced deficits in emotional control and cognitive abilities, we tested WIN- and CTRL offspring of both sexes for their anxiety-like behavior and temporal order memory. No differences between WIN- and CTRL prenatally exposed prepubertal rats were found in the elevated plus-maze. Specifically, there was no change in the percentage of time spent in the open arms of the maze (Figure 3C: [$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.]), in the percentage of open arm entries (Figure 3D: $p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.]) and in the number of closed-arm entries (considered as a measure of locomotion in the maze) ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown). Also the SAP ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), and the HDIPS ([$p_{(sex)}$=n.s.; $p_{(treat)}$<0.05; $p_{(sex\ x\ treat)}$=n.s.], data not shown) were not influenced by in utero WIN exposure in neither the male nor the female offspring.

Regarding their cognitive abilities, prepubertal CTRL and WIN-exposed animals of both sexes displayed identical discrimination index (Figure 3E: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and exploration time (Figure 3F: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) in the temporal order memory task. Thus, prenatal WIN exposure did not induce deficits in social play, anxiety-like behaviors and temporal order memory in the progeny of both sexes at the prepubertal period.

Prenatal exposure to the cannabinoid receptor agonist WIN did not induce deficits in social play, anxiety-like behaviors and changes in temporal order memory in the pubertal progeny

When tested in the pubertal period, no differences in social play behavior were found between WIN- and CTRL prenatally exposed rats. Thus, pinning (Figure 4A: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and pouncing (Figure 4B: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) frequencies were similar in WIN- and CTRL- animals in both sexes, suggesting no main effects of in utero cannabinoid exposure on social play behavior at pubescence. Moreover, the time spent in general social exploration (including non-playful forms of social interaction, like sniffing) was unchanged during the 15-min session ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not showed).

Further, no differences between WIN- and CTRL prenatally exposed rats were found in the elevated plus-maze. Specifically, there was no change in the percentage of time spent in the open arms of the maze (Figure 4C: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]), in the percentage

of open arm entries (Figure 4D: [$p_{(sex)}$<0.05; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]), in the number of closed-arm entries ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), in SAP ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown), and HDIPS ([$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.], data not shown). When tested in the temporal order memory task, pubertal animals displayed identical discrimination index (Figure 4E: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) and exploration time (Figure 4F: [$p_{(sex)}$=n.s.; $p_{(treat)}$=n.s.; $p_{(sex\ x\ treat)}$=n.s.]) suggesting an intact temporal order memory. Collectively, these results show that prenatal WIN exposure did not induce deficits in social play, anxiety-like behaviors and temporal order memory in the progeny at pubescence.

Positive allosteric modulation of mGlu5 receptors corrected the behavioral deficits induced by prenatal exposure to the cannabinoid receptor agonist WIN in the male offspring at infancy

Our previous works demonstrated the ability of mGlu5 positive allosteric modulation to correct synaptic and behavioral deficits induced by prenatal WIN exposure at adulthood (Bara *et al.*, 2018). Along this line, we found that systemic treatment with the positive allosteric modulator of mGlu5 receptors CDPPB (1.5 mg; i.p.) at PND 10 normalized the altered USV profile displayed by WIN-exposed pups but remained without effect in the CTRL group, indicating selectivity of the drug effects to the disease-state (Figure 5A: [$p_{(WIN\ in\ utero)}$=n.s.; $p_{(CDPPB)}$=n.s.; $p_{(WIN\ in\ utero\ x\ CDPPB}$<0.05]). Specifically, post hoc analysis revealed that CDPPB rescued the decrease of USVs induced by in utero WIN treatment at PND10 in the male offspring. Furthermore, we found that potentiating mGlu5 signaling by CDPPB administration normalized the hyper-locomotion induced by prenatal exposure to WIN in the male offspring tested in the homing behavior test at PND 13 (Figure 5B: [$p_{(WIN\ in\ utero)}$<0.05; $p_{(CDPPB)}$=n.s.; $p_{(WIN\ in\ utero\ x\ CDPPB)}$<0.05) without affecting the number of crossing in the CTRL-group. Overall, these data show that potentiating mGlu5 signaling normalized the behavioral deficits induced by prenatal exposure to cannabinoids in the infant offspring.

Discussion

In the present study, we demonstrated for the first time that fetal exposure to cannabinoids causes sex-specific, mGlu5-related behavioral alterations in the progeny at early developmental periods. Specifically, we found that prenatal exposure to the synthetic cannabinoid WIN altered isolation-induced USVs and locomotor activity in the male but not female infant rat offspring. Conversely, the social, emotional and cognitive profile was spared in the offspring of both sexes tested at the prepubertal and pubertal periods. Interestingly, potentiating mGlu5R signaling reverted the early behavioral deficits displayed by WIN-exposed infant male rats. Infant rodents produce USVs in response to separation from the mother and the nest and USVs are a potent tool to detect subtle effects of adverse events during development (Branchi *et al.,* 2006; Branchi *et al.,* 2001; Cuomo *et al.,* 1987; Insel *et al.,* 1986). It has previously been shown that cannabinoid exposure during pregnancy and/or lactation alters isolation-induced USVs (Antonelli *et al.,* 2005; Trezza *et al.,* 2008). These early studies, however, were only performed in the male offspring, while the consequences induced by developmental cannabinoid exposure in the female offspring were not investigated. Here, we report that male but not female WIN-exposed pups display a decreased rate of isolation-induced USVs compared to control rats. Whether the decreased USV emission displayed by WIN-exposed male pups could be the consequence of an altered maternal responsiveness, which is one of the factors tuning the rate of USV emission of the offspring (D'Amato *et al.*, 2005), is an interesting issue that deserves further investigation. Related to this, previous studies reported disrupted maternal behavior in lactating rats exposed to very high doses of THC (Bromley *et al.*, 1978; Navarro *et al.,* 1995). Conversely, other authors failed to detect changes in maternal care in rhesus monkeys exposed to low doses of THC during pregnancy and lactation (Golub *et al.,* 1981). Recently, it has been shown that THC administered to pregnant mice (GD 5.5 – GD 17.5) at a "non-intoxicating" daily dose (3mg/kg, intraperitoneal) did not alter maternal behavior or physical measures (Tortoriello *et al.*, 2014) suggesting that moderate doses of cannabinoid should not alter maternal behavior and in turn influence mum-pup interaction. By this evidence, we cannot certainly exclude that prenatal exposure to low doses of WIN (0.5 mg/kg, subcutaneously) may induce any alteration in maternal behavior which in turn may contribute to the altered pattern of emotionality displayed by WIN-exposed male pups. Therefore, this issue still remains absolutely intriguing.

The synthetic cannabinoid receptor agonist used in this study (i.e. WIN) has effects that are highly comparable to those of the main active principle of Cannabis THC regardless they

differ in affinity at CB1 receptors and profile of action (Compton *et al.*, 1992; Wiley *et al.*, 2002). Therefore, we hypothesize that the sex-specific behavioral deficits we here observed at early life stages after prenatal WIN exposure could be similar to those obtained by administering THC during the prenatal period. In support of this hypothesis, we recently demonstrated that prenatal THC administration (from GD 5–GD 20) induced similar sex-specific synaptic deficits in the prefrontal cortex of adult rats without any sign of toxicity and/or gross malformations in the rat offspring as WIN did (Bara *et al.*, 2018). However, in a recent inhalation mouse study, a dose of ~ 0.5 mg/kg/day THC smoke from GD 5.5–17.5 produced deficits in fetal growth and reduced birth weights in cannabis-exposed male offspring suggesting that low-dose exposure to THC via inhalation can compromise fetal development (Benevenuto *et al.*, 2017). This highlights that differences in the treatment schedule, routes of administration, doses and animal strain may account for different results following in utero cannabinoid exposure.

During the early phases of postnatal life, olfaction, and in particular the learned association between maternal odors and maternal stimulation, is crucial for the development of social behavior and social recognition (Terry *et al.*, 1996). Therefore, we tested the infant offspring in the homing behavior test, which requires intact sensory, olfactory and motor capabilities that allow the pup to recognize the mother's odor among others (Bignami, 1996). Both WIN-exposed male and female pups were able to use olfactory cues to discriminate between a neutral odor and their own home cage odor. Interestingly, however, locomotor activity in the test arena was increased specifically in prenatally WIN-exposed male rats, while females were spared, suggesting a sex-dependent detrimental effects of prenatal WIN exposure on early life locomotor activity. Maternal exposure to cannabinoid drugs during pregnancy and/or lactation might particularly affect the ontogeny of motor behaviors: an age-dependent hyperlocomotion has been reported in the lactating offspring of mothers receiving THC (during GD 6–12) (Borgen *et al.*, 1973). Other studies demonstrated that rats pre- and/or postnatally exposed to cannabinoid displayed motor hyperactivity at infancy and adolescence, but not at adulthood (Bara *et al.*, 2018; Mereu *et al.*, 2003; Navarro *et al.*, 1995; Silva *et al.*, 2012). These preclinical studies are in line with human data showing that children of both sexes prenatally exposed to cannabis are hyperactive and impulsive starting around age 6 (Fried *et al.*, 2001; Goldschmidt *et al.*, 2004; Sharapova *et al.*, 2018). Altogether, the abnormal USV profile and locomotor activity displayed by WIN-exposed male pups indicate the presence of sex-specific deficits in social communication and locomotion at early life stages. Previous evidence suggested that prenatal exposure to WIN permanently alters GABA

and glutamate circuits in the prefrontal cortex and hippocampus of the offspring (Antonelli *et al.*, 2004; Antonelli *et al.*, 2005; Mereu *et al.*, 2003; Saez *et al.*, 2014). Notably, a reduction in cortical glutamatergic neurotransmission and NMDA receptor activity has been reported (Antonelli *et al.*, 2005; Mereu *et al.*, 2003). These alterations might result in an inappropriate assembly of neuronal network that could represent a substrate for the observed emotional and locomotor dysfunctions displayed by the WIN-exposed male offspring. Based on this experimental evidence and the prominent role of mGlu5R in synaptic endocannabinoid-mediated signaling (Araque *et al.*, 2017), we tested the ability of CDPPB, a well-described positive allosteric modulator of mGlu5Rs, to rescue the behavioral deficits displayed by WIN-exposed male pups. We found that systemic administration of CDPPB normalized the altered USVs profile and the increased locomotion induced in male pups by prenatal WIN exposure. This finding extends our previous data demonstrating the ability of mGlu5 positive allosteric modulation to correct synaptic and behavioral deficits induced by prenatal cannabinoid exposure at adulthood (Bara *et al.*, 2018). Female did not show the behavioral deficits displayed by the male offspring at infancy; however we cannot exclude that the administration of CDPPB *per se* could affect their USVs and homing performances since CDPPB is known to affect cognitive and operant responding tasks in rodents (Cleva *et al.*, 2012; Fowler *et al.*, 2013; Lee *et al.*, 2018). In the present study we used a dose of CDPPB (1.5 mg/kg) that did not affect early life behavioral parameters (i.e. USVs and homing behavior) in the CTRL male progeny, therefore we hypothesize that the same dose would not have an effect *per se* in the female progeny. Related to this, it should be considered that prenatal exposure to WIN induced sex-related differences in the postsynaptic mGluR proteins at adulthood (Bara *et al.*, 2018) and that mGlu5R modulate spine plasticity in the nucleus accumbens of female mice depending on estrogen receptors (Peterson *et al.*, 2015) suggesting the importance of sex-dependent specificity of the mGluR signaling in the brain.

Moreover, it remains to clarify how prenatal WIN exposure induces sex-specific detrimental behavioral effects at early life stages. Different studies have focused on the sexual dimorphism of the endocannabinoid system, which could explain at least in part the sex dissimilarities in the consequences induced by in utero cannabinoid exposure. Beside molecular and structural differences (Castelli *et al.*, 2014; Garrett *et al.*, 2009; Rodriguez de Fonseca *et al.*, 1994), prenatal exposure to cannabinoids throughout gestation induces sex-specific effects on dopaminergic neurotransmission in the limbic forebrain (Alpar *et al.*, 2016; Navarro *et al.*, 1995; Rodriguez de Fonseca *et al.*, 1991) and also changes in the ontogenetic expression of tyrosine hydroxylase gene (Navarro *et al.*, 1995). Moreover, sex-differences in

mRNA expression levels for mGlu1R have been reported in the prefrontal cortex of adult rats prenatally exposed to WIN, with an increase in mGlu1R mRNA levels exclusively in the male progeny (Bara *et al.*, 2018). In humans, impaired dopamine D2 receptor expression in amygdala is most evident in males in association with prenatal cannabis exposure suggesting a potential pathway for altered emotional regulation (Wang *et al.*, 2004). Interestingly, 10-year-old boys prenatally exposed to marijuana are more susceptible to behavioral problems than girls (Goldschmidt *et al.*, 2004). However, the neurobiological mechanisms underlying maternal exposure to cannabinoids still remain complex. Changes in the epigenetic role of steroid hormones (both sex-steroids and glucocorticoids) on brain development induced by prenatal cannabinoid exposure could be responsible for some specific behavioral effects that we here found at early life stages. Indeed, it has been proposed that the epigenetic effects of abused drugs including marijuana on brain development might be the result of both drug mimicking or modification of the action of natural hormones which play a very important role in neuronal phase during early stages of brain development and cortical organization during perinatal ages in rodents (Navarro *et al.*, 1995). Moreover, marijuana exposure in early fetal life also decreases the expression of genes (through histone lysine methylation) for dopamine D2 receptors in brain areas mediating rewarding processes (i.e. nucleus accumbens) which may explain higher rates of drug addiction in adults exposed prenatally to marijuana (DiNieri *et al.*, 2011). Prenatal exposure to THC also causes substantial changes in gene expression levels of several other significant systems in the brain that are linked to the endocannabinoid signalosome such as the opioid, glutamate, and GABA systems, which may persist well into adulthood (Jutras-Aswad *et al.*, 2009; Navarro *et al.*, 1995) and sex-dependent affect behavioral outcomes since early life stages.

Profound changes in behavioral repertoire and physiological status occur between weaning and puberty; it is during this stage that mammals progressively achieve sexual maturation and establish a sense of independence from their primary caregivers (Spear, 2000; Vanderschuren *et al.*, 2016). This process of development involves several behavioral processes influenced by endocannabinoid signaling (Hill *et al.*, 2012; Solinas *et al.*, 2008; Zanettini *et al.*, 2011). We here showed that prenatally WIN-exposed animals of both sexes did not exhibit deficits in social play, neither anxious-like behaviors in the elevated plus-maze test nor cognitive deficits in temporal order memory at the prepubertal and pubertal periods. The endocannabinoid system has a strong interaction with different neurotransmitters present from early stages of brain development (Alpar *et al.*, 2016). It could be that in utero WIN administration induced mGluR sex-mediated deficits at early life stages (as we found in the present study) and then

the reorganization of this system occurs and different targets (such as dopamine or opioids) become predominant in mediating specific motivational, rewarding and emotional processes that we here did not explore. For instance, prenatal THC-induced reorganization of the dopamine system occurs within this sensitive period and might disrupt reward circuits by genetic and epigenetic modifications (DiNieri *et al.*, 2011; Spano *et al.*, 2007).

Previous findings from our group demonstrated that perinatal exposure to THC (GD 15 to PND 9) altered social play and induced anxiety-like behaviors in the male rat offspring (Trezza *et al.*, 2008). Moreover, it has been recently shown that the postnatal exposure to the cannabinoid receptor agonist CP 55,940 from PND 4 to PND 10, a period of brain development equivalent to the third trimester in human, increased the time spent in the open arms of the elevated-plus maze in offspring of both sexes at pre-pubertal period (Breit *et al.*, 2019). We here showed that prenatally WIN-exposed animals of both sexes did not exhibit anxious-like behaviors in the elevated plus-maze. The discrepancy with our present findings may depend on the different cannabinoid agonists used (THC or CP 55,940 vs WIN) and the treatment schedule (perinatal or postnatal vs prenatal exposure). Moreover, it is possible that a longer activation of endocannabinoid neurotransmission that extends beyond birth till after the early postnatal period may be required to disrupt social play behavior and to induce an anxious-like phenotype in the elevated plus-maze.

Furthermore, social play behavior (van Kerkhof *et al.*, 2013) and temporal order memory (Barker *et al.*, 2007) are mediated by functional activity in the prefrontal cortex and certain levels of regional frontal specificity to the effects of prenatal cannabinoid exposure have been demonstrated (Bara *et al.*, 2018). The fact that the prefrontal cortex develops late in to postnatal life (i.e. late adolescence/early adulthood) (Arain *et al.*, 2013; Kolb *et al.*, 2012) and that temporal order memory requires cortical more than hippocampal integrity (Barker *et al.*, 2017) may explain the normal behavior of (pre)pubertal WIN-exposed rats in these tasks compared to their impaired performance when tested for other forms of memory (Antonelli *et al.*, 2005; Castaldo *et al.*, 2007; Drazanova *et al.*, 2019; Ferraro *et al.*, 2009; Mereu *et al.*, 2003). Further, it can be hypothesized that perturbations of the fetal endocannabinoid system induced by in utero exposure to WIN predisposed the offspring to abnormalities in memory and altered emotionality later in life (Richardson *et al.*, 2016): thus, WIN in utero induced an imbalanced brain circuit at sub-threshold levels (non-manifested during pre-pubescent and pubescent period) that can precipitate neurodevelopmental disease by otherwise sub-threshold stimuli later in life (Bara *et al.*, 2018; Campolongo *et al.*, 2007; Mereu *et al.*, 2003; Tortoriello *et al.*, 2014; Trezza *et al.*, 2008; Vargish *et al.*, 2017).

Overall our results clearly show previously undisclosed sexual divergence in the consequences of fetal cannabinoids at early stages providing new impetus for the urgent need to investigate the functional and behavioral substrates of prenatal cannabinoid exposure in both male and female offspring.

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Tables

Table 1. Reproduction data following in utero exposure to WIN. Dam weight gain was calculated from GD 1 to GD 21 for n=10 dams per group (WIN vs CTRL). Pup weight at different developmental ages was calculated for n=9 CTRL and n=10 WIN-exposed male and n=8 CTRL- and WIN-exposed female pups from different litters. Data represent mean values ± SEM.

Figures legends

Figure 1. Prenatal WIN exposure induces sex-specific communicative deficits in the infant rat offspring.

At infancy (PND 10) male progeny from dams exposed during gestation to WIN vocalized significantly less compared to CTRL-pups when separated from the dam and siblings. In contrast, the communicative profile of female littermates was normal (males: CTRL n=10, WIN n=8; females: CTRL n=9, WIN n=10). Specifically, prenatally-WIN exposed male but not female showed a decrease in the rate of USVs/15 sec compared to their age-matched male progeny from CTRL-group. Scatter dot plot represents each animal. Error bars indicate SEM. *p<0.05. Student–Newman–Keuls test.

Figure 2. Prenatal WIN exposure induces sex-specific deficits in locomotor activity in the infant rat offspring.

A-D, In male and female progeny at PND13 prenatal WIN exposure did not alter the latency to reach the nest arena (**A**), the total time spent in the nest zone (**B**)and the number of entries in the nest zone (**C**) in the homing test. Conversely, the frequency of crossing in the test arena was increased only in male offspring exposed in utero to WIN, while female were spared (males: CTRL n=9, WIN n=10; females: CTRL n=9, WIN n=9) (**D**). Scatter dot plot represents each animal. Error bars indicate SEM. *p<0.05. Student–Newman–Keuls test.

Figure 3. Prenatal WIN exposure does not affect social, anxious and cognitive behaviors in prepubescent male and female offspring.

A-B, No differences between WIN-exposed and CTRL animals in both sexes were found in the social play behavior in the prepubertal period as expressed in the frequency of pinning (**A**) and pouncing (males: CTRL n=8, WIN n=9; females: CTRL n=9, WIN n=8) (**B**). In the elevated plus-maze test, prenatal cannabinoid exposure did not modify the percentage of time spent in the open arms (**C**) and the percentage of open arm entries (males: CTRL n=9, WIN n=9; females: CTRL n=9, WIN n=10) (**D**). **E-F**, In the temporal order memory task, male and female prepubertal rats exposed in utero to the cannabinoid WIN showed no differences in their discrimination index (**E**) and in the total time exploring the objects during the test phase (males: CTRL n=8, WIN n=10; females: CTRL n=12, WIN n=10) (**F**). Scatter dot plot represents a pair of animals for social behavior (A-B) and each animal for the elevated plus-maze (C-D) and the temporal order task (E-F). Error bars indicate SEM.

Figure 4. Prenatal WIN exposure does not affect social, cognitive and anxious behaviors in pubescent offspring of both sexes.

A-B, Prenatal cannabinoid exposure did not alter social behavior at puberty in WIN-exposed and CTRL animals as expressed in the frequency of pinning (**A**) and pouncing (**B**) (males: CTRL n=9, WIN n=8; females: CTRL n=8, WIN n=8). In the elevated plus-maze test, prenatal cannabinoid exposure did not modify the percentage of time spent in the open arms (**C**) and the percentage of open arm entries (males: CTRL n=8, WIN n=10; females: CTRL n=8, WIN n=8) (**D**). **E-F**, In the temporal order memory task, male and female pubertal rats exposed to the cannabinoid WIN in utero showed no differences in their discrimination index (**E**) and in the total time exploring the objects during the test phase (males: CTRL n=8, WIN n=8; females: CTRL n=8, WIN n=8) (**F**). Scatter dot plot represents a pair of animals for social behavior (A-B) and each animal for the elevated plus-maze (C-D) and the temporal order task (E-F). Error bars indicate SEM.

Figure 5. Positive allosteric modulation of $mGlu_5$ receptors normalizes the communicative and locomotor deficits displayed by male pups prenatally exposed to WIN.

A, Systemic administration of CDPPB (1.5 mg/Kg, i.p.) at PND 10 rescued the decrease in the rate of USVs in male rats prenatally exposed to WIN without affecting the USV frequency in the CTRL-group (males CTRL: VEH n=8, CDPPB n=8; males WIN: VEH n=8, CDPPB n=8). **B,** In the homing behavior, treatment with CDPPB (1.5 mg/Kg, i.p.) at PND 13 corrects the increase in the frequency of crossing in male rats prenatally exposed to WIN without affecting the number of crossing in the CTRL-group (males CTRL: VEH n=8, CDPPB n=8; males WIN: VEH n=8, CDPPB n=10). Scatter dot plot represents each animal. Error bars indicate SEM. *$p<0.05$. Student–Newman–Keuls test.

Group	Dam weight gain %	Pregnancy length (days)	Litter size	Postnatal vitality	Pup weight (g)				
					PND1	PND10	PND13	PND25	PND90
CTRL	34.3 ± 1.91	22.6 ± 0.29	12.9 ± 0.78	87.9 ± 1.048	6.7 ± 0.1	24.6 ± 0.65	30.3 ± 0.82	65.6 ± 0.77	484.2 ± 12.52
WIN	33.1 ± 1.42	22.6 ± 0.17	12.7 ± 0.67	88.4 ± 1.352	6.8 ± 0.2	22.6 ± 0.72	30.5 ± 0.75	66.7 ± 1.55	481.7 ± 17.33

Table 1. Reproduction data following in utero exposure to WIN55,212-2. Dam weight gain was calculated from GD 1 to GD 21 for n=10 dams per group.

Pup weight at different ages was calculated for n=9-10 male and female pups from different litters.
Data represent mean values ± SEM.

Table 1

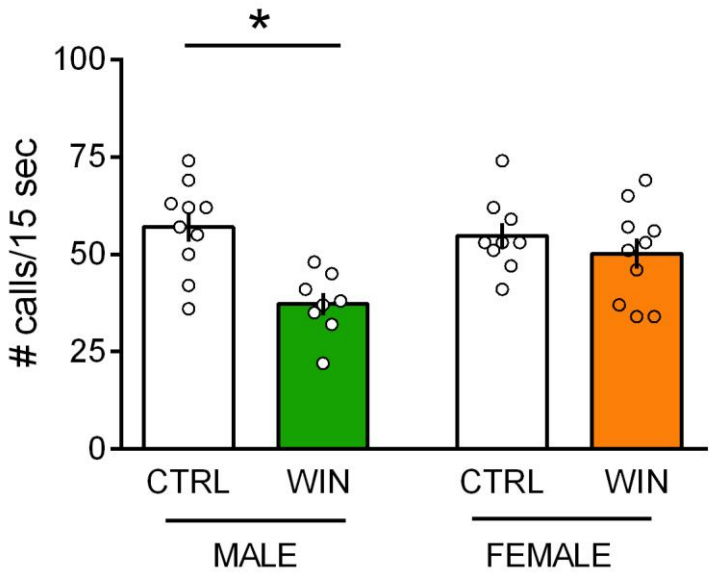


Figure 1

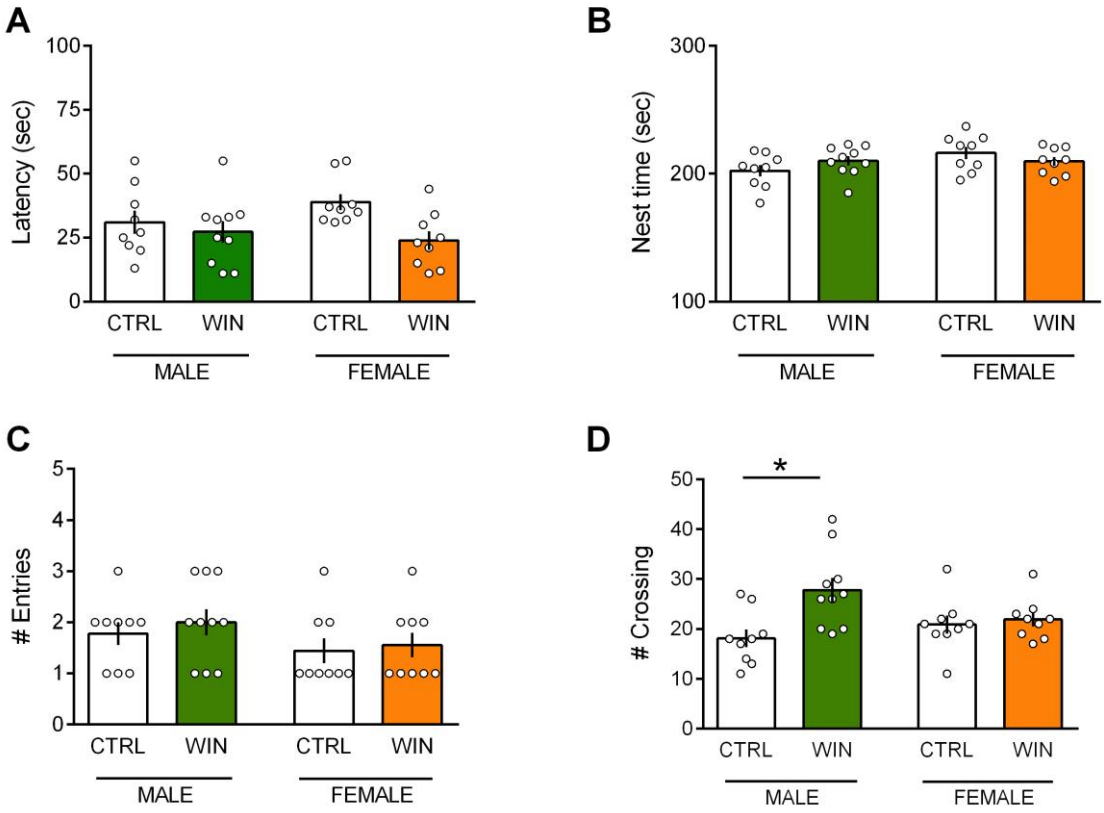
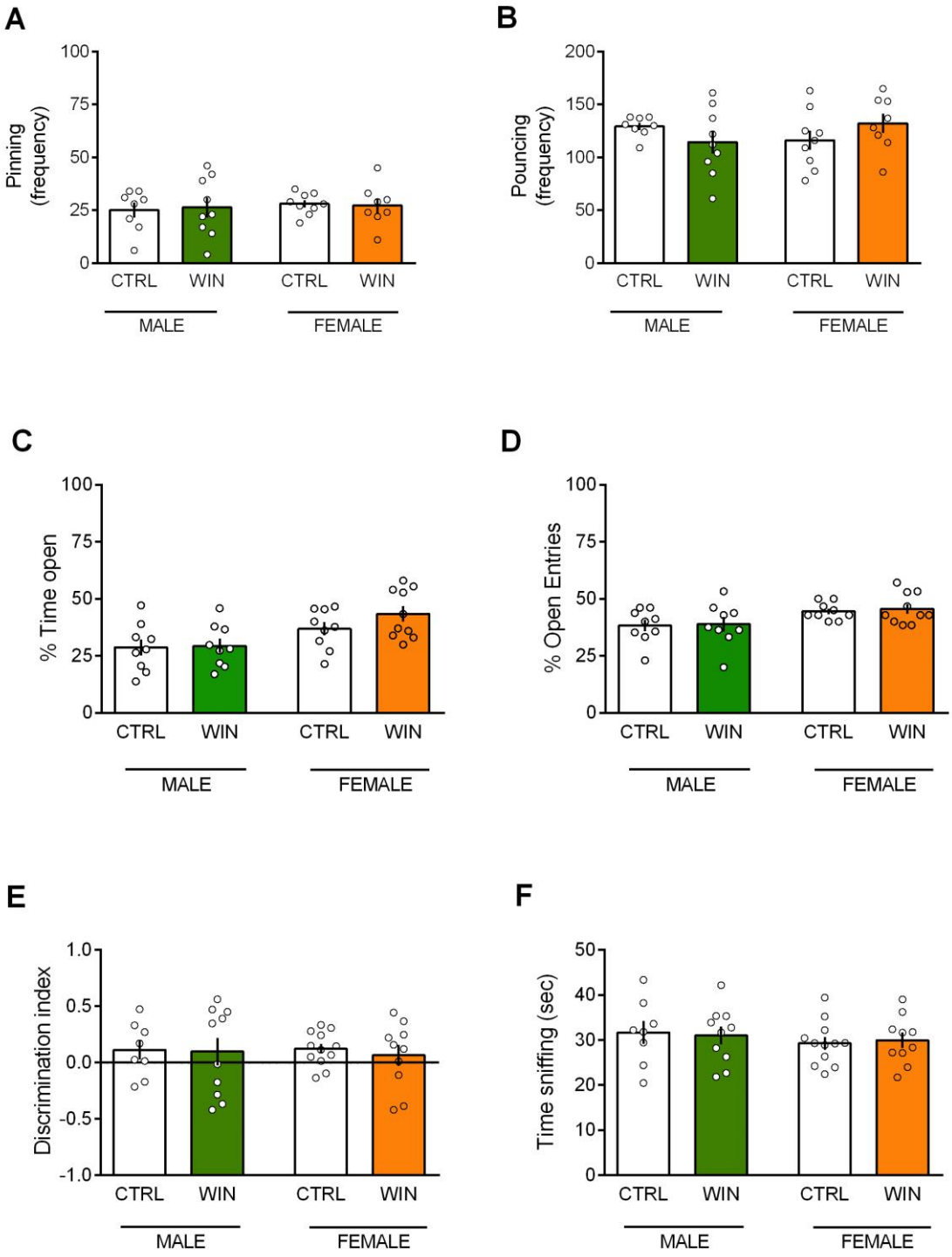


Figure 2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FEMALE

Figure 3

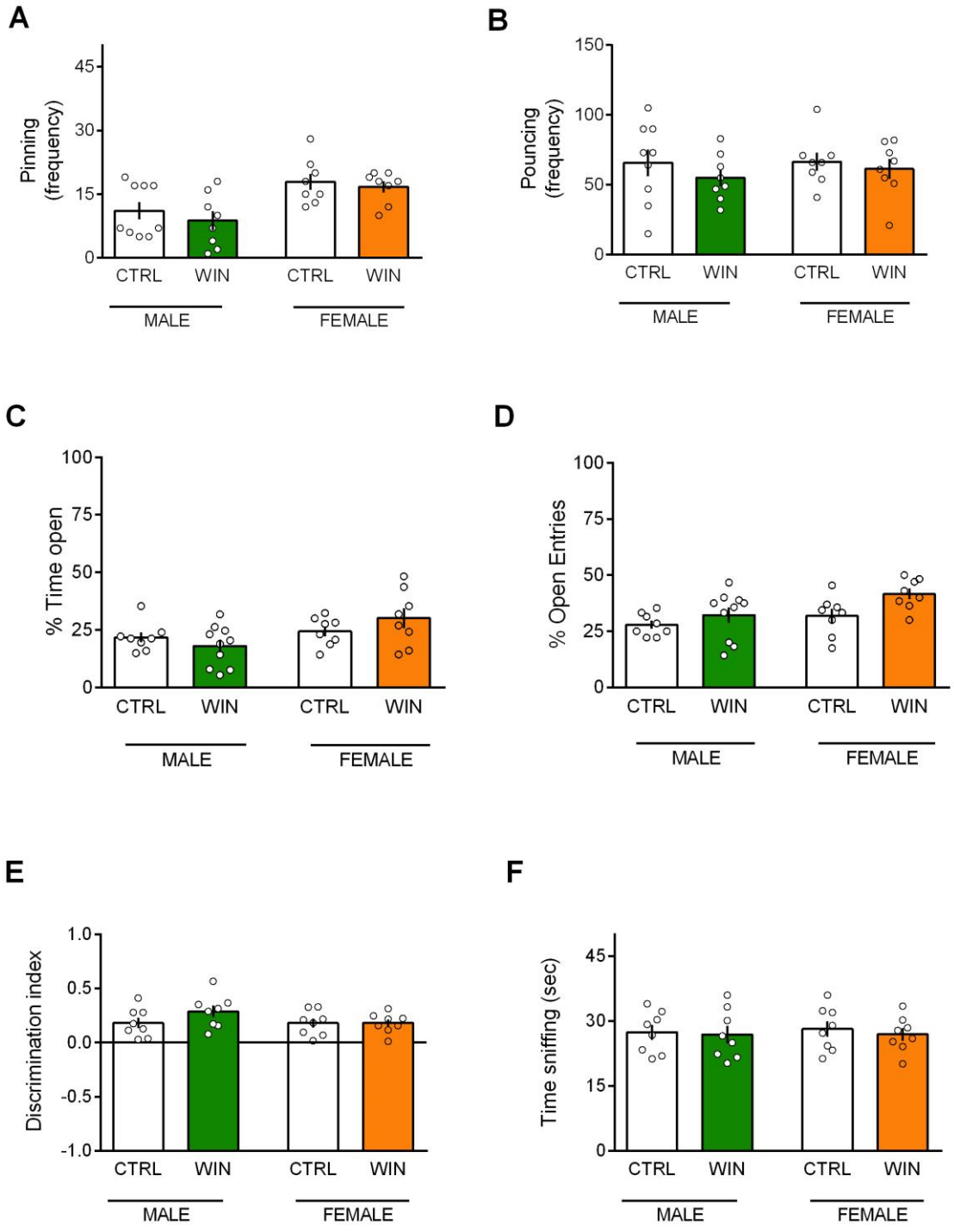
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Figure 4

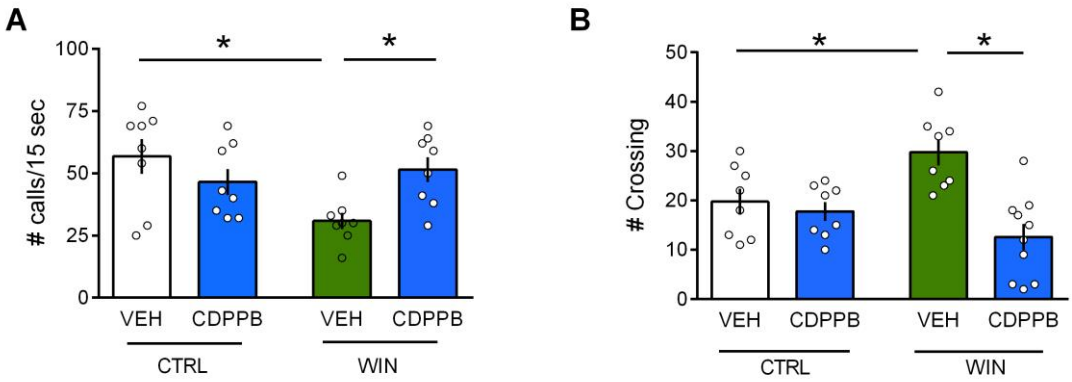
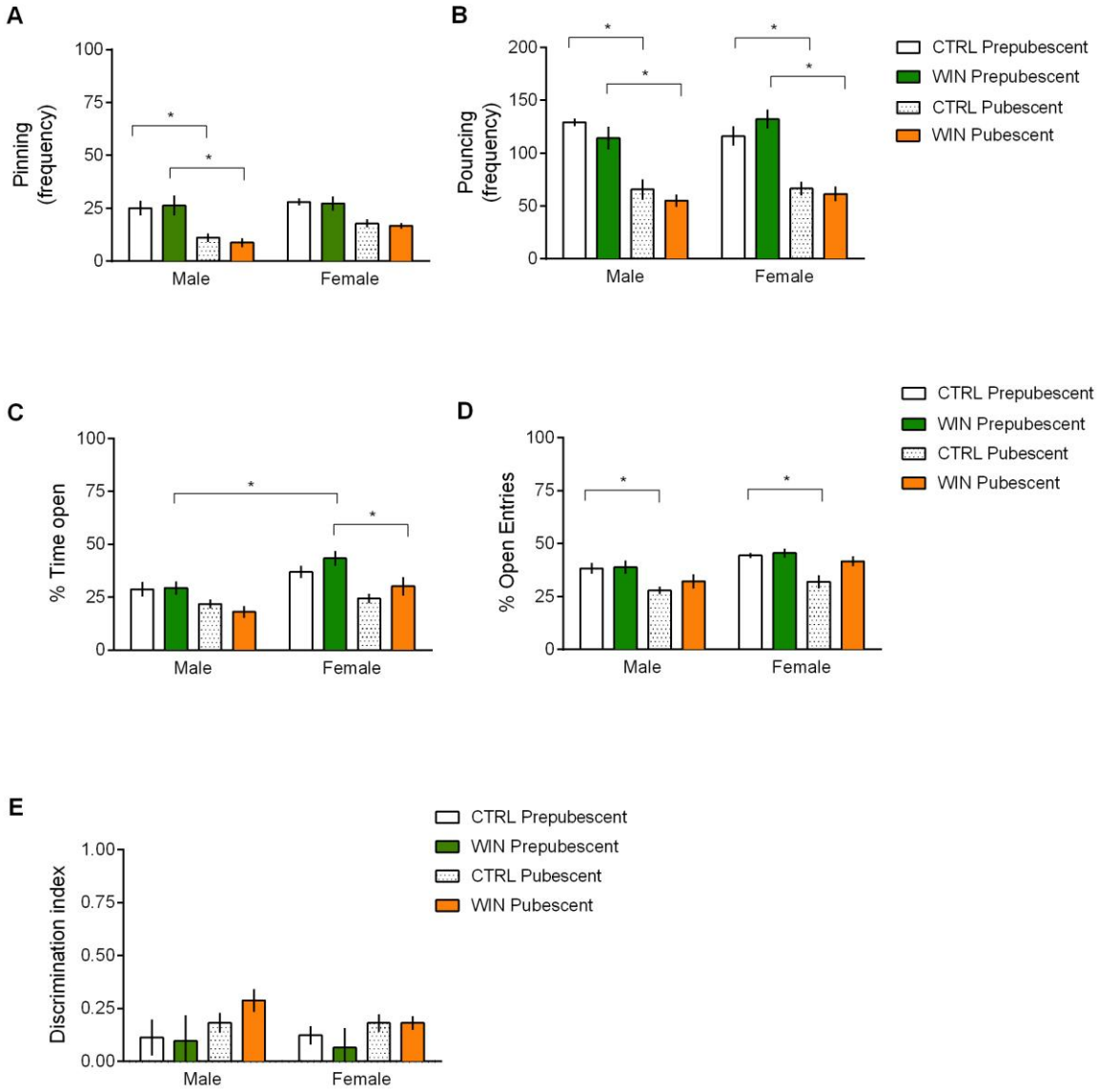


Figure 5



Supplementary Figure 1

CHAPTER 9

1. Concluding remarks

The understanding of the neural mechanisms underlying social behavior is crucial, as several studies suggest that adequate social stimuli during life are critical for developing appropriate socio-emotional and cognitive skills, while adverse social experiences negatively affect the proper development of brain and behavior, increasing, for instance, the susceptibility to develop psychiatric conditions. In this context, studying the neurobiology of social behavior in animal models is essential to provide insights into the neuropathology of several psychiatric disorders.

The research activity realized during my PhD, performed in rodent models, provided important information about the brain mechanisms underlying typical and atypical forms of social behavior. In particular, my first studies (Chapters 3 and 4) clarified the role of neurotransmitter systems classically involved in the regulation of social behavior, and in particular in social play behavior. I focused on play behavior because it is the most characteristic social behavior displayed by young mammals, and it is profoundly impaired in several psychiatric disorders. In rats play behavior contains elements of aggressive, predatory and sexual behavior, performed in a modified or exaggerated form (Panksepp, Siviy et al. 1984, Vanderschuren, Niesink et al. 1997). Moreover, social play is considered the first form of non-maternal-oriented social behavior, whose practice is crucial for social, cognitive, emotional and sensorimotor development (Vanderschuren, Achterberg et al. 2016).

In my first study, entitled "*Unidirectional opioid-cannabinoid cross-tolerance in the modulation of social play behavior in rats*" (Chapter 3), I provided the first evidence for unidirectional cross-tolerance between opioid and endocannabinoid neurotransmission in the modulation of social play behavior. A large body of evidence indicates that endocannabinoid and opioid neurotransmission interact to mediate reward processes, including social play reward (Manzanares, Corchero et al. 1999, Fattore, Deiana et al. 2005, Parolaro, Vigano et al. 2007, Trezza, Baarendse et al. 2010, Wei, Lee et al. 2015, Wei, Allsop et al. 2017). Indeed, it has been shown that the play-enhancing effects of drugs that magnify endocannabinoid activity by interfering with either anandamide or 2-AG degradation are blocked by pretreatment with either cannabinoid or opioid receptor antagonists (Solinas and Goldberg 2005, Trezza and Vanderschuren 2008, Trezza and Vanderschuren 2009, Manduca, Lassalle et al. 2016). This functional interaction seems to be bidirectional, since the play-enhancing effects of opioid receptor agonists are antagonized not only by opioid but also by cannabinoid receptor antagonists (Trezza and Vanderschuren 2008, Trezza and Vanderschuren 2009). Moreover, studies in laboratory animals have also shown that repeated administration of cannabinoid or opioid drugs induces cross-tolerance to their acute behavioral and physiological effects (Maldonado 2002, Robledo, Berrendero et al. 2008). The studies described in Chapter 3 extend this scenario by showing

that opioid-cannabinoid cross-tolerance exists in social play behavior. Indeed, the play-enhancing effects induced by systemic administration of the 2-AG hydrolysis inhibitor JZL184 were suppressed in animals repeatedly pretreated with morphine, indicating that cross-tolerance to the effects of the 2-AG hydrolysis inhibitor JZL184 had occurred after repeated administration of morphine. This cross-tolerance was accompanied by changes in the phosphorylation, and therefore the activation, of both CB1 receptors and their effector Akt in the NAc, amygdala and prefrontal cortex. Conversely, tolerance to the play-enhancing effects of morphine had not occurred after repeated treatment with JZL184, as the acute administration of morphine before testing markedly increased social play in rats pretreated with both JZL184 and vehicle. A possible explanation for this negative finding is that JZL184 is not a cannabinoid receptor agonist. Rather, it acts as an indirect agonist by enhancing local 2-AG signaling through the inhibition of its hydrolysis (Long, Li et al. 2009). Since endocannabinoids are released on demand (Piomelli 2003, Di Marzo 2006, Alger and Kim 2011), i.e., only when appropriate stimuli mobilize them, a direct, impulse-independent activation of CB1 receptors may be needed to induce cross-tolerance to the play-enhancing effects of morphine. A better understanding of opioid-cannabinoid interactions in social play helped me to clarify neurobiological aspects of social behavior at young age giving me hints on therapeutic targets for social dysfunctions.

In my second study, entitled "*Detrimental effects of the abused 'bath salt' Methylenedioxypyrovalerone (MDPV) on social play behavior in adolescent rats*" (Chapter 4), I studied how MDPV, a psychoactive stimulant compound belonging to the synthetic cathinones, pharmacologically interact with the dopaminergic and noradrenergic systems to modulate social play, in order to clarify the involvement of these two neurotransmitter systems in social play behavior. Indeed, among others, the dopaminergic and noradrenergic systems play a prominent role in the modulation of social play (Vanderschuren, Achterberg et al. 2016). The implication of dopaminergic neurotransmission in social play behavior lies in its capability to promote motivational processes and incentive salience, while noradrenergic neurotransmission has been thought to be important in a variety of cognitive processes, including learning, attention, and flexibility, although it also plays a role in the perception of emotions and in reward processes (Berridge and Waterhouse 2003, Aston-Jones and Cohen 2005, Ventura, Alcaro et al. 2005, Robbins and Arnsten 2009, Roozendaal and McGaugh 2011, Bouret and Richmond 2015, Vanderschuren, Achterberg et al. 2016) The studies described in Chapter 4 showed that MDPV reduces social behavior through the simultaneous stimulation of α-2 adrenoceptors and dopamine receptors. In humans, MDPV has been reported to induce pro-social effect (Karila, Lafaye, et al. 2018, Technical Report EMCDDA, 2014), which seems to be not in line with what we found here. It should be noted, however, that we found that MDPV selectively reduced social play in rats, while general sociability and social exploration were

not affected. Thus, it could be possible that the social inhibition caused by MDPV found in this study is restricted to the adolescent periods and does not persist at adult ages. Follow-up studies are needed to clarify this issue.

During the second part of my PhD project (Chapters 5,6 and 7), I studied atypical social behavior in rodents to find hints for new therapeutic opportunities to treat social dysfunctions. To do so, I mainly focused on ASD, a group of neurodevelopmental psychiatric disorders whose core symptoms include impaired communication and social interaction. To date, the etiology of ASD is still unclear, with a complex connection between genetic and environmental factors being at the basis of these disorders. Furthermore, there is a wide degree of phenotypic variation in the severity of the core and associated symptoms displayed by ASD patients. As a result, it is difficult to reproduce in one single laboratory animal all the molecular, cellular, and behavioral features of ASD. Despite that, models of ASD exist, and I used one of the most used environmental model of ASD: the rodent animal model based on prenatal exposure to VPA. VPA is a medication used for epilepsy and mood swings but it is also used off label for pathological states such as migraine (Tartaglione, Schiavi et al. 2019). Numerous studies have reported that prenatal VPA exposure in laboratory animals is an animal model of ASD with both high face and construct validity (Roullet, Lai et al. 2013). Indeed, studies in both rats and mice confirm that prenatal VPA exposure leads to autistic-like behaviors in offspring, including social abnormalities, repetitive behaviors and disrupted communication (Roullet, Lai et al. 2013, Servadio, Melancia et al. 2016, Melancia, Schiavi et al. 2018). Moreover, the behavioral abnormalities displayed by animals exposed to VPA during pregnancy are often accompanied by neural impairments (Rodier, Ingram et al. 1996, Ingram, Peckham et al. 2000, Markram, Rinaldi et al. 2008, Rinaldi, Perrodin et al. 2008, Snow, Hartle et al. 2008, Bringas, Carvajal-Flores et al. 2013). In Chapter 5, I provide an exhaustive review about the VPA preclinical model of ASD, entitled "*Prenatal valproate in rodents as a tool to understand the neural underpinnings of social dysfunctions in Autism Spectrum Disorder*".

Using the VPA preclinical model, I first validated the ASD-like symptoms in both male and female rats prenatally exposed to VPA, and then I investigated the involvement of the endocannabinoid system in the sex-specific ASD-like socio-emotional, cognitive and repetitive symptoms displayed by rats prenatally exposed to VPA (Chapter 6). In this study, entitled "*Sex-specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signaling"*, I found that female rats are somehow less vulnerable to the deleterious effects of prenatal VPA exposure on social communication, emotional reactivity and cognitive performance than male rats. Conversely, VPA-exposed female rats show selective deficits in social play behavior and stereotypies. I also found that prenatal VPA exposure alters the phosphorylation of CB1 receptors in

a sex-, age- and tissue-specific manner. It has been previously shown that the enhancement of anandamide activity by inhibiting its degradation ameliorates the socio-emotional and communicative deficits and the stereotypies displayed by VPA-exposed male rats (Kerr, Downey et al. 2013, Servadio, Melancia et al. 2016). The findings reported in Chapter 6 extended these findings by showing that enhancing anandamide signaling through inhibition of its degradation reversed the behavioral deficits displayed by VPA-exposed animals of both sexes.

Next, as the altered social behavior displayed by VPA-exposed rats could be due to either a deficit in social reward processing or to a more general inability to properly understand and respond to social signals, I determined whether the social deficits displayed by VPA-exposed rats are associated with changes in more specific reward-related behaviors, including social, drug and food rewards (Chapter 7). Thus, in my fifth study entitled "*Reward-related behavioral, neurochemical and electrophysiological changes in a rat model of autism based on prenatal exposure to valproic acid*", I performed behavioral, electrophysiological and neurochemical experiments to test the involvement of the brain reward system in the social dysfunctions displayed by rats prenatally exposed to VPA. Indeed, the pervasive social deficits found in autistic patients have been related to blunted social reward processing, i.e., inability to enjoy and prolong reciprocal social interactions, which has been hypothesized to be the consequence of abnormal activity of the brain reward circuit in social contexts (Chevallier, Kohls et al. 2012, Pellissier, Gandia et al. 2018). Based on this, the social dysfunctions displayed by VPA-exposed rats may be caused by either their inability to properly understand and respond to social signals by the social partner or by a failure of their reward system to assign a positive value to the social experience. I found that VPA-exposed rats showed altered expression of dopamine receptors together with inherent hyperexcitability of medium spiny neurons (MSNs) in the NAc. However, when tested for tasks aimed at analyzing reward-related behavior such as socially-induced conditioned place preference, locomotor response to amphetamine and sucrose preference, control and VPA-exposed rats performed similarly, indicating normal responses to social, drug and food rewards. Thus, it is possible that social dysfunctions displayed by VPA-exposed rats are more likely caused by alterations in cognitive aspects of the social interaction, such as the interpretation and reciprocation of social stimuli and/or the ability to adjust the social behavior of the individual to the changing circumstances in the social and physical environment, rather than to inability to enjoy the pleasurable aspects of the social interaction.

In the last part of my PhD project, I used a model of prenatal exposure to cannabinoids in rats to study how early changes in the endocannabinoid system could interfere with social behavior later in life. Is it known that alterations of the endocannabinoid functionality contribute to the pathogenesis of several psychiatric and neurological disorders (Zamberletti, Gabaglio et al. 2017). As

endocannabinoids are key modulators of neural plasticity (Hallmayer, Cleveland et al. 2011) and brain development (Sandin, Lichtenstein et al. 2014), a variety of pathologies are thought to involve dysregulation of their signaling functions. Recently, studies have documented an impaired endocannabinoid signaling in animal models of neuropsychiatric pathology in which social impairment is a core feature such as schizophrenia and ASD (Wei, Allsop et al. 2017). Thus, in my last study, entitled "*Sex-specific behavioral deficits induced at early life by prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 depend on mGlu5 receptor signaling"* (Chapter 8), I and my group studied the behavioral social deficits induced at early life by prolonged cannabinoid exposure in rats. In particular, we studied the effects of prenatal exposure to the cannabinoid receptor agonist WIN55,212-2 on the emotional reactivity and cognitive performance of male and female rat offspring from infancy through adolescence and tested the role of mGlu5 receptor signaling in the observed effects. We found that prenatally WIN-exposed male infant pups emitted less USVs compared with male control pups when separated from the dam and siblings and showed increased locomotor activity, while females were spared. These effects were normalized when male pups were treated with the positive allosteric modulator of mGlu5 receptor CDPPB. When tested at the prepubertal and pubertal periods, WIN-prenatally exposed rats of both sexes did not show any difference in social play behavior, anxiety, and temporal order memory. These results suggested us that *in utero* WIN administration induced mGluR sex-mediated deficits at early life stages (i.e. on USVs and homing behavior) and then, later in life, the reorganization of this signaling occurs leading to some compensatory mechanisms and perhaps mediating specific motivational, rewarding and emotional processes that we did not explore with the present experiments.

To conclude, the research activity realized during my PhD increased our knowledge about the neurotransmitter systems and brain areas involved in the regulation of social behavior, both in health and disease states. Understanding the neural underpinning of social behavior, and particularly social play, in physiological conditions allowed me to study the foundation of the atypical social behaviors observed in the preclinical model of ASD based on prenatal VPA exposure, and in the model of prenatal exposure to cannabinoids, leading me to identify some of the principal neurotransmitter systems involved in social behavior as new potential targets for the treatment of the social dysfunctions that characterize these disorders. Beside others, the endocannabinoid system emerged as a promising target to deeper understand some of the facets of ASD, given its pivotal role in some of the functions that are disrupted in the disease, and its capability to mediate and participate in neuronal plasticity and brain development. To support the notion that the endocannabinoid system has a key role in the proper maturation of brain and behavior, my studies showed that altering endocannabinoid tone during neural development by administration of a cannabinoid receptor

agonist, WIN55,212-2, leads to social dysfunction in rat's early life. However, my research also unlocked new questions, that could be addressed in follow up studies. First of all, given the complexity of neuropsychiatric disorders such as ASD and the impossibility to represent all its facets in a single animal model, it is necessary to confirm my findings in other animal models of ASD. Furthermore, since the results of the first year of my PhD (Chapters 3 and 4) have disclosed the important role of the endogenous opioid and the noradrenergic systems in social play, it would be interesting to evaluate, both from a biochemical and pharmacological point of view, the involvement of these neurotransmitter systems in the social dysfunctions observed in the VPA animal model of ASD. Concerning the dopaminergic system, follow up studies are needed to understand the precise role of the brain reward system in VPA-exposed animals. Indeed, I found a biochemical alteration in the expression of D1 and D2 dopamine receptors in the NAc and in the hippocampus of VPA-exposed animals compared to control animals, that led me to hypothesize that VPA-exposed rats would show atypical reward-related behaviors. However, when tested in tasks aimed at analyzing reward-related behaviors, control and VPA-exposed rats performed similarly. Thus, follow up studies in which other forms of reward are analyzed are needed, to deeper understand the role of the brain reward system in the VPA animal model of ASD. Regarding the positive effects of URB597 in the autistic-like phenotype displayed by VPA-exposed animals, follow up studies are needed to test the effect of chronic treatment with URB597, in order to explore, as much as possible, the effects of the therapeutic use of the drug. Last, since the endocannabinoid, the endogenous opioid, the dopaminergic and the noradrenergic systems are involved in social behaviors, it would be of interest to study how these different neurotransmitter systems interact between each other to contribute to the disrupted social behaviors displayed by rats prenatally exposed to VPA.

In conclusion, the research performed during my three-year PhD project increased our knowledge about the neurotransmitter systems and brain areas involved in the regulation of social behavior, both in health and disease states. Understanding the neural underpinning of social behavior, and particularly social play, in physiological conditions allowed me to study the foundation of the atypical social behaviors observed in the preclinical model of ASD based on prenatal VPA exposure, and in the model of prenatal exposure to cannabinoids, leading me to identify some of the principal neurotransmitter systems involved in social behavior as new potential targets for the treatment of the social dysfunctions that characterize these disorders. Beside others, the endocannabinoid system emerged as a promising target to deeper understand some of the facets of ASD, given its pivotal role in some of the functions that are disrupted in the disease, and its capability to mediate and participate in neuronal plasticity and brain development.

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LIST OF PUBLICATIONS

List of publications included in the PhD thesis:

***Schiavi S,** *Manduca A, Segatto M, Campolongo P, Pallottini V, Vanderschuren LJMJ, Trezza V. (2019) Unidirectional opioid-cannabinoid cross-tolerance in the modulation of social play behavior in rats. Psychopharmacology (Berl). doi: 10.1007/s00213-019-05226-y; (*These authors contributed equally to the study).

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