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PRECLINICAL STUDIES ON NOVEL PHARMACOLOGICAL TARGETS FOR AUTISM SPECTRUM DISORDER

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Riassunto

Studi preclinici di nuovi target farmacologici per il disturbo dello spettro autistico

I termini "Disturbo dello spettro autistico" si riferiscono a un gruppo di disturbi psichiatrici dello sviluppo caratterizzati da deficit nella comunicazione verbale e non verbale, da un comportamento sociale alterato e dalla presenza di comportamenti ripetitivi e stereotipati.
Nonostante i recenti progressi della ricerca scientifica nella comprensione della fisiopatologia del disturbo dello spettro autistico, ad oggi non ci sono terapie efficaci e, sebbene molti farmaci off-label siano utilizzati per attenuare alcuni dei sintomi, nessun farmaco specifico è stato ancora approvato.
In questo contesto, la ricerca preclinica è di fondamentale importanza. Infatti, i modelli animali consentono di riprodurre sintomi specifici della patologia e di studiare il ruolo dei fattori genetici e ambientali, nonché la loro potenziale interazione, nella patogenesi del disturbo autistico. Inoltre, i modelli animali sono importanti per validare nuovi bersagli terapeutici e l'efficacia di nuovi potenziali farmaci.
I comportamenti tipici del disturbo dello spettro autistico possono essere riprodotti in modo efficace negli animali da laboratorio, specialmente nei roditori, e sono disponibili diversi modelli animali che mostrano le caratteristiche comportamentali tipiche dell'autismo, le cui basi neurali sono simili a quelle osservate nei pazienti autistici. Ad esempio, dal momento che i roditori vocalizzano immediatamente dopo la nascita, i deficit nella comunicazione sociale possono essere rilevati fin dai primi giorni di vita, quando altri comportamenti sono ancora immaturi, attraverso l'analisi delle vocalizzazioni emesse dai cuccioli separati dal nido. Inoltre, i deficit cognitivi possono essere analizzati nei roditori in un'età molto precoce attraverso il test comportamentale di *homing*, che consente di valutare la capacità del roditore di discriminare un odore familiare da un odore neutro. Le abilità sociali possono essere facilmente valutate nei roditori attraverso il *social play test* e il 3-*chamber test* durante l'adolescenza e l'età adulta, mentre i comportamenti ripetitivi e stereotipati possono essere analizzati attraverso i test di *marble burying* e *hole board.*
In questo scenario, l'obiettivo generale del mio progetto di dottorato è stato quello di utilizzare modelli preclinici del disturbo dello spettro autistico allo scopo di 1. fare luce sulle possibili alterazioni neurochimiche che causano questo disturbo; 2. identificare nuovi bersagli terapeutici. A tale scopo, ho focalizzato la mia attenzione sull'analisi delle alterazioni comportamentali tipiche del disturbo dello spettro autistico, osservate nei modelli preclinici della patologia. Durante la prima parte del mio progetto di dottorato, ho effettuato esperimenti

comportamentali per validare nel nostro laboratorio due modelli ambientali di autismo. Il primo modello che ho studiato è basato sull'esposizione prenatale al Metimazolo (MMI). In particolar modo, ho studiato se l'ipotiroidismo prenatale indotto da MMI fosse in grado di indurre nella prole di ratto comportamenti simili a quelli esibiti dai pazienti autistici. I risultati ottenuti hanno dimostrato che l'ipotiroidismo indotto da MMI prenatale non causa nella prole del ratto deficit nella comunicazione sociale, nell'interazione sociale, nella sfera cognitiva e un fenotipo ansioso.

Il secondo modello che ho studiato è basato sull'esposizione prenatale ad acido valproico (VPA) nel ratto. Tale modello sperimentale si basa sull'evidenza clinica di un'associazione esistente tra l'assunzione di VPA in gravidanza e l'insorgenza di autismo nella prole esposta. In particolar modo, ho testato nei ratti gli effetti di un'esposizione a dosi crescenti di VPA durante la gravidanza, valutando le alterazioni comportamentali tipiche del disturbo dello spettro autistico nella progenie. Ho anche valutato la risposta al danno del DNA (*double-strand breaks*, DSB) in embrioni esposti a una dose di VPA che ha indotto nella prole di ratto le alterazioni comportamentali tipiche del disturbo dello spettro autistico, al fine di determinare se le alterazioni comportamentali osservate nei ratti esposti a VPA sono associate al danno al DNA indotto dal farmaco. I risultati dei miei esperimenti dimostrano che solamente la somministrazione prenatale di VPA alla dose di 500 mg/kg induce nella prole di ratto deficit nella comunicazione e discriminazione sociale durante l'infanzia e altera il comportamento sociale e l'emotività nella prole durante l'adolescenza e l'età adulta. Abbiamo inoltre scoperto che a questa dose il VPA provoca negli embrioni di ratto l'inibizione dell'enzima istone deacetilasi e favorisce la formazione di DSB nel DNA, compromettendone la riparazione.

Durante la seconda parte del mio Dottorato, ho eseguito esperimenti comportamentali su due modelli preclinci della sindrome dell'X fragile, la causa monogenetica più comune del disturbo dello spettro autistico. Per prima cosa, ho eseguito test comportamentali su topi e ratti *knock-out* per il gene *FMR1* al fine di determinare se questi animali mostrassero sintomi simili a quelli esibiti dai pazienti autistici. Successivamente, ho utilizzato questi due modelli genetici di autismo per testare la validità di un nuovo bersaglio farmacologico. Infatti, è stato recentemente dimostrato che l'mRNA della fosfodiesterasi 2a (*Pde2a*) è uno dei principali bersagli della *fragile X mental retardation protein* (FMRP), una proteina legante gli mRNA codificata dal gene *FMR1* e mancante nella sindrome dell'X fragile. In particolar modo, in assenza di FMRP, il livello di PDE2A è aumentato sia nella corteccia che nell'ippocampo dei topi *Fmr1*-KO e in queste aree cerebrali il livello di cAMP e cGMP è ridotto. Su queste basi,

ho eseguito test comportamentali per valutare la capacità di un inibitore selettivo di PDE2A chiamato BAY607550 di mitigare i deficit comunicativi, sociali e cognitivi mostrati dai topi *Fmr1*-KO nel corso dello sviluppo. Per validare la PDE2A come bersaglio terapeutico per la sindrome dell'X fragile, ho anche esteso lo studio della sua inibizione ai ratti *Fmr1*-KO durante l'infanzia. È interessante notare che il BAY607550 è stato in grado di ripristinare i deficit comunicativi, cognitivi e sociali mostrati dai ratti *Fmr1*-KO. Inoltre, ho convalidato questo risultato bloccando la PDE2A con un altro inibitore altamente specifico chiamato Lu AF64280 che, come BAY607550, è stato in grado di ripristinare i deficit comunicativi mostrati dai topi *Fmr1*-KO. Gli esperimenti biochimici condotti in collaborazione con la dott.ssa Barbara Bardoni dell'Institut de Pharmacologie Moléculaire et Cellulaire di Valbonne, in Francia, hanno dimostrato alterazioni nella lunghezza degli assoni e nella maturazione delle spine dendritiche dei neuroni corticali di topi *Fmr1*-KO e la normale lunghezza degli assoni e la morfologia delle spine dendritiche è stata ripristinata dopo il trattamento con l'inibitore selettivo della PDE2A, BAY607550. Abbiamo anche dimostrato che l'inibizione farmacologica di PDE2A con il BAY607550 ha invertito l'incremento della LTD dipendente da mGluR nell ippocampo dei topi *Fmr1*-KO. Pertanto, gli esperimenti comportamentali, biochimici ed elettrofisiologici descritti rappresentano un buon razionale preclinico per la validazione della PDE2A come nuovo bersaglio farmacologico per attenuare i sintomi osservati nella principale forma genetica del disturbo dello spettro autistico.

In conclusione, l'attività di ricerca realizzata durante il mio triennio di dottorato ha contribuito ad ampliare le attuali conoscenze riguardo i meccanismi neurochimici che sono alla base del disturbo dello spettro autistico e il ruolo dei fattori ambientali e genetici coinvolti nella sua patofisiologia, introducendo nuovi bersagli terapeutici che meritano ulteriori approfondimenti.

Abstract

Preclinical studies on novel pharmacological targets for autism spectrum disorder

The terms "Autism Spectrum Disorder" (ASD) refer to a group of developmental psychiatric disorders characterized by deficits in verbal and non verbal communication, altered social behavior, restricted and repetitive behavior and interests. Currently, clinical research for ASD, and for neuropsychiatric disorders in general, is slowly progressing, primarily due to the ethical problems related to research in human subjects. Furthermore, despite the recent advances in our understanding of ASD pathophysiology, effective targeted therapies are still lacking and, although many off-label medications are used to mitigate some of the symptoms, no specific drug is yet approved.

In this context, preclinical research in appropriate animal models of the human disease is of pivotal importance. Indeed, animal models allow to mimic specific symptoms and to study the role of genetic and environmental factors, as well as their potential interaction, in the pathogenesis of these disorders. In addition, animal models are important to validate new therapeutic targets and the efficacy of potential new drugs. Behaviors relevant to the core and associated symptoms of ASD can be easily and effectively assessed in laboratory animals, especially in rodents, and several animal models are available that show specific autism-relevant behavioral features, whose neural underpinnings resemble those found in autistic patients. For example, since rodent pups vocalize immediately after birth, deficit in social communication can be detected from the first days of life, when other behaviors are still immature, through the analysis of the vocalizations emitted by the pups isolated from the nest. Furthermore, deficits in cognitive processing can be evaluated in rodents at a very early developmental age through the homing behavior test, that allows to assess the ability of rodent pups to discriminate a familiar from a neutral odor. Social abilities can be easily assessed in rodent through the social play behavior test, the social interaction test and the 3-chamber test, during adolescence and adulthood, while repetitive and stereotyped behaviors can be evaluated in rodents through the marble burying and the hole board tests. Animal models of ASD can be assessed in these behavioral tests in order to evaluate the ability of new potential therapeutic compounds to improve behavioral deficits that resemble the core and associated symptoms of ASD.

In this scenario, the overall aim of my PhD project was to use preclinical animal models of ASD in order to 1. shed light on possible neurochemical alterations causing this disorder; 2. find new therapeutic targets. To these aims, I focused my attention on the analysis of

behavioral alterations observed in rodent models of ASD relevant to the core and secondary symptoms displayed by autistic patients. During the first part of my PhD project, I performed behavioral experiments to validate in our laboratory two preclinical environmental animal models of ASD. The first model I studied was based on prenatal exposure to Methimazole (MMI). In particular, I investigated if prenatal MMI-induced hypothyroidism was able to cause in the rat offspring behaviors that resemble core and associated ASD symptoms. I found that prenatal MMI-induced hypothyroidism did not cause in the rat offspring deficit in social communication, social interaction, anxiety and cognition.

The second model I studied was based on prenatal exposure to valproic acid (VPA) in rats. In particular, I tested the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. I also evaluated the double strand breaks (DSB) response in embryos exposed to a dose of VPA that induced autistic-like features in the rat offspring, in order to determine whether the autistic-like features displayed by VPA-exposed rats are associated with drug-induced DNA damage. My results extended the existing literature about the effects of different doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. In particular, I found that only prenatal administration of VPA at the dose of 500 mg/kg induced deficits in communication and social discrimination in rat pups, and altered social behavior and emotionality in the adolescent and adult offspring. I also found that at this dose of VPA inhibited histone deacetylase in rat embryos and favored the formation of DNA DSB, but impaired their repair.

During the second part of my PhD project, I performed behavioral experiments on rodent models of Fragile X syndrome (FXS), the most common monogenetic cause of ASD. I first performed behavioral tests in *Fmr1*-KO rats and mice in order to determine if these two genetic animal models of ASD were able to reproduce core and associated symptoms of ASD. Next, I used these animal models to test the validity of a novel pharmacological target. Indeed, it has been recently demonstrated that the mRNA of *Phosphodiesterase 2 a* (*Pde2a*) is one prominent target of the fragile X mental retardation protein (FMRP), which is a mRNA binding protein encoded by the *FMR1* gene and missing in FXS. In particular, in the absence of FMRP, the level of PDE2A is elevated both in cortex and hippocampus and the level of cAMP and cGMP is reduced in those brain areas of *Fmr1*-KO mice. On these bases, I performed behavioral tests to assess the ability of a selective inhibitor of PDE2A called BAY607550 to rescue the atypical communicative, social and cognitive behaviors displayed by *Fmr1*-KO mice in the course of development. To validate PDE2A as a therapeutic target for FXS, I also extended the study of its inhibition to *Fmr1*-KO infant rats. Interestingly,

BAY607550 was able to revert the communicative, cognitive and social deficits displayed by *Fmr1*-KO animals. Moreover, I validated this result by inhibiting PDE2A with another highly specific PDE2A inhibitor called Lu AF64280 that, similar to BAY607550, was able to revert the altered communicative pattern displayed by *Fmr1*-KO mice. The biochemical experiments performed in collaboration with Dr. Barbara Bardoni from the Institut de Pharmacologie Moléculaire et Cellulaire in Valbonne, France, showed alterations in axonal length and spine maturation in cultured cortical neurons of *Fmr1*-KO mice and that the normal length of growing axons and dendritic spine morphology were restored after treatment with the specific PDE2A inhibitor BAY607550. We also demonstrated that the pharmacological inhibition of PDE2A with the specific inhibitor BAY607550 rescued the exaggerated mGluR-dependent LTD in *Fmr1*-KO hippocampal slices. Thus, the behavioral, biochemical and electrophysiology experiments described here provided a preclinical rationale about the validity of PDE2A as a new pharmacological target to mitigate the symptoms observed in the leading genetic form of ASD.

In conclusion, the research performed during my three-year PhD project increased the current knowledge about the neurochemical mechanisms underlying ASD, about the role of environmental and genetic factors in its pathophysiology and introduced new therapeutic targets that are worth of further investigation.

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Chapter 1

Introduction

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Introduction

1. Autism spectrum disorder (ASD)

Autism spectrum disorder (ASD) is a group of heterogeneous neurodevelopmental disorders characterized by early-onset impairment in verbal and non verbal communication, altered social behavior, restricted and repetitive behavior and interests (American Psychiatric Association, 2013). Due to a more precise definition of diagnostic criteria, the prevalence of ASD has increased in the last 30 years, and recent epidemiological studies reported a prevalence of about 1% (Lai et al., 2014). According to the last edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013), the syndrome is characterized by two core diagnostic criteria: criterion A, that includes social/communicative deficits; criterion B, that includes restricted, repetitive patterns of behaviors, interests, or activities (American Psychiatric Association, 2013). ASD patients may also present some associated symptoms such as anxiety, hyperactivity, impulsivity, seizures or gastrointestinal disorders (Lai et al., 2014). The wide degree of variation in both the core symptoms and the comorbid features has led to the use of the term 'spectrum' to indicate this syndrome. As early as infancy, babies with ASD may demonstrate the following symptoms: (i) lack of or weak response to their parents' voice; (ii) not using their voice to attract attention to themselves, express emotions, or establish contact; and (iii) 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 impoverished (Jones et al., 2014; Lai et al., 2014; Zachor and Curatolo, 2014). For these reasons parents are usually the first to notice symptoms of social dysfunction and impaired social communication in their children (Young et al., 2003).

No specific and effective drug treatment is currently available for ASD (Lai et al., 2014; Mohiuddin and Ghaziuddin, 2013). Current approaches focus on behavioral therapy and off-label medications that mitigate only a limited set of symptoms: antidepressant drugs are often prescribed to ameliorate symptoms of anxiety, depression, or compulsive and aggressive behaviors. Antipsychotic medications are used to treat stereotypies, irritability, and self-injuring behaviors. Seizures can be treated with one or more anticonvulsant drugs. Stimulant

drugs are sometimes used effectively to decrease impulsivity and hyperactivity (Mohiuddin and Ghaziuddin, 2013; Zachor and Curatolo, 2014).

Given the diverse set of symptoms displayed by ASD patients, it is not surprising that multiple etiological components are thought to be involved in the pathogenesis of the disease, including genetic and environmental factors (Jeste and Geschwind, 2014). Genetic factors clearly play an important role, as the concordance rate for ASD ranges from ~ 2–6% in dizygotic twins to 60–95% in monozygotic twins (Abrahams and Geschwind, 2008). Accordingly, single-gene mutations, polymorphisms, and copy number variations have been associated with ASD. At the same time, the evidence that the concordance is less than 100% in monozygotic twins prompted investigation of environmental and epigenetic factors as causes of ASD (Chaste and Leboyer, 2012). Several prenatal and perinatal risk factors have been identified, including exposure to intrauterine infections, maternal treatment with drugs such as valproic acid (VPA) and thalidomide, or exposure to toxicants such as organophosphate insecticides or heavy metals (Chaste and Leboyer, 2012).

The most common genetic form of ASD is the Fragile X syndrome (FXS). Indeed, FXS is the most common monogenetic cause of intellectual disability (ID) and ASD. FXS patients show developmental delay, deficits in executive functioning and a wide range of psychiatric problems such as repetitive behavior, social deficits, hyperactivity, anxiety and irritability (Garber et al., 2008; Hersh and Saul, 2011; Lozano et al., 2014). FXS is associated with an unstable expansion of a CGG trinucleotide repeat within the 5′untranslated region (5′UTR) of the fragile X mental retardation 1 (*FMR1*) gene, located in the X chromosome (Oberle et al., 1991; Verkerk et al., 1991). In the normal population, the number of CGG triplets in the *FMR1* gene varies from 6 to 54, allowing transcription and translation of the gene. When the number of CGG triplets expands between 55 and 200, the premutation (PM) state occurs, while more than 200 CGG repeats cause the full mutation and the fragile site at Xq27.3. This expansion induces hypermethylation and other epigenetic modifications of the CpG island in the promoter region, causing the heterocromatinization of the FMR1 locus and consequently with the loss of the protein product, the fragile X mental retardation protein (FMRP) (Coffee et al., 2002). FMRP is an RNA-binding protein involved in different steps of RNA metabolism ranging from nuclear export to transport of mRNA along neurites and translational control in the soma and at synapses (Bardoni et al., 2006). FMRP is expressed in many tissues and it is particularly abundant in the brain, where it is essential for neural development and plasticity (Ferron, 2016; Jin and Warren, 2000). In the absence of FMRP,

the deregulation of translation/transport/stability of these mRNAs affects multiple neuronal pathways, generating the altered phenotype displayed by FXS patients.

Approximately 30% of patients with FXS meet the full diagnostic criteria for ASD (Harris et al., 2008), while over 90% of individuals with FXS display some ASD symptoms (Hernandez et al., 2009). FXS patients usually exhibit long narrow face, prominent ears and forehead, flat feet, hypotonia and joint hypermobility, and macroorchidism in about 95% of postpuberal males (Lachiewicz and Dawson, 1994). At the behavioral level, FXS is characterized by a progressive decline of cognitive abilities (Huddleston et al., 2014). Other common behavioral features displayed by FXS patients involve poor eye contact or gaze aversion, excessive shyness, anxiety, stereotypic movements, unusual speech, self-injury, hand flapping and hand biting, hyperactivity, tactile defensiveness, attention deficits, problems in impulse control, hyperarousal and oversensitivity to tactile, auditory, olfactory or visual stimuli (Abbeduto and Hagerman, 1997; Cohen et al., 1989; Cordeiro et al., 2011; Hall et al., 2008; Reiss and Dant, 2003). The behavioral patterns emerging during the early life of individuals affected by FXS led to associate FXS with ASD, suggesting to consider the FM of *FMR1* gene as the most common monogenic cause of syndromic ASD (Hatton et al., 2006).

2. Animal models of ASD

Despite the recent advances in our understanding of ASD pathophysiology, effective targeted therapies are still lacking and, although many off-label medications are used to mitigate some of the symptoms, no specific drug is yet approved.

In this scenario, the use of animal models substantially contributed to increase our understanding of pathophysiological aspects ASD. Indeed, animal models mimicking the specific symptoms of a certain disease are excellent translational research tools, providing the possibility to study the neurobiological bases of a particular normal or abnormal phenotype (physiologically, genetically or environmentally induced). Furthermore, animal models allow to understand the role of both genetic and environmental factors possibly involved in ASD, and their potential interactions. Last, animal models are essential to validate new therapeutic targets and the efficacy of new potential drug candidates.

2.1 Validation of animal models

In 1969, McKinney and Bunney described the main criteria for the definition of an animal model (McKinney and Bunney, 1969). According to McKinney and Bunney, an ideal animal model should mimic a certain disease in its etiology, biochemistry, symptomatology and

treatment. The McKinney and Bunney (1969) criteria laid the foundation for the concept of face, construct and predictive validity of animal models (Blanchard et al., 2013; Willner, 1984). In animal models of neuropsychiatric disorders, face validity refers to the similarities between the symptoms displayed by the patients and the behaviors observed in the animal model of the disease. Construct validity refers to the similarity between the neurobiological mechanisms underlying the behavior in the model and those underlying the behavior in the pathological state that is being modeled. The criterion of predictive validity is related to the ability of the model to predict interventions that will be successful (or unsuccessful) in the clinical setting (Blanchard et al., 2013; Willner, 1984), including sensitivity of the proposed model to pharmacological manipulations affecting the disease in humans, either in a positive or in a negative direction (Blanchard et al., 2013). A valid animal model should also be reliable – that is, the results obtained through the model should be the same between one time and another within the same laboratory and between one laboratory and another (van der Staay et al., 2009; Willner, 1997).

Given the multifactorial etiology of ASD and its great phenotypic variation, there is no animal model that can capture, at once, all the molecular, cellular, and behavioral features of ASD. Rather, as ASD is defined by specific behavioral characteristics, a useful approach in ASD research is to focus on animal behaviors that are relevant to the core diagnostic symptoms of the disease, in order to study both the underlying neural mechanisms and the potential pharmacological targets to mitigate the phenotype. Thus, a valid animal model would show behaviors relevant to each category of the diagnostic symptoms of ASD.

2.2 Rodent models for the study of ASD

Rodent animal models of ASD are essential to understand the impact of genetic and environmental factors on the phenotype displayed by patients, to shed light on the neurobiological aspects underlying this phenotype, and to assess potential therapeutic targets. Behaviors relevant to the core and associated symptoms of ASD can be easily and effectively assessed in laboratory animals, and several rodent models are available that show specific autism-relevant behavioral features, whose neural underpinnings resemble those found in autistic patients. Rodent models of ASD based on genetic factors include the BTBR T+tf/J mouse model, the model based on the mutations in neuroligin-3 (NLGN-3) and neuroligin-4 (NLGN-4) genes, the model based on the mutations in the *SHANK* genes, the model based on the mutations in the tuberous sclerosis (TSC) 1 and 2 genes and the model based on the mutation in the *FMR1* gene.

In addition to the role of genetic factors in ASD (Kim et al., 2014), there is extensive literature to show correlations between non-heritable factors and the disease. In particular, several environmental factors have been correlated with ASD (Arndt et al., 2005), such as maternal infection (Chess, 1971) or maternal exposure to ethanol (Nanson, 1992), thalidomide (Stromland et al., 1994), VPA (Moore et al., 2000), and maternal hypothyroidism (Gillberg et al., 1992). The two most widely used environmental animal models of ASD are the rodent models based on maternal exposure to VPA or to maternal infection. The first model is based on the prenatal exposure to VPA, around the time of neuronal tube closure, which in rodents occurs at around gestational day 12.5.

Rodent models of ASD induced by maternal infection are based on maternal respiratory infection with influenza virus or maternal immune activation (MIA) induced by polyinosine:cytosine, poly(I:C), a synthetic double-stranded RNA evoking an antiviral-like immune reaction, or by lipopolysaccharide (LPS), which evokes an antibacterial-like immune reaction (Patterson, 2011).

Another environmental model of ASD is based on hormonal dysfunction during pregnancy. For example, the animal model of ASD based on the gestational hypothyroidism is generated by the administration of Methimazole (MMI), which affects thyroid hormone synthesis and deiodinase activity by inhibiting thyroid peroxidase, to rat dams.

At the behavioral level, all the rodent models described here recapitulate the behavioral symptoms displayed by autistic patients (for a review see (Servadio et al., 2015)).

2.3 ASD relevant behavioral phenotypes in the laboratory setting

At the behavioral level, the most common symptoms displayed by ASD patients are impaired social communication, altered social behaviors and stereotypies. Patients may also present some associated symptoms such as anxiety and hyperactivity. These behavioral features can be modelled in laboratory animals, and deficits in these domains are observed in animal models of ASD.

2.3.1 Deficits in social communication

One of the most widely used means to study social communication in rodents is the analysis of ultrasonic vocalization (USV) emission in social settings. Like many other vertebrates, rodents emit USVs in different social situations across their entire lifespan: for instance, to communicate distress due to separation from mother and siblings or following a predator attack, to communicate pleasure in the anticipation of and during mating or playful

interactions, or to communicate food location to other group members (Cuomo et al., 1988; D'Amato, 1991; Portfors, 2007; Scattoni et al., 2009). The transmission of different types of information through USVs depends on the specific frequency and temporal properties of the acoustic signals (Portfors, 2007): in rodents, low frequency (around 22kHz) USVs have been associated with negative social experiences (e.g. exposure to predator odor, inter male fighting), whereas high-frequency (around 50kHz) USVs have been detected in social contexts involving potential reward (e.g. sexual approach, play fighting) (Burgdorf et al., 2011). Although rodent USVs are generally inaudible to humans, they can be studied in laboratory settings using specific equipment. A very useful tool in rodent models is the analysis of the USVs emitted by rodent pups in response to separation from the mother and the nest. When separated from their mother and siblings, rats and mice emit USVs with frequencies between 30 and 90kHz (Zippelius and Schleidt, 1956). These USVs play an essential communicative role in mother–offspring interaction and are crucial for pup survival (Hofer and Shair, 1991). They are, indeed, a potent stimulus for maternal retrieval and elicit care-giving behaviors in the dam (Branchi et al., 2001; Scattoni et al., 2009; Trezza et al., 2011). The idea that USV emission in pups serves a communicative function originated from early findings showing that dams selectively retrieve vocalizing pups, but not anesthetized or killed pups (Zippelius and Schleidt, 1956). Since this first study, many experiments have confirmed that maternal retrieval of pups can be induced by solely presenting pup USVs (for a review see (Ehret, 2005)). This early vocalization response is observed in most mammalian species, including humans, when infants separated from their familiar surroundings cry to elicit maternal care. As rodent pups vocalize immediately after birth, isolation-induced USVs can be detected from the first days of life, when other behaviors are still immature (Portfors, 2007). The most widely used and simplest 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. Although USVs can be counted manually by the experimenter by listening to the audible output through earphones, nowadays specific software is available to analyze each spectrogram for the number and shape of the USVs emitted. As low temperatures also elicit USV emission, the testing room is maintained around 25°C and the body temperature of each pup is controlled before and after the test. Furthermore, when the pups get older, USV emission can be detected in other social contexts, such as during juvenile social play behavior, during adult social

interaction, or in response to female odors (for reviews, see (Portfors, 2007; Wohr and Schwarting, 2013))

2.3.2 Deficits in social behavior and sociability

Social play behavior is the first form of non-mother directed social behavior displayed by most developing mammals. The opportunity to engage in social play is crucial both for children and young mammals, since it helps to develop communicative skills, social competence, and behavioral and mental flexibility (Vanderschuren and Trezza, 2014). Social play behavior can be easily assessed in laboratory animals. In particular, the rat is an ideal species to study this behavior, since social play is the most commonly occurring form of social behavior displayed by rats between weaning and sexual maturation (Vanderschuren and Trezza, 2014). In rats, social play can be easily quantified by measuring frequency and duration of specific behaviors (Trezza et al., 2010). Social behaviors primarily related to play, such as pouncing (one rat soliciting another animal to play, by attempting to nose or rub the nape of its neck), pinning (the animal that is pounced upon fully rotates to its dorsal surface with the other animal standing over it) and chasing (the animal that is pounced upon responds by evading and the soliciting rat may start to chase it) occur frequently when young rats are interacting with each other and these behavioral patterns can be easily recognized (Trezza et al., 2010). As the rats get older, the structure of social play changes: rather than pinning, the most frequent response to pouncing become evasion and partial rotation (Trezza et al., 2010). Since social play is a rewarding social activity, it can be associated with the emission of high frequency USVs. Indeed, some researchers reported that rats emit more 50-kHz USVs while playing together than while alone (Knutson et al., 1998), although other studies failed to correlate the performance of social play behavior in adolescent rats with the emission of 50-kHz USVs (Manduca et al., 2014a; Manduca et al., 2014b; Willey and Spear, 2012). Since mice usually engage in very rudimentary forms of social play (Pellis and Pasztor, 1999), other behavioral tests are usually used to assess social behavior in mouse models of ASD. A common behavioral paradigm used to assess social approach in both mice and rats is the 3-chamber test (Nadler et al., 2004). The apparatus usually consists of a three-chamber box with openings between the lateral chambers and the central compartment. The two lateral chambers are identical, except for the fact that, during the testing session, one chamber contains an unfamiliar stimulus animal inside a cage, while the other chamber contains an identical but empty cage. During the habituation phase, the experimental subject is left free to explore the empty apparatus and to enter in the two lateral identical empty chambers. During the testing

session, the experimental subject is placed in the central compartment and can choose to spend time either in the chamber that contains the cage with the unfamiliar stimulus animal or in the chamber that contains the empty cage. During the test session, “sociability” is defined as the propensity of the experimental subject to spend time in the chamber containing the stimulus animal, as compared to time spent alone in the identical but empty opposite chamber. Another session may follow to assess the so called “preference for social novelty”. During this second session, the stimulus animal (stranger-1) used in the test session is confined again in the cage located in the same compartment as in the test session, while a new unfamiliar stimulus animal (stranger-2) is placed inside the cage in the opposite chamber, which was empty in the previous session (Liu and Smith, 2009). Usually, rodents prefer to spend time with a peer rather than alone, and with an unfamiliar rather than a familiar animal. Compared to control animals, rodents showing social dysfunctions tend to enter less and spend less time in the chamber with the stimulus (during the first test session) or in the chamber with the stranger-2 (during the “preference for social novelty” session) (Baronio et al., 2015; McFarlane et al., 2008; Moy et al., 2013; Yang et al., 2007).

2.3.3 Repetitive and stereotyped behaviors

Rodents show particular behaviors that can be cataloged as stereotyped behaviors, such as high levels of vertical jumping, circling, digging, rearing and excessive self-grooming. The marble burying test is commonly used to detect repetitive digging behavior in rodents. In this test, the animal is placed in a cage containing clean bedding and some marbles equidistant from each other. The test consists of a 10–20- minutes exploration period, after which the animal is removed from the cage and the number of marbles buried with bedding up to 2/3 of their depth is counted (Deacon, 2006). Stereotypies and perseverative behaviors in rodents can also be detected through the hole board test. The apparatus is a square metal table with several evenly spaced holes, inserted in a Plexiglas arena. Rats or mice are individually placed in the apparatus and their behavior is observed for few minutes. Dipping behavior is scored by the number of times an animal inserts its head into a hole at least up to the eye level. High frequencies of dipping behavior indicate stereotyped behavior.

2.3.4 Cognitive deficits

Deficits in cognitive processing can be evaluated in rodents at a very early developmental age through the homing behavior test, that allows to assess the ability of rodent pups to discriminate a familiar from a neutral odor. This test exploits the strong tendency of immature

rodent pups to maintain body contact with the dam and siblings and it is based on the learned association between maternal odors and maternal stimulation, which is crucial for the development of social behavior and social recognition (Melo et al., 2006; Servadio et al., 2016). The experimental procedure involves a brief isolation of the pup prior to testing; then, the pup is placed for few minutes in a cage with the floor covered for two parts by clean sawdust and for one part by sawdust from the pup's 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., 2008; Servadio et al., 2016).

One of the most popular tests routinely used to assess cognitive performance in rodent models of ASD is the novel object recognition (NOR) test. The test is based on the observation that when rodents are exposed to two objects, one familiar and one novel, they approach and spend more time exploring the novel than the familiar object (Ennaceur, 2010). The test is therefore used to assess recognition memory, defined as the ability of rodents to recognize and discriminate an object previously explored (and therefore familiar) compared to a new (and therefore unfamiliar) object. The test usually consists of two phases: in the first phase, the animal is placed in an open arena and left free to explore two identical objects. Then, the animal is returned to its home cage for a retention period. In the second phase, the animal is returned to the experimental arena and presented with two objects: the previously experienced object and a novel object. Object recognition is measured by the difference in the exploration time of the novel and familiar objects (Bevins and Besheer, 2006).

The inhibitory avoidance test is commonly used to study emotional memory processing in rodents. During inhibitory avoidance training, rodents receive a single mildly aversive footshock after stepping from a lighted compartment into a darkened compartment. Memory retention is tested usually 24 or 48 h later by placing the animal in the lighted compartment and measuring its latency to enter the dark compartment where the aversive footshock was originally delivered. Longer retention latencies during the test session indicate better memory for the emotionally salient event (i.e., the footshock) (McGaugh and Roozendaal, 2009).

The Morris water maze, described for the first time by Richard Morris in 1981 (Morris, 1981) relies on the ability of rodents to use visual external cues to locate a submerged escape platform in an open circular swimming arena. The most basic experimental procedure allows to assess spatial learning, i.e., the ability of the animals, learned over several training sessions, to use external visual cues to navigate a direct path to the hidden platform when started from different, random locations around the perimeter of the pool. Besides this basic procedure, many variations of this test have been described (Vorhees and Williams, 2006).

2.3.5 Anxiety-like behaviors

Several behavioral tasks are available to assess anxiety-like behaviors in rodents. The Elevated Plus-Maze (EPM) test is the most widely used one. The test is based on the innate conflict of rodents between the natural fear for open spaces and the innate interest for the exploration of new environments. The apparatus is an elevated cross-shaped platform, with two open and two closed arms. The animal is placed in the apparatus for a 5-min session, and the time spent in the open and closed arms and the number of open and closed arm entries are scored. An unusually high time spent in the closed arms indicates a more pronounced anxious phenotype (Pellow and File, 1986). The choice between sheltered and exposed regions is also at the bases of the lightdark box test, in which animals are allowed to freely move between a small dark (and therefore perceived as safe) compartment and a large illuminated (and therefore perceived as aversive) compartment of a two-chambered apparatus (Crawley and Goodwin, 1980).

2.4 Environmental animal models of ASD

Most of the rodent models of ASD based on environmental factors are supported by strong epidemiological data (Gardener et al., 2009; Newschaffer et al., 2007; Veiby et al., 2013). Two common rodent models of ASD based on environmental factors that are known to induce the disease in humans are related to prenatal exposure to pharmaceutical agents at the time of neural tube closure and to hormonal dysfunctions during pregnancy. Below, I discuss two environmental animal models that are used for the study of ASD: the first model is based on the prenatal exposure to VPA, while the second model is based on the gestational hypothyroidism induce by administration of MMI during pregnancy.

2.4.1 The rat model based on prenatal exposure to valproic acid (VPA)

VPA is an anticonvulsant and mood-stabilizing drug primarily prescribed to treat epilepsy and bipolar disorder (Johannessen and Johannessen, 2003). However, the use of VPA during pregnancy is related to the onset of several minor and major malformations in the offspring, such as neural tube defects, cleft lip and palate, cardiovascular abnormalities, genitourinary defects, endocrinological disorders, limb defects, developmental delay, and also ASD (Jentink et al., 2010; Kozma, 2001). Specifically, several epidemiological studies showed that some behavioral alterations found in children exposed to VPA during pregnancy had several aspects in common with the symptoms displayed by autistic patients (Christianson et al., 1994; Williams et al., 2001; Williams and Hersh, 1997). Indeed, the behavioral phenotype observed

in these children was characterized by impaired communication, reduced sociability, and stereotyped and repetitive behaviors and interests, providing evidence that prenatal VPA exposure is a risk factor for ASD. On the basis of these epidemiological findings, prenatal exposure to VPA in rodents has been validated and standardized in many laboratories and to date has become the most widely used environmentally triggered model of ASD, in both rats (Markram et al., 2008; Rodier et al., 1996) and mice (Gandal et al., 2010; Wagner et al., 2006).

One of the first studies demonstrating that prenatal VPA exposure reproduces some of the symptoms of ASD in laboratory animals was performed by Rodier et al. (1996). The authors of this study treated pregnant rats with 350mg/kg of VPA on gestational day 11.5, 12, or 12.5. Postmortem brain analysis revealed that the offspring exposed to VPA during gestation showed a decrease in the number of motor nuclei, comparable to the damage found in autistic patients, with no overt signs of other physical malformation (Rodier et al., 1996). Concerning the validity of this rodent model of ASD, VPA-exposed animals exhibit most of the behavioral and anatomical abnormalities observed in autistic patients. At the behavioral level, they show impairments in social interaction, repetitive and stereotyped behaviors, increased anxiety, alteration in fear memory, and compromised emission of pup isolation-induced USVs. In particular VPA-exposed animals show impaired social behavior when tested in the social play behavior (Chomiak et al., 2010; Schneider and Przewlocki, 2005), three-chamber (Baronio et al., 2015; Kerr et al., 2013; Kim et al., 2011), resident-intruder (Felix-Ortiz and Febo, 2012) and social interaction tests (Felix-Ortiz and Febo, 2012; Markram et al., 2008; Schneider and Przewlocki, 2005). Furthermore, several studies have shown alterations in the number of USVs emitted by pups prenatally exposed to VPA when isolated from their nests (Dufour-Rainfray et al., 2010; Gandal et al., 2010; Schneider and Przewlocki, 2005). 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 (Baronio et al., 2015; Kim et al., 2014; Mehta et al., 2011). These findings show that this animal model of ASD displays the characteristic symptoms essential for the diagnosis of the disease, together with comorbid features, such as increased anxiety and cognitive deficits (Dufour-Rainfray et al., 2010; Kim et al., 2011; Mychasiuk et al., 2012; Roullet et al., 2013; Schneider and Przewlocki, 2005). 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 et al., 1996; Tashiro et al., 2011), a rearrangement of the dendritic morphology in

several limbic and cortical regions (Bringas et al., 2013; Snow et al., 2008), hyperreactivity of pyramidal neurons after electrical stimulation and increased synaptic plasticity in the amygdala (Markram et al., 2008), decreased excitability with a reduction in putative synaptic contacts in pyramidal neurons (Rinaldi et al., 2008), and a reduced number of Purkinje cells in the posterior lobes of the cerebellum (Ingram et al., 2000). Altogether, these findings suggest that the VPA rodent model of ASD has face and construct validity. As for predictive validity, some studies tested the ability of 'off-label' drugs commonly prescribed to autistic patients to ameliorate the behavioral and brain abnormalities displayed by VPA-exposed animals. For instance, Choi et al. (2014) found that the attention deficit hyperactivity disorder (ADHD) medication atomoxetine mitigated the hyperlocomotion displayed by VPA-exposed rats (Choi et al., 2014). Kim et al. (2014) reported that the acetylcholinesterase inhibitor donepezil improved sociability and prevented repetitive behavior and hyperactivity in VPA-exposed mouse offspring (Kim et al., 2014), a result that is in line with the dysregulation of the brain cholinergic system frequently reported in ASD patients (Lam et al., 2006). Last, the histamine receptor 3 antagonist ciproxifan attenuated the social deficits and stereotypic behaviors displayed by VPA-exposed mice (Baronio et al., 2015).

2.4.2 The rat model based on prenatal exposure to Methimazole (MMI)

Thyroid hormones play a fundamental role in the development of the central nervous system, since they are involved in many processes underlying brain development and maturation (Moog et al., 2017): neuronal and glial cell differentiation and proliferation, axonal and dendritic growth, synapse formation, cell migration and myelination (Bernal, 2007; van Wijk et al., 2008). Optimal thyroid functioning is required during pregnancy. The fetal thyroid gland is not functioning until mid-gestation, being active at 16–20 weeks post-conception in humans and at gestational day 17.5 in rats (Moog et al., 2017; Morreale de Escobar et al., 2004). However, even before the onset of fetal thyroid function, the fetal cerebral cortex is capable of generating T3 from maternal T4 by local deiodination, and significant levels of T3 and thyroid hormone receptors (TRs) can be found in the fetal brain (Chan et al., 2002; Moog et al., 2017). Thus, active transport of maternal thyroid hormones across the placenta has to occur during the early phases of gestation and it is essential for the development of the mammalian central nervous system (Moog et al., 2017). Maternal thyroid hormone deficiency has adverse consequences on pregnancy outcome and offspring neurodevelopment. Overt hypothyroidism is associated with increased prevalence of abortion, anemia, pregnancy-induced hypertension, preeclampsia, placental abruption, postpartum hemorrhage, premature

birth, low birth weight, intrauterine fetal death and neonatal respiratory distress, associated with long term effect on the cognitive function of the offspring (Nazarpour et al., 2015). Recent evidence suggests that even more moderate forms of maternal thyroid dysfunction, particularly during early gestation, may have a long-lasting influence on child development, impairing the offspring's cognitive and motor development and increasing the risk of neurodevelopmental disorders (Moog et al., 2017). In particular, thyroid hormone deficiencies during brain development, either due to a genetic deficiency of the thyroid receptor interacting protein, which codes for a transcriptional regulator associated with nuclear TRs (Castermans et al., 2007), or due to maternal hypothyroidism, have been associated with an increased risk of ASD (Andersen et al., 2014; Berbel et al., 2014; Getahun et al., 2018; Roman et al., 2013). The animal model of ASD based on the gestational hypothyroidism is generated by the administration of MMI to rat dams. MMI affects thyroid hormone synthesis and deiodinase activity by inhibiting thyroid peroxidase and it has been extensively used to model maternal hypothyroidism in laboratory animals (Darbra et al., 2003; Sala-Roca et al., 2002; Santos et al., 2012). The model is based on the administration of 20 mg of MMI dissolved in tap water (100 ml) (Darbra et al., 2003; Sala-Roca et al., 2002; Santos et al., 2012), from gestational day (GD) 9 until the day of the birth (Darbra et al., 2003). Indeed, it has previously been shown that MMI, given for 10 days to pregnant rats from GD9, is able to cause hypothyroidism in the dams by inducing a decrease of circulating maternal total T3 and T4 levels (Ahmed et al., 2010; Santos et al., 2012). Despite MMI affects thyroid hormone synthesis and deiodinase activity by inhibiting thyroid peroxidase and it has been extensively used to model maternal hypothyroidism in laboratory animals (Darbra et al., 2003; Sala-Roca et al., 2002; Santos et al., 2012), there is not a scientific evidence that show the ability of this preclinical animal model to reproduce all the core and associated symptoms of ASD. Several studies show altered pattern of behavior in rodents prenatally exposed to MMI which can be related to ASD: compared to control animals, MMI-exposed animals show deficits in the prepulse inhibition of the acoustic startle response, that is used as a measure of sensorimotor gating mechanisms, hyperactivity, lower level of anxiety, and deficit in the emotional learning (Darbra et al., 2003; Navarro et al., 2015; Sala-Roca et al., 2002).

2.5 Genetic animal models of ASD: *Fmr1*-KO mice and rats

FXS is the most common form of inherited mental retardation and the most frequent monogenic cause of syndromic ASD. The syndrome is caused by the loss of FMRP, a key RNA-binding protein involved in synaptic plasticity and neuronal morphology. At the

behavioral level, the most common symptoms displayed by FXS patients are impaired cognition and altered social behaviors. These behavioral features can be modelled in laboratory animals, and deficits in these domains are observed in animal models of FXS. These animal models are essential to understand the impact of the FMR1 mutation on the phenotype displayed by FXS patients, to shed light on the neurobiological aspects underlying this phenotype, and to assess potential therapeutic targets for the syndrome. The first and most widely used animal model for FXS is the *Fmr1*-KO mice, obtained by the inactivation of the murine gene that causes the loss of FMRP production (Bakker et al., 1994). This animal model reproduces many of the behavioral symptoms displayed by FXS patients: cognitive deficits, such as impairments in spatial and reversal learning, social anxiety, reduced social interaction, repetitive behaviors and hyperactivity (Dahlhaus and El-Husseini, 2010; Ding et al., 2014; Gomis-Gonzalez et al., 2016; Michalon et al., 2014; Mineur et al., 2002; Pardo et al., 2017; Pietropaolo et al., 2011; Veeraragavan et al., 2011) but also recapitulates the synaptic alterations which characterize FXS (Dolen et al., 2007; Galvez and Greenough, 2005; He and Portera-Cailliau, 2013; McKinney et al., 2005). Over the past decade, the *Fmr1*-KO mouse was the only animal model available to study FXS. More recently, *Fmr1*-KO rats have been generated by zinc-finger nuclease (ZFN) methodologies (Geurts et al., 2009; Hamilton et al., 2014) and by CRISPR technology (Tian et al., 2017). *Fmr1*-KO rats display a number of behavioral alterations which characterized FXS in humans, like altered patterns of social interaction (Tian et al., 2017) and social play behavior (Hamilton et al., 2014), defects in visual attention (Berzhanskaya et al., 2016), and auditory dysfunctions (Engineer et al., 2014). Since rats have bigger brains than mice, are easier to train, can learn sophisticated behaviors and have an elaborated social repertoire, nowadays *Fmr1*-KO rats are considered a valuable tool to study neurobiological aspects of FXS and to test potential new treatments (Hamilton et al., 2014).

3. *Phosphodiesterase 2a (Pde2a)* as a new therapeutic target for ASD

3.1 cAMP and cGMP signaling pathway

3′,5′-cyclic monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are two cyclic nucleotides activated by extracellular chemical substances (first messengers) which cannot enter cells directly (Yan et al., 2016). Particularly, these first messengers translate physical and chemical signals into cAMP and cGMP within the cells via cell surface receptors and the recognition process between these intracellular second messengers and extracellular

receptors gives rise to a series of biochemical reactions that result in several physiological effects. cAMP is generated by the activation of Adenylate cyclase (AC). AC converts adenosine triphosphate (ATP) into cAMP, which stimulates cAMP-dependent protein kinase A (PKA) (Taylor et al., 2013). Subsequently, specific proteins are phosphorylated by PKA to evoke cellular reactions. The phosphorylation of the cAMP response-element binding-protein (CREB), a transcription factor, is important in the regulation of gene transcription (Altarejos and Montminy, 2011; Yamamoto et al., 1988). At the same time, endogenous and exogenous compounds, including hormones, neurotransmitters and toxins, produce cellular responses through cGMP (Lucas et al., 2000). In particular, exogenous compounds can activate the membrane-bound forms of guanylate cyclases (GC) while endogenous compounds, such as nitric oxide (NO), can activate the soluble form of guanylate cyclases (sGC). Both convert guanosine triphosphate (GTP) into cGMP, which stimulates cGMP-dependent protein kinase G (PKG). PKG, in turn, phosphorylates specific protein in order to evoke cellular reactions (Lucas et al., 2000). Phosphodiesterases (PDEs) are a superfamily of enzymes that have been demonstrated to catalyze the hydrolysis of cAMP and cGMP (Maurice et al., 2014; Zaccolo and Movsesian, 2007). PDE families 1, 2, 3, and 10 hydrolyze both cGMP and cAMP, PDE families 4, 7, and 8 preferentially cleave cAMP and PDE families 5, 6, and 9 specifically hydrolyze cGMP. The activity of PDEs is crucial for cellular signaling because metabolism of cyclic nucleotides modulates their intracellular concentrations and affects subsequent cellular and behavioral responses, such as cardiac and neuronal functions, adrenal steroidogenesis and phototransduction (Juilfs et al., 1999). In the CNS, cAMP and cGMP are second messengers at the crossroad of many signaling pathways which modulate a large array of intracellular processes in neurons strongly impacting memory and cognition (Gomez and Breitenbucher, 2013).

3.2 *Pde2a* mRNA as a target of FMRP

FMRP is an RNA binding protein encoded by the *FMR1* gene. FMRP is involved in different steps of RNA metabolism, ranging from nuclear export to transport of mRNA along neurites and translational control in the soma as well as at synapses (Maurin et al., 2014). FMRP deficiency impacts the size of brain regions, synaptogenesis and alters the morphology of dendritic spines as well as some forms of synaptic plasticity (Bassell and Warren, 2008; Sidorov et al., 2013). The silencing of the *FMR1* gene causes FXS. The understanding of the physiopathology of FXS and the development of a specific therapy are intimately linked to the understanding of FMRP function and then to the identification of FMRP mRNA targets.

For this reason, the search for molecular interactors (proteins and mRNAs) of FMRP has been very active (Maurin et al., 2014). By using high-throughput sequencing-crosslinking immunoprecipitation (HITS-CLIP) on mRNAs associated with polyribosomes in whole brains of 11–25 day-old mice, it has been demonstrated that 842 mRNAs are targets of FMRP (Darnell et al., 2001). More recently, a CLIP study using microarray performed in 8-day in vitro cultured neurons resulted in the identification of one predominant mRNA target of FMRP (Tabet et al., 2016) but it is not clear if this target has a critical role in the physiopathology of FXS and in which cells it interacts with FMRP.

A recent study investigated the FMRP targets at an early mouse developmental stage (PND 13). At this time FMRP is most highly expressed (Bonaccorso et al., 2015; Davidovic et al., 2011) and synaptogenesis peaks (Semple et al., 2013). This study identified the largest set of brain mRNA targets of FMRP to date. This allowed to dissect the role of FMRP in different brain regions and cell types. On the basis of these findings, Maurin and colleagues were able to identify a predominant motif bound by FMRP in the brain and a prominent mRNA target (Maurin et al., 2018). In particular, they showed that FMRP negatively modulates both translation and transport of the *Pde2a* mRNA at the synapses and that *Pde2a* is only bound by FMRP in cortex and hippocampus (Maurin et al., 2018).

3.3 Role of PDE2A in the physiopathology of Fragile X Syndrome (FXS)

The recent study performed by Maurin and colleagues showed that the cAMP/cGMP pathway is one the most prominent deregulated pathways in the *Fmr1*-KO mouse brain and that the *Pde2a* mRNA is one prominent target of FMRP: in the absence of FMRP the level of PDE2A is elevated both in cortex and hippocampus, two brain areas which are most likely causative to cognitive and behavior deficits in FXS. Consequently, the level of cAMP and cGMP is reduced in those brain areas of *Fmr1*-null mice (Maurin et al., 2018). In neurons, PDE2A is mainly synaptic and exerts both pre- and post-synaptic functions (Fernandez-Fernandez et al., 2015; Maurin et al., 2018) being involved in synaptogenesis (Boess et al., 2004) and synaptic plasticity (Domek-Lopacinska and Strosznajder, 2008; Fernandez-Fernandez et al., 2015). Considering that the most common altered phenotype observed in animal models of FXS and in FXS patients is the loss of cognitive abilities, the increased expression of a subset of synaptic proteins and subsequent alteration in synaptic plasticity is considered a distinctive feature of FXS (Huber et al., 2002).

All these considerations suggest that PDE2A is a putative therapeutic target for an effective treatment of FXS.

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Chapter 2

Aims

Chapter 2

Aims

The general aim of my PhD project was to use preclinical animal models of ASD in order to 1. shed light on possible neurochemical alterations causing this disorder; 2. find new therapeutic opportunities. To these aims, I focused my attention on the analysis of the behavioral alterations observed in rodent models of ASD. Indeed, mice and rats are essential to shed light on the neurobiological alterations underlying the autistic phenotype, to understand the impact of the genetic and environmental factors on the phenotype displayed by patients and to assess potential therapeutic targets. Behaviors relevant to the core and associated symptoms of ASD can be easily and effectively reproduced in laboratory animals, and several rodent models are available that show specific autism-relevant behavioral features.

During the first year of my PhD project, I aimed at validating in our laboratory two preclinical environmental animal models of ASD in order to analyze if they were able to reproduce all the core and associated symptoms of ASD. The first model I studied was based on the prenatal exposure to Methimazole, a thyroid peroxidase inhibitor drug able to induce gestational hypothyroidism. In particular, I analyzed if prenatal MMI-induced hypothyroidism was able to cause in the rat offspring behaviors that resemble core and associated ASD symptoms. The second model I studied was based on prenatal exposure to VPA in rats, a widely used preclinical model of ASD. In particular, I tested the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. Since there is limited information about the precise biochemical mechanisms underlying the link between ASD and VPA, I evaluated the double-strand breaks (DSB) response in embryos exposed to a dose of VPA that induced autistic-like features in the rat offspring, in order to determine whether the autistic-like features displayed by VPA-exposed rats are associated with drug-induced DNA damage.

During the second year of my PhD project, my research mainly focused on the behavioral study of rodent models of FXS, the most common monogenetic cause of ASD. To this aim, I performed behavioral tests using *Fmr1*-KO rats and mice in order to analyze if these two genetic animal models of ASD were able to reproduce core and associated symptoms of ASD, in particular communicative deficits, social and cognitive alterations.

In the last year of my PhD project I studied, in collaboration with the Dr. Barbara Bardoni from the Institut de Pharmacologie Moléculaire et Cellulaire in Valbonne, France, the

involvement of the PDE2A on the altered behavioral phenotype displayed by mouse and rat models of FXS. It has been demonstrated that PDE2A levels and activity are increased in *Fmr1*-KO mice, resulting in reduced levels of cAMP and cGMP. In particular, in the absence of FMRP the level of PDE2A is elevated both in cortex and hippocampus, implying a reduced level of cAMP and cGMP in those brain areas in *Fmr1*-null mice. On these bases, I tested the ability of two selective inhibitor of PDE2A called BAY607550 and Lu AF64280, to rescue the atypical communicative, social and cognitive behaviors displayed by *Fmr1*-KO mice in the course of development. To validate PDE2A as a therapeutic target for FXS I also extended the study of its inhibition to *Fmr1*-KO infant rats.

Chapter 3

Testing the correlation between experimentally-induced hypothyroidism during pregnancy and autistic-like symptoms in the rat offspring

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Chapter 3

Testing the correlation between experimentally-induced hypothyroidism during pregnancy and autistic-like symptoms in the rat offspring

One of the first aims of my PhD project was to validate preclinical animal models of ASD in our lab. Maternal thyroid deficiency has been associated with a higher incidence of neurodevelopmental disorders in the newborns but the relationship between maternal hypothyroidism and the onset of ASD in the offspring, however, is not still clear. To address this issue, I used an animal model of prenatal hypothyroidism based on the administration of the thyroid peroxidase inhibitor methimazole (MMI) to rat dams from gestational day 9 up to delivery. I performed behavioral tasks in the offspring during infancy and adolescence to capture some of the core and associated symptoms of ASD.

I found that prenatal MMI-induced hypothyroidism does not cause in the rat offspring behaviors that resemble core and associated ASD symptoms: indeed, during infancy MMI-exposed pups were able to vocalize as controls and showed intact social discrimination abilities in the homing behavior test; at adolescence, MMI-exposed rats did not show an anxious-phenotype in the elevated plus maze test and showed intact object recognition. At the same time, MMI-exposed male rats solicited their partner to play more and showed more interest for novel rather than familiar objects compared to control rats, showing increased novelty-directed exploratory behaviors.

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Research report

Testing the correlation between experimentally-induced hypothyroidism during pregnancy and autistic-like symptoms in the rat offspring

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HIGHLIGHTS

- We tested the association between maternal hypothyroidism and autism in the offspring.
- We used an experimental model based on methimazole (MMI) administration to rat dams.
- MMI-exposed rats had no deficits in communication, social interaction and anxiety.
- MMI-exposed rats showed increased novelty-directed exploratory behaviors.

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ABSTRACT

Thyroid hormones are important for the development of the central nervous system. Since the fetal thyroid gland is not functioning until mid-gestation, transport of maternal thyroid hormones across the placenta is essential during the early phases of gestation. Maternal thyroid deficiency has been associated with a higher incidence of neurodevelopmental disorders in the newborns. The relationship between maternal hypothyroidism and the onset of autism spectrum disorders (ASD) in the offspring, however, is still debated. To address this issue, we used a validated animal model of prenatal hypothyroidism based on the administration of the thyroid peroxidase inhibitor methimazole (MMI, 0.02 g/100 ml in tap water) to rat dams from gestational day 9 up to delivery. The offspring was tested in behavioral tasks during infancy (PNDs 5, 9, 13) and adolescence (PND 35-40) to capture some of the core and associated symptoms of ASD. MMI-exposed pups were able to vocalize as controls when separated from the nest, and showed intact social discrimination abilities in the homing behavior test. At adolescence, the offspring from both sexes did not show an anxious-phenotype in the elevated plus maze and showed intact object recognition. However, MMI-exposed male rats showed increased novelty-directed exploratory behaviors: they solicited their partner to play more and showed more interest for novel rather than familiar objects compared to control rats. Our results show that prenatal MMI-induced hypothyroidism does not cause in the rat offspring behaviors that resemble core and associated ASD symptoms, like deficits in communication and social interaction and anxiety.

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1. Introduction

Thyroid hormones play a fundamental role in the development of the central nervous system, since they are involved in many processes underlying brain development and maturation [1]: neuronal and glial cell differentiation and proliferation, axonal and dendritic growth, synapse formation, cell migration and myelination [2,3].

Optimal thyroid functioning is required during pregnancy. Thus, hyperthyroidism during pregnancy is associated with severe maternal tachycardia, thyromegaly and exophthalmia, along with several adverse effects on pregnancy outcomes, such as miscar-

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riage, stillbirth, preterm delivery, intrauterine growth retardation, preeclampsia [4]. Compared to hyperthyroidism, hypothyroidism is more common during pregnancy, with 0.3–0.5% of pregnant women showing overt hypothyroidism and 2–2.5% subclinical hypothyroidism [4,5].

Several factors like genetic mutations, infections, nutrients and environmental contaminants can affect thyroid function during gestation and early postnatal development. The most common cause of maternal and fetal thyroid dysfunction is iodine deficiency from inadequate alimentary habits [6]: it has been estimated that nearly 2 billion individuals globally have an insufficient iodine intake that can result in inadequate thyroid hormone production [7]. The fetal thyroid gland is not functioning until mid-gestation, being active at 16–20 weeks post-conception in humans and at gestational day 17.5 in rats [1,8]. However, even before the onset of fetal thyroid function, the fetal cerebral cortex is capable of generating T3 from maternal T4 by local deiodination, and significant levels of T3 and thyroid hormone receptors (TRs) can be found in the fetal brain [1,9]. Thus, active transport of maternal thyroid hormones across the placenta has to occur during the early phases of gestation and it is essential for the development of the mammalian central nervous system [1]. Conversely, thyroid hormone deficiency, present at the time of birth through low maternal iodine levels, is a major contributing factor to congenital hypothyroidism in newborns [10]. Maternal thyroid hormone deficiency has adverse consequences on pregnancy outcome and offspring neurodevelopment. Overt hypothyroidism is associated with increase in prevalence of abortion, anemia, pregnancy-induced hypertension, preeclampsia, placental abruption, postpartum hemorrhage, premature birth, low birth weight, intrauterine fetal death and neonatal respiratory distress, associated with long term effect on the cognitive function of the offspring [4]. Recent evidence suggests that even more moderate forms of maternal thyroid dysfunction, particularly during early gestation, may have a long-lasting influence on child development, impairing the offspring's cognitive and motor development and increasing the risk of neurodevelopmental disorders [1]. In particular, thyroid hormone deficiencies during brain development, either due to a genetic deficiency of the thyroid receptor interacting protein, which codes for a transcriptional regulator associated with nuclear TRs [11], or due to maternal hypothyroidism, have been associated with an increased risk of autism spectrum disorders (ASD) [6,12,13]. However, other studies found no association between neonatal T4 levels and ASD [14], or reported an inverse correlation between mid-pregnancy thyroid stimulating hormone levels and ASD risk [15]. Thus, it is still unclear whether maternal hypothyroidism is a risk factor for ASD and can therefore become a target for effective public health risk reduction efforts. The aim of this study was to test this possibility at the preclinical level, by investigating whether maternal hypothyroidism induces in rats behavioral features that resemble some of the core and associated symptoms of ASD. To this aim, we used a validated animal model of prenatal hypothyroidism based on methimazole (MMI) administration to rat dams. In Europe and many other countries, MMI is used to treat Graves' disease, the most common cause of hyperthyroidism in pregnancy [16]. MMI affects thyroid hormone synthesis and deiodinase activity by inhibiting thyroid peroxidase and it has been extensively used to model maternal hypothyroidism in laboratory animals [17–19]. Since ASD patients typically show poor communication and social interaction [20], deficits in social play [21], increased interests for objects and intact object recognition [22], we tested the offspring born from MMI-exposed and control (CTRL) dams in the following behavioral tests: 1. the pup isolation-induced ultrasonic vocalizations (USVs) test, that provides quantitative and qualitative measures of the USVs emitted by rodent pups when isolated from the nest, which play an essential communicative role in mother-offspring interaction [23,24]; 2. the homing behavior test, that provides an early measure of social discrimination, since it allows to detect the pups' cognitive, sensory and motor ability necessary to discriminate between a neutral odor and their own nest odor [23,25]; 3. the social interaction test at adolescence, to detect social behaviors both related and unrelated to play [23,26]; 4. the object recognition test, a two trial cognitive paradigm that assesses object recognition memory in rodents [27]. Last, since anxiety is a frequent symptom displayed by autistic patients [28], we also tested whether MMI prenatal exposure affected the behavior of the offspring in the elevated plus-maze test, the most common behavioral paradigm used to assess anxiety-like behaviors in rodents.

2. Materials and methods

2.1. Animals and treatments

Primiparous female Wistar rats (Charles River, France), 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*. Gestational hypothyroidism was induced by adding 20 mg of MMI to tap water (100 ml) [17–19], from gestational day (GD) 9 until the day of the birth [18]. Indeed, it has previously been shown that MMI, given for 10 days to pregnant rats from GD9, is able to cause hypothyroidism in the dams by inducing a decrease of circulating maternal total T3 and T4 levels [19,29]. CTRL females had access to the same volume of tap water. Fluid consumption was recorded daily throughout gestation to ensure that both CTRL and MMI-exposed dams had a similar water intake. The mean dose of MMI consumed daily by each pregnant dam was 12 mg. On PND 1, the litters were culled to eight animals (4 males and 4 females). On PND 21, rats were weaned and housed in group of three with same litter and same sex partners. Experiments were carried out on the male and female offspring during infancy (PNDs 5, 9 and 13) and adolescence (PND 35-40). The total number of litters used for the behavioral experiments was 8 CTRL- and 9 MMI-exposed dams. For the USV and homing behavior tests, we used two male and two female pups per litter; for the behavioral tests performed during adolescence, one male and one female rat per litter from different litters per treatment group were used in each experiment.

All the experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

2.2. Measurements of thyroid hormones serum levels

Thyroid status was evaluated through the determination of total T3 and T4 serum levels in pregnant rats. For the measurement of thyroid hormones, at GD21, pregnant rats (n = 6 animals/group) were decapitated and trunk blood was collected in plastic tubes. Serum samples were obtained after clotting and were stored at −80 °C. Total T3 and T4 levels in serum were measured by competitive enzyme-linked immunosorbent assay (ELISA) complete kit according to the manufacturer's instructions sheet (Interteck, Katal, MG, Brazil). The final hormone concentration was calculated based on a standard curve constructed for each assay using recombinant hormone standards. All hormone assays were conducted at the same time using supplies from the same assay.

2.3. Length of pregnancy, litter size at birth and weight gain of pups

During gestation, each cage was checked daily for the presence of newborn pups. On the day of parturition, the number of living pups were recorded. Newborn litters found up to 05:00 p.m. were considered to be born on that day (Postnatal day (PND) 0). The body weight was measured at PND1, PND5, PND9, PND13 and PND30. Survival rate was defined as the number of live pups at weaning divided by the number of live pups at birth.

2.4. Behavioral tests

2.4.1. 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 [24]. On PNDs 5, 9 and 13, the isolation-induced USVs emitted by CTRL and MMI-exposed pups were recorded as previously described [30]. 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, with a camera positioned above the arena to monitor the activity of the pup during the test. Pup USVs were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Berlin, Germany) sensitive to frequencies between 10 and 200 kHz and fixed at 15 cm above the arena. Pup axillary temperature was measured before and after the test by a digital thermometer. The emission of USVs was analyzed, both quantitatively (total number of USVs emitted in the 3 min of the test) and qualitatively, using Avisoft Recorder software (Version 5.1). The USVs were classified in 6 different groups according to number of syllables, frequency modulation and duration [25,31]: flat calls (calls with constant frequency with a maximum variation of ±5 kHz); up/downward (single syllable calls emitted at ±5 kHz with a single frequency modulation); flat high frequency calls (calls with similar constant frequency, but emitted at >75 kHz); syllable calls (calls composed by two or more syllables); complex calls (calls displaying complex frequency modulation); short calls (calls with durations shorter than 5 ms). The values obtained were expressed as percentage of the total USVs emitted.

2.4.2. Homing behavior

The homing behavior test was performed as previously described [25]. 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 videorecorded 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.

2.4.3. Social play behavior

The experiments were performed in a sound attenuated chamber under dim light conditions. The testing arena consisted of a Plexiglas cage measuring 40 × 40 × 60 cm (l × w × h), with approximately 2 cm of wood shavings covering the floor. The behavior of the animals was recorded using a video camera with zoom lens, DVD recorder and LCD monitor.

Social play behavior was assessed as previously described [32–34]. At 35–40 days of age, rats were habituated to the experimental apparatus on 2 consecutive days in which they were individually placed into the test cage for 10 min. On the test day, the animals were isolated for 3 h before testing, to enhance their social motivation and thus facilitate the expression of social play behavior during testing [35]. The test consisted of placing two animals belonging to the same experimental group into the test cage for 15 min. The animals in a 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 and analyzed by a trained observer who was unaware of treatment condition using the Observer XT software (Noldus, The Netherlands). The data obtained are presented in Fig. 2.

The following parameters were scored [26,36]:

- frequency of pinning: one animal lying with its dorsal surface on the floor with the other animal standing over it. This is the most characteristic posture in social play in rats and occurs when one animal is solicited to play by its test partner and rotates to its dorsal surface;
- frequency of pouncing: one animal is soliciting the other to play, by attempting to nose or rub the nape of the neck of the test partner;
- time spent on social exploration: sniffing any part of the body of the test partner, including the anogenital area;
- following: running forward in the direction of or pursuing the other subject, who moves away;
- boxing: Rearing in an upright position towards the other subject combined with rapidly pushing, pawing, and grabbing at each other;
- Partial rotation: upon contact of the nape, the recipient animal begins to rotate 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 contact with the nape by leaping, running, or turning away from the partner.

2.4.4. Object recognition test

The novel object recognition task examines a rodent's ability to recognize a previously explored object and the presence of an unfamiliar novel object over time [37]. The experimental apparatus was a gray open-field box (40 × 40 × 40 cm (l × w × h)), with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). The test was performed as previously described [38]. On the training trial, two identical objects (A1 and A2) were placed in the experimental apparatus, equidistantly from each other and from two adjacent corners [39]. During this phase, each rat (35-40-day-old) was individually placed in the arena at the opposite end from the objects and allowed to explore the objects for 5 min. To avoid the presence of olfactory trails, sawdust was stirred and the objects were cleaned with 70% ethanol after each trial. A video camera positioned above the experimental apparatus was used to record rat's behavior. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. The time spent exploring the two objects was taken as a measure of object exploration and the locomotor activity was analyzed by the total number crossings [38]. Retention was tested 30 min after the training trial. On the retention test trial, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial. Each rat was placed in the apparatus for 5 min, and its behavior was recorded. The time spent exploring each object and the total time spent exploring both objects were recorded and videos were analyzed by a trained observer who was unaware of treatment condition. The discrimination index was calculated as the difference in

Table 1
Total serum T3 and T4 levels in CTRL (n = 6) and MMI (n = 6)-treated dams at GD 21. Data represent mean values ± SEM.

Total serum T4 (μg/dl)		Total serum T3 (ng/ml)	
CTRL dams	MMI-treated dams	CTRL dams	MMI-treated dams
11.22 ± 0.57	5.18 ± 0.87***	7.16 ± 0.30	6.09 ± 0.27*

* p < 0.05.
*** p < 0.001 vs. CTRL.

Table 2
Length of pregnancy, litter size at birth and weight gain of pups. Data represent mean values ± SEM.

Group	Pregnancy length (day)	Litter size at birth (n)	Litter weight PND1 (g)	Pups weight (g)					Survival rate (%)
					PND 5	PND 9	PND 13	PND 30	
CTRL	21.75 ± 0.16	11.25 ± 1.07	7.43 ± 0.29	Male	13.56 ± 0.44	22.94 ± 0.84	33.96 ± 0.43	110.75 ± 1.88	100
				Female	13.38 ± 0.43	22.86 ± 0.89	31.56 ± 0.42	107.00 ± 4.08	100
MMI	22.78 ± 0.022**	10.56 ± 1.39	6.20 ± 0.30**	Male	10.89 ± 0.58***	19.06 ± 0.49***	30.03 ± 0.55***	99.00 ± 1.41**	100
				Female	10.28 ± 0.36***	18.17 ± 0.55***	28.27 ± 0.57***	96.78 ± 1.83**	100

** p < 0.01.
*** p < 0.001 vs. CTRL (n dams and litters = 8 CTRL, 9 MMI; n male pups = 8 CTRL, 9 MMI; n female pups = 8 CTRL, 9 MMI).

time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects.

2.4.5. Elevated plus-maze test

The elevated plus-maze apparatus comprised two open ($50 \times 10 \times 40\,cm^3$; $l \times w \times h$) and two closed arms ($50 \times 10 \times 40\,cm^3$; $l \times w \times h$) that extended from a common central platform ($10 \times 10\,cm^2$). The test was performed as previously described [35,40]. Rats (35-40-day-old) 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, 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;
- % 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;
- frequency of head dipping (HDIPS).

2.5. Statistical analysis

Data are expressed as mean ± SEM. To assess the effects of the prenatal treatments (MMI or CTRL) on the behavior of the male and female offspring, data were analyzed with Two-way ANOVA, with treatment and sex of the offspring as factors. Two-way ANOVA for repeated measures was used to assess the effects of prenatal treatments (CTRL or MMI) on the number of USVs emitted by male and female pups on PNDs 5, 9 and 13. Two-way ANOVA was followed by Student-Newman-Keuls post-hoc test where appropriate. To assess the effects of the treatment (MMI or CTRL) on pregnancy length, litter size at birth and litter weight at PND1, data were analyzed with Student *t*-tests.

3. Results

3.1. Thyroid hormones serum levels

At GD 21, MMI-treated dams (n = 6) showed decreased serum T4 ($t = 5.78$; $p < 0.001$; df = 10) and T3 ($t = 2.6$; $p = 0.027$; df = 10) levels compared to CTRL dams (n = 6, Table 1).

3.2. Length of pregnancy, litter size at birth and weight gain of pups

Pregnancy length was higher in MMI-exposed dams than in CTRL animals ($t = 3.64$; $p = 0.0024$; df = 15, Table 2). Litter size at birth did not differ between the two experimental groups ($t = 0.40$; p = n.s.; df = 15). At PND 1, litter weight was higher in CTRL dams ($t = 3.03$; $p = 0.0085$; df = 15); at PNDs 5, 9, 13 and 30, offspring born from MMI-exposed dams showed a reduced body weight compared to the offspring born from CTRL dams (PND5: [$F_{(treat.)1,30} = 39.15$, $p < 0.001$; $F_{(sex)1,30} = 0.75$, p = n.s.; $F_{(treat\times sex)1,30} = 0.21$, p = n.s.]; PND 9: [$F_{(treat.)1,30} = 38.02$, $p < 0.001$; $F_{(sex)1,30} = 0.47$, p = n.s.; $F_{(treat\times sex)1,30} = 0.35$, p = n.s.]; PND 13: [$F_{(treat.)1,30} = 50.83$, $p < 0.001$; $F_{(sex)1,30} = 16.91$, $p < 0.001$; $F_{(treat\times sex)1,30} = 0.39$, p = n.s.]; PND 30: [$F_{(treat.)1,30} = 20.24$, $p < 0.001$; $F_{(sex)1,30} = 1.47$, p = n.s.; $F_{(treat\times sex)1,30} = 0.10$, p = n.s.]). Post hoc analysis showed that, compared to CTRL offspring, both male and female MMI-exposed animals had reduced body weight: (PND 5: males CTRL vs. males MMI $p < 0.001$, females CTRL vs. females MMI $p < 0.001$; PND 9: males CTRL vs. males MMI $p < 0.001$, females CTRL vs. females MMI $p < 0.001$; PND 13: males CTRL vs. males MMI $p < 0.001$, females CTRL vs. females MMI $p < 0.001$; PND 30: males CTRL vs. males MMI $p = 0.002$, females CTRL vs. females MMI $p = 0.006$; Table 2).

3.3. Behavioral tests

3.3.1. Effects of prenatal MMI exposure on the isolation-induced USVs emitted by the male and female offspring

The analysis of the number of the USVs emitted by male and female pups removed from the nest at PNDs 5, 9 and 13 revealed no differences between MMI-exposed and CTRL pups (males: $F_{(treat.)1,26} = 2.22$, p = n.s.; $F_{(PND)1,26} = 0.69$, p = n.s.; $F_{(treatxPND)1,26} = 0.57$, p = n.s.; females: $F_{(treat.)1,28} = 0.75$, p = n.s.; $F_{(PND)1,28} = 0.87$, p: n.s.; $F_{(treatxPND)1,28} = 1.41$, p = n.s.) (Fig. 1). No qualitative differences between groups have been found in the percentage of the different call categories emitted by male and female pups (PND 5: flat calls [$F_{(treat.)1,56} = 0.470$, p = n.s.; $F_{(sex)1,56} = 1.230$, p = n.s.; $F_{(treat\times sex)1,56} = 1.341$, p = n.s.]; flat high frequency calls [$F_{(treat.)1,56} = 0.694$, p = n.s.; $F_{(sex)1,56} = 5.627$, p = n.s.; $F_{(treat\times sex)1,56} = 1.330$, p = n.s.]; syllable [$F_{(treat.)1,56} = 0.171$, p = n.s.; $F_{(sex)1,56} = 2.365$, p = n.s.; $F_{(treatxsex)1,56} = 2.942$, p = n.s.]; short calls [$F_{(treat.)1,56} = 0.681$, p = n.s.; $F_{(sex)1,56} = 1.803$, p = n.s.; $F_{(treat\times sex)1,56} = 2.236$, p = n.s.]; up/downward calls [$F_{(treat.)1,56} = 0.421$, p = n.s.; $F_{(sex)1,56} = 0.089$, p = n.s.;

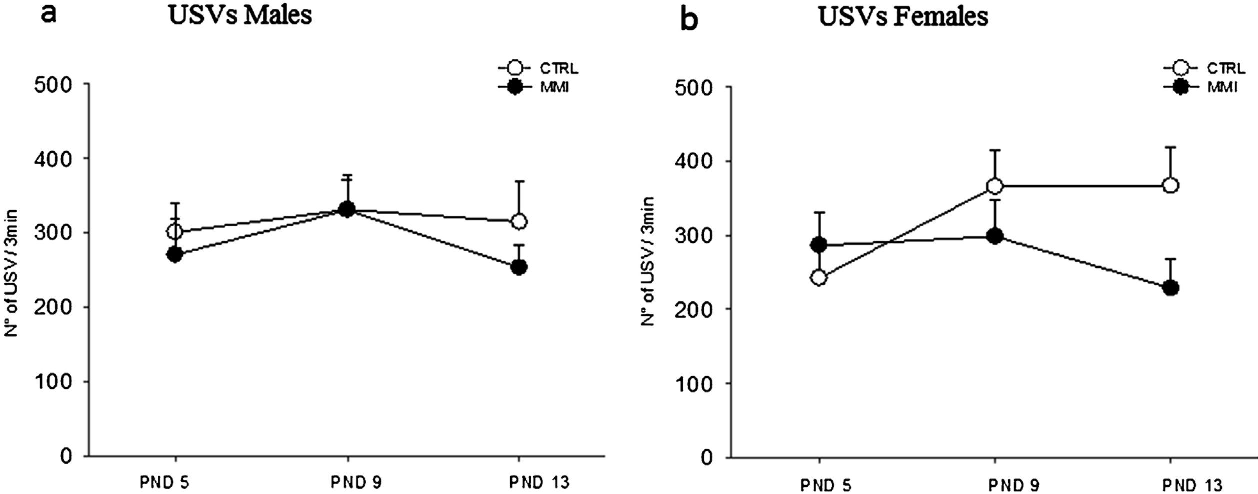

Fig. 1. Effects of prenatal MMI exposure on the isolation-induced USVs emitted by the male and female offspring on PNDs 5, 9 and 13. Prenatal MMI exposure did not alter the number of USVs emitted by male (a, n = 14 CTRL, 18 MMI) and female pups (b, n = 15 CTRL, 16 MMI). Data represent mean values ± SEM.

$F_{(treatxsex)1,56} = 1.562$, p = n.s.]; complex calls [$F_{(treat.)1,56} = 0.00$, p = n.s.; $F_{(sex)1,56} = 0.00$, p = n.s.; $F_{(treat\times sex)1,56} = 0.00$, p = n.s.]. PND 9: flat calls [$F_{(treat.)1,56} = 3.766$, p = n.s.; $F_{(sex)1,56} = 0.032$, p = n.s.; $F_{(treatxsex)1,56} = 0.564$, p = n.s.]; flat high frequency calls [$F_{(treat.)1,56} = 3.857$, p = n.s.; $F_{(sex)1,56} = 0.00$, p = n.s.; $F_{(treat\times sex)1,56} = 0.00$, p = n.s.]; syllable calls [$F_{(treat.)1,56} = 1.208$, p = n.s.; $F_{(sex)1,56} = 0.419$, p = n.s.; $F_{(treat\times sex)1,56} = 0.667$, p = n.s.]; short calls [$F_{(treat.)1,56} = 0.00$, p = n.s.; $F_{(sex)1,56} = 0.00$, p = n.s.; $F_{(treat\times sex)1,56} = 0.00$, p = n.s.]; up/downward calls [$F_{(treat.)1,56} = 0.538$, p = n.s.; $F_{(sex)1,56} = 0.106$, p = n.s.; $F_{(treatxsex)1,56} = 0.480$, p = n.s.]; complex calls [$F_{(treat.)1,56} = 0.448$, p = n.s.; $F_{(sex)1,56} = 1.007$, p = n.s.; $F_{(treat\times sex)1,56} = 0.771$, p = n.s.]. PND13: flat calls [$F_{(treat.)1,56} = 2.253$, p = n.s.; $F_{(sex)1,56} = 0.150$, p = n.s.; $F_{(treat\times sex)1,56} = 0.098$, p = n.s.]; flat high frequency calls [$F_{(treat.)1,56} = 0.00$, p = n.s.; $F_{(sex)1,56} = 0.00$, p = n.s.; $F_{(treat\times sex)1,56} = 0.00$, p = n.s.]; syllable [$F_{(treat.)1,56} = 7.485$, p = n.s.; $F_{(sex)1,56} = 0.138$, p = n.s.; $F_{(treat\times sex)1,56} = 0.347$, p = n.s.]; short calls [$F_{(treat.)1,56} = 0.00$, p = n.s.; $F_{(sex)1,56} = 0.00$, p = n.s.; $F_{(treatxsex)1,56} = 0.00$, p = n.s.]; up/downward calls [$F_{(treat.)1,56} = 1.697$, p = n.s.; $F_{(sex)1,56} = 0.930$, p = n.s.; $F_{(treatxsex)1,56} = 0.717$, p = n.s.]; complex calls [$F_{(treat.)1,56} = 1.428$, p = n.s.; $F_{(sex)1,56} = 1.745$, p = n.s.; $F_{(treat\times sex)1,56} = 0.479$, p = n.s.]; data not shown).

3.3.2. *Effects of prenatal MMI exposure on the performance of the male and female offspring in the homing behavior test*

The male and female offspring prenatally exposed to MMI did not show deficits in the homing behavior test on PND 13 (Table 3). Indeed, no differences between groups have been found in the latency to reach the home-cage bedding [$F_{(treat.)1,46} = 0.106$, p = n.s.; $F_{(sex)1,46} = 0.507$, p = n.s.; $F_{(treat\times sex)1,46} = 1.895$, p = n.s.], in the time spent in the nest area [$F_{(treat.)1,46} = 0.363$, p = n.s.; $F_{(sex)1,46} = 0.358$, p = n.s.; $F_{(treatxsex)1,46} = 2.772$, p = n.s.] and in the total number of entries into the nest bedding area [$F_{(treat.)1,46} = 0.855$, p = n.s.; $F_{(sex)1,46} = 0.034$, p = n.s.; $F_{(treat\times sex)1,46} = 0.461$, p = n.s.]. Furthermore, no significant differences in locomotor activity (number of crossing) were found between the experimental groups [$F_{(treat.)1,30} = 0.015$, p = n.s.; $F_{(sex)1,30} = 0.303$, p = n.s.; $F_{(treat\times sex)1,30} = 1.391$, p = n.s.] (Table 3)

3.3.3. *Effects of prenatal MMI exposure on the social play behavior of the male and female adolescent offspring*

The adolescent male and female offspring prenatally exposed to MMI did not differ from the CTRL offspring in the frequency of pinning [$F_{(treat.)1,21} = 0.0054$, p = n.s.; $F_{(sex)1,21} = 6.034$, p = 0.023; $F_{(treat\times sex)1,21} = 1.00$, p = n.s.] (Fig. 2a), in the fre-

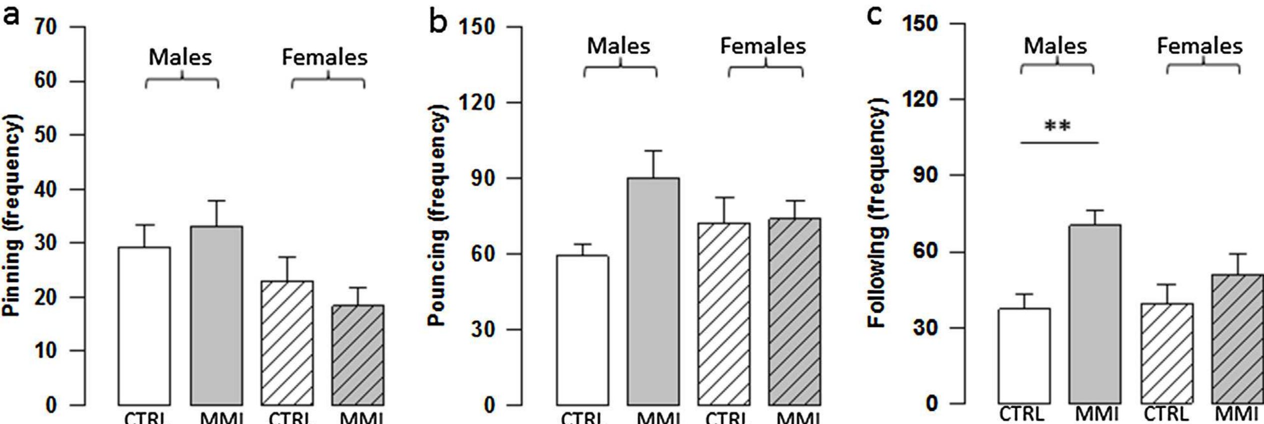

Fig. 2. Effects of prenatal MMI exposure on the social play behavior of the male and female adolescent offspring. Male rats prenatally exposed to MMI (n = 7) showed an increase in the frequency of following (c) compared to CTRL male animals (n = 5). All other parameters were unaffected in the MMI-exposed male and female offspring (a,b; females n = 6 CTRL, 7 MMI). Data represent mean values ± SEM. ** p < 0.01 vs. CTRL. (Student–Newman–Keuls post hoc test).

Table 3
Effects of prenatal MMI exposure on the performance of the male and female offspring in the homing behavior test. Data represent mean values ± SEM (n males = 11 CTRL, 13 MMI; n females = 14 CTRL, 12 MMI).

Group	Latency (s)	Nest time (s)	No Entries	No Crossing
CTRL ♂	26.00 ± 14.49	208.00 ± 15.09	1.27 ± 0.18	32.37 ± 6.54
MMI ♂	49.23 ± 13.33	175.77 ± 13.88	1.23 ± 0.17	25.67 ± 6.17
CTRL ♀	54.50 ± 12.84	181.64 ± 13.38	1.36 ± 0.16	21.37 ± 6.54
MMI ♀	40.17 ± 13.87	196.75 ± 14.45	1.08 ± 0.17	29.67 ± 6.17

quency of pouncing [$F_{(treat.)1,21} = 3.147$, p = n.s.; $F_{(sex)1,21} = 0.03$, p = n.s.; $F_{(treat\times sex)1,21} = 2.630$, p = n.s.] (Fig. 2b), in the frequency of boxing [$F_{(treat.)1,21} = 0.461$, p = n.s.; $F_{(sex)1,21} = 4.317$, p = n.s.; $F_{(treat\times sex)1,21} = 2.485$, p = n.s.] (data not shown) and in the total time spent in social exploration [$F_{(treat.)1,21} = 0.789$, p = n.s.; $F_{(sex)1,21} = 0.73$, p = n.s.; $F_{(treat\times sex)1,21} = 0.67$, p = n.s.] (data not shown). Compared to CTRL rats, rats prenatally exposed to MMI followed the play partner more [$F_{(treat.)1,21} = 9.470$, $p = 0.006$; $F_{(sex)1,21} = 1.422$, p = n.s.; $F_{(treat\times sex)1,21} = 2.361$, p = n.s.] (Fig. 2c). This was confirmed by the post hoc analysis ($p = 0.005$, Fig. 2c).

3.3.4. *Effects of prenatal MMI exposure in the male and female adolescent offspring tested in the object recognition task*

CTRL and MMI-exposed animals did not differ in the discrimination index [$F_{(treat.)1,27} = 0.0000142$, p = n.s.; $F_{(sex)1,27} = 0.0715$, p = n.s.; $F_{(treat\times sex)1,27} = 1.924$, p = n.s.] (Fig. 3a). However, a two-way ANOVA analysis for the time spent sniffing the old and new object by males rats in the test trial gave the following results: [$F_{(treat.)1,26} = 5.93$, $p = 0.022$; $F_{(object)1,26} = 50.17$, $p < 0.001$; $F_{(treatxobject)1,26} = 7.18$, $p = 0.013$]. Post hoc analysis showed that male rats of both groups spent more time sniffing the novel object in the retention trial (CTRL $p = 0.004$; MMI $p < 0.001$ Fig. 3b). Compared to CTRL animals, MMI-exposed animals showed an increase in the time spent sniffing the novel object ($p = 0.001$), while no differences between CTRL and MMI-exposed animals were found in the time spent exploring the old object (p = n.s. Fig. 3b).

A two-way ANOVA for the time spent by females rats sniffing the old and new object in the test trial gave the following results: [$F_{(treat.)1,28} = 0.04$, p = n.s.; $F_{(object)1,28} = 31.22$, $p < 0.001$; $F_{(treat\times object)1,28} = 1.00$, p = n.s.]. Post hoc analysis showed that female rats of both groups spent more time sniffing the novel object in the retention trial (CTRL $p < 0.001$; MMI $p = 0.003$. Fig. 3c) and no differences between CTRL and MMI-exposed animals were found in the time spent exploring the old and the new object (p = n.s. Fig. 3c). Compared to CTRL rats, no significant differences in the locomotor activity of MMI exposed animals were found during the retention trial. The only difference was found between MMI-exposed male and female rats [$F_{(treat.)1,27} = 0.546$, p = n.s.; $F_{(sex)1,27} = 7.843$, $p = 0.009$; $F_{(treat\times sex)1,27} = 0.718$, p = n.s.]. Post hoc analysis showed an increased locomotor activity of MMI female rats compared to MMI male rats ($p = 0.017$, data not shown).

3.3.5. *Effects of prenatal MMI exposure on the performance of the male and female adolescent offspring in the elevated plus-maze test*

No differences between MMI-exposed and CTRL animals were found in the percentage of open arm entries [$F_{(treat.)1,27} = 1.943$, p = n.s.; $F_{(sex)1,27} = 2.156$, p = n.s.; $F_{(treat\times sex)1,27} = 1.351$, p = n.s.] (Fig. 4a), in the percentage of time spent in the open arms [$F_{(treat.)1,27} = 1.520$, p = n.s.; $F_{(sex)1,27} = 0.292$, p = n.s.; $F_{(treat\times sex)1,27} = 1.067$, p = n.s.] (Fig. 4b), in the number of closed arms entries [$F_{(treat.)1,27} = 1.722$, p = n.s.; $F_{(sex)1,27} = 0.095$, p = n.s.; $F_{(treat\times sex)1,27} = 0.169$, p = n.s.] (Fig. 4c) and in the frequency of head-dippings [$F_{(treat.)1,27} = 0.467$, p = n.s.; $F_{(sex)1,27} = 0.015$, p = n.s.; $F_{(treat\times sex)1,27} = 1.867$, p = n.s.] (Fig. 4d).

4. Discussion

Thyroid hormones affect multiple maturational processes of the CNS since very early developmental ages. It is known that the fetus is completely dependent on maternal supply of thyroid hormones in the first half of gestation, and a significant transfer of thyroid hormones from the mother to the fetus also persists during the second half of gestation [1]. The source of T4 reaching the fetal brain up to mid gestation is mostly of maternal origin [8]. Both T4 and T3 cross the placenta and reach the fetal organs, but only T4 appears to cross the fetal blood-brain barrier. Thus, the administration of T3 by constant infusion to pregnant rat dams increased T3 concentrations in all maternal and fetal tissues except the fetal brain [41]. In contrast, the administration of T4 increased T3 content in fetal brain as well as in other tissues. As in fetal brain, when either T4 or T3 was administered to hypothyroid rats, the T3 content of the brain was maintained within control values only by T4, but not by T3, infusion [8]. Transfer of thyroid hormone through the choroid plexus achieves only limited diffusion to the brain parenchyma after passage to the cerebrospinal fluid but would allow uptake of T4 by astrocytes and subsequent T3 generation in these cells [42].

Clinical and preclinical studies have shown that maternal thyroid dysfunction is associated with a wide range of adverse neurodevelopmental outcomes and increased risk for neurodevelopmental disorders in the offspring [1]. Recently, maternal hypothyroidism and maternal hypothyroxinemia have been related to a higher incidence of ASD in the children [6,12,13], although controversial data about this association are also available [15]. The aim of our study was to clarify this issue at the preclinical level.

In particular, by using a validated animal model of prenatal hypothyroidism based on MMI administration to rat dams, we investigated whether maternal hypothyroidism induces in rats behavioral features that resemble some of the core and associated symptoms displayed by ASD patients.

At the end of gestation, T3 and T4 serum levels were significantly lower in MMI-treated than CTRL dams, thus confirming the validity of prenatal MMI administration as an animal model of gestational hypothyroidism [17–19].

On average, pregnancy length was one day longer in MMI-treated dams compared to CTRL animals, with no differences between the two groups in litter size at birth and postnatal mortality. In line with what previously reported [3,17,18,43,44], pups born from MMI-treated mothers had reduced body weight at PNDs 1, 5, 9, 13 and 30, but did not show any physical abnormality. The reduced body weight of the MMI-exposed offspring could be due to hypothyroidism-induced growth hormone (GH) deficiency [43]. Indeed, thyroid hormones have a direct effect on GH gene transcription [45] and the levels of pituitary GH mRNA are reduced in hypothyroid rats and restored to normal values after 3 weeks of T3 and T4 treatment [43,45,46].

The emission of USVs by rodent pups plays a fundamental communicative role in mother-offspring interactions. USVs are crucial for pup survival: when infants are separated from their mother, they emit USVs to induce maternal retrieval and to elicit caregiv-

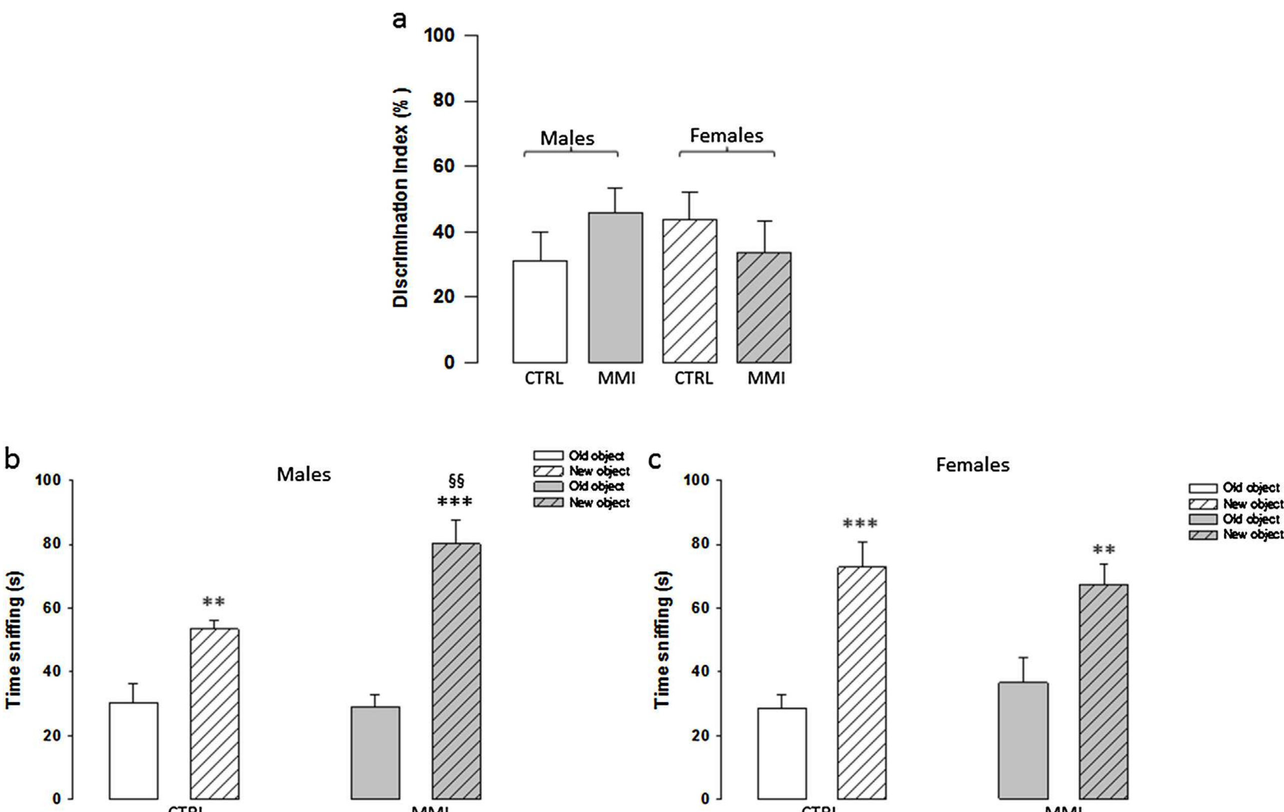

Fig. 3. Effects of prenatal MMI exposure in the male and female adolescent offspring tested in the object recognition task. No differences between groups were found in the discrimination index (a). Adolescent male rats of both groups spent more time sniffing the novel object in the retention trial. Male rats prenatally exposed to MMI (n = 7) showed an increase in the time spent sniffing the novel object (b) compared to CTRL animals (n = 8). Adolescent female rats of both groups spent more time sniffing the novel object in the retention trial (n = 8 CTRL, 8 MMI). Data represent mean values ± SEM. Males (b): ** < 0.01 vs. old object CTRL, ***<0.001 vs. old object MMI, §§ < 0.01 vs. new object CTRL. Females (c): ***<0.001 vs. old object CTRL, **<0.01 vs. old object MMI. (Student–Newman–Keuls post hoc test).

ing behaviors in the dam [23,24,47–49]. Our results indicate that MMI-exposed pups were able to vocalize as CTRL pups: indeed, we found no significant differences, neither quantitative nor qualitative, in the USVs emitted by male and females MMI-exposed and CTRL pups when isolated from the nest at PNDs 5, 9 and 13, albeit a slight decrease in the number of USVs emitted by female MMI-exposed pups compared to CTRL pups was observed on PND13, which may be due to the inter-individual variability often observed in the USVs test.

Furthermore, we found that MMI-exposed pups were able to use olfactory cues to discriminate between a neutral odor and their own home cage odor in the homing behavior test. Olfaction, and in particular the learned association between maternal odors and maternal stimulation, is crucial for the development of social behavior and social recognition [50]. Altogether, the normal USV profile and homing behavior displayed by MMI-exposed pups indicate their lack of deficits in social communication and social discrimination since the first days of life.

ASD patients show marked deficits in social interaction, including lack or atypical social play behavior [20]. Social play has a crucial role in the identification and diagnosis of ASD [21]. In ASD, play patters are characterized not only by deficient cognitive complexity, but also by a typical asocial dimension. Children with ASD are able to engage in certain forms of play. However, these are passive, stereotyped and rigid, with no adjustment of play patterns to involve others and fewer opportunities to join and share play scenarios. As a result, the play of children with ASD is less likely to engage the interest of other children. The lack of socialized patterns of play and the failure to engage in play with peers lead, in turn, to further social isolation. Since the opportunity to engage in social play is crucial to acquire proper social and cognitive skills, 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. To investigate whether maternal hypothyroidism affected the social repertoire and the social play behavior of the offspring, we tested MMI-exposed adolescent rats in a free dyadic social encounter with a same-age social partner. We found that, compared to CTRL animals, MMI-exposed male but not female rats followed their play partner more. Furthermore, MMI-exposed males showed a slight increase in play solicitation compared to CTRL animals. The two experimental groups, however, did not differ in their response to play solicitation, neither in the total time they spent in non playful forms of social interaction.

ASD patients often show increased interests for objects and intact object recognition [22]. Therefore, we tested the adolescent offspring born from MMI and CTRL dams in the object recognition task. We found that both CTRL and MMI-exposed animals were able to discriminate a new versus a familiar object during the test trial. Interestingly, however, the time spent exploring the novel rather than the familiar object was higher in MMI-exposed than CTRL rats. This finding is reminiscent of the increased novelty-directed exploratory behavior displayed by rats exposed to MMI during the perinatal period [18].

Last, a two-way ANOVA analysis performed on the data collected in the elevated plus-maze test, taking both the sex of the offspring and the prenatal treatment into account, did not reveal statistically significant differences between MMI-exposed and CTRL male and

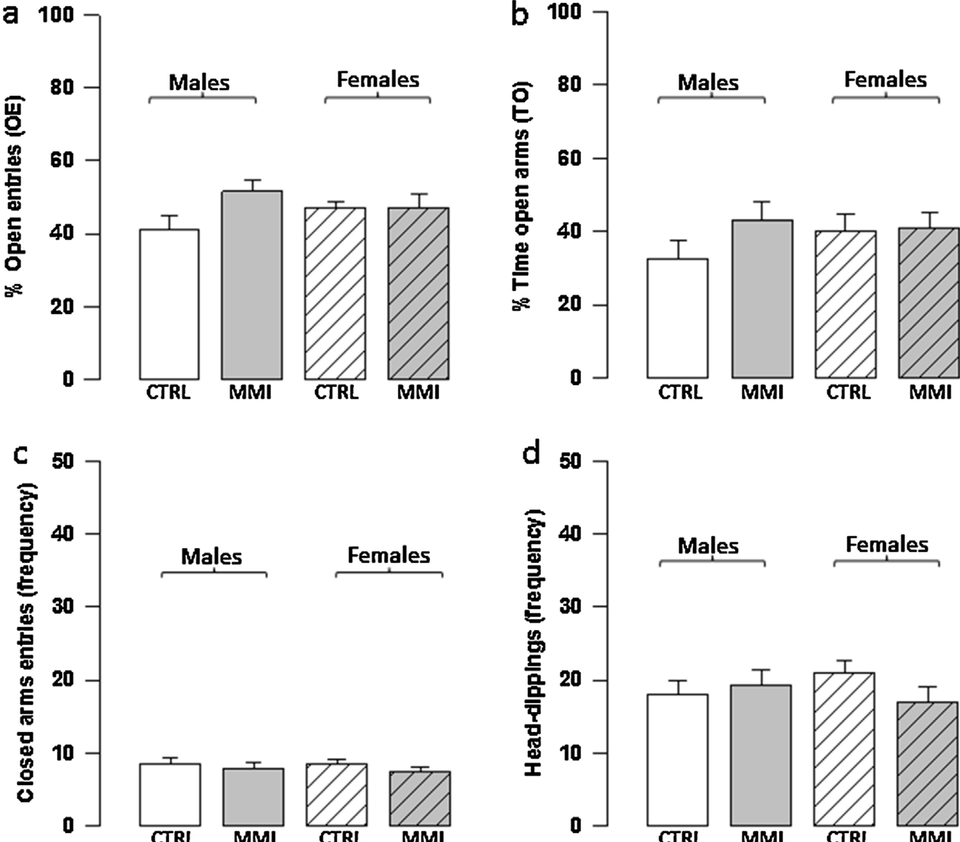

Fig. 4. Effects of prenatal MMI exposure on the performance of the male and female adolescent offspring in the elevated plus-maze test. No differences between groups were found in the percentage of entries in the open arms of the maze (a), in the percentage of time spent in open arms (b), in the number of closed arms entries (c) and in the frequency of head-dippings (d) both in the male (n = 8 CTRL, 8 MMI) and female (n = 7 CTRL, 8 MMI) offspring. Data represent mean values ± SEM.

female rats. It should be noted, however, that when we analyzed the results of the elevated plus-maze tests separately for the two sex by Student's *t*-test, taking only the prenatal treatment into account, a higher percentage of open arms entries in males prenatally exposed to MMI than in CTRL male subjects ($p < 0.05$) was observed. This finding obtained is in line with previous studies that reported reduced anxious-like behaviors displayed by male rats prenatally exposed to MMI in both the elevated plus-maze and social interaction tests [17,51]. The reduced anxious-phenotype observed in MMI-exposed male animals may be the result of hypothyroidism-induced changes in neurotransmitter systems involved in anxiety, such as GABA and serotonin: thus, it has been shown that thyroid hormones regulate GABA receptor activity, and that the serotoninergic system is depressed by hypothyroidism [17,52]. The behavior displayed in the elevated plus-maze test represents a combination of exploratory and avoidance behaviors. Therefore, an alternative explanation is that male rats prenatally exposed to MMI showed less anxiety to novelty, a mechanism that could alter the proper recognition of risky situations and induce lower reactivity under stress, as reported in other studies [18].

ASD are a group of severe developmental psychiatric disorders emerging in the early life, characterized by altered social interaction, compromised verbal and nonverbal communication, stereotyped and repetitive behaviors, often associated with comorbid features, such as social and generalized anxiety [53]. No specific treatments for ASD are currently available. For this reason, it is fundamental to obtain animal models that can reproduce the core and associated symptoms of ASD.

Our results show that prenatal hypothyroidism induced by MMI administration to pregnant rats does not induce in the offspring behaviors that resemble some of the core and associated ASD symptoms. Indeed, rats born from MMI-treated dams did not show deficits in social communication, social discrimination and social play, neither anxious-like behaviors in the elevated plus-maze test. However, gestational hypothyroidism caused reduced body weight in the offspring of both sexes; furthermore, it increased following during social play test and reduced anxiety-like behaviors in the male offspring only but also novel object exploration in both sexes. These effects may be related to the increased novelty-directed exploratory behaviors displayed by MMI-treated rats [18].

Furthermore, since the critical window during which hypothyroidism may affect brain development includes the early postnatal period in humans [54–56] and the third week postnatal in rats [57–59], follow-up experiments are needed to determine whether hypothyroidism during both the prenatal and early postnatal period has a causative role in the onset of neurological diseases, including ASD.

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Chapter 4

Impaired repair of DNA damage is associated with autistic-like traits in rats prenatally exposed to valproic acid

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Chapter 4

Impaired repair of DNA damage is associated with autistic-like traits in rats prenatally exposed to valproic acid

The second preclinical model of ASD I studied was based on prenatal exposure to VPA in rats. Prenatal VPA exposure in rodents is a widely used preclinical model of ASD but there is limited information about the precise biochemical mechanisms underlying the link between ASD and VPA. In the present study I investigated the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. In particular, I tested the effects of three doses of VPA (350, 400, 500 mg/kg) and I found that only at the dose of 500 mg/kg, prenatal VPA induced deficits in communication and social discrimination in rat pups, and altered social behavior and emotionality in the adolescent and adult offspring in the absence of gross malformations. I also found that at this dose of VPA inhibited histone deacetylase in rat embryos and favored the formation of DNA double strand breaks (DSB), but impaired their repair.

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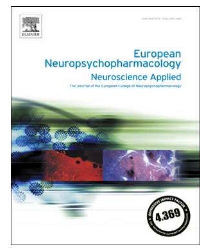

Impaired repair of DNA damage is associated with autistic-like traits in rats prenatally exposed to valproic acid

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Abstract
Prenatal exposure to the antiepileptic and mood stabilizer valproic acid (VPA) is an environmental risk factor for autism spectrum disorders (ASD), although recent epidemiological studies show that the public awareness of this association is still limited. Based on the clinical findings, prenatal VPA exposure in rodents is a widely used preclinical model of ASD. However, there is limited information about the precise biochemical mechanisms underlying the link between ASD and VPA. Here, we tested the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. Only when administered prenatally at the dose of 500 mg/kg, VPA induced deficits in communication and social discrimination in rat pups, and altered social behavior and emotionality in the adolescent and adult offspring in the absence of gross malformations. This dose of VPA inhibited histone deacetylase in rat embryos and favored the formation of DNA double strand breaks (DSB), but impaired their repair. The defective DSB response was no more visible in one-day-old pups, thus supporting the hypothesis that unrepaired VPA-induced DNA damage at the time of neural tube closure may underlie the autistic-like traits displayed in the course of development by rats prenatally exposed to VPA. These experiments help to understand the neurodevelopmental trajectories affected by prenatal VPA exposure and identify a biochemical link between VPA exposure during gestation and ASD.
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1. Introduction

Autism Spectrum Disorders (ASD) are among the most severe psychiatric disorders in childhood in terms of prevalence, outcome, impact on families and cost to society. They are characterized by altered social interaction, compromised communication, stereotyped and repetitive behaviors (American Psychiatric Association, 2013) often associated with comorbid features, such as social and generalized anxiety (Lai et al., 2014).

ASD result from the combination of both genetic and environmental factors, including prenatal exposure to valproic acid (VPA), a widely used and effective antiepileptic and mood stabilizer drug. When given during gestation, VPA not only increases the risk for various congenital malformations (Kini, 2006; Kozma, 2001), but also induces autistic-like features in the offspring, such as impaired communication, reduced sociability and stereotyped behaviors (Williams et al., 2001; Williams and Hersh, 1997). However, the public awareness of the deleterious consequences induced by prenatal VPA exposure in the offspring is still limited. A study published in 2011 revealed that VPA was still the second most common antiepileptic drug taken by pregnant women in Quebec (Kulaga et al., 2011). A recent study showed that only 13.2% of all the reproductive-aged female patients in the US Midwest who were prescribed VPA was documented about the teratogenicity of the drug (Gotlib et al., 2016). Similarly, it has been reported that 17.9% of monotherapy pregnant Australian patients received VPA (Vajda et al., 2012). In line with this scenario, in March 2016, 450 cases of malformations in French children born between 2006 and 2014 from mothers who had taken VPA during pregnancy have been reported (IGAS, 2016). These reports demonstrate that a considerable number of patients across many Countries continues to take VPA during pregnancy, and that more effective public health risk reduction efforts are warranted.

Based on the clinical observations, prenatal VPA exposure in rodents is a widely used preclinical model of ASD (Ranger and Ellenbroek, 2016; Roullet et al., 2013). A wide range of VPA doses has been administered to rodents at different embryonic time points, with the most susceptible period of exposure being around the time of neural tube closure (Bertelsen et al., 2017; Codagnone et al., 2015; Dufour-Rainfray et al., 2010; Favre et al., 2013; Ingram et al., 2000; Kim et al., 2011; Markram et al., 2007, 2008; Rodier et al., 1996; Schneider and Przewlocki, 2005; Sabers et al., 2014; Servadio et al., 2016). Although the majority of these studies used protocols based on a single administration of moderate-to-high VPA doses during the prenatal period, and therefore caution should be paid when comparing these dose regimens with the clinical exposure, all doses tested alter neurodevelopment at a certain extent, in both rats and mice (Roullet et al., 2013; Ranger and Ellenbroek, 2016). Since the diagnosis of ASD is based on specific behavioral characteristics, VPA should be administered at a dose able to induce behaviors in the offspring that are relevant to the core diagnostic symptoms of the disease, and eventually to some of its secondary symptoms (Crawley, 2012; Servadio et al., 2015), without inducing any gross morphological malformation, which would compromise the behavioral analysis.

VPA is a histone deacetylase inhibitor that counteracts key steps in the cellular response to DNA double strand break (DSB) formation by affecting checkpoint activation, DSB repair, and stability of crucial enzymes involved in resection of DNA ends (Shubassi et al., 2012). In the presence of high levels of VPA-induced DNA damage, apoptotic mechanisms are activated, leaving an insufficient number of neuroepithelial cells to undergo neural tube closure and resulting in neural tube defects (Tung and Winn, 2011a). However, the relation between VPA-induced DNA damage and autistic-like features is still unclear.

Here, we first tested the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. Furthermore, to determine whether the autistic-like features displayed by VPA-exposed rats are associated with drug-induced DNA damage, we evaluated the DSB response in embryos exposed to the dose of VPA that induced autistic-like features in the rat offspring, sacrificed either 24 h after VPA administration or after birth.

2. Experimental procedures

2.1. Animals

Female Wistar rats (Charles River, France), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day 1 (GD1). Pregnant rats were singly housed in Macrolon cages, under controlled conditions (temperature 20–21 °C, 55–65% humidity and 12/12 h light cycle with lights on at 07:00 a.m.). Food and water were available *ad libitum*. On GD12.5, females received a single intraperitoneal injection of either VPA or saline (SAL). Newborn litters found up to 5.00 p.m. 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). On PND 21, the pups were weaned and housed in groups of three. Experiments were carried out on the male offspring in the course of development (Figure S1). One pup per litter from different litters per treatment group was used in each experiment. 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 guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

2.2. Drugs

VPA (Cayman, Italy) was dissolved in saline at the concentration of 250 mg/ml and administered at doses (350, 400, 500 mg/kg) and at a time (GD 12.5) that have been shown to induce autistic-like features in the rodent offspring (Roullet et al., 2013).

2.3. Reproduction data

Body weights of the dams were taken daily throughout pregnancy and the length of pregnancy was determined. Litter size, male/female ratio, weight gain of pups and postnatal vitality were also measured. At birth, the occurrence of any physical malformation was recorded.

2.4. Isolation-induced ultrasonic vocalizations (USVs)

The test was performed as previously described (Servadio et al., 2016). On PNDs 5 and 9, pups were individually removed from the nest and

placed into a Plexiglas arena located inside a sound-attenuating and temperature-controlled chamber, with a camera positioned above the arena. The USVs emitted by the pup were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Germany) sensitive to frequencies between 10 and 200 kHz. Pup axillary temperature was measured before and after the test by a digital thermometer. The test was performed in the morning (between 9.00 a.m. and 12.00 a.m.) at a light condition of 650 lux. The USVs were analyzed quantitatively using Avisoft Recorder software (Version 5.1).

2.5. Homing Behavior

The test was performed as previously described (Scattoni et al., 2008). On PND13, the litter was separated from the dam for 30 min. The cage was placed on a heating pad set at a temperature of 35 °C to maintain normal body temperature of the pups in the nest. Then, each pup was placed into a box whose floor was covered for 1/3 with bedding from the pup 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 videorecorded for 4 min (duration of each session). The following parameters were scored by an observer using the Observer 3.0 software (Noldus Information Technology, The Netherlands): 1. latency (sec) to reach the home-cage bedding area; 2. total time (sec) spent in the nest bedding area (Scattoni et al., 2008; Servadio et al., 2016). The test was performed in the morning (between 9.30 a.m. and 12.30 a.m.) at a light condition of 650 lux.

2.6. Social play behavior

The test was performed as previously described (Trezza and Vanderschuren, 2008). 35-day-old rats were individually habituated to the test cage for 10 min on each of the 2 days before testing. On the test day, the animals were isolated for 3 h before testing. The test consisted of placing VPA- or SAL-exposed rats together with an untreated animal for 15 min. The test was performed between 9 a.m. and 2 p.m. under low light condition (2 lux). Behavior was assessed per each individual animal of a pair separately using the Observer 3.0 software (Noldus Information Technology, 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. 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 and Pellis, 2009).

We determined: 1. frequency of pinning; 2. frequency of pouncing; 3. evasion (the animal that is pounced upon does not prolong the playful interaction but rather runs away); 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)); 5. 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).

2.7. Social interaction in adult rats

The test was performed as previously described (Manduca et al., 2015). 90-day-old rats were individually habituated to the test cage for 5 min on each of the two days prior to testing. Then, they were socially isolated for 24 h, to enhance their motivation to interact with a social partner. On the following day, VPA- or SAL-exposed rats were placed in the test cage for 10 min together with an untreated animal. The test was performed between 9 a.m. and 2 p.m. under low light condition (2 lux).

The behavior of the animals was videorecorded and assessed per each individual animal of a pair separately using the Observer 3.0 software (Noldus Information Technology, The Netherlands).

The following behavioral parameters were scored: 1.frequency and time spent in social exploration (*i.e.*, sniffing any part of the body of the test partner; social grooming, when one rat licks and chews the fur of the conspecific); 2. mean duration of social exploration (*i.e.*, total time spent in social exploration/total frequency of social exploration).

2.8. Elevated plus-maze

The apparatus comprised two open ($50 \times 10 \times 40$ cm^3; l × w × h) and two closed ($50 \times 10 \times 40$ cm^3; l × w × h) arms that extend from a common central platform (10×10 cm^2). The test was performed as previously described (Manduca et al., 2015). Rats were individually placed on the central platform of the maze for 5 min. Each session was videorecorded for subsequent behavioral analysis performed using the Observer 3.0 software (Noldus Information Technology, The Netherlands). The following parameters were analyzed: 1.% open arm entries (% OE): (the number of entries into the open arms of the maze/number of entries into open + closed arms) × 100; 2. % time spent in open arms (%TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100; 3. number of closed arm (CE) entries. The test was performed between 9 a.m. and 2 p.m. under low light condition (2 lux).

2.9. Biochemical investigation

Twenty-four hours after injection of dams with either SAL or 500 mg/kg VPA, three embryos per experimental group were sacrificed and rapidly frozen. Moreover, three male infants (PND1) per experimental group, prenatally exposed to SAL or to 500 mg/kg VPA, were sacrificed and their whole brain was rapidly frozen. Biochemical assays were performed as previously described (di Masi et al., 2006). Primary antibodies were: anti-actin (Sigma Aldrich, USA); anti-ATM, anti-ATR, anti-CHK2, anti-HDAC1, anti-HDAC2, anti-MRE11, anti-p53, anti-RAD50, anti-RAD51 (Santa Cruz Biotechnology, CA, USA); anti-acetyl-histone H3, anti-pSer1981-ATM, anti-pSer428-ATR, anti-pSer1524-BRCA1, anti-pThr68-CHK2, anti-pSer343-NBN, anti-pSer15-p53 (Cell Signaling Technology, MA, USA); anti-pSer2056-DNA-PK, anti-BRCA1 (Abcam, UK); anti-NBN (GeneTex, CA, USA); anti-53BP1, anti-γ-H2AX (Millipore, MA, USA) antibodies. HRP-conjugated secondary antibodies were from Bio-Rad Laboratories (CA, USA). Proteins were visualized using the ChemiDoc™ Imaging System (Bio-Rad Laboratories, CA, USA). Densitometric analyses were performed using the ImageLab software (version 5.2.1; Bio-Rad) by quantifying the band intensity of the protein of interest with respect to the relative loading control band.

2.10. Statistical analysis

Data are expressed as mean ± SEM. To assess the effects of different doses of VPA (350, 400, and 500 mg/Kg) on the behavior of the offspring, data were analyzed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test. Latencies in the homing behavior test showed a non-parametric distribution and therefore were analyzed by Kruskal-Wallis analysis of variance and the Mann-Whitney U test was used to assess the significance of differences between treatment groups using the Bonferroni's correction for multiple comparisons (*post hoc* comparisons). Biochemical data were analyzed by Student's *t*-test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Prenatal VPA exposure did not alter reproduction parameters and did not induce overt signs of toxicity in the dam and the offspring

At all doses, prenatal VPA exposure did not affect pregnancy length, dam weight gain, litter size at birth, male/female ratio, pup weight gain, and postnatal vitality (Table 1). In line with what has been previously reported (Favre et al., 2013), VPA-exposed rats exhibited good health. In particular, ear malformations were never observed in VPA-exposed rats. Overall, the 2.4 % of the offspring exposed to VPA 500 mg showed tail kinks (data not shown).

3.2. Prenatal VPA exposure induced dose-dependent deficits in social communication and social discrimination in the infant offspring

Since communicative deficits in ASD appear already in infancy, a very useful tool in rodent models of ASD is the analysis of the USVs emitted by pups in response to separation from the mother and the nest. These USVs play an essential communicative role in mother-offspring interaction and are crucial for pup survival (Wohr and Schwarting, 2013).

VPA, administered at the doses of 400 and 500 mg/kg, affected the USVs of the offspring at PND9 [$F_{3,25}=11.94$, $p<0.001$, Figure 1b] but not at PND5 (Figure 1a). Pups prenatally exposed to VPA at the doses of 400 ($p<0.01$) and 500 ($p<0.001$) mg/Kg vocalized significantly less on PND9 compared to SAL-exposed pups, while the dose of 350 mg/kg was ineffective (Figure 1b).

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 (Tonkiss et al., 1996). A one-way ANOVA analysis performed on the time spent into the nest area gave the following result: [$F_{3,24}=4.67$, $p=0.01$]. A Kruskal-Wallis analysis of variance performed to analyze the latencies to reach the home-cage bedding area gave the following result: [$H_3=9.04$, $p<0.05$]. *Post hoc* analysis revealed that only pups prenatally exposed to 500 mg/kg VPA showed a longer latency to reach the familiar bedding ($p<0.05$; Figure 1c) and spent less time in the nest area ($p<0.01$; Figure 1d) compared to SAL-exposed pups.

3.3. Prenatal VPA exposure induced dose-dependent deficits in social play and anxiety-like behaviors in the adolescent offspring

Social play behavior is the first form of non-mother directed social behavior displayed by most developing mammals (Vanderschuren et al., 2016) and it is profoundly impaired in ASD (Jordan, 2003).

Prenatal VPA dose-dependently altered social play in the offspring. In particular, compared to controls, rats exposed prenatally to VPA 500 mg/kg more often responded to play solicitation by evading the social contact ($p<0.05$ vs control rats, Table 2). As a consequence, the percentage of response to play solicitation was lower in the offspring born from VPA 500 mg/kg-treated dams (percentage of response to play solicitation [$F_{3,40}=7.62$, $p<0.001$]; time spent in social exploration [$F_{3,40}=2.98$, $p<0.05$]). *Post hoc* analysis revealed that only rats prenatally exposed to 500 mg/kg VPA showed reduced responsiveness to play solicitation compared to SAL-exposed rats ($p<0.05$, Figure 2a), with no changes in general social exploration (Figure 2b).

A one-way ANOVA analysis performed on the parameters measured in the elevated plus-maze test gave the following results: percentage of open arm entries (% OE) [$F_{3,38}=4.96$, $p<0.01$; Figure 2c]; percentage of time spent in the open arms (% TO) [$F_{3,38}=2.97$, $p<0.05$; Figure 2d]; frequency of closed arm entries (CE) [data not shown]. *Post hoc* analysis showed that only rats exposed to the 500 mg/Kg VPA made less open arm entries and spent less time in the open arms of the maze compared to SAL-exposed rats ($p<0.05$). All VPA doses did not affect closed arm entries.

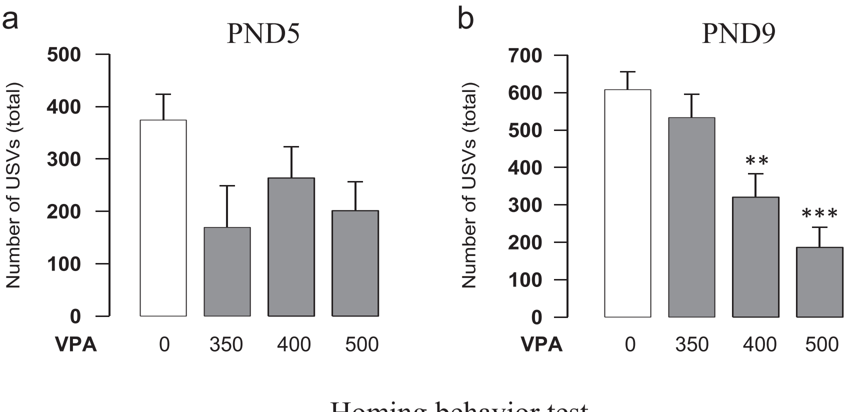
3.4. Prenatal VPA exposure dose-dependently induced deficits in social interaction and anxiety-like behaviors in the adult offspring

Prenatal VPA exposure altered social behavior in the adult offspring (mean duration of social exploration: [$F_{2,50}=3.71$, $p<0.05$; Figure 2e]; time spent in social exploration: [p=n.s.; Figure 2f]). *Post hoc* analysis revealed that rats prenatally exposed to 500 mg/kg VPA showed reduced mean

Table 1 Reproduction data.

Group	Dam weight gain %[a]	Pregnancy length, days	Litter size	Pup weight PND1	Pup weight PND12	Pup weight PND28	Male/ female ratio	Postnatal vitality (%)	Number of Dams
SAL	35.5±0.9	22.4 ± 0.1	11 ± 0.8	6.5±0.2	33±0.5	82.7±2.7	09±0.1	89.1±2.4	13
VPA 350	32.3±1.9	22.6 ± 0.2	12.9 ± 0.7	6.7±0.1	34.6±1	81.5±3.5	1.2±0.2	86.5±3.5	11
VPA 400	33.1±1.4	22.6 ± 0.1	11.7 ± 0.6	6.8±0.2	34.3±0.8	81.7±1.9	1.1±0.2	86.1±3.5	12
VPA 500	31.7±1.3	22.6 ± 0.4	10.7 ± 0.4	6.8±0.1	33.6±0.7	82.4±2.6	1±0.1	85.1±2.9	12

Data represent mean values±SEM.
[a]From GD 1 to GD 21.



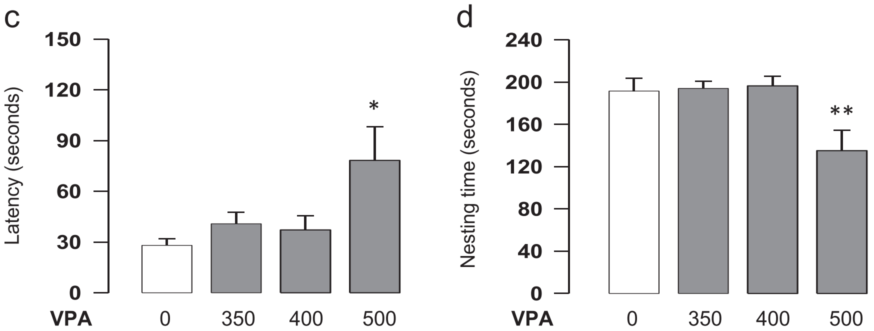


Figure 1 Prenatal VPA exposure induced dose-dependent deficits in social communication and social discrimination in the infant offspring. Prenatal VPA exposure at the doses of 500 and 400 mg/Kg reduced isolation-induced USV emission at PND 9 (b), but not at PND5 (a). Prenatal VPA exposure altered homing behavior only at the dose of 500 mg/Kg (c, d). Data represent mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *versus* SAL-exposed pups; Student-Newman-Keuls *post hoc* test. USV test: SAL=8, VPA350= 5, VPA400= 8, VPA500= 8 pups. Homing behavior test: SAL= 8, VPA350= 5, VPA400= 7, VPA500= 8 pups.

Table 2 Social play behavior parameters.

Group	Pouncing	Pinning	Evasion
SAL	50.4±8	23.2±4.0	3.2±0.5
VPA 350	41.4±2.1	27.2±1.2$^{\#}$	3.4±0.5
VPA 400	47.6±5.1	28.9±4.0$^{\#}$	3.4±1
VPA 500	45.4±4.3	14.4±1.7	6.1±1*

Data represent mean values±SEM.
Statistical Analysis: one way ANOVA followed by Student-Newman-Keuls *post hoc* test.
$^{\#}$p<0.05 versus VPA500.
*p<0.05 versus SAL.

duration of social exploration compared to SAL-exposed rats ($p<0.05$).

Prenatal VPA exposure induced anxiety-like behaviors in the adult offspring without affecting locomotor activity (% OE: [$F_{2,41}=5.8$, $p<0.01$; Figure 2g]; %TO: [$F_{2,41}=3.5$, $p<0.05$; Figure 2h]; frequency of CE: [p=n.s.; data not shown]). *Post hoc* analysis revealed that animals prenatally exposed to VPA at the doses of 400 and 500 mg/kg made less open arm entries compared to SAL-exposed rats, while only rats exposed to VPA at the dose of 500 mg/kg spent less time in the open arms of the maze compared to SAL-exposed rats. All VPA doses did not affect closed arm entries.

3.5. Reduction of HDAC1 and HDAC2 protein levels and histone H3 acetylation in VPA-treated embryos

The behavioral experiments showed that VPA, administered to pregnant rats at the dose of 500 mg/kg, induced core and associated autistic-like symptoms in the offspring. Therefore, we investigated the efficacy of this dose of VPA in inhibiting HDACs. Both HDAC1 ($p<0.01$) and HDAC2 ($p<0.05$) were significantly downregulated in VPA-treated embryos compared to SAL-treated ones (Figure 3a,b), while a statistically significant VPA-induced increase of histone H3 acetylation ($p<0.01$) was observed (Figure 3c).

3.6. Response to DNA damage in VPA-treated embryos

DSB represents one of the most harmful DNA lesions. DSB repair requires the activity of damage sensor proteins belonging to the phosphatidylinositol-3-kinase protein kinase-like (PI3K) family. This family includes the ataxia-telangiectasia mutated (ATM) kinase, the ataxia-telangiectasia and Rad3-related (ATR) kinase, and the

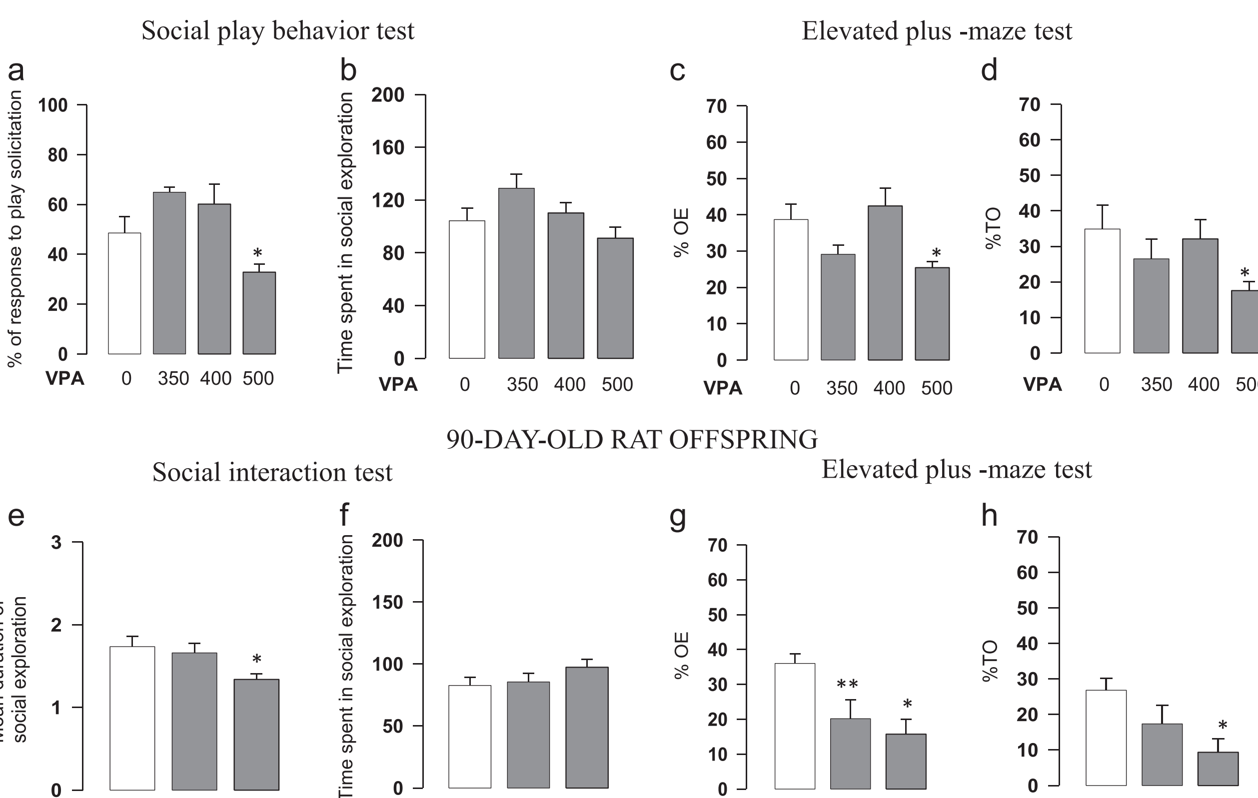


Figure 2 Prenatal VPA exposure dose-dependently induced social deficits and anxiety-like behaviors in the adolescent and adult offspring. Prenatal VPA exposure at the dose of 500 mg/Kg altered social play behavior (a) at PND 35 without affecting the total time spent in social exploration (b). At this dose, prenatal VPA reduced the percentage of open arm entries (% OE, c) and the percentage of time spent on the open arms (% TO, d) in the elevated plus-maze test. Prenatal VPA exposure at the dose of 500 mg/Kg reduced the mean duration of social exploration (e) without affecting the total time spent in social exploration (f) at PND 90. In adult rats tested in the elevated plus-maze test, prenatal VPA reduced the percentage of open arm entries (% OE, g) at the doses of 400 and 500 mg/kg, and the percentage of time spent on the open arms (% TO, h) at the dose of 500 mg/kg only. Data represent mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$ *versus* SAL-exposed rats; Student-Newman-Keuls *post hoc* test. PND35: Social play behavior test: SAL= 12, VPA350= 11, VPA400= 9, VPA500= 12. Elevated plus-maze test: SAL= 12, VPA350= 9, VPA400= 10, VPA500= 11. PND90: Social Interaction test: SAL= 19, VPA400= 16, VPA500= 19. Elevated plus-maze test: SAL= 17, VPA400= 17, VPA500= 10.

DNA-dependent protein kinase (DNA-PK) that phosphorylate the histone H2AX at the Ser139 residue (the phosphorylated H2AX protein being named γ-H2AX). After the PI3K-dependent H2AX phosphorylation, a plethora of proteins (*e.g.*, the MRE11-RAD50-NBN complex, 53BP1, BRCA1, and CHK2) accumulate at the DSB in order to signal the damage to the DSB repair proteins (Abraham, 2004; Shiloh and Ziv, 2013). Noteworthy, most of these proteins are ATM substrates (Shiloh and Ziv, 2013).

Since γ-H2AX and 53BP1 are well-known markers of the DSB that mediate accumulation of various signaling and repair proteins to the damaged sites (Panier and Boulton, 2014; Scully and Xie, 2013), the phosphorylation status of the Ser139 residue of H2AX and the levels of 53BP1 in VPA-exposed and control embryos have been determined. 500 mg/kg VPA induced DSB, as indicated by the increased levels of γ-H2AX ($p<0.05$) and 53BP1 ($p<0.01$) (Figure 4a, b), and caused the activation (*i.e.*, the phosphorylation) of the three PI3K kinases. Indeed, we detected increased levels of pSer2056-DNA-PK ($p<0.05$, Figure 4d), pSer1981-ATM ($p<0.01$, Figure 4e), and pSer428-ATR ($p<0.01$, Figure 4g) in VPA-treated embryos compared to SAL-treated ones. As expected, no significant variation in ATM and ATR total levels were observed (Figure 4f,h). To evaluate the activity of these kinases, and in particular of ATM that plays a pivotal role in the DSB response (Shiloh and Ziv, 2013), the phosphorylation status of several ATM substrates was examined. The phosphorylation of NBN at Ser343 ($p<0.01$, Figure 4i), BRCA1 at Ser1524 ($p<0.01$, Figure 4k), CHK2 at Thr38 ($p<0.05$, Figure 4m), and p53 at Ser15 ($p<0.05$, Figure 4o) increased in VPA-treated embryos compared to SAL-treated ones. In turn, the expression of RAD51 protein, which plays a key role in the regulation of the DSB repair via the homologous recombination pathway (Chapman et al., 2012), was downregulated in VPA-treated embryos ($p<0.05$) (Figure 4c). As expected on the basis of their phosphorylation status, the total levels of NBN ($p<0.05$, Figure 4j), BRCA1 (Figure 4l), CHK2 (Figure 4n) and p53 ($p<0.05$, Figure 4p) increased following VPA treatment. Overall, these results indicate the induction of DSB following embryos exposure to 500 mg/kg VPA, the activation of the

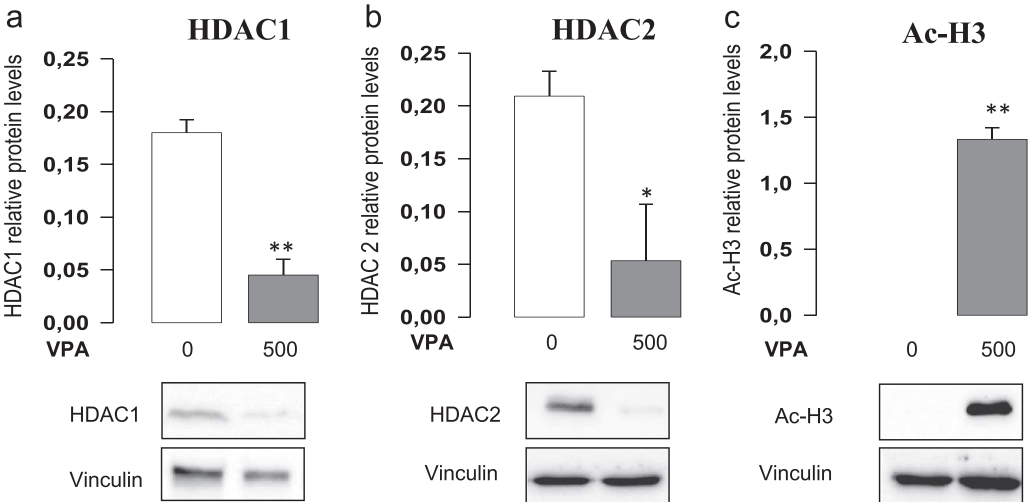


Figure 3 Reduction of HDAC1 and HDAC2 protein levels and histone H3 acetylation in 500 mg/kg VPA-treated embryos. Western blotting and relative densitometric analyses of HDAC1 (a), HDAC2 (b), and histone H3 acetylation (c) in rat embryos exposed to SAL or to VPA at the dose of 500 mg/Kg. Twenty-five micrograms of total protein lysates were used to perform immunoblot experiments; filters were blotted with anti-HDAC1, anti-HDAC2, and anti-acetylated-H3 antibodies. The analysis of vinculin expression in the same filters was used as loading control. Graphs express the mean values derived from the analysis of 3 SAL-treated *versus* three 500 mg/kg VPA-treated embryos ± SEM. *$p<0.05$, **$p<0.01$ *versus* SAL-exposed embryos.

DSB-induced damage response, and a defective capability to repair the DSB.

3.7. Lack of histone H3 acetylation, HDAC activation, and DNA damage response induction in infant rats prenatally exposed to VPA

In one-day-old pups prenatally exposed to 500 mg/kg VPA, no differences in the histone H3 acetylation status and in HDAC1 and HDAC2 expression levels were observed compared to SAL-treated infants (Figure 5a-c). Moreover, neither H2AX phosphorylation nor increased 53BP1 levels were observed in PND1 VPA-treated animals (Figure 5d,e). ATM and ATR kinases were not activated (Figure 5f,h,i), although ATM levels slightly increased in VPA-treated animals ($p<0.05$, Figure 5g). The activated form of DNA-PK increased in VPA-treated pups ($p<0.05$, Figure 5j), possibly as an effect of the physiological metabolism of the endogenous damage. These results suggest that VPA activates the DNA damage response soon after treatment, and that no alteration in histone H3 acetylation, HDAC expression, and in the overall DNA damage induction occurred after birth in VPA-treated animals.

4. Discussion

VPA is an effective and widely prescribed antiepileptic and mood stabilizer drug. However, *in utero* exposure to VPA in humans is problematic, since it may induce a wide range of abnormalities in the offspring, ranging from structural malformations to more subtle autistic-like behaviors. This led to the inclusion of prenatal VPA exposure among the environmental etiological factors of ASD, and to the validation of a rodent model of ASD based on prenatal VPA exposure (Ranger and Ellenbroek, 2016; Roullet et al., 2013). However, there is still limited public awareness of the deleterious consequences induced by *in utero* exposure to VPA, despite the recent warnings by various Medicines Agencies (AIFA, 2016; EMA, 2014). Furthermore, there is limited information about the precise biochemical mechanisms underlying the link between ASD and VPA.

In animal studies, the effects of prenatal VPA are dose-dependent, ranging from subtle behavioral abnormalities to structural malformations. Here, we tested the effects of increasing doses of VPA on behavioral features resembling core and secondary symptoms of ASD in rats. Next, we tested the ability of the dose of VPA (500 mg/kg) that induced autistic-like features in the rat offspring to inhibit histone deacetylase and to counteract key steps in the cellular response to DSB formation by affecting checkpoint activation, DSB repair, and the stability of crucial enzymes involved in resection of DNA ends.

At the behavioral level, VPA dose-dependently induced deficits in social communication and social discrimination in the rat infant offspring. Prenatal VPA, administered at the doses of 400 and 500 mg/kg, altered the emission of USVs at PND9. These results are in line with previous studies that tested a higher dose of VPA (600 mg/kg) (Felix-Ortiz and Febo, 2012; Gandal et al., 2010; Mehta et al., 2011; Tyzio et al., 2014). In the homing behavior test, only 500 mg/kg VPA induced deficits in social discrimination, altering the ability of the pups to discriminate between a neutral odor and their home cage odor, as previously reported in studies that used both the 600 (Degroote et al., 2014; Roullet et al., 2010; Schneider and Przewlocki, 2005) and 500 (Favre et al., 2013; Servadio et al., 2016) mg/kg doses. Both USVs and olfaction are crucial instruments for communication and social interaction in rodent pups. USVs play an essential communicative role to elicit care giving behaviors in the dam (Wohr and Schwarting, 2013), while olfaction, and in

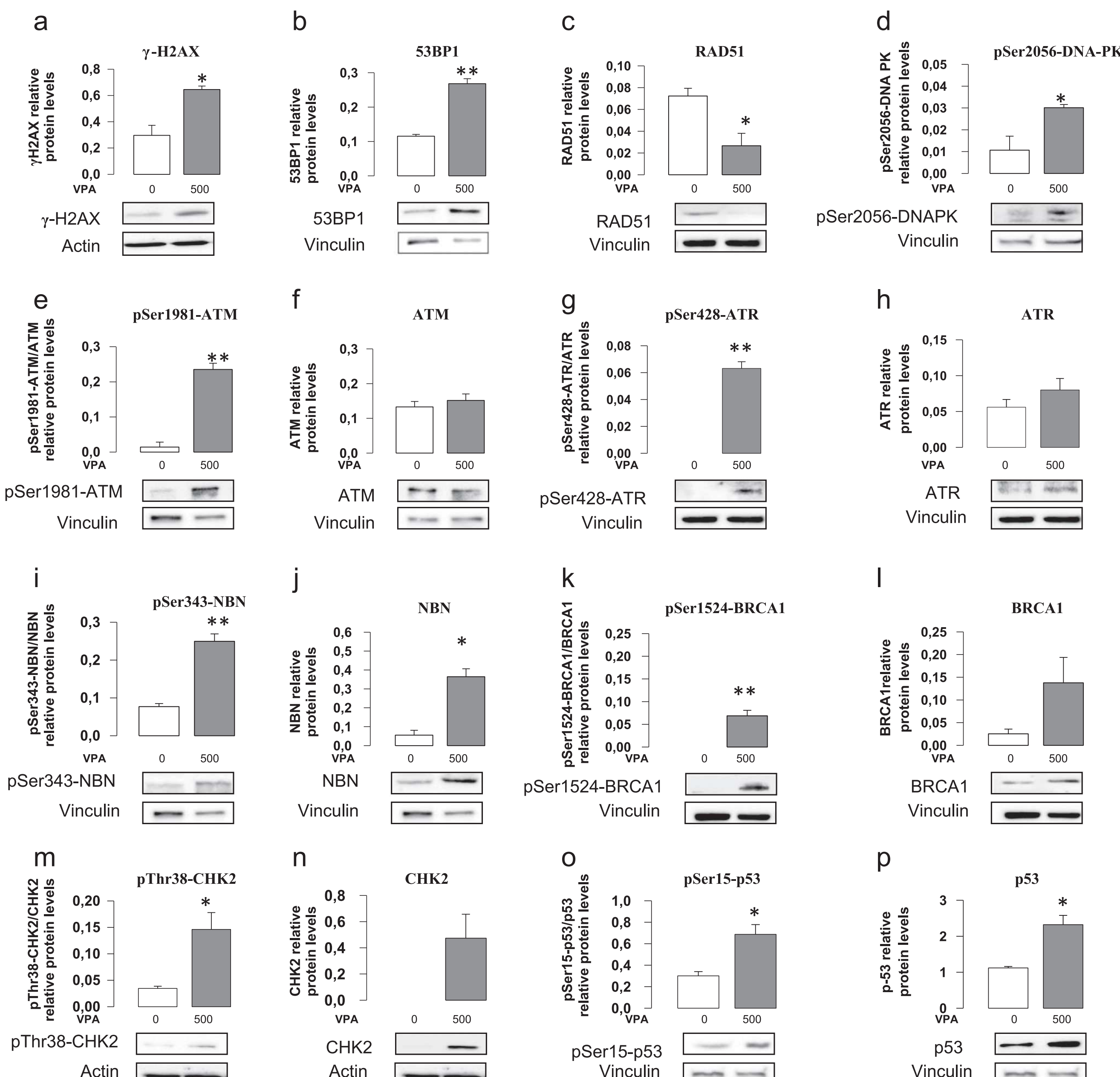


Figure 4 DSBs damage response in 500 mg/kg VPA- exposed embryos. Western blotting and relative densitometric analyses of a panel of proteins involved in sensing, signaling, and repair of DSBs in rat embryos exposed to SAL or to 500 mg/Kg VPA. Twenty-five micrograms of total protein lysates were used to perform immunoblot experiments; filters were blotted with the anti-γ-H2AX (a), anti-53BP1 (b), anti-RAD51 (c), anti-pSer2056-DNA-PK (d), anti-pSer1981-ATM (e), anti-ATM (f), anti-pSer428-ATR (g), anti-ATR (h), anti-pSer343-NBN (i), anti-NBN (j), anti-pSer1524-BRCA1 (k), anti-BRCA1 (l), anti-pThr68-CHK2 (m), anti-CHK2 (n), anti-pSer15-p53 (o), and anti-p53 (p) antibodies. Where phosphorylated proteins were analyzed, the filter was stripped and reprobed with the non-phosphorylated antibody. The analysis of actin or vinculin expression was used as loading control. Graphs express the mean values derived from the analysis of three SAL-treated *versus* three 500 mg/kg VPA-treated embryos ± SEM. *$p<0.05$ and **$p<0.01$ *versus* SAL-exposed embryos.

particular the learned association between maternal odors and stimulation, is crucial for the development of social behavior and social recognition (Terry and Johanson, 1996). Thus, the altered USV profile and homing behavior displayed by pups exposed to 500 mg/Kg VPA indicate a reduced ability to communicate with their mother and deficits in social recognition.

At adolescence, only 500 mg/kg VPA affected the social behavior and emotional reactivity of the offspring. In line with the social deficits reported in previous studies performed with higher doses of VPA (Felix-Ortiz and Febo, 2012; Schneider and Przewlocki, 2005; Schneider et al., 2006), adolescent rats prenatally exposed to 500 mg/kg VPA showed decreased responsiveness to play solicitation. The social

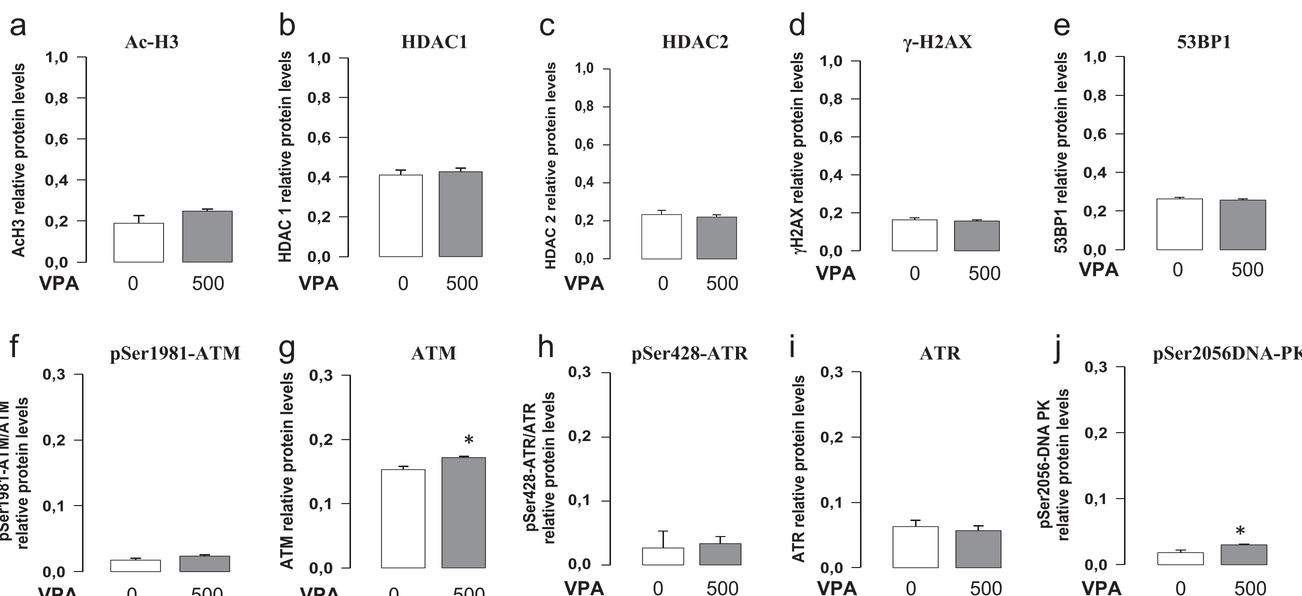


Figure 5 Evaluation of histone H3 acetylation, HDAC induction, and activation of the DNA damage response in the whole brain of infant (PND1) rats prenatally exposed to SAL or 500 mg/Kg VPA. Twenty-five micrograms of total protein lysates were used to perform immunoblot experiments; filters were blotted with anti-acetylated-H3 (a), anti-HDAC1 (b), anti-HDAC2 (c), anti-γ-H2AX (d), anti-53BP1 (e), anti-pSer1981-ATM (f), anti-ATM (g), anti-pSer428-ATR (h), anti-ATR (i), and anti-pSer2056-DNA-PK (j). Where phosphorylated proteins were analyzed, the filter was stripped and reprobed with the non-phosphorylated antibody. Actin expression was used as loading control. Graphs express the mean values derived from the analysis of the whole brain of three SAL-*versus* three 500 mg/kg VPA-treated infant (PND1) rats ± SEM. *$p<0.05$ *versus* SAL-exposed infant rats.

deficits induced by prenatal exposure to 500 mg/kg VPA were long-lasting. Indeed, adult rats exposed to this dose of VPA showed a decreased mean duration of social exploration, which is in line with previous findings reporting social fear and social avoidance in VPA-exposed adult rodents (Baronio et al., 2015; Kataoka et al., 2013; Markram et al., 2008).

Anxiety is a frequent symptom displayed by autistic patients (Lai et al., 2014). Accordingly, as previously reported (Ellenbroek et al., 2016; Kumar et al., 2015; Markram et al., 2008; Schneider et al., 2008; Schneider et al., 2006), rats exposed to 500 mg/kg VPA showed an anxious phenotype in the elevated plus-maze test, both during adolescence and adulthood.

Collectively, the behavioral experiments showed that VPA, administered to pregnant rats at the dose of 500 mg/kg, induced core and associated autistic-like symptoms in the exposed offspring, in the absence of overt signs of toxicity. Conversely, VPA was ineffective or only partially effective in inducing autistic-like behavioral features when administered at the doses of 350 and 400 mg/kg, respectively.

VPA is a histone deacetylase inhibitor that induces the relaxation and opening of chromatin conformation by histone hyperacetylation; this increases cell susceptibility to DNA damage with the activation of the cell cycle checkpoints (Sha and Winn, 2010; Shubassi et al., 2012). To determine whether the autistic-like features observed in this rodent model of ASD are associated with VPA-induced DNA damage, the DSB response was evaluated in embryos exposed to the dose of VPA that induced core and associated autistic-like features in the rat offspring. Maternal exposure to VPA induced DSB in embryos, as indicated by the significant increase in γ-H2AX and 53BP1 expression compared to SAL-treated embryos. These results are in line with previous findings showing that maternal exposure to VPA produced a rapid increase of γ-H2AX in Chinese hamster ovary cells (Sha and Winn, 2010) and in mouse embryos (Tung and Winn, 2011b). The induction of γ-H2AX and 53BP1 in VPA-treated embryos was sustained by the activation of the three PI3K (*i.e.*, ATM, ATR, and DNA-PK), which often act by redundant mechanisms, and by the phosphorylation of well-known ATM substrates (*i.e.*, NBN, BRCA1, CHK2, and p53) (Shiloh and Ziv, 2013). However, despite the activation of the DSB response machinery, the repair capacity was impaired in VPA-treated embryos, as demonstrated by the low levels of expression of the DSB repair protein RAD51. Interestingly, the defective DSB response was no more visible in VPA-exposed rats at PND1. This supports the notion that, in addition to the VPA-induced down-regulation of HDACs, VPA-induced DNA damage may contribute to developmental defects in embryos (Tung and Winn, 2011b; Wells et al., 2009), particularly at the level of the neural tube (Tung and Winn, 2011a).

Altogether, the following conclusions can be drawn from this study. First, we correlated a dose of VPA that, given prenatally, induces long-lasting core and associated autistic-like features in the offspring, without overt signs of toxicity, with early DNA damage. Thus, this study provides a guide for experimental design choices for researchers willing to use the VPA rodent model of ASD in future studies. Second, the altered profile displayed by VPA-exposed rats from

infancy till adolescence and adulthood in ASD-relevant behavioral domains confirms, at the preclinical level, the long-lasting deleterious effects induced by prenatal VPA exposure. The clinical data clearly show that VPA is still often prescribed to pregnant women. This prompted Medicine Agencies to sensitize health personnel and patients about the risk induced by prenatal VPA exposure, and researchers to perform targeted studies on causative environmental risk factors for ASD. Last, and most importantly, these experiments help to understand the neurodevelopmental trajectories that are affected by prenatal VPA exposure. Indeed, besides the well-known VPA-induced down-regulation of HDACs, we identified the formation of DBS and their impaired repair as a biochemical substrate underlying the link between VPA exposure during gestation and ASD.

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Contributors

Authors M. Servadio, A. Manduca, F. Melancia, S. Schiavi, P. Campolongo and M. Palmery performed, analyzed and contributed to the design of the behavioral experiments. Authors A. di Masi, L. Leboffe and P. Ascenzi performed, analyzed and contributed to the design of the biochemical experiments. Authors M. Servadio, A. di Masi and P. Ascenzi wrote the manuscript. Author V. Trezza supervised the project, designed the experiments and wrote the manuscript. All authors contributed to and have approved the final 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 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.euroneuro.2017.11.014.

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Chapter 5

Modelling fragile X syndrome in the laboratory setting: A behavioral perspective.

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Chapter 5

Modelling fragile X syndrome in the laboratory setting: A behavioral perspective.

The last two papers of this thesis were mainly focused on the study of genetic animal models of ASD, in particular of Fragile X syndrome, the most common monogenetic cause of autism. In particular, the present review paper provides an overview of the behavioral features, diagnostic criteria and off-label pharmacotherapy of FXS. Then I described the preclinical models of FXS commonly used in laboratories to study this syndrome. I focused my attention on the description of the FXS-relevant behavioral features and how they can be modelled in laboratory animals in the course of development. I paid particular attention to the rodent models of FXS, particularly to the *Fmr1*-KO mice and rats, that are the most widely used animal models of FXS currently available, and the animal models that I used in the last part of my PhD project.

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Review

Modelling fragile X syndrome in the laboratory setting: A behavioral perspective

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ABSTRACT

Fragile X syndrome is the most common form of inherited mental retardation and the most frequent monogenic cause of syndromic autism spectrum disorders. The syndrome is caused by the loss of the Fragile X Mental Retardation Protein (FMRP), a key RNA-binding protein involved in synaptic plasticity and neuronal morphology. Patients show intellectual disability, social deficits, repetitive behaviors and impairments in social communication. The aim of this review is to outline the importance of behavioral phenotyping of animal models of FXS from a developmental perspective, by showing how the behavioral characteristics of FXS at the clinical level can be translated into effective, developmentally-specific and clinically meaningful behavioral readouts in the laboratory setting. After introducing the behavioral features, diagnostic criteria and off-label pharmacotherapy of FXS, we outline how FXS-relevant behavioral features can be modelled in laboratory animals in the course of development: we review the progress to date, discuss how behavioral phenotyping in animal models of FXS is essential to identify potential treatments, and discuss caveats and future directions in this research field.

1. Introduction

In 1943, Martin and Bell described for the first time a sex-linked form of mental retardation affecting eleven sons of a family, all born from mothers with normotypic levels of intelligence [1]. More than 25 years later, the existence of a constriction near the end of the long arm of the X chromosome of males affected by intellectual disabilities was reported by Lubs [2]. Later, this variant was localized to Xq27.3 [3] and become known as "fragile site" on the X chromosome. Seven members of the original family of sex-linked mental retardation reported by Martin and Bell in 1943 were re-examined: five of them were found to carry this fragile site, together with typical facial features (i.e., long face, large ears and prominent jaw) and macroorchidism [4]. This condition was named as "Martin-Bell syndrome", now known as Fragile X Syndrome (FXS) [5]. In 1991, the gene responsible of FXS was cloned and named *Fragile X Mental Retardation 1* gene (*FMR1*) [6].

FXS is the most commonly inherited form of developmental and intellectual disability [7,8]. Several population-based studies estimated the prevalence of the disease as 1 in 4000 males and 1 in 8000 females [8,9] although it has recently been suggested that the best estimate for the frequency of the full mutation (FM) is 1/2500 for both males and females [10].

FXS is associated with an unstable expansion of a CGG trinucleotide repeat within the 5′untranslated region (5′UTR) of the *FMR1* gene, located in the X chromosome [6,11]. In the normal population, the number of CGG triplets in the *FMR1* gene varies from 6 to 54, allowing transcription and translation of the gene. When the number of CGG triplets expands between 55 and 200, the premutation (PM) state occurs, while more than 200 repeats characterize the FM condition. PM alleles are unstable and, upon maternal transmission, tend to expand to FM alleles [12]. The probability of having a FM allele in the offspring born from mothers carrying the PM depends on the size of the maternal CGG repeat tract. Conversely, the presence of AGG interruptions in the CGG repeat locus of the *FMR1* gene increases the stability of PM alleles during prenatal transmission, thus decreasing the risk of expansion of a PM allele to a FM allele [13].

Patients carrying the PM have normal intellectual abilities but some of them show fragile X tremor ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder affecting approximately one in 3000 men and a smaller number of women in the general population [14]. In addition, fragile X–associated primary ovarian insufficiency (POI), which consists in menopause appearance prior to the age of 40, occurs in women carrying the PM [15].

More than 200 CGG repeats cause the FM and the fragile site at Xq27.3 [6]. This expansion triggers hypermethylation and other epigenetic modifications of the CpG island in the promoter region, resulting with the heterocromatinization of the *FMR1* locus and consequently with the loss of the protein product, the fragile X mental

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retardation protein (FMRP) [16].

FMRP is an RNA-binding protein with four major isoforms between 70 and 80 kDa. FMRP is involved in different steps of RNA metabolism ranging from nuclear export to transport of mRNA along neurites and translational control in the soma and at synapses [17,18].

FMRP is expressed in many tissues and it is particularly abundant in the brain, where it is essential for neural development and plasticity [19,20]. FMRP binds a large number of mRNAs, also called the FMRP transcriptome, and many of them code for proteins involved in neuronal excitability and synaptic transmission [18]. In the absence of FMRP, the deregulation of translation/transport/stability of these mRNAs affects multiple neuronal pathways, generating the altered phenotype displayed by FXS patients.

Not only FXS is the most frequent heritable form of intellectual disability, but it is also the most common monogenic cause of syndromic autism spectrum disorders (ASD) [21,22]. Approximately 30% of patients with FXS meet the full diagnostic criteria for ASD [22], while over 90% of individuals with FXS display some ASD symptoms [23].

In addition to severe intellectual disability and autistic-like features, FXS is characterized by seizures, attention deficit and hyperactivity, sleep disturbances, craniofacial abnormalities and macroorchidism. Females typically display milder symptoms than males due to compensation by the second non-affected X chromosome.

Approved therapies for FXS are not yet available: current approaches focus on behavioral therapy and off-label medications that mitigate only a limited set of symptoms, such as hyperactivity, seizure and anxiety. Thus, the search for new treatments able to modify the lifetime course of FXS and to improve its symptoms is urgent. In this context, animal models are excellent research tools for two main reasons: 1. to understand how loss of *FMR1* and FMRP contributes to FXS symptomatology; 2. to validate new therapeutic targets and potential drug candidates.

The aim of this review is to outline the importance of animal models in FXS research, by showing how the behavioral characteristics of FXS at the clinical level can be translated into effective, developmentally-specific and clinically meaningful behavioral readouts in the laboratory setting. After introducing the clinical diagnosis and current pharmacotherapy of FXS, we outline how FXS-relevant behavioral features can be modelled in laboratory animals in the course of development: we review the progress to date, discuss how behavioral phenotyping in animal models of FXS is essential to identify potential treatments, and discuss caveats and future directions in this research field.

2. Diagnosis of fragile X syndrome

In general, FXS testing should be considered in: 1. individuals of either sex with mental retardation and developmental delay, especially if they have other physical or behavioral signs of FXS, or a family history of FXS, or relatives with undiagnosed mental retardation; 2. individuals seeking reproductive counseling or women who are experiencing fertility problems, especially if they have a family history of premature ovarian failure, a family history of FXS, or male or female relatives with undiagnosed mental retardation; 3. men and women who are experiencing late onset intention tremor and cerebellar ataxia of unknown origin, in particular if they have a family history of movement disorders, a family history of FXS, or male or female relatives with undiagnosed mental retardation [24]. In all these cases, a family history of intellectual disabilities (ID), ASD and neurological problems such as dementia, ataxia and tremor, assists in the diagnosis of FXS.

2.1. Genetic diagnosis

Prior to the identification of the *FMR1* gene, the diagnosis of FXS was based on the cytogenetic evaluation of the presence of the fragile site at Xq27.3 (FRAXA) in peripheral blood lymphocytes. To improve the detection rate of FRAXA, cytogenetic methods have been replaced by Southern blot analyses and, more recently, by Polymerase Chain Reaction (PCR)-based techniques, which are more sensitive and specific than the karyotype observation and allow the identification of PM carriers [25].

Southern blot analysis detects all *FMR1* alleles including normal, larger-sized PM and FM; furthermore, it allows to determine the methylation status of the *FMR1* promoter region [26]. This technique is time consuming, relatively expensive and difficult to interpret. Standard PCR is based on the direct amplification of the CGG-repeat, it is fast and highly sensitive to detect *FMR1* repeats in the normal and PM range. It can only reveal alleles with up to ~300 repeats in males and up to ~160 repeats in females but it fails to identify larger CGG expansions [27]. Thus, in the past ten years, the combination of traditional PCR plus Southern blot analysis has been considered the best procedure for the molecular diagnosis of the *FMR1* mutation.

The limitations of the PCR plus Southern blot technique lead to the development of a new procedure able to detect all *FMR1* alleles, called Triplet primed PCR (TP-PCR). TP-PCR is the evolution of previous PCR protocols and allows to simultaneously amplify both the full-length *FMR1* alleles and CGG triplets in the same PCR reaction [28].

To detect the normal or mutant *FMR1* gene in the fetus, early prenatal diagnosis tests can be done in pregnant women trough chorionic villus sampling as a predictive test at 10–12 weeks of gestation [24]. However, the methylation status of the *FMR1* gene is often not yet established at this pregnancy stage. For this reason, DNA testing performed on cultured amniocytes obtained by amniocentesis after 15 weeks of gestation is considered the follow-up test to resolve possible ambiguities.

2.2. Physical and behavioral diagnosis

FXS patients usually exhibit long narrow face, prominent ears and forehead, irregular teeth, flat feet, hypotonia and joint hypermobility, and macroorchidism in about 95% of postpuberal males [29].

At the behavioral level, FXS is characterized by mental retardation and by a progressive decline of cognitive abilities [30]. Other common behavioral features displayed by FXS patients involve poor eye contact or gaze aversion, excessive shyness, anxiety, stereotypic movements, unusual speech, self-injury, hand flapping and hand biting, hyperactivity, tactile defensiveness, attention deficits, problems in impulse control, hyperarousal and oversensitivity to tactile, auditory, olfactory or visual stimuli [31–36].

Physical, behavioral and cognitive manifestations of FXS are dissimilar in males and females. The majority of female FXS carriers exhibit normal physical appearance, moderate ID or normal IQ and the severity of cognitive impairment depends on the X activation ratio (the percentage of cells in which the normal X chromosome is the active X chromosome) [37]. Compared to males, girls with FXS display a higher rate of emotional disturbance including mood lability, social withdrawal, inappropriate affect, poor modulation of verbal tone and depression [38,39].

During early infancy, FXS is typically identified through delayed or abnormal development of the infant. Parents report lack of motor coordination, hyperactivity or irritability and delayed developmental milestones as crawling, first speech and walking [40–42]. Male children affected by FXS exhibit severe deficits in motor skills and a significant cognitive delay [43,44]. They often manifest hyperarousal resulting in quick loss of capacity for self-regulation, aggressive outbursts or self-abusive behaviors, gaze avoidance, stereotypies, unusual speech and tactile defensiveness [31,32,34,43–45]. In addition, boys affected by FXS can display hypersensitivity to auditory, tactile, visual and olfactory stimuli [46]. In the social domain, FXS children appear excessively shy and anxious and avoid unfamiliar people [32,47,48]. During the school age, social maladaptive behaviors became more problematic and FXS children demonstrate social avoidance and autistic-like behaviors [35]. The prevalence of attention deficit/hyperactivity disorder

(ADHD) symptoms in children affected by FXS is high [49,50]. During the transition from the preschool to the school age, the cognitive and speech domains are particularly affected, together with executive functions including goal directed and future oriented behaviors [30,51]. The communicative deficits displayed by FXS males may become more severe in adolescence, when language skills and executive functions associated with working memory and planning activities become compromised [33,35]. Social anxiety, shyness and avoidant behavior are risk factors for depression: indeed, depression is often displayed at adulthood by both male and female patients [39,52].

The behavioral patterns emerging during the early life of individuals affected by FXS led to associate FXS with ASD, suggesting to consider the FM of *FMR1* gene as the most common monogenic cause of syndromic ASD [53].

3. Animal models of FXS

Animal models are essential to understand the impact of the *FMR1* mutation on the phenotype displayed by FXS patients, to shed light on the neurobiological aspects underlying this phenotype, and to assess potential therapeutic targets for FXS.

The first and most widely used animal model for FXS is the *Fmr1*-KO mice, obtained by the inactivation of the murine gene that causes the loss of FMRP production [54]. This animal model reproduces many of the behavioral symptoms displayed by FXS patients: cognitive deficits, such as impairments in spatial and reversal learning, social anxiety, reduced social interaction, repetitive behaviors and hyperactivity [55–73], but also recapitulates the synaptic alterations which characterize FXS [74–79]. Over the past decade, the *Fmr1*-KO mouse was the only animal model available to study FXS. More recently, *Fmr1*-KO rats have been generated by zinc-finger nuclease (ZFN) methodologies [80,81] and by CRISPR technology [82]. *Fmr1*-KO rats display a number of behavioral alterations which characterized FXS in humans, like altered patterns of social interaction [82] and social play behavior [81], defects in visual attention [83], and auditory dysfunctions [84]. Since rats have bigger brains than mice, are easier to train, can learn sophisticated behaviors and have an elaborated social repertoire, nowadays *Fmr1*-KO rats are considered a valuable tool to study neurobiological aspects of FXS and to test potential new treatments [81].

Drosophila melanogaster is widely used to study developmental and neurological diseases. The genetic plasticity, electrophysiological and behavioral characteristics of *Drosophila* make it a powerful model system to specifically investigate neurodevelopmental disorders. Compared with rodent models of FXS, the use of *Drosophila* has some advantage: low maintenance cost and rapid generation time, excellent predisposition to genetic analysis and the possibility to analyze different genetic elements in single animals. All these characteristics make this organism a particularly attractive model system to study the neurobiological alterations which characterize FXS [85]. *Drosophila* has a single conserved *FMR1* gene, called *dfmr1* gene, and the gene product, *Drosophila* Fragile X Mental Retardation Protein (dFMRP), is 35% identical and 60% similar to the human FMRP. The *Drosophila* FXS model system was established in 2000 based on loss-of-function mutants of *dfmr1* [86].

Zebrafish is a small tropical freshwater teleost used in biological research since 1981 [87]. It is a simple vertebrate species commonly used for developmental and genetic studies [88] for several reasons: the genomic and physiological homology with humans, the external fertilization and large numbers of fertilized eggs, the transparency of the zebrafish embryo, the small size and low maintenance efforts [89]. The *Fmr1*-KO zebrafish animal model was generated in 2009 [90] and it reproduces the behavioral and synaptic characteristics of the disease [89,91,92].

3.1. Measuring FXS-relevant behavioral phenotypes in the laboratory setting

At the behavioral level, the most common symptoms displayed by FXS patients are impaired cognition and altered social behaviors. These behavioral features can be modelled in laboratory animals, and deficits in these domains are observed in animal models of FXS.

3.1.1. Cognitive deficits

When modeling developmental psychiatric disorders in animal models, it is important to assess any potential behavioral deficit in the early postnatal period. Deficits in cognitive processing can be evaluated in rodents at a very early developmental age through the homing behavior test, that allows to assess the ability of rodent pups to discriminate a familiar from a neutral odor. This test exploits the strong tendency of immature rodent pups to maintain body contact with the dam and siblings and it is based on the learned association between maternal odors and maternal stimulation, which are crucial for the development of social behavior and social recognition [93,94]. The experimental procedure involves a brief isolation of the pup prior to testing; then, the pup is placed for few minutes in a cage with the floor covered for two parts by clean sawdust and for one part by sawdust from the pup's own nest (Fig. 1A). The latency to reach the familiar bedding and the total time spent by the pup in the familiar bedding are scored [94–96].

One of the most popular tests routinely used to assess cognitive performance in rodent models of FXS is the novel object recognition (NOR) test (Fig. 1B). The test is based on the observation that when rodents are exposed to two objects, one familiar and one novel, they approach and spend more time exploring the novel than the familiar object [97]. The test is therefore used to assess recognition memory, defined as the ability of rodents to recognize and discriminate an object previously explored (and therefore familiar) compared to a new (and therefore unfamiliar) object. The test usually consists of two phases: in the first phase, the animal is placed in an open arena and left free to explore two identical objects. Then, the animal is returned to its home cage for a retention period. In the second phase, the animal is returned to the experimental arena and presented with two objects: the previously experienced object and a novel object. Object recognition is measured by the difference in the exploration time of the novel and familiar objects [98].

The inhibitory avoidance test is commonly used to study emotional memory processing in rodents. During inhibitory avoidance training, rodents receive a single mildly aversive footshock after stepping from a lighted compartment into a darkened compartment (Fig. 1C). Memory retention is tested usually 24 or 48 h later by placing the animal in lighted compartment and measuring its latency to enter the dark compartment where the aversive footshock was originally delivered. Longer retention test latencies indicate better memory for the emotionally salient event (i.e., the footshock) [99].

The Morris water maze, described for the first time by Richard Morris in 1981 [100] relies on the ability of rodents to use visual external cues to locate a submerged escape platform in an open circular swimming arena (Fig. 1D). The most basic experimenal procedure allows to assess spatial learning, i.e., the ability of the animals, learned over several training sessions, to use external visual cues to navigate a direct path to the hidden platform when started from different, random locations around the perimeter of the pool. Besides this basic procedure, many variations of this test have been described [101].

The two most popular tests used to assess cognition in *Drosophila dfmr1* mutants are a classical avoidance conditioning paradigm and a conditioned courtship paradigm [102]. In the first test, also known as the odor-shock paradigm, memory is measured by the ability of the flies to recognize an odor associated with an adverse event (a mild electric footshock), from another associated with a neutral event. In this test, flies are placed inside a cylinder with a copper surface able to propagate the electric shock, and are presented to the first odor along with electric

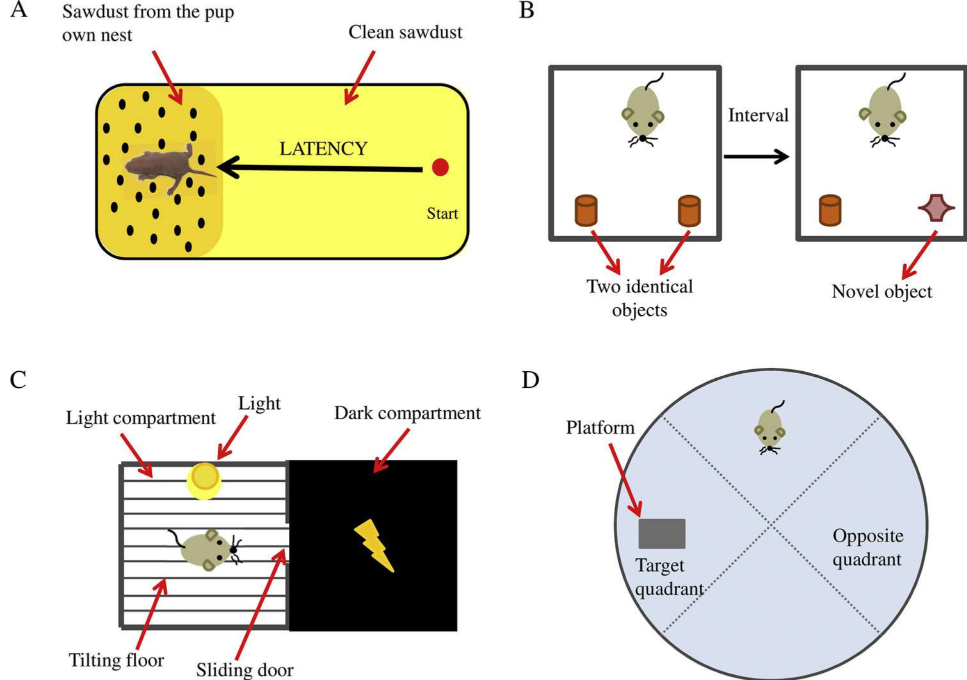

Fig. 1. Schematic representation of common behavioral tests performed to assess cognitive performance in rodents. (A): The homing behavior test (A). (B): the Novel Object Recognition test. (C): the inhibitory avoidance test. (D): the Morris water maze test. See the text for details about each behavioral test.

pulses. Then, they are presented to a control odor, without shock. After an interval (that usually ranges from 60 min, to assess short-term memory, to one day after training, to assess long-term memory) flies are tested in a T-maze in which the two odors are presented in each of two arms. The flies that avoid the arm with the odor previously associated with the electric footshock, preferring the arm with the other odor, show intact learning and memory abilities [103,104].

The conditioned courtship paradigm test [105] is based on the typical sexual behavior displayed by flies. During courtship, male and female flies exchange visual, olfactory, gustatory and auditory stimuli. Although fertilized Drosophila females stimulate males to court them [106], they are not receptive to further mating and produce anti-aphrodisiac pheromones. In response to this rejection, male flies reduce their courtship behavior. Thus, when the male courts a fertilized female, he learns to associate courtship-stimulating and courtship-inhibiting cues, so that when subsequently presented with a virgin female (that only produces courtship-stimulating cues), his courtship response is repressed in anticipation of an aversive cue. The test therefore consists of a training phase, when the male courts a fertilized female, and a retention phase, when the male is placed with a receptive, virgin female. Males with intact memory abilities reduce the levels of courtship behavior toward a virgin female for 2–3 h after training [102]

The T-maze test is often used to assess cognitive performance in zebrafish [107,108]. The experimental apparatus is composed of one long and two short arms. One of the short arms is connected to a deeper square chamber, which serve as a favorable environment for the fish. Zebrafish are first individually trained to explore and travel to the deeper and more favorable end of the T-maze. Incorrect choices result in getting small congested environment confinement. The latency to reach the deeper chamber is then recorded at different time points after training [109].

The inhibitory avoidance task can also be applied to zebrafish. The test is similar to its rodent version: the experimental subject is placed into a chamber which is divided into two equal-size compartments (the shallow compartment and the deep compartment) by a guillotine door. The deep compartment contains two metal plates to serve as electrodes for delivering a mild electric shock as an aversive stimulus. During the habituation session, the experimental subject is placed in the shallow chamber for 5 min and let free to swim across the compartments. During the training session, the fish is placed in the shallow compartment and, when the guillotine door is opened, it enters the deep compartment, where it receives a mild electric shock. During the test session on the next day, the fish is placed in the shallow chamber with the guillotine door opened and the latency to enter the deep compartment is recorded as an index of memory retention [110]. Other tasks used to assess cognitive performance in zebrafish are the spatial alternation test, that allows to assess the ability of zebrafish to learn a simple spatial alternation paradigm in a short period of time [111], and the spatial and visual discrimination task [112].

3.1.2. Deficits in social communication

Communication deficits are characteristic of both males and females with FXS, with males being more severely affected than females [33]. Importantly, most patients have difficulties in using language effectively since childhood [113,114].

Rodents are very useful animal models to study possible alteration in social communication, since they emit ultrasonic vocalizations (USVs) in different social context across the entire lifespan. For example, rodent pups emit USVs to induce maternal retrieval, while at older ages they vocalize to communicate pleasure during mating or playful interactions, to communicate distress due to a predator attack, or to communicate food location to other group members [115–118]. 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 emitted in social contexts involving potential reward (e.g., sexual approach, play fighting) [119].

Since communicative deficits in FXS appear already in infancy, an important research tool in rodent models of FXS is the analysis of the USVs emitted by the pups when separated from the mother and the nest. Such USVs, emitted at frequencies between 30 and 90 kHz, play a fundamental communicative role in mother-offspring interaction and are crucial for pups survival, since they elicit retrieval and caregiving behavior in the dam [96,118,120–122].

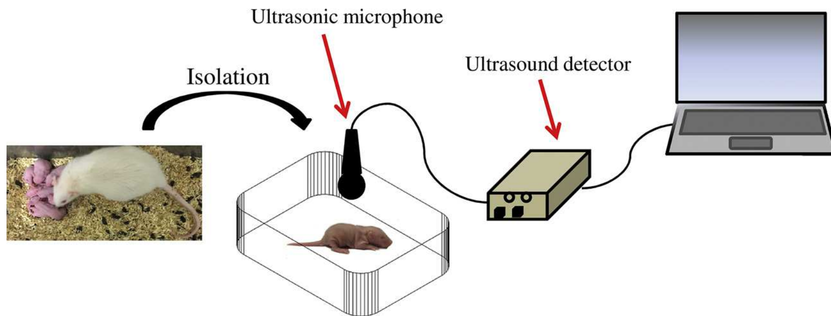

Fig. 2. Schematic representation of the isolation-induced USVs test in rodents. See the text for more details about the test.

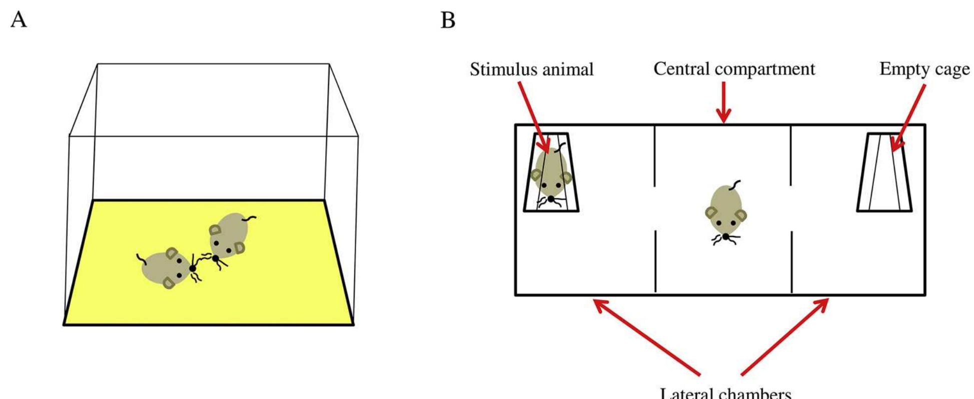

Fig. 3. Schematic representation of two behavioral tests commonly performed to assess deficits in social behavior in rodents. (A): The social play behavior test. (B): The 3-chamber test. See the text for details about each behavioral test.

The test starts with the isolation of the pup from its mother and littermates for few minutes. The USVs emitted by the pup are detected by an ultrasonic microphone connected to an ultrasound detector and specific software are available to analyze qualitatively and quantitatively the USVs emitted (Fig. 2). Since low temperatures also elicit USV emission, the testing room is maintained around 25 °C and the body temperature of each pup is controlled before and after the test.

Rodent USVs can also be detected in other social contexts, such as during social play with peers or during sexual behavior [115–118].

As in a variety of mammals, odors allow rodents to recognize and communicate with individuals of the same species and olfaction, along with other senses, is considered an important modality of intraspecific communication as well as predator detection [123]. Rodents use urinary scent marks to communicate in many social contexts and display high interest in urinary scents from other conspecifics. Olfactory communication can be measured by analyzing the number of scent marks and countermarkings in close proximity to urinary olfactory cues [124]. Another test used to assess olfactory communication abilities in rodents is the olfactory habituation/dishabituation test. This test consists of sequential presentations of different odors. After repeated presentations of a cotton swab with a certain odor, rodents spend less time sniffing the swab (habituation phase). Subsequent introduction of a cotton swab saturated with a new odor reinstates a high level of sniffing (dishabituation). The sniffing time is recorded and the shapes of the habituation and dishabituation curves indicate the ability of the animals to discriminate familiar and unfamiliar non-social and social odors, while the height of the peaks of the curves provides a measure of interest in the social and nonsocial odors [125,126].

Communicative abilities in a social context can also be assessed in *Drosophila. Drosophila* males show an innate ability to interact with females through courtship rituals, performing a characteristic sequence of behaviors to mate. The courtship starts when the male, after a period of orienting, follows and taps the female to pick up pheromonal cues. Then, the male starts to emit acoustic signals by extending and vibrating a wing. Later, it licks the female abdomen and, if the female displays receptive behavior, copulation can start [102]. The emission of acoustic signals during courtship behavior plays a fundamental communicative role between males and females [127,128] and it has been extensively studied in many *Drosophila* species [128–132].

3.1.3. *Deficits in social behavior and sociability*

While interacting with peers, individuals with FXS often exhibit social deficits, social avoidance and gaze aversion, and the lack of these socialized patterns leads, in turn, to further social withdrawal [32,36,47,133,134]. Thus, it is important to assess any potential deficit displayed by animal models of FXS in the social domain, particularly at a young age.

Boys with FXS show impairments in social play behavior [133,135]. Social play behavior is the first form of non-mother directed social behavior displayed by most developing mammals. The opportunity to engage in social play is crucial both for children and young mammals, since it helps to develop communicative skills, social competence, and behavioral and mental flexibility [136]. Social play behavior can be easily assessed in laboratory animals (Fig. 3A). In particular, the rat is an ideal species to study this behavior, since social play is the most commonly occurring form of social behavior displayed by rats between weaning and sexual maturation [136]. In rats, social play can be easily quantified by measuring frequency and duration of specific behaviors [137]. Social behaviors primarily related to play, such as pouncing (one rat soliciting another animal to play, by attempting to nose or rub the nape of its neck), pinning (the animal that is pounced upon fully rotates to its dorsal surface with the other animal standing over it) and chasing (the animal that is pounced upon responds by evading and the soliciting rat may start to chase it) occur frequently when young rats are interacting with each other and these behavioral patterns can be easily recognized [137]. As the rats get older, the structure of social play changes: rather than pinning, the most frequent response to pouncing become evasion and partial rotation [137]. Since social play is a

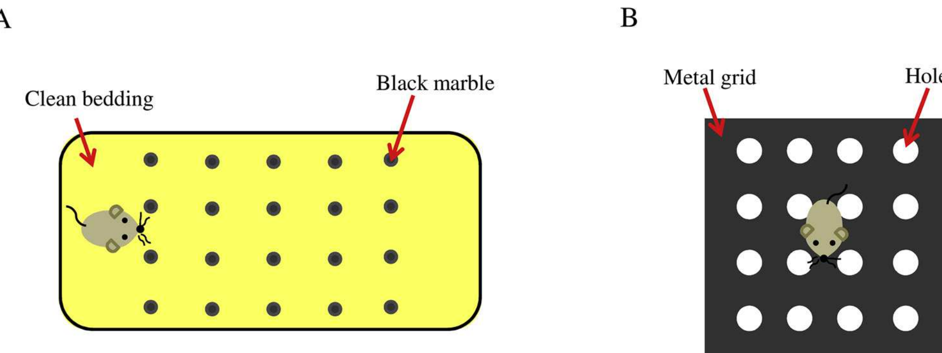

Fig. 4. Schematic representation of two behavioral tests performed to assess repetitive and stereotyped behaviors in rodents. (A): The marble burying test. (B): The hole board test. See the text for details about each behavioral test.

rewarding social activity, it can be associated with the emission of high frequency USVs. Indeed, some researchers reported that rats emit more 50-kHz USVs while playing together than while alone [138], although other studies failed to correlate the performance of social play behavior in adolescent rats with the emission of 50-kHz USVs [139–141].

Since mice usually engage in very rudimentary forms of social play [142], other behavioral tests are usually used to assess social behavior in mouse models of FXS. A common behavioral paradigm used test to assess social approach in both mice and rats is the 3-chamber test (Fig. 3B) [143]. The apparatus usually consists of a three-chamber box with openings between the lateral chambers and the central compartment. The two lateral chambers are identical, except for the fact that, during the testing session, one chamber contains an unfamiliar stimulus animal inside a cage, while the other chamber contains an identical but empty cage. During the habituation phase, the experimental subject is left free to explore the empty apparatus and to enter in the two lateral identical empty chambers. During the testing session, the experimental subject is placed in the central compartment and can choose to spend time either in the chamber that contains the cage with the unfamiliar stimulus animal or in the chamber that contains the empty cage. During the test session, "sociability" is defined as the propensity of the experimental subject to spend time in the chamber containing the stimulus animal, as compared to time spent alone in the identical but empty opposite chamber. Another session may follow to assess the so-called "preference for social novelty". During this second session, the stimulus animal (stranger-1) used in the test session is confined again in the cage located in the same compartment as in the test session, while a new unfamiliar stimulus animal (stranger-2) is placed inside the cage in the opposite chamber, which was empty in the previous session [144]. Usually, rodents prefer to spend time with a peer rather than alone, and with an unfamiliar rather than a familiar animal. Compared to control animals, rodents showing social dysfunctions tend to enter less and spend less time in the chamber with the stimulus (during the first test session) or in the chamber with the stranger-2 (during the "preference for social novelty" session) [125,145–148].

The social performance of *Drosophila* is often assessed calculating the courtship index, which corresponds to the percentage of time a male spends in courtship activity during a 10-minute test session.

Another simple approach used to study social behavior in *Drosophila* is the measurement of the distance between two flies within a social group (the so-called social space). To measure this parameter, flies are introduced in a vertical triangle test chamber and the distance between individual flies and their closest neighbor (i.e., the social space) is measured as an index of a fundamental form of social interaction [149].

Zebrafish are highly social animals and exhibit robust social behaviors, such as shoaling and social preference [150,151]. Shoaling is an aggregation behavior that leads to conspecifics being distributed in a given area of space, i.e. they form groups. The shoaling test is used to assess the overall social competence of a group of zebrafish and different parameters of the social interaction are analyzed, such as the distance between the body center of every member of the shoal, the distance between the body center of each fish and the farthest neighboring fish and the percent of fishes in the upper half of the tank [151–153].

Similarly to rodents, zebrafish can be tested in the social preference test, in which zebrafish sociability can be assessed by the analysis of its preference for conspecifics. The test starts with the experimental subject in the central arena of a three chamber apparatus and a conspecific confined in one of the two lateral boxes. Then, the experimental subject can choose to spend time in the compartment of the apparatus containing the conspecific fish, or in the empty part of the apparatus. The time spent in the conspecific arm, in the empty arm, in the center of apparatus, and the number of entries in each arm are considered an index of sociability [151,153]. One commonly used modification of this test combines the shoaling and social preference tests to assess preference of an individual fish for shoals of zebrafish or for another species [154].

3.1.4. Repetitive and stereotyped behaviors

Perseverative and stereotyped behaviors are predominant symptoms of FXS: 85–100% of males with the FM show repetitive behaviors, rigidity, hand-flapping and hand-biting [155]. As in humans, rodents also show particular behaviors that can be cataloged as stereotyped behaviors, such as high levels of vertical jumping, circling, digging, rearing and excessive self-grooming. The marble burying test is commonly used to detect repetitive digging behavior in rodents. In this test (Fig. 4A), the animal is placed in a cage containing clean bedding and some marbles equidistant from each other. The test consists of a 10–20-min exploration period, after which the animal is removed from the cage and the number of marbles buried with bedding up to 2/3 of their depth is counted.

Stereotypies and perseverative behaviors in rodents can also be detected through the hole board test. The apparatus is a square metal table with several evenly spaced holes, inserted in a Plexiglas arena (Fig. 4B). Rats or mice are individually placed in the apparatus and their behavior is observed for few minutes. Dipping behavior is scored by the number of times an animal inserts its head into a hole at least up to the eye level. High frequencies of dipping behavior suggest a pronounced stereotyped phenotype.

Stereotyped behaviors can be observed in *Drosophila* analyzing grooming activity, a behavior commonly performed by flies that lasts few seconds [156], while they can be assessed in zebrafish analyzing the presence of meaningless repetitive behavioral patterns (e.g., continuous swimming back and forth along one side of the tank) maintained for longer than 1 min [157].

3.1.5. Anxiety-like behaviors

In addition to cognitive deficits and social withdrawal, anxiety is a

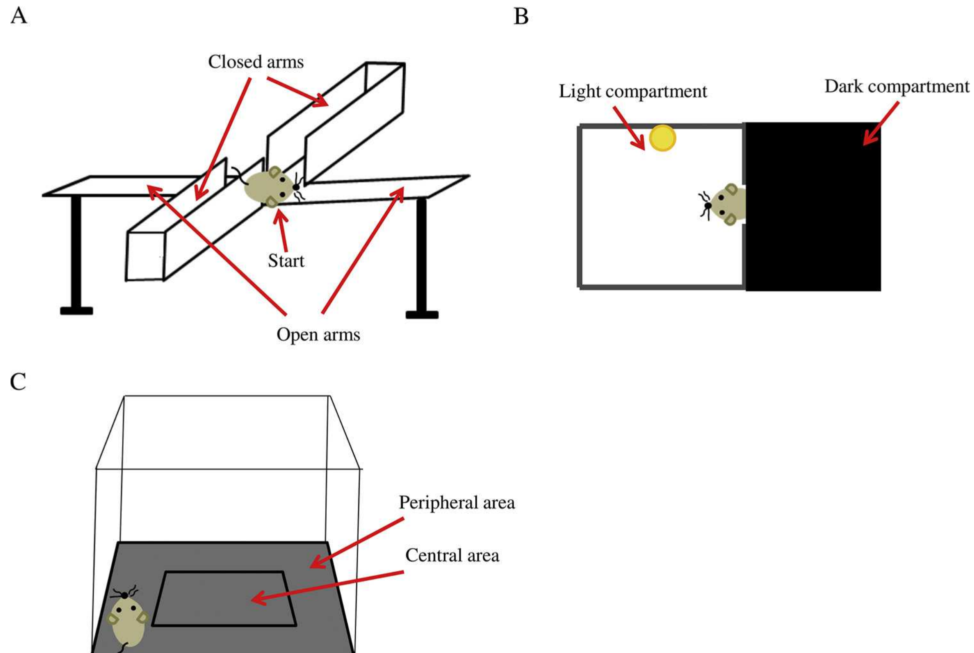

Fig. 5. Schematic representation of common behavioral tests performed to assess anxiety-like behaviors in rodents. (A): The Elevated Plus-Maze test. (B): The Light-dark box test. (C): The open field test. See the text for details about each behavioral test.

frequent symptom displayed by both young and adult FXS patients [36,158].

Several behavioral tasks are available to assess anxiety-like behaviors in rodents. Considering the sensitive nature of these tasks, it is important to use similar testing protocols across laboratories, trying to exclude factors, such as housing conditions or stress levels, that can alter rodent performance during testing [159]. The Elevated Plus-Maze (EPM) test is the most widely used test to detect anxiety-like behaviors in rodents. The test is based on the innate conflict of rodents between the natural fear for open spaces and the innate interest for the exploration of new environments. The apparatus is an elevated cross-shaped platform, with two open and two closed arms (Fig. 5A). The animal is placed in the apparatus for a 5-min session, and the time spent in the open and closed arms and the number of open and closed arm entries are scored. An unusually high time spent in the closed arms indicates a more pronounced anxious phenotype [160]. The choice between sheltered and exposed regions is also at the bases of the light-dark box test (Fig. 5B), in which animals are allowed to freely move between a small dark (and therefore perceived as safe) compartment and a large illuminated (and therefore perceived as aversive) compartment of a two-chambered apparatus [161].

The open field test (Fig. 5C), in which the behavior of the animal in a large empty arena is scored for a variable period of time, is often used to assess, at the same time, the occurrence of anxiety-like and stereotyped behaviors.

Attempts have been made to use *Drosophila* to study the genetic and molecular basis of emotional behavior [162]. When flies are in enclosed chambers, they spend a large proportion of time near the walls, avoiding the center of the arena. This behavior, called wall-following, resembles the avoidance of open spaces displayed by rodents [163]. Interestingly, GABAergic and serotonergic neurotransmission affect wall-following behavior in *Drosophila* in a way strikingly concordant with their modulation of rodent anxiety. Therefore, it has been suggested that open space avoidance in Drosophila represents a primordial form of anxiety-related behaviors [163].

The conflict between the innate aversion of animals for brightly illuminated areas and their spontaneous exploratory behavior in novel environments is also used to model anxiety-like behaviors in zebrafish. Indeed, the light-dark test and the novel tank task are two commonly used tests to evaluate an anxious-like phenotype in zebrafish. In the light-dark test, the experimental subject is allowed to swim freely between the white and a black compartments of a rectangular tank, and a reduced exploration of the white compartment reflects high anxiety-like states [91,151]. Similarly, the novel tank task is based on the typical response of zebrafish when exposed to a new tank: they dive to the bottom and do not initially explore the environment until they feel safe enough [164]. In this test, the experimental subject is allowed to swim freely in a new tank and the percentage of time spent in the upper zone, the latency to enter the upper half, the time spent in the top, erratic movements and freezing are indicative of the anxiety-like behavior of the fish [91,164].

4. Behavioral and synaptic features displayed by *Fmr1*-KO animal models

4.1. Fmr1-KO mice: behavioral features

At the cognitive level, *Fmr1*-KO mice show deficits in different behavioral tasks. As for inhibitory avoidance learning, some studies showed that the mean latency to enter the dark compartment is shorter in *Fmr1*-KO mice compared to WT animals twenty-four hours after the training session [55–57], while no differences between *Fmr1*-KO and WT mice were found in others studies [54,79,165,166].

Several studies have shown that *Fmr1*-KO mice are not able to discriminate between a familiar and a novel object in the NOR test [58–61], although Yan and colleagues did not find differences between the two genotypes in this test [167]. Furthermore, it has been reported that Fmr1 KO mice are not able to differentiate between a familiar and a novel mouse in the social discrimination task [168]. Other studies demonstrated that *Fmr1*-KO mice display deficit in the Morris water maze during the acquisition and reversal (i.e. when the location of the platform is changed) phases of the task [62,63], while other studies did not find differences between *Fmr1*-KO and WT mice [167,169].

As for the social domain, several studies revealed that *Fmr1*-KO mice show social avoidance: they show no preference for the stimulus animal compared to WT controls in the 3-chamber test [64,65] and they spend

less time engaging in affiliative behaviors toward a female mouse in the social interaction test [66]. Other studies, however, did not show social deficits in *Fmr1*-KO mice in both the 3-chamber [65,66,72,144] and the social interaction test, neither when the social partner was a juvenile male mice [66] nor a female mouse [170].

Fmr1-KO mice show communicative alterations throughout lifespan: they show different patterns of USV emission compared to WT animals when separated from the mother and the nest at infancy [171,172] and during courtship and mating at adulthood [67,170]. However, Pietropaolo and colleagues did not find differences in the emission of USVs between genotypes, in terms of both frequency and duration, during a direct social interaction with an adult female [66].

Regarding the occurrence of stereotyped behaviors, *Fmr1*-KO mice show higher levels of self-grooming [65,66] and they bury a higher number of marbles in the marble burying test [67,166,173] compared to WT animals. Many studies in which locomotor activity is analyzed through the open field test reveal that *Fmr1*-KO mice show hyperactivity compared to WT animals [55,64,66–73,168,174] and this altered phenotype is in line with the hyperactive behavior displayed by FXS patients [155,175,176].

Fmr1-KO mice do not show an anxious phenotype in the elevated plus-maze test. Rather, they spend more time in the open arms of the maze compared to WT animals [71,72,177–179], although this may likely be due to their hyperactivity. In line with this possibility, Ding and colleagues did not find differences between *Fmr1*-KO and WT mice in the time spent in the light compartment in the light-dark box test [55].

The contrasting behavioral data obtained with *Fmr1*-KO mice can be explained by the influence of genetic and environmental factors, which need to be carefully considered in behavioral experiments. Genetic background, general heath, prenatal environment, stress factors, diet and age at testing can indeed influence the mouse performance in the behavioral tests outlined above. Given the sensitive nature of behavioral experiments, similar experimental protocols should be used across labs to determine the predominant phenotype of *Fmr1*-KO mice, trying to avoid any confounding variable.

The age and the sex of the tested animals and the environmental conditions (like housing, the temperature and the light of the testing room) should be taken into consideration, together with a number of practical factors affecting the successful outcome of the behavioral tests outlined above. For example, when manipulating rodent pups to measure the isolation-induced USVs, it is important to maintain the testing room at around 25 °C and to control the body temperature of each pup before and after the test.

4.2. Fmr1-KO rats: behavioral features

Despite *Fmr1*-KO rats have been generated only recently [81], some behavioral tests have already been performed to define their behavioral phenotype. Till and colleagues found that *Fmr1*-KO rats show deficits in the object-place-context task, which involves the associative recognition of objects, their spatial locations and the local context. They did not find differences in the spatial learning abilities of *Fmr1*-KO rats in the Morris water maze [180], while a recent study has showed that *Fmr1*-KO rats exhibit altered learning abilities in the same test [82]. Regarding the social domain, *Fmr1*-KO rats show social deficits in the 3-chamber test and lower frequency of social play behavior compared to WT animals [81].

No differences between *Fmr1*-KO rats and WT controls have been found in the number of USVs emitted during the social interaction test and in the locomotor activity in an open field arena [81]. Last, compared to WT animals, *Fmr1*-KO rats show neither anxiety-like behaviors in the elevated plus-maze test level nor stereotyped behaviors [82].

4.3. dfmr1 mutant Drosophila melanogaster: behavioral features

At the behavioral level, *dfmr1* mutant flies show cognitive alterations, social deficits, stereotyped behaviors, altered circadian rhythm, sleep disorders and locomotor defects (reviewed in [181]). Learning and memory deficits have been detected in both the odor-shock paradigm and in the conditioned courtship test [182,183].

dfmr1 mutant males also display deficits in the social domain: compared to control males, the courtship index of *dfmr1* mutant males paired with virgin females is significantly reduced. *Dfmr1* mutant males fail to sustain courtship, resulting in a lower percent of flies that proceed to the last steps of copulation [182–185]. *Dfmr1* mutants also show excessive grooming behavior, a feature that increases with age: during a 5 min observation period, 5-day-old *dfmr1* flies groomed, on average, for 19% of the time while control flies of the same age groomed for approximately 7% of the time. This excessive grooming was more pronounced at 35 days, when *dfmr1* mutant flies groomed on average 79% of the total time, whereas WT flies showed no changes in grooming activity over time [156].

4.4. Fmr1-KO zebrafish: behavioral features

Fmr1-KO zebrafish show cognitive and social deficits, hyperactivity and a variable anxious-like phenotype. *Fmr1*-KO fish exhibit learning and memory impairments in the inhibitory avoidance task, showing the same latency to enter the dark compartment of the apparatus both during training and during test days [89]. *Fmr1*-KO zebrafish spend more time in the white compartment of a light-dark apparatus compared to wild-type fish, although this finding may be secondary to the hyperlocomotion displayed by *Fmr1*-KO zebrafish in both the light-dark box and in an open tank [89]. To support this possibility, a recent study conducted by Wu and colleagues demonstrates that the level of shoaling behavior is elevated in *Fmr1*-KO zebrafish compared to WT fish and that this change may result from the hyperactivity and increased anxiety-like behavior found by the authors in the novel tank task [91]. In another study performed by Kim and colleagues, *Fmr1*-KO zebrafish responded atypically to a novel environment. Indeed, when placed in an open field with two white and two transparent walls, the *Fmr1*-KO zebrafish did not show the hypo-activity usually displayed by intact fish in the first minutes spent in an unfamiliar environment (neophobic response). Whether this was due to reduced open-space aversion or altered spatial information processing was not clear [92].

4.5. Synaptic correlates

Considering the importance of FMRP for the regulation of synaptic functions, it is predictable that the lack of this protein results in abnormalities in the functionalities of synapses, producing alterations in normal synaptic activity which contributes to the behavioral phenotype that characterizes FXS. Despite the identification of numerous FMRP target mRNAs in last few years, [18,186–189], how the changes in the expression of their protein products contribute to different features of FXS pathology remains to be elucidated. Considering that the most common altered phenotype observed in animal models of FXS and in FXS patients is the loss of cognitive abilities, the increased expression of a subset of synaptic proteins and subsequent alteration in synaptic plasticity is considered a distinctive feature of FXS [190].

Synaptic plasticity (a molecular mechanism underlying memory and cognition) is the ability of neurons to persistently strengthen (long-term potentiation, LTP) or weaken (long-term depression, LTD) synaptic efficacy [191]. It has been shown that both LTD, which is dependent on protein synthesis and mGluR activation [192,193], and LTP, which requires an early phase with neither transcription nor protein synthesis, an intermediate mGluR-dependent phase with only protein synthesis, and a late phase requiring transcription and protein synthesis [194,195], are altered in *Fmr1*-KO mice. In particular, LTD is enhanced

in hippocampus and hippocampal neuron cultures [190,196,197] while LTP is impaired in cortex, anterior cingulate cortex and amygdala [198,199]. Consistent with the increased density of dendritic spines, most of which appear elongated and immature, found through post-mortem analysis of human brain tissue of individuals with FXS [200–202], *Fmr1*-KO mice show deficits in spine number and morphology. Dendritic spines are small protrusions along neuronal dendrites that are essential for synaptic neurotransmission since they contain receptors and signaling molecules. In *Fmr1*-KO mice they appear to be thin, elongated, immature and of increased density compared to WT animals [74–79]. The abnormal synaptic plasticity and alterations in the morphology of dendritic spines widely demonstrated in *Fmr1*-KO mice are also found in the other animal models of FXS. *Fmr1*-KO rats show abnormal synaptic plasticity and alterations in the morphology of dendritic spines of hippocampal pyramidal neurons [180]. *Drosophila dfmr1* mutants also show a mGluR dysregulation [183] which reflects the enhanced mGluR activity found in the brains of *Fmr1*-KO mice. It has been demonstrated the treatment with mGluR antagonists or lithium can restore the cognitive deficits displayed by *dfrm1* mutant flies [183]. *dfrm1* mutant flies show dendritic alterations, in particular an increased number of terminal dendritic processes compared to WT flies [203].

Fmr1-KO zebrafish show markedly reduced LTP and enhanced LTD compared with control fish [89]. It has been also demonstrated that the loss of FMRP in zebrafish is related to abnormal axonal branching, neuronal guidance, and defasciculation defects [204].

5. Pharmacotherapy of FXS

To date, no specific drug is approved for the treatment of FXS although many off-label medications are used to mitigate some of its symptoms (Fig. 6).

The ADHD symptoms frequently displayed by FXS boys can be reduced by methylphenidate and dextroamphetamine [205,206]; α-adrenergic receptor agonists, such as clonidine and guanfacine [7], or L-acetyl-carnitine [207] can be effective in children who do not respond properly to stimulants. Selective serotonin reuptake inhibitors (SSRIs) alleviate selective mutism and aggressive behavior [7]. It has been reported that intranasal administration of oxytocin may ameliorate some symptoms of social anxiety in patients with FXS [208]. Sleep difficulties are significantly improved in children treated with melatonin [209]. To resolve aggression or mood instability, new-generation antipsychotics are often prescribed [7,206]. Lithium is used off-label to treat aggression and mood instability [209].

Nowadays, the efforts of the research community are devoted to explore different treatment strategies, such as: 1. by restoring *FMR1* expression acting on the epigenetic mechanisms involved in its transcriptional inactivation; 2. by compensating for the lack of FMRP acting on the pathways in which FMRP is involved; 3. by restoring the excitatory/inhibitory neurotransmission balance [7,209,210]. In this context, the definition of disease-specific endpoints, adequate sample size and subgroup heterogeneity are of critical importance when evaluating the results of the clinical studies.

The first approach is based on the possibility to restore the activity of the *FMR1* gene using epigenetic modulators able to revert the epigenetic changes responsible of its silencing, such as DNA methylation. For instance, 5-aza-deoxycytidine (5-azadC), a methyltransferase inhibitor, restores transcription and translation of the *FMR1* gene in lymphoblastoid cell lines from FXS patients [211]. The reactivation makes the inactive methylated allele similar to the active unmethylated allele. However, there are several limitations to the possible clinical use of 5-azadC: 1. it may affect other methylated genes; 2. it can induce apoptosis; 3. it is apparently effective only on dividing cells [211]. Other epigenetic approaches have therefore been explored. Studies in vitro on lymphoblastoid cells from FXS patients showed that valproic acid (VPA), an anticonvulsant and mood stabilizer drug that inhibits

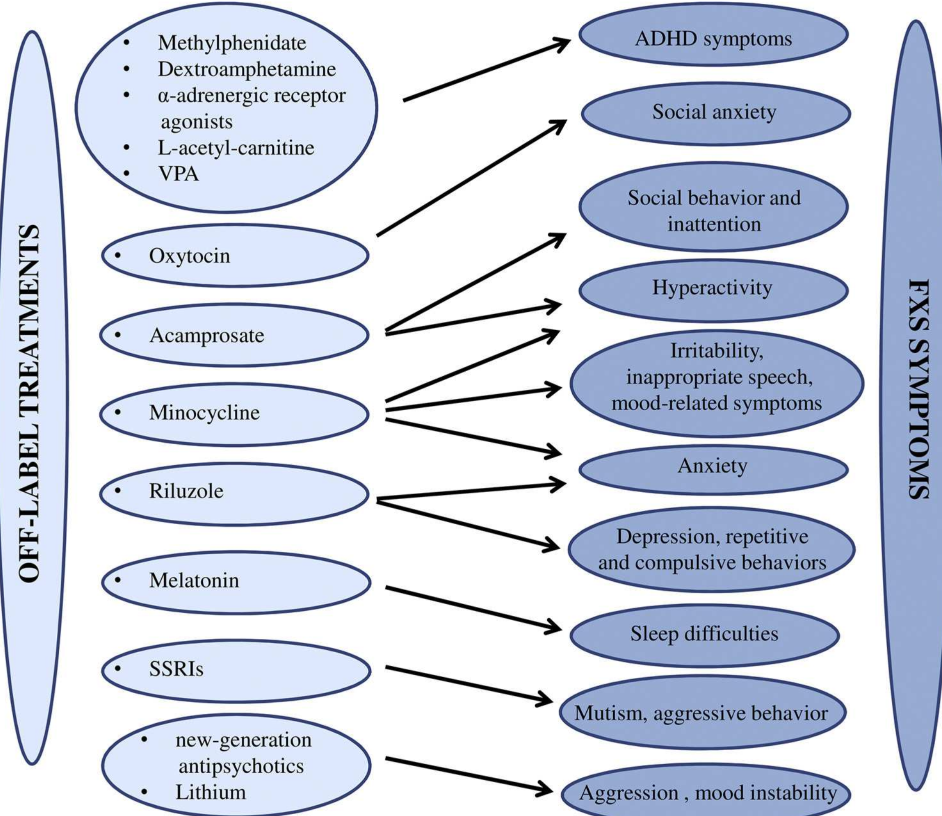

Fig. 6. Off-label medications used for FXS and the symptoms they mitigate.

histone deacetylases, induced a modest reactivation of *FMR1* transcription [212]. On this basis, a preliminary safety clinical trial tested the efficacy of VPA on ADHD symptoms in FXS patients. After 6 months of treatment, a decrease in the hyperactivity displayed by the patients was observed [213].

L-Acetyl-carnitine inhibits cytogenetic expression of the fragile X site by modifying histone acetylation [214], and two clinical trials tested its ability to ameliorate ADHD symptoms in FXS children. The results of these studies showed that L-Acetyl-carnitine improved hyperactivity and adaptive behavior, although the effects were more remarkable in the parent reports than in the teacher reports [207,215].

A second therapeutic strategy that has been explored is the pharmacological modulation of FMRP downstream targets. Indeed, since FMRP regulates the synthesis of several proteins, multiple targets for therapeutic intervention have been identified, some of which have already been tested in the clinical setting. For example, the amount of matrix metalloproteinase 9 (MMP9), a protein involved in synaptic physiology and plasticity, is elevated in the hippocampus of *Fmr1* knock-out (KO) mice [216]. Minocycline, a broad-spectrum antibiotic that inhibits MMP9, reduces anxiety in Fmr1 KO mice and improves their immature dendritic spine phenotype [217]. In line with these findings, FXS patients treated with minocycline showed reduced irritability, hyperactivity and inappropriate speech [218], and less anxiety and mood-related symptoms [219].

Loss of FMRP is associated with high levels of excitatory glutamatergic neurotransmission, together with deficits in gamma-aminobutyric acid (GABA)-mediated inhibitory neurotransmission [220]. According to the metabotropic glutamate receptor (mGluR) theory of FXS, excessive signaling through mGluRs is responsible of the behavioral, electrophysiological, and molecular dysfunctions associated with FXS [220]. On the basis of this hypothesis, preclinical studies from several laboratories led to the identification of metabotropic glutamate receptor 5 (mGluR5) as a potential pharmacological target for FXS. A number of mGluR5 antagonists have been tested [221,222]. However, clinical trials were discontinued because the mGluR5 antagonists tested were either not effective or they were showing side effects [221–223].

Another pharmacological approach that has been investigated is based on the correction of the GABA/glutamate imbalance observed in FXS by modifying ionotropic glutamate receptor activity. Studies in vitro showed that memantine, an uncompetitive antagonist of *N*-methyl-d-aspartic acid receptors (NMDARs), has stimulatory effects on dendritic spine maturation and excitatory synapse formation in *Fmr1* KO-cultured cerebellar granule cells [224]. The efficacy and tolerability of memantine have been tested in a pilot study involving 6 patients with FXS [225]. The results of this study showed that memantine was well tolerated and modestly effective in 4 out of 6 patients [225].

GABAergic neurotransmission is downregulated in FXS, and it has been suggested that impaired GABAergic activity in different brain regions, such as the amygdala, striatum and cerebral cortex, contributes to the behavioral abnormalities that characterize FXS [226,227]. Riluzole, that inhibits glutamate release and potentiates post-synaptic $GABA_{-A}$ receptor activity, has been suggested to be useful to mitigate depression and anxiety [228]. A pilot study tested the ability of riluzole to control the repetitive and compulsive behaviors displayed by 6 FXS adult patients. While riluzole was well tolerated, it did not induce significant clinical improvement [229].

Acamprosate, a drug used in alcohol dependence that interferes with both $GABA_{-A}$- and mGluR-mediated neurotransmission, improved the social behavior, inattention and hyperactivity displayed by 9 out of 12 young FXS patients [230]. The efficacy of ganaxolone, a synthetic neurosteroid modulator of $GABA_{-A}$ receptors, and arbaclofen, a $GABA_{-B}$ receptor agonist, has also been investigated. Ganaxolone has been found to rescue audiogenic seizures [177] and to reduce repetitive behaviors [231] in *Fmr1*- KO mice. However, a phase II trial in children with FXS showed no significant behavioral improvements induced by the drug in the overall study population. Some beneficial effects were only observed in subgroups of children affected by FXS with higher anxiety or lower cognitive abilities [232]. Arbaclofen reduced susceptibility to audiogenic seizures and normalized dendritic spine density and protein synthesis in *Fmr1*-KO mice [233]. This drug was well tolerated in a clinical trial completed in 2010 [234], although no significant improvements were detected in two phase III placebo-controlled trials [235].

In recent years, several lines of evidence led to the hypothesis that the endocannabinoid system may also be involved in the pathogenesis of FXS [236,237]: 1. endocannabinoids are key modulators of cognition, socio-emotional responses, seizure susceptibility, nociception and neuronal plasticity [238], all of which are affected in FXS; 2. Synaptic activation of mGluR5 promotes the synthesis of endocannabinoids, triggering CB1 receptor-mediated long-term depression (LTD) of excitatory and inhibitory neurotransmission [239]; 3. Endocannabinoids modulate GABAergic and glutamatergic neurotransmission and regulate the mTOR pathway [240]; 4. FMRP-deficient mice show altered mGluR5-dependent endocannabinoid activity and synaptic plasticity [241,242]; 5. drugs that target the endocannabinoid system can normalize some behavioral, biochemical and electrophysiological changes displayed by *Fmr1*-KO mice [61,179,236,242,243]. Thus, it would be interesting to determine whether changes in the endocannabinoid system are displayed by FXS patients, and whether drugs targeting the endocannabinoid system ameliorate FXS symptoms in the clinical setting.

Environmental interventions at very early developmental ages are critical to mitigate the behavioral abnormalities observed in neurodevelopmental disorders [244]. Both a high-quality home environment and an efficient educational service have been associated with fewer autistic behaviors and higher IQ scores in children affected by FXS [7,245,246]. These clinical findings are in line with preclinical studies showing that housing in an enriched environment [70] and early social enrichment [247] can rescue many behavioral and neuronal abnormalities displayed by Fmr1 KO mice [70,247].

6. Conclusions

Despite the recent advances in our understanding of FXS pathophysiology, effective targeted therapies are still lacking. To date, the medications used to attenuate the behavioral deficits displayed by FXS patients rarely have a positive effect on the whole symptomatology. In this context, animal models are excellent translational research tools able to mimic specific symptoms of the syndrome: their development has increased our understanding of the biological mechanisms underlying FXS, and their use allowed us to understand the relationships between altered brain function and behavior in FXS. Furthermore, animal models are essential to validate new therapeutic targets and the ability of potential drug candidates to ameliorate FXS symptoms.

The severity of the symptoms displayed by FXS patients may be quite variable between different individuals, and this makes it difficult to reproduce in one single laboratory animal all the molecular, cellular and behavioral features of human FXS. Furthermore, FXS-relevant behavioral patterns are obviously more elaborated in humans than in rodents. Regardless, in line with the concept of face validity of animal models, single behaviors with similarity to the symptoms displayed by FXS can be effectively assessed in laboratory animals, and several tests can be performed to study behavioral features whose neural underpinnings resemble those found in FXS. Interestingly, behavioral phenotyping in rodent models of FXS can be performed already from the first days of life. For instance, cognitive abilities can be assessed few days after birth through the homing behavior test, while early deficits in social communication can be detected by measuring the USVs emitted by the pups separated from the nest. The competence in the cognitive, social and communicative domains can be measured and followed throughout the entire development of rodents, using age-specific behavioral tests. As an example, social behaviors before

puberty mainly consist of playful forms of social interaction that prepare, as the animal gets older, to sexual, agonistic and affiliative behaviors. Longitudinal behavioral phenotyping of animal models can thus provide evidence of a developmental trajectory of pathological traits relevant to the human disease. Furthermore, this approach allows testing potential pharmacological targets at very early developmental ages. This is relevant, since an increasing amount of data suggests the need to treat patients affected by neurodevelopmental disorders at the earliest possible age [248]. Thus, in order to translate potential drug targets into effective treatments, preclinical drug testing in developing laboratory animals is of the utmost importance. However, it should be noted that 100% predictive validity of efficacy in humans cannot be expected, since species differences may exist in drug metabolism, biochemical pathways involved, and toxicology. For instance, studies from several laboratories have led to the identification of mGluR5 as a potential pharmacological target for FXS. A number of mGluR5 antagonists have been tested at the preclinical level [221,222], but clinical trials were discontinued because the tested compounds were either ineffective or showing side effects.

Although much has been done in the field of research of FXS, much remains still unanswered. Continuous study of the behavioral and molecular assays relevant to FXS and combined preclinical and clinical approaches should be pursued to further clarify the role of FMRP, the molecular mechanisms used to repress the translation of its target mRNAs, the effects of its mutation on the synaptic function and which brain areas are particularly involved, in order to identify different potential neurobiological mechanisms, and to find new pharmacological targets able to ameliorate the whole symptomatology of the disease.

Declarations of interest

None.

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Chapter 6

Involvement of Phosphodiesterase 2A activity in the pathophysiology of Fragile X Syndrome.

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Chapter 6

Involvement of Phosphodiesterase 2A activity in the pathophysiology of Fragile X Syndrome.

This work capitalizes on the aim of my PhD project, i.e., find new therapeutic opportunities to treat ASD. In particular, I investigated the involvement of the Phosphodiesterase 2A in the pathophysiology of FXS. I tested the ability of an acute administration of a selective PDE2A inhibitor BAY607550 to rescue the atypical communicative, social and cognitive behaviors displayed by *Fmr1*-KO mice. To further analyze the involvement of PDE2A in the pathophysiology of FXS, I tested the ability of BAY607550 to revert the behavioral alterations displayed by *Fmr1*-KO infant rats. In order to validate PDE2A as a new therapeutic target for FXS, the results were confirmed by using another PDE2A selective inhibitor, Lu AF64280. Similarly to BAY607550, Lu AF64280 was able to revert the communicative deficits displayed by *Fmr1*-null mice. Importantly, chronic inhibition of PDE2A in newborn *Fmr1*-KO mice rescued the altered social behavior displayed by these mice at adolescence. Collectively, these results reveal the key role of PDE2A in the physiopathology of FXS and indicate that PDE2A pharmacological inhibition may represent a novel therapeutic approach for FXS.

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ORIGINAL ARTICLE

Involvement of Phosphodiesterase 2A Activity in the Pathophysiology of Fragile X Syndrome

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Abstract

The fragile X mental retardation protein (FMRP) is an RNA-binding protein involved in translational regulation of mRNAs that play key roles in synaptic morphology and plasticity. The functional absence of FMRP causes the fragile X syndrome (FXS), the most common form of inherited intellectual disability and the most common monogenic cause of autism. No effective treatment is available for FXS. We recently identified the *Phosphodiesterase 2A* (*Pde2a*) mRNA as a prominent target of FMRP. PDE2A enzymatic activity is increased in the brain of *Fmr1*-KO mice, a recognized model of FXS, leading to decreased levels of cAMP and cGMP. Here, we pharmacologically inhibited PDE2A in *Fmr1*-KO mice and observed a rescue both of the maturity of dendritic spines and of the exaggerated hippocampal mGluR-dependent long-term depression. Remarkably, PDE2A blockade rescued the social and communicative deficits of both mouse and rat *Fmr1*-KO animals. Importantly, chronic inhibition of PDE2A in newborn *Fmr1*-KO mice followed by a washout interval, resulted in the rescue of the altered social behavior observed in adolescent mice. Altogether, these results reveal the key role of PDE2A in the physiopathology of FXS and suggest that its pharmacological inhibition represents a novel therapeutic approach for FXS.

Key words: autism spectrum disorder, *Fmr1*-KO mice, *Fmr1*-KO rats, fragile X syndrome, phosphodiesterase 2A

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Introduction

Fragile X syndrome (FXS) is a rare genetic neurodevelopmental disorder with a prevalence of 1:4000 males and 1:7000 females representing the most common form of inherited intellectual disability (ID) and a leading genetic cause of autism spectrum disorder (ASD). Patients may also exhibit a range of disabling neurological problems including hyperactivity, attention deficit, anxiety and epileptic seizures in addition to facial dysmorphims and physical abnormalities (Bassell and Warren 2008; Maurin et al. 2014; Castagnola et al. 2017). FXS is caused by the absence of expression of the *FMR1* gene, which ultimately leads to the lack of its product, the fragile X mental retardation protein (FMRP), a translational modulator of synaptic proteins and a regulator of mRNA transport at the synapse. Consequently, neurons of both FXS patients and *Fmr1*-KO mice exhibit abnormal dendritic spines associated with altered forms of synaptic plasticity (Bassell and Warren 2008; Maurin et al. 2014; Castagnola et al. 2017). Furthermore, altered volumes of specific brain structures that develop prenatally or early postnatally in young FXS children (Gothelf et al. 2008; Hoeft et al. 2010) and *Fmr1*-KO pups (Lai et al. 2016) have been described. The abundance of many synaptic proteins is altered in the absence of FMRP and, consequently, multiple molecular pathways are dysregulated in *Fmr1*-KO neurons (Maurin et al. 2018). However, despite the research efforts made both at preclinical and clinical levels, approved therapies are not yet available for FXS (Budimirovic et al. 2017; Castagnola et al. 2017; Erickson et al. 2017). Towards this goal, it is essential to have a better understanding of the pathophysiology of FXS and of the role played by FMRP during brain development. Therefore, we used High Throughput Sequencing-Cross Linking Immuno-Precipitation (HITS-CLIP) to identify FMRP RNA targets at postnatal day (PND) 13, an early developmental stage of mouse brain, when FMRP is most highly expressed and synaptogenesis peaks (Maurin et al. 2018). At this age, in hippocampus and in cortex a prominent target of FMRP is the *Phosphodiesterase 2A* (*Pde2a*) mRNA (Maurin et al. 2018), which encodes an enzyme involved in cAMP and cGMP degradation (Maurice et al. 2014). PDE2A levels and activity are increased (Maurin et al. 2018 and this study) in *Fmr1*-KO, resulting in reduced levels of cAMP and cGMP, 2 intracellular secondary messengers having key roles in neuronal differentiation, development and function (Shelly et al. 2010; Park et al. 2014). Here, we unravel the pathophysiological relevance of PDE2A activity in FXS by combining in vitro, ex vivo, and in vivo experiments and using 2 rodent models of FXS. We conclud that PDE2A represents a novel therapeutic target to treat children affected by FXS.

Materials and Methods

Neuronal Cultures and Spine Morphology Analysis

Primary cortical neurons were prepared from embryos at E15.5 obtained from pregnant C57Bl/6 *Fmr1*-KO and wild type (WT) mice as previously described (Khayachi et al. 2018). Neurons (17 days in vitro) were treated with 0.2 µM BAY607550 or DMSO (control) for 24 h in total. After 5 h of pharmacological treatment, neurons were transduced with attenuated Sindbis viral particles pSinRep5(nsP2726)-expressing GFP at a multiplicity of infection (MOI) of 0.1. Transduced neurons (18 days in vitro) were washed twice in PBS at room temperature (RT) after 19 h of transduction, and then fixed (Devader et al. 2015). Sequential confocal images (512 × 220 pixels; Zoom 3.0; Average 4; Speed 7) of GFP-expressing neurons were acquired with a 63X oil-immersion lens (Numerical Aperture NA 1.4) on an inverted Zeiss LSM780 confocal microscope. Z-series of 7–8 images of randomly selected secondary dendrites (3 independent cultures, 24–30 neurons per condition) were analyzed using NeuronStudio software, which allows for the automated detection of immature and mature spines (Rodriguez et al. 2008; Devader et al. 2015).

The dendritic spine morphology analysis was scored and analyzed by trained observers who were unaware of treatment conditions.

cAMP and cGMP Detection

1) ELISA test: Frozen ground hippocampi from PND 13 mice, were resuspended in 10 volumes of 0.1 N HCl and centrifuged to remove debris. Supernatants were used directly for cGMP measurement. ELISA was then carried out according to the manufacturer's instructions (Direct cGMP ELISA kit; Enzo Life Science). 2) cAMP-Glo Max assay: Primary cortical neurons (17–21 days in vitro) cultured in 96 wells plates were stimulated in 2 biological replicates with 10 µM Forskolin and 1 µM BAY607550 in dPBS supplemented with $CaCl_2$ and $MgCl_2$ for 30 min at 37 °C. cAMP concentration was measured with the cAMP-Glo Max assay (Promega) according to the manufacturer's indications. 3) cAMP Biosensors: Brain slices were prepared from male mice at PND 7–11, transduced with Sindbis viral particles to express the cAMP biosensor Epac-S^{H150} (Polito et al. 2013). Wide-field images were obtained with an Olympus BX50WI or BX1WI upright microscope with a 40× 0.8 NA water-immersion objective and an ORCA-AG camera (Hamamatsu). Images were acquired with iVision (Biovision, Exton, PA, USA). The excitation and dichroic filters were D436/20 and 455dcxt. Signals were acquired by alternating the emission filters with a filter wheel (Sutter Instruments, Novato, CA, USA), HQ480/40 for the donor, and D535/40 for the acceptor. Images were analyzed with custom routines according to the IGOR Pro environment (Wavemetrics, Lake Oswego, OR, USA). The emission ratio was calculated for each pixel as F480/F535. The pseudocolor images display the ratio value coded in hue and the fluorescence of the preparation coded in intensity. The amplitudes of responses were quantified for each neuron as the fractional change in ratio from its own baseline and maximal final ratio response (in the presence of forskolin and IBMX). Responses obtained from CA1 neurons were averaged for each experiment (i.e., brain slice). Data were analyzed with SPSS statistical software version 22.0 (Chicago, IL, USA). Normality in variable distributions and homogeneity of variances across groups were assessed with the Shapiro–Wilk and Levene tests, respectively.

Animals

The experiments were performed following the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny et al. 2010). *Fmr1*-knockout (KO) and WT mice on a C57BL/6J congenic background were obtained from Prof. R. Willemsen (Mientjes et al. 2006), while *Fmr1*-KO and WT rats on a Sprague-Dawley background were purchased from Horizon Discovery (formerly SAGE Labs, USA). All animals were generated and housed in groups of 4 in standard laboratory conditions (22 °C, 55 ± 10% humidity, 12-h light/12-h dark diurnal cycles) with food and water provided ad libitum.

Behavior

Experimental testing was performed between 12:00 and 16:30 each day during the 12-h light period. Only male mice and rats were used. Animal care was conducted in accordance with the

European Community Directive 2010/63/EU. The experiments were approved by the local ethics committee (Comité d'Ethique en Expérimentation Animale CIEPAL-AZUR N. 00 788.01; APAFIS#4985-2 016 032 314 169 426 v4APAFIS#8100-2 016 112 217 148 206 v3), by the French Ministry of Research and by the Italian Ministry of Health. The number of animals used in each experiment is indicated in the figure legends.

Electrophysiology

Hippocampal slices were prepared from WT and *Fmr1*-KO mice on a C57BL/6J genetic background at PND 13 as previously described (Costa et al. 2012), following protocols approved by local ethics committee (OPBA, University of Catania) and by the Italian Ministry of Health (N. 35212016-PR). Data were acquired and analyzed with the Signal software (Cambridge Electronic Design, England). Excitatory Post Synaptic Current (EPSC) amplitude was measured as the difference between peak and baseline current. EPSC amplitude values were averaged over 1 min and expressed as % of baseline (mean EPSC amplitude calculated from EPSCs recorded during at least 15 min before [S]-3,5-Dihydroxyphenylglycine [DHPG] application). Different sets of values were compared using the appropriate statistical tests indicated in the corresponding figure legend. The amount of long-term depression (LTD) induced by metabotropic group I glutamate receptor (mGluR) was calculated 40 min after LTD induction by DHPG application and is expressed by indicating EPSC amplitude as percentage of baseline (% EPSC).

Drug Treatment

BAY607550 (Cayman) was dissolved in 10% DMSO/8.75% Tween 80/8.75% polyethylene glycol/saline. For the behavioral experiments, BAY607550 or Lu AF64280 (or their vehicles) were administered intraperitoneally (i.p.) 30 min before testing. BAY607550 was administrated at the doses of 0.05 mg/kg at infancy and 0.1 mg/kg at adolescence, while Lu AF64280 was a administered at 0.5 mg/kg. Drug doses and pretreatment intervals were based on literature (Boess et al. 2004; Masood et al. 2008, 2009; Ding et al. 2014; Redrobe et al. 2014; Wang et al. 2017) and our pilot data showing that, at the doses used in the present study, drugs did not affect the behavior of WT animals. In one experiment, chronic treatment was carried out by a daily i.p. injection of 0.05 mg/kg BAY607550 to mice from PND 5–21 and mice were tested for social interaction after a washout interval of 9 days. One pup per litter from different litters per treatment group was used in the behavioral experiments, to control for any potential litter effect. Animals were randomly allocated to each treatment group. Coding of the drug solutions ensured that both during experimentation and behavior analysis, the experimenter was unaware of the treatment of the animals. The number of animals per group is indicated in the figure legends.

The Isolation-Induced Ultrasonic Vocalizations Test

The test was performed as previously described (Servadio et al. 2016). Briefly, each pup (at PND 10 for mice and PNDs 5 and 9 for rats) was individually removed from the nest and placed into a black Plexiglas arena, located inside a sound-attenuating and temperature-controlled chamber. Pup ultrasonic vocalizations (USVs) were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Germany) sensitive to frequencies between 10 and 250 kHz and fixed at 10 cm above the arena. Pup axillary temperature was measured before and after the test by a digital thermometer. The emission of USVs was analyzed using Avisoft Recorder software (Version 5.1).

Homing Behavior Test

At PND 14, the litter was separated from the dam and kept for 30 min in a temperature-controlled holding cage. Then, each mouse 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 videorecorded for 4 min for subsequent analysis. The following parameters were scored using the Observer 3.0 software (Noldus Information Technology): latency (s) to reach the home-cage bedding area; total time (s) spent by the pup in the nest bedding area.

Social Interaction Test

The test was performed as previously described (Terranova and Laviola 2005; Jamain et al. 2008). The 28–30-day-old mice were individually habituated to the experimental apparatus (a Plexiglas cage measuring 30 × 30 × 30 cm^3) for 5 min the day before testing. On the test day, the animals were isolated for 2 h before testing, to enhance their social motivation and thus facilitate the expression of social interaction during testing. The test consisted of placing 2 animals (same treatment and weight) into the test cage for 10 min.

The behavior of the animals was recorded using a video camera with zoom lens, DVD recorder and LCD monitor. Behavior was assessed per single animal and analyzed by a trained observer who was unaware of genotype and treatment conditions using the Observer XT software (Noldus, The Netherlands).

The following parameters were scored (Terranova and Laviola 2005; Jamain et al. 2008):

a. Social activities:
   1. Social sniffing: sniffing any part of the body of the partner, including the anogenital area.
   2. Following: moving in the direction of or pursuing the partner, who moves away.
   3. Mutual circle: partners are mutually sniffing each other's anogenital region, while describing tight circles with their reciprocal following movements.
   4. Pushing past: the focal animal passes between the wall of the cage and the body of the partner by pushing its own body through the narrow space available.
   5. Crawling under/over: the focal animal crawls underneath or over the partner's body, crossing it transversely from one side to the other.
   6. Social grooming: chewing and licking the fur of the partner.
   7. Social rest: the focal animal is being groomed by the partner.
   8. Pushing under: the focal animal pushes its own snout or the whole anterior part of its body under the partner's body, and rests for at least 3 s.
   9. Social inactivity: the focal animal is lying flat or standing still (eyes closed or open) while maintaining close physical contact with the partner.

b. Nonsocial activities:
   1. Running: the focal animal performs a sudden, rapid, vigorous, and erratic darting, characterized by frequent and sharp changes in direction and without any obvious target.
   2. Inactive: Self-explanatory.
   3. Exploring: Self-explanatory.

4. Digging: the focal animal is digging in the sawdust, pushing and kicking it around, using the snout and/or both the forepaws and hindpaws.

The average frequency of total social activities, quantified as number of events during the 10 min testing session, was graphed.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). All statistical analyses were based on biological replicates. Appropriate statistical tests used for each experiment are described in the corresponding figure legends. All statistical analyses were carried out using the GraphPad Prism Version 6.0e.

Results

PDE2A Dysregulation is Involved in the Physiopathology of FXS

Pde2a is expressed both in cortex and hippocampus (e.g., supragranular layer of neocortex, CA1 and CA3 regions of hippocampus) (Stephenson et al. 2009, 2012), with a high and homogenous expression in the mouse CA1. To assess whether the increased abundance of the PDE2A protein in the absence of FMRP that we described (Maurin et al. 2018) is associated with its elevated activity in hippocampus, we measured cAMP levels in single neurons of the CA1 area in *Fmr1*-KO and WT mouse brain slices. For this purpose, we used the Epac-S^{H150} fluorescent biosensor that detects an increase in cAMP levels by a decrease in FRET between the donor and acceptor fluorophores (Polito et al. 2013). These changes were monitored in real-time by ratiometric fluorescence imaging. cAMP synthesis was first stimulated using forskolin, leading to a steady-state biosensor emission ratio. PDE2A was then activated using the NO donor DEANO, which decreased the biosensor ratio. PDE2A activity was then blocked by the addition of the potent and specific PDE2A inhibitor BAY607550 (Boess et al. 2004), which increased the biosensor ratio, revealing the effective contribution of PDE2A in cAMP degradation. A final application of forskolin and the nonselective phosphodiesterase inhibitor IBMX increased the biosensor ratio to its maximum. An example of this analysis in WT neurons is reported in Figure 1*A*, *B*. Then we performed these assays in hippocampal slices obtained from PND 7–11 WT and *Fmr1*-KO mice. cAMP levels elicited by forskolin and DEANO stimulation were significantly lower in the absence of *Fmr1* expression, consistent with an elevated PDE2A activity in the *Fmr1*-KO hippocampus (Fig. 1C). We confirmed these findings by a detailed analysis of cAMP

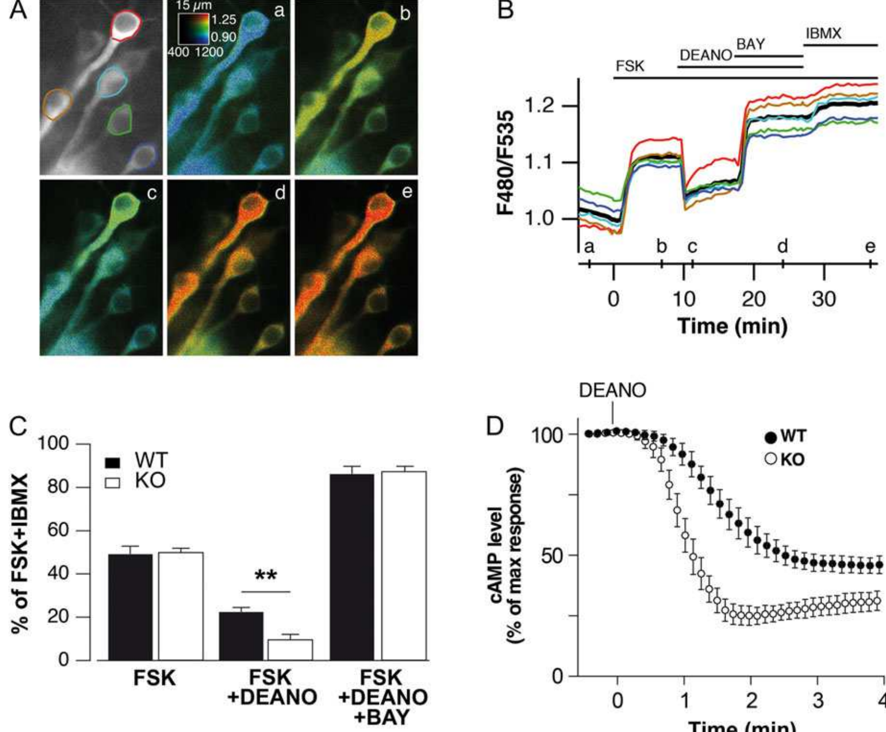


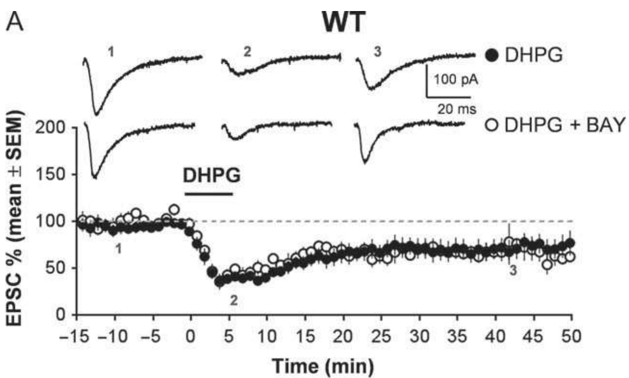
Figure 1. The increased activity of PDE2A results into decreased cAMP levels. (*A*) Hippocampal brain slices expressing the Epac-S^{H150} biosensor were imaged with wide-field fluorescence microscopy (here exemplified with a measure in a WT brain slice). Images show the raw fluorescence intensity at 535 nm (in gray scale) and the ratio (in pseudocolor), reporting changes in cAMP concentration before stimulation and at different times of the recording during the different treatments (a–e), as indicated by the corresponding lines on the graph in panel "B". The calibration square on the pseudocolor image indicates from left to right increasing fluorescence intensity levels, and from bottom to top increasing ratio values. The width of the square is used as a scale bar. Its size is indicated above it in micrometers. (*B*) Each trace on the graph indicates the F480/F535 emission ratio measured on the regions (neuron) delimited by the color contour drawn on the gray scale image (upper left panel). The black trace corresponds to the mean of the 5 colored traces. (*C*) Quantification of cAMP in WT and *Fmr1*-KO hippocampal slices: the successive responses to forskolin, DEANO and BAY607550 were quantified as a fraction of the maximal response measured in the presence of IBMX. (*C*) Average ratio response to forskolin (10 μM), forskolin + DEANO (10 μM) and forskolin + DEANO + BAY607550 (0.2 μM) for WT and *Fmr1*-KO. Mean ± SEM is shown. In each experiment (i.e., brain slices tested, one slice per animal) a variable number of neurons have been considered (between 1 and 6). Results from $n = 12$ WT and $n = 16$ *Fmr1*-KO experiments are shown. Two-way ANOVA followed by Bonferroni multiple comparisons post hoc test revealed a significantly lower level of cAMP in *Fmr1*-KO slices for the FSK + DEANO condition ($F_{treatment(1,26)} = 640$, $P < 0.001$; $F_{genotype(1,26)} = 1.25$, $P = 0.278$; $F_{genotype \times treatment(1,26)} = 15.76$, $P = 0.001$) followed by Bonferroni multiple comparisons post hoc test (adjusted P value $^{**}P < 0.01$). (*D*) Time course measurement of cAMP levels after DEANO application in the presence or in the absence of FMRP.

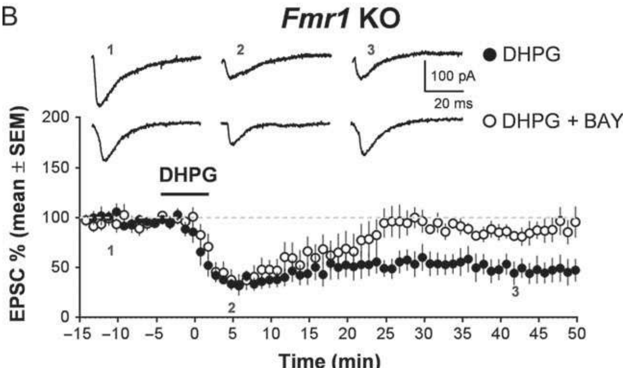
degradation kinetics in the presence and in the absence of FMRP upon PDE2A activation with DEANO: the decrease in biosensor ratio upon PDE2A stimulation with DEANO was significantly faster in *Fmr1*-KO neurons than in WT (Fig. 1D).

In addition, using an ELISA immuno-assay, we showed that cGMP levels are also significantly decreased in *Fmr1*-KO hippocampi (Supplementary Fig. S1). Collectively, these data indicate that there is an elevated activity of PDE2A in hippocampal *Fmr1*-KO neurons.

Blocking PDE2A Activity Suppresses the Exaggerated LTD in *Fmr1*-KO hippocampus

A hallmark of FXS is the exaggerated LTD induced by mGluR activation in the hippocampal CA3-CA1 synapses (Huber et al. 2002). To assess whether inhibition of PDE2A can prevent the exaggerated synaptic plasticity characterizing FXS hippocampi, we measured LTD expression in the presence and in the absence of BAY607550. AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) were recorded from CA1 pyramidal neurons under whole-cell patch clamp following stimulation of Schaffer collaterals, in the continuous presence of D-AP5 (50 μM) and bicuculline (5 μM). Bath application of DHPG (100 μM, 5 min), an agonist of group I mGluRs, induced a LTD of EPSC amplitude that in WT was not modified in the presence of BAY607550 (50 nM; Fig. 2A,C). The amount of mGluR-LTD is exaggerated in *Fmr1*-KO hippocampi (EPSC amplitude: 40 ± 9% vs. 78 ± 9% in *Fmr1* KO vs. WT, $P < 0.05$; Fig. 2B,C), as previously reported (Costa et al. 2012; Castagnola et al. 2017). Intracellular BAY607550 (50 nM) in *Fmr1*-KO mouse hippocampal neurons reverted the exaggerated mGluR-LTD to a level that is not statistically different from WT control recordings (EPSC % amplitude: 78 ± 9% vs. 86 ± 7% in WT vs. *Fmr1*-KO treated with BAY607550; Fig. 2B,C). Remarkably, BAY607550 treatment (50 nM) had no effect on mGluR-LTD in WT slices (Fig. 2A–C). These results clearly show the implication of PDE2A-mediated regulation of cAMP and cGMP in the exaggerated mGluR-dependent LTD in *Fmr1*-KO mice.





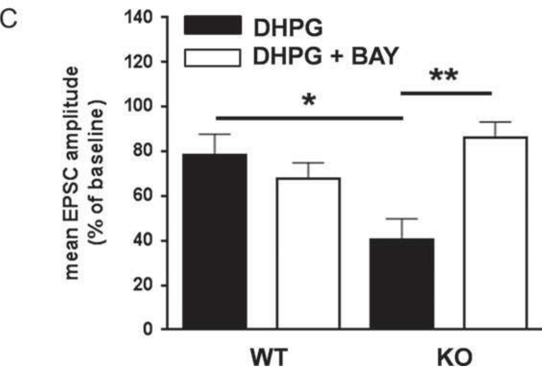


Figure 2. Blockade of PDE2A rescues the exaggerated hippocampal mGluR-dependent LTD in the *Fmr1*-KO brain. (A) DHPG (100 μM, 5 min) induces a mGluR-LTD of EPSCs recorded from CA1 pyramidal neurons obtained from WT mouse slices ($n = 6$). BAY607550 (50 nM, added intracellularly in the recording pipette) did not modify the amount of mGluR-LTD of EPSCs recorded from CA1 pyramidal neurons in WT slices ($n = 6$). (B) In mouse *Fmr1*-KO slices ($n = 9$), mGluR-LTD was reversed in the presence of intracellular BAY607550 (50 nM). (C) Bar graphs show % EPSC amplitude (mean ± SEM from groups of neurons) 40 min after application of DHPG in control conditions or in the presence of intracellular BAY607550 (50 nM). Bar graphs show the mean ± SEM values of EPSC % after the indicated pharmacological treatments. Two-way ANOVA were computed ($F_{genotype(1,22)} = 1.159$, $P = 0.2933$; $F_{treatment(1,22)} = 3.919$, $P = 0.2933$; $F_{genotype \times treatment(1,22)} = 10.24$; $P = 0.0041$) with Bonferroni's post-tests for multiple comparisons of data sets, using genotype (*Fmr1*-KO or WT) and treatment (BAY607550 or vehicle) as between-subjects factor (adjusted P value: *$P = 0.0276$; **$P = 0.0037$).

Inhibiting PDE2A Activity Restores Axonal Length and Spine Maturation in Cultured Cortical *Fmr1*-KO Neurons

The presence of abnormal immature dendritic spines in the brain of FXS patients and in primary neuronal cultures of mouse *Fmr1*-KO models of FXS (Comery et al. 1997; Irwin et al. 2000; Nimchinsky et al. 2001; Antar et al. 2005, 2006) is another hallmark associated with the functional absence of FMRP. Importantly, both cAMP and cGMP have been reported to exert an important role in axonal growth and dendritic spine maturation (Shelly et al. 2010; Shen and Cowan 2010; Averaimo and Nicol 2014; Akiyama et al. 2016). Therefore, to assess the involvement of PDE2A in synaptic morphology, we first analyzed cAMP levels in cultured cortical neurons in the absence or in the presence of PDE2A inhibitors (Supplementary Fig. S2). We then assessed the impact of an inhibition of PDE2A activity on the maturation of dendritic spines (Fig. 3) and on axonal growth (Fig. 4) in *Fmr1*-KO cultured cortical neurons. To quantify the activity of PDE2A in FXS neurons, we measured cAMP levels in cultured *Fmr1*-KO upon forskolin stimulation associated with pan-PDE inhibition with IBMX (Supplementary Fig. S2). This latter treatment led to a significant increase in cAMP levels both in the presence and in the absence of FMRP expression (Supplementary Fig. S2A), while treatment with Rolipram (a specific inhibitor of PDE4) did not (Supplementary Fig. S2B). Conversely, the specific blockade of PDE2A activity by BAY607550 promoted a significant increase in cAMP levels in *Fmr1*-KO neurons without affecting its abundance in WT neurons (Supplementary Fig. S2C). This finding suggests a PDE2A-dependent regulation of cAMP levels in *Fmr1*-KO neurons.

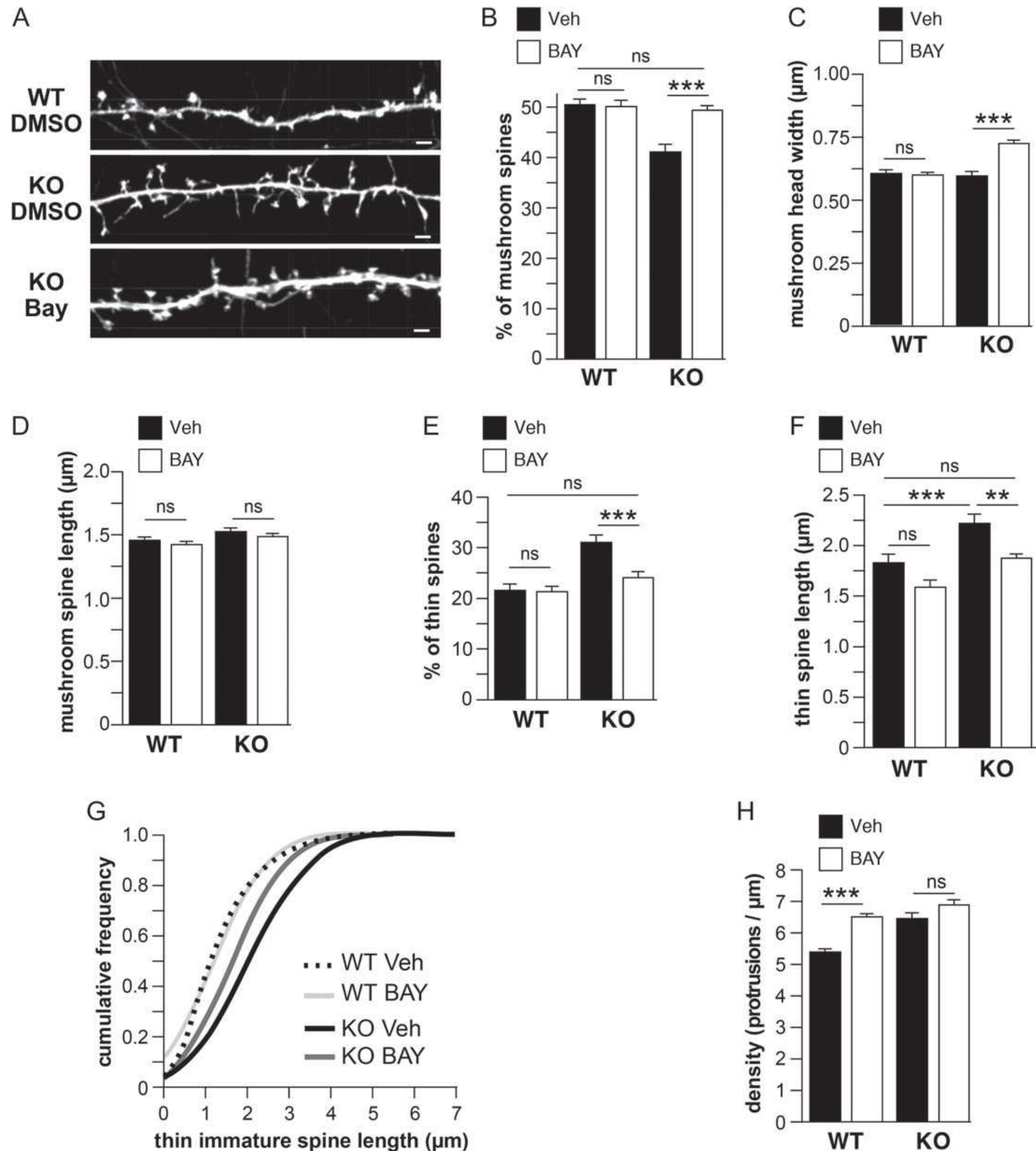


Figure 3. Inhibition of PDE2A activity improves *Fmr1*-KO dendritic spine morphology in cultured cortical neurons. (*A*) Representative high-resolution confocal images showing GFP-expressing WT and *Fmr1*-KO mouse secondary dendrites treated or not for 24 h with 0.2 μM BAY607550 to block PDE2A activity. Spine morphology was assessed using NeuronStudio software 19 h post-transduction and compared with the measurement obtained from control DMSO-treated neurons. Scale bars: 2 μm. All summary histograms present mean ± SEM values, statistical significance was assessed with 2-way ANOVA. The percentage of mature mushroom spines ($F_{genotype(1,110)} = 10.98$, $P = 0.0012$; $F_{treatment(1,110)} = 7.042$, $P = 0.0091$; $F_{genotype \times treatment(1,110)} = 8.984$; $P = 0.0034$), (*B*) mushroom head width ($F_{genotype(1,1678)} = 18.15$, $P < 0.0001$; $F_{treatment\ (1,\ 1678)} = 22.06$, $P < 0.0001$; $F_{genotype \times treatment(1,1678)} = 28.41$; $P < 0.0001$), (*C*) and mushroom spine length ($F_{genotype(1,1599)} = 7.217$, $P = 0.0073$; $F_{treatment(1,1599)} = 3.665$, $P = 0.0558$; $F_{genotype \times treatment(1,\ 1599)} = 0.002329$; $P = 0.9615$) (*D*) measured in secondary dendrites of control and BAY607550-treated neurons. The percentage of immature thin spines ($F_{genotype(1,\ 111)} = 30.25$, $P < 0.0001$; $F_{treatment(1,111)} = 12.09$, $P = 0.0007$; $F_{genotype \times treatment(1,111)} = 8.911$; $P = 0.0035$) (*E*) and thin spine length ($F_{genotype\ (1,\ 878)} = 23.16$, $P < 0.0001$; $F_{treatment(1,878)} = 17.37$, $P < 0.0001$; $F_{genotype \times treatment(1,878)} = 0.6166$; $P = 0.4325$) (*F*) is presented. The consequence of the BAY607550 treatment on the distribution of the thin spine length is depicted as cumulative frequency curves in (*G*). (*H*) Histograms showing the mean ± SEM values of protrusion frequency after the indicated pharmacological treatments. Two-way ANOVA were computed with Bonferroni post hoc test to assess the treatment effect in neurons from each genotype. (Adjusted *P* value: $^{**}P < 0.01$; $^{***}P < 0.001$. $N = {\sim}1000$.) Protrusions per condition (3 independent mouse cortical neuron cultures; 24–30 neurons per condition). ns, not significant.

In cultured *Fmr1*-KO neurons (Fig. 3*A*), the specific inhibition of PDE2A by BAY607550 strongly promoted spine maturation by increasing the number of mushroom spines (Fig. 3*B*) and reduced the number of immature filopodia (Fig. 3*E*) to normal WT levels. BAY607550 also had a positive impact on the maturity of *Fmr1*-KO neurons by increasing the head size of mushroom spines (Fig. 3*C*) and concomitantly reducing the length of thin immature spines to WT levels (Fig. 3*F*–*G*). Finally, the BAY607550 treatment affected neither the length of mature spines (Fig. 3*D*) nor the density of dendritic protrusions (Fig. 3*H*) in *Fmr1*-KO neurons.

Abnormal axonal growth has been observed in the fly model of FXS (Morales et al. 2002). Since both cAMP and cGMP fulfill critical roles in axonal growth (Shelly et al. 2010; Shen and Cowan 2010; Averaimo and Nicol 2014; Akiyama et al. 2016), we assessed whether PDE2A activity regulates the length of axons.

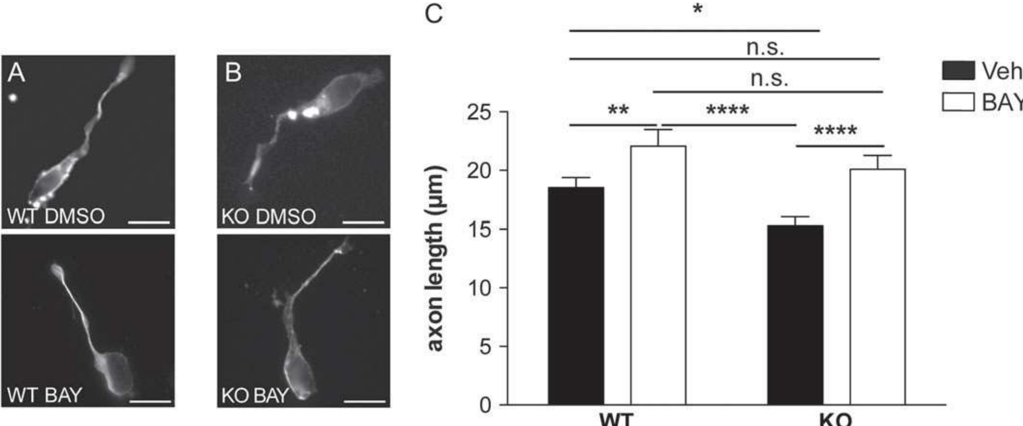


Figure 4. PDE2A activity is associated to axon growth regulation. (*A*, *B*) Representative pictures of 2 days in vitro cultured WT (*A*) and *Fmr1*-KO (*B*) primary cortical neurons treated with vehicle or 1 μM BAY607550 as indicated (scale bar: 10 μm). (*C*) Histogram of axon length of WT and *Fmr1*-KO neurons treated with vehicle or with 1 μM BAY607550 for 24 h. Results show the mean axon length ± SEM from 3 independent cultures on 73 randomly selected cells for each condition. Two-way ANOVA with Tukey post hoc test: DMSO:KO versus DMSO:WT. *Adjusted *P* value = 0.0256; BAY:WT versus DMSO:WT. **Adjusted *P* value = 0.0081; BAY:KO versus DMSO:WT. ns: Adjusted *P* value = 0.4114; BAY:WT versus DMSO:KO. ****Adjusted *P* value < 0.0001; BAY:KO versus DMSO:KO. ****Adjusted *P* value < 0.0001; BAY:KO versus BAY: WT. ns: Adjusted *P* value = 0.3547; ns, not significant.

Using an immunocytochemistry-based approach, we measured axon length of 2 days in vitro neurons and showed that *Fmr1*-KO neurons had significantly shorter axons than WT cells (Fig. 4A–C). The blockade of PDE2A for 24 h with BAY607550 was sufficient to fully rescue the axonal growth defect of FXS neurons (Fig. 4).

Critical Role of PDE2A Activity in Defining Social Deficits Displayed by 2 Rodent Models of FXS

The *Fmr1*-KO mouse model of FXS recapitulates the main behavioral traits initially described in FXS patients, such as cognitive deficit and social interaction impairments (Mientjes et al. 2006; Maurin et al. 2014). Since we identified *Pde2a* mRNA as a target of FMRP during the early postnatal life in the mouse brain and since we showed that PDE2A expression was increased in *Fmr1*-KO brains (Maurin et al. 2018), we investigated whether an acute PDE2A blockade in vivo rescued the altered phenotype of *Fmr1*-KO infant (PND 10-14) and adolescent (PND 30) mice in FXS-relevant behaviors (Fig. 5). At infancy, *Fmr1*-KO pups displayed early communicative deficits, since they vocalized significantly less compared with WT pups when separated from the dam and siblings at PND 10 (Fig. 5*A*,*B*). Furthermore, *Fmr1*-KO animals showed early deficits in social discrimination, since they were unable to use olfactory cues to discriminate between a neutral odor and their own cage odor in the homing behavior test (Fig. 5*C*,*D*). BAY607550 has been shown to efficiently cross the blood-brain barrier when administered i.p., promoting comparable PDE2A inhibition levels as the intracranial injection route (Wang et al. 2017). Our results showed that inhibition of PDE2A activity through i.p. administration of BAY607550 normalized the altered USV profile displayed by PND 10 *Fmr1*-KO mice (Fig. 5*B*). Remarkably, we validated this result by inhibiting PDE2A with Lu AF64280, another highly specific PDE2A inhibitor (Redrobe et al. 2014). Similar to BAY607550, Lu AF64280 was able to revert the altered USV frequency displayed by *Fmr1*-null pups (Supplementary Fig. S3). This confirms that PDE2A blockade is able to rescue the communicative deficit displayed by *Fmr1*-KO mice in the USV test. Furthermore, we found that treatment with BAY607550 improved the performance of *Fmr1*-KO pups in the homing behavior test (Fig. 5C–*E*) without altering the performance of WT pups. Our results pointed out that altered social behavior is a core phenotypic characteristic of the FXS mouse model. Accordingly, compared with WT animals, adolescent *Fmr1*-KO mice showed reduced social interaction, a phenotype that was rescued by PDE2A inhibition (Fig. 5*F*).

PDE2A is a Therapeutic Target for FXS

The elevated activity of PDE2A may underlie the deficits in communicative and social domains displayed by *Fmr1*-KO mice throughout development. To confirm this possibility, we chronically treated *Fmr1*-KO mice with BAY607550 from PND 5 to PND21, and tested their social abilities after a washout interval of 9 days. Strikingly, early treatment with BAY607550 reversed the social deficits displayed by *Fmr1*-KO mice at PND 30, showing that the beneficial effects of early PDE2A pharmacological blockade are long-lasting (Fig. 5*G*). Importantly, the administration of BAY607550 had no effect on the behavior of WT mice (Fig. 5*B*,*D*–*G*), further indicating the specificity of this treatment for the FXS phenotype. Finally, chronically administered BAY607550 rescued the abnormal dendritic spine length in the CA1 region of the hippocampus of *Fmr1*-KO mice (Supplementary Fig. S4). To validate PDE2A as a therapeutic target for FXS, we extended the behavioral analysis to *Fmr1*-KO infant rats. Similar to *Fmr1*-KO mice, *Fmr1*-KO rats vocalized less than WT controls when separated from their mother and siblings at PND 5 and PND 9 (Fig. 6*A*,*B*). Remarkably, acute administration of BAY607550 also normalized their altered USV pattern (Fig. 6*A*,*B*) and the ability of these infant rats to communicate, without affecting the behavior of WT control animals (Fig. 6*A*,*B*).

Discussion

Role of PDE2A in Hippocampus and Cortex Development

We have shown that an elevated amount of the PDE2A protein is present in *Fmr1*-null cortex and hippocampus (Maurin et al. 2018). Due to the heterogeneous pattern of the expression level of PDE2A in these brain regions (Stephenson et al. 2009, 2012), we measured here the PDE2A activity at the single cell level demonstrating that the activity of PDE2A is also significantly increased

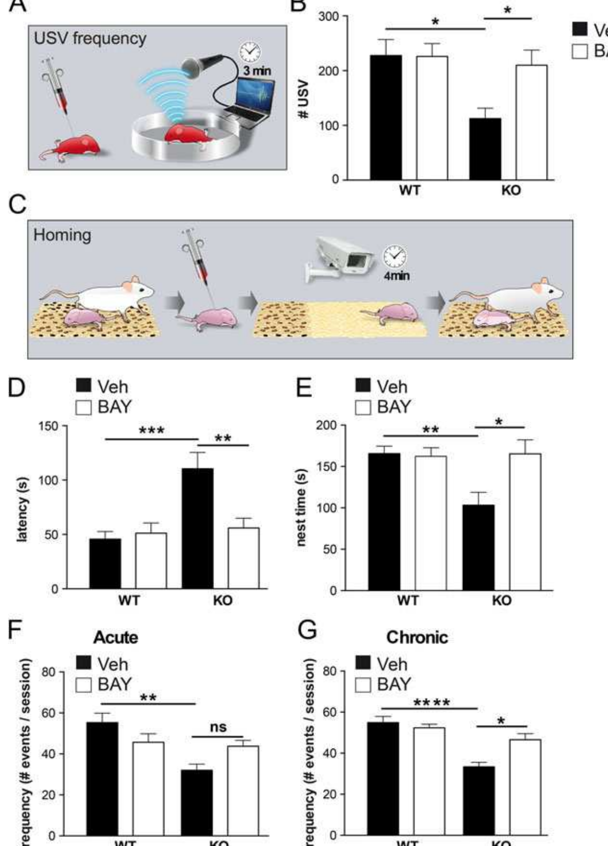


Figure 5. Inhibition of PDE2A activity rescues abnormal behaviors in infant and adolescent *Fmr1*-null mice. (A) Scheme of the USV test performed at PND 10. The 30 min after treatment with BAY607550 or vehicle, pups were separated from the dam and ultrasonic vocalizations (USV) were recorded for 3 min. (B) *Fmr1*-KO mice emit less USVs when removed from the nest at PND 10, and this communicative deficit is reversed upon BAY607550 injection ($F_{genotype(1,63)}$ = 7.07, P = 0.01; $F_{treatment(1,63)}$ = 3.80, P = 0.05; $F_{genotype{\times}treatment(1,63)}$ = 4.09; P = 0.04); *n*: WT-VEH = 17; WT-BAY = 17; KO-VEH = 16; KO-BAY = 17); (C) presentation of the homing test performed at PND 14; *Fmr1*-KO mice show (D) longer latency to reach the home-cage bedding ($F_{genotype(1,45)}$ = 11.79, P = 0.001; $F_{treatement(1,45)}$ = 5.86, P = 0.02; $F_{genotype{\times}treatment(1,45)}$ = 8.81; P = 0.005) and (E) spend less time in the nest area ($F_{genotype(1,45)}$ = 5.51, P = 0.02; $F_{treatment(1,45)}$ = 5.37, P = 0.02; $F_{genotype{\times}treatment(1,45)}$ = 6.63; P = 0.013) in the homing behavior test at PND 14 (*n*: WT-VEH = 14; WT-BAY = 16; KO-VEH = 9; KO-BAY = 10); both these parameters are normalized when *Fmr1*-KO mice are treated with BAY 607 550. (F, G) Social interaction was evaluated at PND 30, results are reported for acute (F) (*n*: WT-Veh = 18; WT-Bay = 22; KO-Veh = 12; KO-Bay = 9) ($F_{genotype(1,57)}$ = 7.53, P = 0.008; $F_{treatment(1,57)}$ = 0.05, P = 0.82; $F_{genotype{\times}treatment(1,57)}$ = 5.43; P = 0.02), and chronic treatment (G) (*n*: WT-Veh = 11; WT-Bay = 14; KO-Veh = 8; KO-Bay = 7) ($F_{genotype\ (1,36)}$ = 28,71 P < 00001; $F_{(treatment)\ 1,36}$ = 4.358 P = 0.0440; $F_{genotype{\times}treatment\ (1,36)}$ = 9.539 P = 0.0039). Data represent mean ± SEM (adjusted P value: *P < 0.05, **P < 0.01, ***P < 0.001). Two-way ANOVA was used to assess the effects of BAY607550 in *Fmr1*-KO and WT mice, using genotype (*Fmr1*-KO or WT) and treatment (BAY607550 or vehicle) as between-subjects factor, followed by Tukey multiple comparison post hoc test where appropriate.

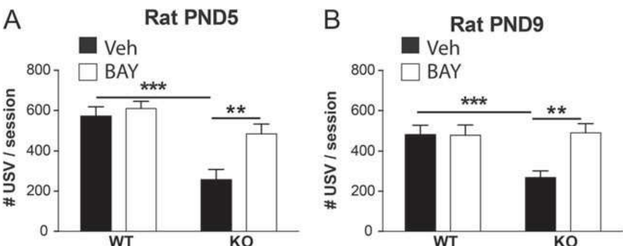


Figure 6. Inhibition of PDE2A activity rescues communication deficit in infant *Fmr1*-KO rats. *Fmr1*-KO rats emit less USVs when removed from the nest at PND 5 and 9, and this communicative deficit is reversed upon BAY607550 injection at (A) PND 5 (*n*: WT-Veh = 8; WT-Bay = 8; KO-Veh = 9; KO-Bay = 9) ($F_{genotype(1,32)}$ = 5.67, P = 0.023; $F_{treatment(1,32)}$ = 6.76, P = 0.014; $F_{genotype{\times}treatment(1,32)}$ = 7.12; P = 0.012) and (B) at PND 9 (*n*: WT-Veh = 8; WT-Bay = 8; KO-Veh = 10; KO-Bay = 10) ($F_{genotype(1,30)}$ = 22.72, P < 0.001; $F_{treatment(1,30)}$ = 8.27, P = 0.007; $F_{genotype{\times}treatment\ (1,30)}$ = 4.28; P = 0.047). Data represent the mean number of emitted USV (±SEM) per 3 min-recording session. (Adjusted P value **P < 0.01, ***P < 0.001.) Two-way ANOVA was used to assess the effects of BAY607550 in *Fmr1*-KO and WT rats, using genotype (*Fmr1*-KO or WT) and treatment (BAY607550 or vehicle) as between-subject factors, followed by Tukey multiple comparison post hoc test where appropriate.

in the absence of FMRP. Since an important fraction of PDE2A is localized at the synapse (Russwurm et al. 2009; Maurin et al. 2018), we reasoned that its elevated activity in FXS neurons could impact local cAMP and cGMP homeostasis. It has been reported that the levels of both cAMP and cGMP have critical roles in axon elongation and guidance (Shelly et al. 2010; Akiyama et al. 2016) and in regulating the morphology and growth of dendritic spines (Dityatev and El-Husseini 2011). Indeed, *Fmr1*-KO neurons show an increased PDE2A activity as well as an increased density of spines compared with WT neurons. Blocking the excess of PDE2A activity in *Fmr1*-KO cells leads to spine maturation without decreasing the protrusion density. In WT cells, there is no effect of the BAY607550 on spine maturation but there is an increase in the density of protrusion suggesting separated roles of PDE2A activity in spine formation and maturation but not in spine elimination. This latter result is consistent with our recent findings showing that the density of spines is restored in *Fmr1*-KO olfactory neurons by depleting CYFIP1, a protein connecting FMRP with actin remodeling *via* Rac1 (Abekhoukh et al. 2017). Moreover, the reduced length of growing axons that we found in 2 DIV *Fmr1*-KO neurons may result from the elevated levels of PDE2A that lead, in turn, to a reduced concentration of axonal cAMP and cGMP. Indeed, the normal length of growing axons is restored after treatment with the specific PDE2A inhibitor BAY607550. Furthermore, it is known that cGMP stimulates synthesis of glutamate via phosphoglycerate kinase (PGK) (Neitz et al. 2011). Consistent with a reduction of cGMP, glutamate levels are reduced in the *Fmr1*-KO cortex (Davidovic et al. 2011) and hippocampus (Hebert et al. 2014). PDE2A is the only PDE identified so far at the presynaptic active zone, associated with docked vesicles and illustrating the importance of such a compartmentalized action (Maurin et al. 2018). Indeed, cAMP abundance coupled to PKA signaling is critical to modulate assembly/disassembly/priming/recycling of neurotransmitter vesicles and, consequently, for synaptic transmission and plasticity events (Crawford and Mennerick 2012) and basal synaptic transmission (Gomez and Breitenbucher 2013). Here we show that the pharmacological inhibition of PDE2A activity rescues the exaggerated mGluR-dependent LTD in *Fmr1*-KO hippocampal slices, a well-characterized hallmark of *Fmr1*-KO brain (Huber et al. 2002).

As we already explained, cGMP and cAMP are involved in axonal growth, spine maturation and synaptic plasticity. Since PDE2A modulates the level of both cAMP and cGMP, we can suggest that its elevated activity in the absence of FMRP contributes to the definition of a neuronal FXS phenotype characterized by altered dendritic morphology, altered axonal length and exaggerated mGluR-LTD.

PDE2A has a Crucial and Evolutionarily Conserved Role in the Physiopathology of FXS

We show here that *Fmr1*-KO pups have profound deficits in both social communication and social discrimination from the first days of life, as revealed by their altered USV profile and impaired homing behavior, respectively. This, in turn, may alter the proper development of social behavior and social recognition (Terry and Johanson 1996; Melo et al. 2006). In line with this hypothesis, *Fmr1*-KO mice show deficits in social interaction at the adolescent age (Liu and Smith 2009; Dahlhaus and El-Husseini 2010; Kazdoba et al. 2014) that mirror the phenotype observed in FXS patients. Considering the impact of the pharmacological inhibition of PDE2A on the in vitro and ex vivo FXS phenotypes, we assessed whether the PDE2A inhibitor BAY607550 could revert the altered phenotype displayed by *Fmr1*-KO animals in social communication, social discrimination, and social interaction. Administration of BAY607550 normalizes the USV profile displayed by *Fmr1*-KO mice and rats. In addition, this treatment rescues the altered performance of *Fmr1*-KO pups in the homing behavior test, and increases the frequency of social interactions observed in *Fmr1*-KO mice to similar levels as WT animals. While PDE2A was previously linked to cognitive processes (Gomez and Breitenbucher 2013; Redrobe et al. 2014; Lueptow et al. 2016), here, for the first time, we associate its increased activity with altered social deficits. It is interesting to notice that PDE2A is coexpressed with FMRP in a specific class of neurons in the olfactory bulb (Korsak et al. 2017). These neurons have been shown to play a role in thermosensing and detecting stress in congeners as well as in pheromone sensing behavior (Juilfs et al. 1997). This can lead to the speculation that modulating PDE2A activity in these neurons may influence the social behavior. In conclusion, PDE2A may be an attractive target to simultaneously treat the social and communicative dysfunctions characterizing FXS patients. Importantly, mice chronically treated with BAY607550 during the early postnatal development period clearly benefit of the positive effect of this therapy when tested at adolescence. This result strongly suggests that inhibition of PDE2A during infancy has long-term positive effects and provides a strong preclinical rationale for a new therapeutic strategy for FXS patients. Remarkably, we used very low doses of BAY607550 that do not affect the behavior of WT animals. This is important not only because our approach targets a pediatric population but also because low doses should reduce possible toxic side effects of the drug. Interestingly, several trials have been performed in the past with various formulations and dosages of PF-05 180 999 to treat migraine. We note that the trial in which the highest dosage (360 mg) was tested was discontinued for safety issues, nevertheless former trials using lower doses (30 or 120 mg) were completed but results are yet to be published (https://clinicaltrials.gov; #NCT01429740 and #NCT01981499). These studies are however encouraging for future therapeutic intervention for central nervous system (CNS) disorders. Indeed, a phase 1 study has been conducted to investigate the pharmacological properties of TAK915 (another PDE2A inhibitor from Takeda pharmaceuticals (Nakashima et al. 2018)) in order to guide dosage in future clinical studies in schizophrenia (https://clinicaltrials.gov/ct2/show/results/NCT02584569). We would suggest that TAK915 may also be used to investigate other CNS disorders in the future.

Our data clearly highlight that PDE2A abundance has a pivotal role in the physiopathology of FXS (Maurin et al. 2018). While cGMP metabolism had never been studied in FXS, it has already been proposed that the convergence of altered pathways in FXS neurons is responsible for an altered abundance of cAMP in this syndrome. All these pathways are mostly postsynaptic signaling cascades and their relevance for FXS up to date was only studied in adult *Fmr1*-KO mice (Choi et al. 2016; Sethna et al. 2017). Inhibition of *Pde4D* was recently shown to have positive effects on LTD, learning and memory in adult *Fmr1*-KO mice (Gurney et al. 2017). These results provide evidence for the crucial role of cAMP abundance in synaptic plasticity in *Fmr1*-KO mice. However, the PDE4 enzyme family modulates cAMP but not cGMP levels and does not appear to be directly involved in the pathophysiology of FXS since its expression is not deregulated in the synaptosomal preparations obtained from both young and adult *Fmr1*-KO mice (Tang et al. 2015). Furthermore, *Pde4D* mRNA is not a prominent FMRP target in all the CLIP assays that have been performed so far (Darnell et al. 2011; Ascano et al. 2012; Tabet et al. 2016; Maurin et al. 2018).

Also, up to now, most of the treatments proposed for FXS have been tested in adult mice and, even when successful, their translation to the clinic failed (Budimirovic et al. 2017; Castagnola et al. 2017; Erickson et al. 2017). Considering these unsuccessful results, an increasing amount of data suggests the need to treat patients affected by neurodevelopmental disorders at the earliest possible age (Khalfallah et al. 2017 for review, and this study) and for a long period of time (Erickson et al. 2017). Remarkably, BAY607550 treatment rescues the communication deficits in both *Fmr1*-KO mice and rats. This strongly argues in favor of a conserved contribution of PDE2A activity in the regulation of processes and/or communication circuits underpinning social behaviors. In the same direction, our findings showing the rescue of social interaction after 9 days of washout suggest that the chronic treatment performed in infancy of these mice was sufficient to modify circuits for an extended time-period. Even if these processes should be investigated in depth in the near future, overall these findings further reinforce the translation potential of this targeted therapeutic approach for FXS.

Conclusions

PDE2A is an overlooked phosphodiesterase previously linked to cognitive processes (Boess et al. 2004; Redrobe et al. 2014; Mikami et al. 2017; Nakashima et al. 2018). Here, for the first time, we establish a relationship between its altered expression and defects in axonal growth, maturation of dendritic spines, mGluR-dependent hippocampal LTD and altered social communication, social discrimination and social interaction behaviors at early developmental ages in *Fmr1*-KO animals. FXS is the leading inherited cause of ID and ASD and the *Fmr1*-KO mouse and rat models are not only widely recognized animal models of FXS but also genetic models of ASD. Since we highlight here that PDE2A abundance has a pivotal role in the pathophysiology of FXS, an implication of PDE2A in other forms of autism can be hypothesized and therefore, targeting PDE2A could be considered a generalized pharmacological target to treat social deficits common to both ASD and FXS.

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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Author's Contribution

Li.C. and E.M. performed biosensor cAMP experiments under the guidance of P.V. T.M. performed cAMP and cGMP measures in cultured neurons as described in M&M. La.C. and L.S. peformed LTD studies under the supervision of Lu.C. S.C. and M.D. performed cortical neuronal cultures under the guidance of T.M. and B.B. S.C. and M.J. performed RT-qPCR studies under the guidance of B.B. A.K. and G.P. performed dendritic spines analysis under the guidance of S.M. M.J. performed axonal studies under the guidance of T.M. and B.B. M.J., S.D., F.M., M.S., and S.S. performed behavioral studies under the supervision of V.T. M.J., S.D., and T.M. performed Golgi staining under the supervision of B.B. A.D.G. synthetized Lu AF64280 under the guidance of S.A. T.M. and B.B. designed the study, T.M., V.T., L.C., P.V., S.M., and B.B. analyzed the data and wrote the article.

Notes

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Chapter 7

Concluding remarks

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Concluding remarks

The research activity realized during my PhD, performed in rodent models, provided new and important information about ASD. In particular, my first studies clarified the role of environmental and the genetic factors in ASD, using different preclinical models mimicking core and associated autistic-like features. Next, I studied the involvement of PDE2A in the altered behavioral phenotype displayed by two genetic animal models of ASD, in order to test new potential drug targets for ASD.

To date effective targeted therapies for ASD are still lacking and, despite the recent advances in our understanding of its pathophysiology, only off-label medications are used to mitigate some of the symptoms.

Preclinical studies give us the opportunity to increase our understanding of pathophysiological aspects of neuropsychiatric disorders and allow us to test the validity of potential drug targets. In particular, animal models are excellent translational research tools in ASD research, for several reasons: they are able to mimic the specific symptoms of the disease, they allow to understand the role of both genetic and environmental factors possibly involved in ASD and their potential interactions and they allow to validate new therapeutic targets. At the same time, given the multifactorial etiology of ASD and the great phenotypic variation in each core symptom domain, there is no animal model that can capture, at once, all the molecular, cellular, and behavioral features of ASD. Rather, as ASD is defined by specific behavioral characteristics, a useful approach in ASD research is to focus on animal behaviors that are relevant to the core diagnostic symptoms of the disease.

The involvement of both genetic and environmental factors (such us maternal treatment with drugs, maternal infections and pathologies, or exposure to toxicants), and their potential interactions in the onset of ASD has been already documented in previous studies (Chaste and Leboyer, 2012; Hertz-Picciotto et al., 2006; Karimi et al., 2017; London, 2000; Matsuzaki et al., 2012; Tordjman et al., 2014). My research extended these previous findings. In particular, my first studies were performed to clarify the association between the effects of different environmental factors during pregnancy and the higher incidence of ASD in the offspring. Since maternal thyroid deficiency has been associated with a higher incidence of neurodevelopmental disorders in the newborns but the relationship between maternal hypothyroidism and the onset of ASD in the offspring is not still clear, I used a validated animal model of prenatal hypothyroidism based on MMI administration to rat dams to

investigate whether maternal hypothyroidism induced in rats behavioral features that resemble some of the core and associate symptoms displayed by ASD patients. This first study showed that prenatal MMI-induced hypothyroidism did not cause in the rat offspring behaviors that resemble core and associated ASD symptoms. My results indicate that MMI-exposed pups did not show communicative deficits during infancy: we found no significant differences, neither quantitative nor qualitative, in the USVs emitted by male and females MMI-exposed and CTRL pups when isolated from the nest. Furthermore, MMI-exposed pups were able to use olfactory cues to discriminate between a neutral odor and their own home cage odor in the homing behavior test, showing intact social discrimination abilities in the homing behavior test. The normal USV profile and homing behavior displayed by MMI-exposed pups indicate their lack of deficits in social communication and social discrimination since the first days of life. ASD patients show marked deficits in social interaction, including lack or atypical social play behavior (American Psychiatric Association, 2013). Social play has a crucial role in the identification and diagnosis of ASD (Jordan, 2003). We tested MMI-exposed adolescent rats in a social encounter with a same-age social partner in order to investigate whether maternal hypothyroidism affected the social play behavior of the offspring. MMI-exposed male rats followed their play partner more and they showed a slight increase in play solicitation compared to CTRL animals. At the same time the two experimental groups did not differ in their response to play solicitation, neither in the total time they spent in non playful forms of social interaction. Both CTRL and MMI-exposed animals were able to discriminate a new versus a familiar object during the test trial of the object recognition test. However, the time spent exploring the novel rather than the familiar object was higher in MMI-exposed than CTRL rats. At the same time, prenatal exposure to MMI did not induce an anxiogenic-like profile in adolescent rats tested in the elevated plus maze test. Collectively, these results show that prenatal hypothyroidism induced by MMI administration to pregnant rats did not induce in the offspring behaviors that resemble some of the core and associated ASD symptoms.

Next, I investigated the relationship between maternal use of the anticonvulsant drug VPA during pregnancy and the risk of ASD in the exposed offspring. To this aim, I used a preclinical rodent model of ASD based on the prenatal VPA exposure. VPA is a widely used and effective antiepileptic and mood stabilizer drug. It is also a histone deacetylase inhibitor that counteracts key steps in the cellular response to DNA DSB formation by affecting checkpoint activation, DSB repair, and stability of crucial enzymes involved in resection of DNA ends (Shubassi et al., 2012). When given during gestation, VPA not only increases the risk for various congenital malformations (Kini et al., 2006; Kozma, 2001), but also induces

autistic-like features in the offspring (Williams et al., 2001; Williams and Hersh, 1997). Based on the clinical observations, prenatal VPA exposure is included among the environmental etiological factors of ASD and prenatal VPA exposure in rodents is a widely used preclinical model of ASD (Ranger and Ellenbroek, 2016; Roullet et al., 2013): I aimed at testing the effects of increasing doses of VPA on core and secondary autistic-like features in the rat offspring, and at investigating the relation between VPA-induced DNA damage and autistic-like features, which was still unclear.

At the behavioral level, I found that VPA dose-dependently induced deficits in social communication and social discrimination in the rat infant offspring. Prenatal VPA, administered at the doses of 400 and 500 mg/kg, altered the emission of USVs at PND9. In the homing behavior test, only 500 mg/kg VPA induced deficits in social discrimination, altering the ability of the pups to discriminate between a neutral odor and their home cage odor as previously reported in studies that used both the 600 (Degroote et al., 2014; Roullet et al., 2013; Schneider and Przewlocki, 2005) and 500 (Felix-Ortiz and Febo, 2012; Servadio et al., 2016) mg/kg doses. The altered USV profile and homing behavior displayed by pups exposed to 500 mg/kg VPA indicate a reduced ability to communicate with their mother and deficits in social recognition. At adolescence, only 500 mg/kg VPA affected the social behavior and emotional reactivity of the offspring. In line with the social deficits reported in previous studies performed with higher doses of VPA (Felix-Ortiz and Febo, 2012; Schneider and Przewlocki, 2005; Schneider et al., 2006), adolescent rats prenatally exposed to 500 mg/kg VPA showed decreased responsiveness to play solicitation. The social deficits induced by prenatal exposure to 500 mg/kg VPA were long-lasting: adult rats exposed to this dose of VPA showed a decreased mean duration of social exploration, which is in line with previous findings reporting social fear and social avoidance in VPA-exposed adult rodents (Baronio et al., 2015; Kataoka et al., 2013; Markram et al., 2008). Rats exposed to 500 mg/kg VPA showed an anxious phenotype in the elevated plus-maze test, both during adolescence and adulthood, as previously reported (Ellenbroek et al., 2016; Kumar et al., 2015; Markram et al., 2008; Schneider et al., 2008; Schneider et al., 2006). The biochemical experiments showed that maternal exposure to VPA at the dose of 500 mg/kg (the dose that induced core and associated autistic-like features in the rat offspring) induced DSB in embryos, as indicated by the significant increase in γ-H2AX and 53BP1 expression compared to SAL-treated embryos. These results are in line with previous findings showing that maternal exposure to VPA produced a rapid increase of γ-H2AX in Chinese hamster ovary cells (Sha and Winn, 2010) and in mouse embryos (Tung and Winn, 2011b). The induction of γ-H2AX and 53BP1 in

VPA-treated embryos was sustained by the activation of the three PI3K (i.e., ATM, ATR, and DNA-PK), which often act by redundant mechanisms, and by the phosphorylation of well-known ATM substrates (i.e., NBN, BRCA1, CHK2, and p53) (Shiloh and Ziv, 2013). My results showed that, despite the activation of the DSB response machinery, the repair capacity was impaired in VPA-treated embryos, as demonstrated by the low levels of expression of the DSB repair protein RAD51. Interestingly, the defective DSB response was no more visible in VPA-exposed rats at PND1. This supports the notion that, in addition to the VPA-induced down-regulation of HDACs, VPA-induced DNA damage may contribute to developmental defects in embryos (Tung and Winn, 2011b; Wells et al., 2009), particularly at the level of the neural tube (Tung and Winn, 2011a).

Collectively, the behavioral experiments showed that VPA, administered to pregnant rats at the dose of 500 mg/kg, induced core and associated autistic-like symptoms in the exposed offspring, in the absence of signs of toxicity. VPA was ineffective or only partially effective in inducing autistic-like behavioral features when administered at the doses of 350 and 400 mg/kg, respectively. In addition, the biochemical experiments helped to understand the neurodevelopmental trajectories that are affected by prenatal VPA exposure: we identified the formation of DBS and their impaired repair as a biochemical substrate underlying the link between VPA exposure during gestation and ASD.

During the second part of my PhD project, I used two genetic models of ASD, i.e., rats and mice knock-out for the *FMR1* gene. These animals are rodent models of FXS, the most common monogenetic cause of ASD. The recent study conducted by Maurin and colleagues showed that the cAMP/cGMP pathway is one of the most prominent deregulated pathways in the *Fmr1*-KO mouse brain and that the *Pde2a* mRNA is one prominent target of FMRP: in the absence of FMRP the level of PDE2A is elevated both in cortex and hippocampus and the level of cAMP and cGMP is reduced in those brain areas of *Fmr1*-KO mice. On the basis of these findings, in collaboration with Dr. Barbara Bardoni from the Institut de Pharmacologie Moléculaire et Cellulaire in Valbonne, France, my experiments tested whether manipulation of cAMP and cGMP signaling pathway through pharmacological inhibition of PDE2A was able to restore the altered phenotype displayed by *Fmr1*-KO mice and rats. In particular, we tested the ability of the selective inhibitor of PDE2A called BAY607550 to rescue the cellular and behavioral alterations displayed by *Fmr1*-KO mice and rats.

Since an important fraction of PDE2A is localized at the synapse (Maurin et al., 2018; Russwurm et al., 2009), we reasoned that its elevated activity in FXS neurons could impact local cAMP and cGMP homeostasis. We demonstrated that the increased activity of PDE2A

results into decreased cAMP and cGMP levels. It has been reported that the levels of both cAMP and cGMP have critical roles in axon elongation and guidance (Akiyama et al., 2016; Shelly et al., 2010) and in regulating the morphology and growth of dendritic spines (Dityatev and El-Husseini, 2011). Indeed, *Fmr1*-KO neurons show an increased PDE2A activity as well as an increased density of spines compared to WT neurons. We demonstrated that the normal length of growing axons and dendritic spine morphology in cultured cortical neurons were restore after treatment with the specific PDE2A inhibitor BAY607550. We also demonstrated that e pharmacological inhibition of PDE2A with the specific inhibitor BAY607550 rescued the exaggerated mGluR-dependent LTD in *Fmr1*-KO hippocampal slices, a well-characterized hallmark of *Fmr1*-KO brain (Huber et al., 2002). Since cGMP and cAMP are involved in axonal growth, spine maturation and synaptic plasticity and PDE2A modulates the level of both cAMP and cGMP, we hypothesized that its elevated activity in the absence of FMRP contributes to definition of neuronal FXS phenotype characterized by altered dendritic morphology, altered axonal length and exaggerated mGluR-LTD.

At the behavioral level, I found that *Fmr1*-KO mice showed deficits in both social communication and social discrimination during infancy, as revealed by their altered USV profile and impaired homing behavior: *Fmr1*-KO pups displayed early communicative deficits, since they vocalized significantly less compared to WT pups when separated from the dam and siblings and they showed early deficits in social discrimination, since they were unable to use olfactory cues to discriminate between a neutral odor and their own cage odor in the homing behavior test. Furthermore we demonstrated that, compared to WT animals, adolescent *Fmr1*-KO mice showed reduced social interaction in the social interaction test. Our results showed that inhibition of PDE2A activity through intraperitoneal administration of BAY607550 normalized the altered USV profile displayed by *Fmr1*-KO mice. We also validated this result by inhibiting PDE2A with Lu AF64280, another highly specific PDE2A inhibitor (Redrobe et al., 2014). Similar to BAY607550, Lu AF64280 was able to revert the altered USV frequency displayed by *Fmr1*-KO mice. This confirms that PDE2A blockade is able to rescue the communicative deficit displayed by *Fmr1*-KO mice in the USV test. Furthermore, we found that treatment with BAY607550 improved the performance of *Fmr1*-KO pups in the homing behavior test. The altered social profile displayed by *Fmr1*-KO adolescent mice was rescued by PDE2A inhibition after acute administration of BAY607550. Furthermore, we chronically treated *Fmr1*-KO mice with BAY607550 from PND5 to PND21, and we tested their social abilities after a wash-out interval of nine days. Early chronic treatment with BAY607550 reversed the social deficits displayed by *Fmr1*-KO mice at

PND30, showing that the beneficial effects of early PDE2A pharmacological blockade are long-lasting. We also found that the administration of BAY607550 had no effect on the behavior of WT mice, further indicating the specificity of this treatment for the FXS phenotype. We extended the behavioral analysis to *Fmr1*-KO infant rats, in order to validate PDE2A as a therapeutic target for FXS. Similar to *Fmr1*-KO mice, *Fmr1*-KO rats vocalized less than WT controls when separated from their mother and siblings at PND5 and PND9. Remarkably, acute administration of BAY607550 also normalized their altered USV pattern without affecting the behavior of WT control animals. Overall these findings further reinforce the translation potential of this targeted therapeutic approach for FXS.

In conclusion, the research activity performed during my PhD increased our knowledge about pathogenetic and neurochemical aspects of ASD, and provided hints for new therapeutic opportunities. I clarified the link between environmental factors, such as maternal hypothyroidism or VPA use during pregnancy, and higher ASD risk in the offspring. While maternal hypothyroidism did not induce autistic-like traits in the rat offspring, maternal VPA exposure did. A more in-depth study revealed that DNA damage and impaired repair may be responsible of the autistic-like traits displayed by the VPA-exposed offspring. My studies also identified an interesting pharmacological target for ASD. Indeed, we established a relationship between PDE2A altered expression and defects in axonal growth, maturation of dendritic spines, mGluR-dependent hippocampal LTD and altered social communication, social discrimination and social interaction behaviors at early developmental ages in *Fmr1*-KO animals. Since we highlight that PDE2A abundance has a pivotal role in the pathophysiology of FXS, an implication of PDE2A in other forms of autism can be hypothesized and therefore, targeting PDE2A could be considered a generalized pharmacological target to treat social deficits common to both ASD and FXS. However, my research also opened new questions, that will be addressed in follow up studies. Regarding the first model I studied based on maternal hypothyroidism, since the critical window during which hypothyroidism may affect brain development includes the early postnatal period in humans (DeLong, 1993; Kooistra et al., 1994; Raiti and Newns, 1971) and the third week postnatal in rats (Nathaniel et al., 1988; Oppenheimer and Schwartz, 1997; Weller et al., 1996), further experiments are needed to determine whether hypothyroidism during both the prenatal and early postnatal period has a causative role in the onset of neurological diseases, including ASD. Given the complexity of ASD and the related impossibility to represent all its facets in a single animal model, it is necessary to confirm my findings about the positive effects of PDE2A inhibition in other animal models of ASD. Indeed, ongoing experiments in

my lab are testing whether PDE2A inhibition is also able to revert the behavioral deficits displayed by VPA exposed rats. Furthermore, since FXS is characterized by a progressive decline of cognitive abilities, the effect of PDE2A inhibition should be evaluated in behavioral tests specifically designed to assess the cognitive performance of *Fmr1*-KO animals.

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LIST OF PUBLICATIONS

List of accepted publications

Melancia F., Servadio M., Schiavi S., Campolongo P., Giusti-Paiva A., Trezza V. (2017). Testing the correlation between experimentally-induced hypothyroidism during pregnancy and autisticlike symptoms in the rat offspring. *Behav Brain Res*. 321:113-122

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