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Motor determinants of autism spectrum disorder (ASD)

Tareq Al Sagheer

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THÈSE

Pour l'obtention du grade de
DOCTEUR DE L'UNIVERSITÉ DE POITIERS
UFR des sciences fondamentales et appliquées
Laboratoire de neurosciences expérimentales et cliniques - LNEC (Poitiers)
(Diplôme National - Arrêté du 25 mai 2016)

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Secteur de recherche : Neurosciences

Présentée par :
Tareq Mohammad Al Sagheer

Déterminants moteurs des troubles du spectre autistiques (TSA)

Directeur(s) de Thèse :
Mohamed Jaber

Soutenue le 18 décembre 2017 devant le jury

Jury :

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THESE

Pour l'obtention du Grade de

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Faculté des Sciences Fondamentales et Appliquées)
(Diplôme National - Arrêté du 25 mai 2016)

Ecole Doctorale : Biosanté n°524
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**Déterminants Moteurs des Troubles du Spectre
Autistiques (TSA)**

**Motor Determinants of Autism Spectrum Disorder
(ASD)**

Directeur de Thèse : Mohamed JABER

Inserm UMR_S 1084

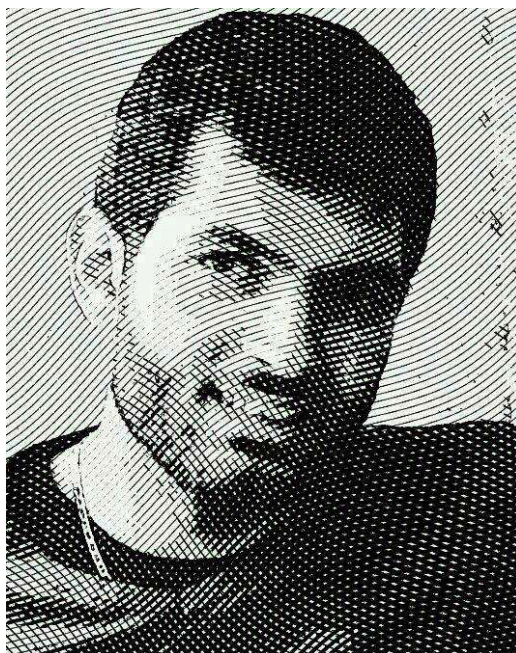
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Soutenue le 18 décembre 2017

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*To my Parents,
To Kassem,*



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Part of the work presented in this thesis is currently under submission:

Article 1: *Motor behavior disturbances are correlated with social impairments and cerebellar and cortical cell loss in an environmental animal model of autism.* **AL SAGHEER T***, HAIDA O*, BALBOUS A, FRANCHETEAU M, MATAS E, FERNAGUT PO, JABER M.

Parts of the work presented in this thesis are in preparation:

Article 2: *Motor behavior and their anatomical correlates in Poly I:C mouse model of Autism.* HAIDA O*, **AL SAGHEER T***, BALBOUS A, FRANCHETEAU M, MATAS E, FERNAGUT PO, JABER M.

Article 3: *Molecular biochemical pathways implicated in the development of autism spectrum disorders.* MATAS E*, BALBOUS A*, **AL SAGHEER T**, HAIDA O, FRANCHETEAU M, FERNAGUT PO, JABER M.

** contributed equally*

Some of these resulted have been presented in poster sessions of various scientific meetings:

- «Motor determinants of Autism Spectrum Disorders» NeuroFrance International meeting, Bordeaux (France) May 2017. Poster Presentation: P1. 164.
- «Motor determinants of Autism Spectrum Disorders» SFR Tours-Poitiers meeting, Poitiers (France) June 2017. Poster Presentation: P.31.
- «Motor behavior alterations in animal models of Autism Spectrum Disorders» SFR Tours-Poitiers meeting, Tours (France) May 2016. Poster Presentation.

- «Motor Behavior in Mice Models of ASD and Underlying Cellular and Molecular Mechanisms» 10'th FENS forum of Neuroscience, Copenhagen (Denmark) July 2016. Poster Presentation: C-058.

I also presented my results in two of the doctoral school colloquiums:

1. "Séminaires thématiques: Genomics and Beyond". Pouligny Notre-Dame February 2015. Poster presentation.
2. "Séminaires thématiques: Médecine Personnalisée". Pouligny Notre-Dame March 2016. Oral communication.

I also had the opportunity to present this work in an oral communication at the scientific day SFR Tours-Poitiers. The talk entitled: «Motor behavior alterations in animal models of Autism Spectrum Disorders» SFR Tours-Poitiers meeting, Poitiers (France) June 2017 Oral Presentation.

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ABBREVIATIONS

- 5'UTR** 5' gene untranslated region
ABC Aberrant Behavior Checklist
ADHD Attention Deficit Hyperactivity Disorder
AMPA α -amino-3-hydroxy-5-methylisoxazol-4-propionate
ANOVA analysis of variance
APA American Psychiatric Association
ASD Autism Spectrum Disorder
BOT Bruininks-Oseretsky Test of motor proficiency
CDC Centers for Disease Control and Prevention (USA)
CNS Central Nervous System
CNV Copy Number Variants
CR Conditioned response
CROIs Cytogenetic Regions of Interest
CS Conditioned stimulus
DSM the American Diagnostic and Statistical Manual
E Embryonic day
FMRP fragile X mental retardation protein
FXS Fragile X syndrome
G0 gestational day zero
GABA Acide γ -aminobutyrique
GTP Guanosine triphosphate
I.P. Intraperitoneal
I.V. Intravenous
ICD 10 The International Classification of Diseases, tenth edition
ID Intellectual Disability
IQ Intelligence Quotient
KO Knock-out
LA Locomotor Activity
LTD Long-Term Depression
LTP Long-term potentiation
M-ABC The Movement Assessment Battery for Children
MeCP2 Methyl CpG binding protein 2
MIA Maternal Immune Activation
MRI Magnetic Resonance Imaging
MRN the magnocellular part of the red nucleus
mToRC mammalian target of rapamycin complex
NETBAG NETwork-Based Analysis of Genetic associations
NF1 Neurofibromatosis type 1
NLGN Neuroligins
NMDA Acide N-méthyl-D-aspartate
NRXN Neuroxins
NSC Non-Social Chamber
P Postnatal day
PC Purkinje cells
PI3K Kinase phosphoinositide-3
PMS Phelan Mc-Dermid syndrome
Poly I:C l'acide polyinosinique-polycytidylique/ polyinosinic-polycytidylic acid
PRN the parvocellular part of the red nucleus
PSD Postsynaptic density
Pten Phosphatase and tensin homolog

RBS-R Repetitive Behavior Scale Revised
ROS Reactive Oxygen Species
RTT Rett syndrome
S.C. subcutaneous
SC Social Chamber
SEM Standard Error to the Mean
Sert serotonin transporter
SHANK SH3 and multiple ankyrin repeat domains protein
SI Sociability Index
SSS Stereotypy Severity Scale
TLR Toll Like Receptor
TS Timothy Syndrome
TSA Les troubles du spectre de l'autisme
TSC Tuberous sclerosis complex
VPA l'acide valproïque/ valproic acid
VPL ventro-lateral nucleus of the thalamus
WII home video game console released by Nintendo (Japan)

Résumé

Les troubles du spectre de l'autisme (TSA) sont des troubles neurodéveloppementaux associés à des déficits persistants dans la communication sociale et à des comportements répétitifs. Notre travail est basé sur l'exploration des troubles moteurs associés à cette pathologie avec comme hypothèse de travail la possibilité que cette approche puisse offrir des éléments de diagnostic précoce. Pour cela, nous avons utilisé deux modèles animaux de toxicité prénatale (E12,5) chez la souris suite à l'administration d'un agent antiépileptique, l'acide valproïque (VPA), ou d'un analogue synthétique de l'ARN double brin viral l'acide polyinosinique-polycytidylique (Poly I:C). Nous avons réalisé une évaluation générale du développement post-natal et une exploration précise de la sociabilité et du comportement moteur fin. Nos résultats montrent: 1) un dysfonctionnement moteur et sensorimoteur précoce dans le modèle VPA, indiquant que ce modèle est approprié pour étudier la contribution des altérations motrices à l'étiologie des TSA; 2) des effets différentiels sur la sociabilité en fonction du sexe ; 3) la présence de déficits moteurs mais pas de déficits sociaux chez les femelles. Nos résultats suggèrent que le comportement moteur peut offrir un outil de diagnostic précoce et quantitatif des TSA. Ce travail ouvre la voie vers des explorations cliniques de ces déficits et potentiellement vers des pistes thérapeutiques ciblées vers le cervelet.

Mot clés : *Troubles du Spectre de l'Autisme ; Troubles du Comportement; Cervelet; Acide Valproïque ; Ataxie.*

Abstract

Autism spectrum disorders (ASD) are neurodevelopmental disorders associated with persistent disturbances in social communication and repetitive pattern of behavior. Our work is based on the exploration of the motor disorders associated with this pathology, with a working hypothesis that this approach can offer elements of early diagnosis. For this, we used two animal models of prenatal toxicity (E12.5) in mice following administration of an antiepileptic agent, valproic acid (VPA) or a synthetic analogue of the double-stranded viral RNA polyinosinic-polycytidylic acid (Poly I:C). We carried out a general evaluation of postnatal development and a detailed exploration of sociability and fine motor behavior. Our results show: 1) an early motor and sensorimotor deficits in the VPA model, indicating that this model is appropriate for studying the contribution of motor impairments to the etiology of ASD; 2) differential effects on sociability with regards to sexes; 3) the presence of motor deficits but not of social deficits in females. Our results suggest motor behavior can offer an early and quantitative diagnostic tool for ASD. They pave the way to clinical explorations of these deficits and potentially to therapeutic pathways targeted towards the cerebellum.

Key words: *Autism spectrum disorders; Behavioral disorders; Cerebellum; Valproic Acid; Ataxia*

Part I. INTRODUCTION

The social and cognitive approaches have dominated the research of Autism Spectrum Disorder (ASD), ignoring the motor deficits although movement atypies, coordination deficits, and disturbed gait were reported (Gowen & Hamilton, 2013). Deficits in motor skills in patients with ASD were reported in earliest clinical descriptions of autism by Leo Kanner who described early delays in motor development, including clumsy gait and gross motor performance (Kanner, 1943). Moreover, Hans Asperger reported motor clumsiness and underdeveloped gross and fine motor skills in all cases he observed (Asperger, 1944). Increasing interest in motor deficits and their interplay with cognitive and social functions has recently been addressed. Several clinical reports suggested that motor deficits can precede or intertwine with higher cognitive deficits in ASD (Leary et al., 1996). The cerebellum has long been known to be a critical structure for the coordination and control of movement. However, both recent and early evidence indicate that the cerebellum also plays a role in cognitive and emotional functions (see Schmahmann, 1997 for a comprehensive review of early work). A growing number of evidence indicates a major role of cerebellar dysfunction in the development of abnormalities seen in ASD (D'Mello, 2015). Following an initial case report (Bauman et al., 1985), the cerebellum is now admittedly the most abnormal brain region consistently found in ASD. Indeed, a significant decrease in the number of Purkinje cells (PC) was reported in almost all of the postmortem brains of ASD individuals studied to date, (Arin et al., 1991; Bailey et al., 1998); this loss is found primarily in the posterolateral neocerebellar cortex and adjacent archicerebellar cortex of the cerebellar hemispheres. The striatum and the cerebellum influence multiple aspects of motor, cognitive and affective behavior (Graybiel, 1998; Schmahmann, 1997). Both structures are densely interconnected with the cerebral cortex. The cerebellum projects to the striatum through the thalamus (Ichinohe et al., 2000; Hoshi et al., 2005). Thus, these structures, along with the motor cortex seem as appropriate targets to investigate in this disorder.

Advances in neuroimaging and postmortem studies have provided crucial indications for the autistic brain phenotype. Nevertheless, it is difficult in these cases to distinguish between

causes and consequences of the pathology, and these studies often offer only a vague perspective of the pathology. In addition, observations can only be made late (after diagnosis or after the death of the patient), whereas ASD is a neurodevelopmental pathology and a study of the trajectory of the disorder is a building block to better manage the symptoms as early as possible. Finally, postmortem brains are difficult to obtain, slowing discoveries on the subject.

Because of the existence of differences between mice and humans, whether from the genetic background, the complexity of regulation of genes and proteins, neural development or brain composition, the animal model necessarily brings a bias to the understanding of the pathophysiology of ASD in humans and the discovery of new drugs. In addition, autism is a very complex pathology, with specific human behaviors, making animal modeling difficult. Thus, using animal models, it is essential to develop new screening questions that can address quantitative behavior that can be easily translated to human population of ASD. Moreover, these models should meet the criteria of validity. Behavior is translated from activity and action, the later demands skilled abilities, including motor development, spontaneous activity, motor control, coordination and balance of the body, and learning. Models of ASD include inbred rodent strains that mimic ASD behaviors as well as models obtained following environmental insults such as prenatal exposure to sodium valproate, maternal autoantibodies, and maternal immune activation (MIA). To test the physiological disturbances underlying motor symptoms in animal models of ASD we chose two of the most widely used environmental risk factors of ASD in line with human findings. Prenatal exposure to VPA and poly I:C as our model.

Exposure to uterine infections increased the risk for ASD. Many studies have reported that MIA plays an important role in the development of ASD (Dufour-Rainfry et al., 2011; Kinney et al., 2008; Newschaffer et al., 2007). Numerous clinical studies suggest a correlation between immune activation and incidence of giving birth to a child with ASD. Recent studies modeling MIA have reported that fetal infections during early development cause alteration in behavior in the postnatal life (Hsiao and Patterson, 2011; Patterson, 2009; Smith et al., 2007).

Several lines of evidence support the fact that environmental risk factors are directly implicated in the etiology of ASD. Prenatal exposure to VPA, a molecule used to treat epilepsy in the first trimester of pregnancy is a known risk factor of ASD (see review: Nicolini & Fahnestock, 2017). The VPA mouse model of autism has been previously described and proven to be a model that shows face, construct, and predictive validity. These two models have been studied employing the “cognitive approach”; new approaches and alternative hypotheses on the etiology are needed, especially that many recent clinical reports are demonstrating persistent “motor symptoms” of ASD. These phenotypes, that often appear early even before the development of the core deficits in sociability, communication and repetitive behavior, are:

1. Quantitative.
2. Easily modeled in animal models.
3. Feasibly assessed in animals by validated and standardized gross and fine motor tests.
4. Related to brain structures that are well identified and that can be studied in animals.

Hypothesis: *the identification of motor signatures associated with ASD open the door on early diagnosis and better therapeutic interventions in ASD. Indeed, the characterization of these phenotypic elements will improve our understanding of the neurobiological elements of ASD and this should be targeted by studying the cerebellar dysfunctions and the contribution of other brain motor systems such as the striatum and the motor cortex.*

Our aims in this study were to:

- 1- Establish non-genetic animal models of ASD.
- 2- Determine social and cognitive deficits in VPA and poly I:C mouse models of ASD.
- 3- Unpin fine and early motor disturbances in these models.
- 4- Elucidate sex differences in mouse models of ASD (high male prevalence in clinical settings).

Chapter 1: Autism Spectrum Disorder

The term ASD refers to a group of neurodevelopmental disorders defined by the presence of anomalies in three areas: (i) social interaction, communication or use verbal and non-verbal language and a mode of behavior, as well as (ii) stereotyped, limited or repetitive interests and activities (American Psychiatric Association, 2013). Symptoms generally become evident before the age of three, but in most regions of the world, these disorders are diagnosed only a few years later. ASD is currently not only a public health major disease but also a challenge for the scientific community because of: (i) the increasing recognition of these disorders, (ii) the emotional impact they have on families, and (iii) the treatment and daily support required. Current treatments, being behavioral or pharmacological, do provide a better quality of life to patients and in a greater extent to what was available a quarter of a decade from now. However, it should be noted that ASD cannot yet be cured and that most patients with ASD do not receive specialized treatment.

This chapter attempts to describe current knowledge in the classification, epidemiology, etiology, clinical evaluation, and the prognosis of ASD.

1.1 Historical background

Eugen Bleuler (1857-1939), a Swiss psychiatrist, introduced the terms *Schizophrenia* and *Autism*. He derived the second term from the Greek word *autos* (meaning "self") to describe the state of withdrawal of schizophrenic patients in their own fantasized life as an effort to confront intolerable external experiences and perceptions (Kuhn, 2004). The use of the term *Autism* in its present meaning began 30 years later when the austrian pediatrician Hans Asperger adopted Bleuler's terminology of autistic psychopaths in his lecture course at the University Hospital of Vienna (Asperger, 1938). Asperger published his second doctoral thesis in 1944 (the first transcription of 1943) in which he described a group of children and adolescents suffering from deficits in communication and social skills, as well as having a pattern of repetitive and restrictive behavior (Asperger, 1944). At the same period, in 1943 (apparently unaware of each

other's' work, separated by distance, and World War II) Leo Kanner, at the Johns Hopkins University Hospital in the United States, described in his famous paper " Autistic disturbances of affective contact ", 11 children with similar behaviors to those depicted by Asperger. Most of the characteristics described by Kanner, such as "autistic detachment" and "sameness", are still among the criteria used to diagnose autism in modern classifications (Kanner, 1943). The children described by Asperger differed from those of Kanner in the fact that they had no noticeable delays in cognitive development or in language.

The Asperger paper remained largely unknown until Uta Frith translated it into English (Asperger, 1944) and thus became available. Rutter's later modification of the disease's definition presented the notion of deficits in three core areas: impaired social relations, impaired communication skills, and "insistence on sameness" (Rutter et al., 1978). These thoughts were then extrapolated by Lorna Wing (Wing, 1997), and as a result, appeared a progressive awareness that autism was a spectrum with a continuum ranging from mild to severe symptoms and that the disorders presented by Asperger were also of this continuum.

By misfortune, the original term by Bleuler and its theoretical relation with schizophrenia, combined with the dominant psychoanalytic theories in the mid-20th century, contributed to a mix between ASD and psychotic disorders, classifying them under the heading of schizophrenia in childhood. The apparent autistic withdrawal was misinterpreted as being similar to the negative symptoms in schizophrenia, namely defensive withdrawal to counter an intolerable external situation (as it was largely conceptualized).

1.2 Definition

The International Classification of Diseases, tenth edition (ICD 10; World Health Organization, 1990) classifies ASD as pervasive developmental disorders defined as "A group of disorders characterized by qualitative abnormalities in reciprocal social interactions and in patterns of communication, and by a restricted, stereotyped, repetitive repertoire of interests and activities. These qualitative abnormalities are a pervasive feature of the individual's functioning

in all situations”. A revised eleventh edition (ICD-11) is expected in 2018 and is probably going to nearly line up with the most recent version (the fifth) of the American Diagnostic and Statistical Manual (DSM).

The American Psychiatric Association (APA, 2013) defines ASD as follow:

A. Persistent deficits in communication and social interactions in different contexts, manifested by the following three symptoms (present or in the past):

1. Lack of social and emotional reciprocity, ranging from abnormal social status and the lack of alternation in speaking up to the lack of initiation of social response, and the reduction of the sharing of interests, emotions and affects.
2. Failure to use nonverbal communicative behaviors used for social interaction, ranging from poor integration of verbal and non-verbal communication, to complete absence of facial expressions and gestures, eye contact and corporate language or a lack of understanding and use of gestures.
3. Failure to develop, maintain, and understand social relations, ranging from difficulties in adjusting one's behavior to adapt to different contexts to a lack of interest in people, to share imaginative games or make friends.

B. Restricted, repetitive, and stereotyped behavior, interests and activities, as evidenced by at least two of the following (Current or past):

1. Stereotyped or repetitive character of motor movements, use of objects, or language (such as simple motor stereotypies, toy alignments, object swings, echolalia, idiosyncratic phrases).
2. Resistance to change, excessive adhesion to routines, or ritualized verbal or non-verbal behaviors (such as extreme distress in small changes, difficulty with transitions, rigid thought patterns, greeting rituals, insistence on taking the same path or consuming the same food every day).

3. Very limited, fixed interest, with an abnormal character in their intensity or content (such as strong attachment or concern for unusual objects, overly circumscribed or persistent interests).
4. Hyper- or hypo-reactivity to sensory stimuli, or unusual interest in sensory characteristics of the environment (such as apparent indifference to pain, cold or heat, a negative response to specific sounds, textures, or excessive light or touch of objects, visual fascination for lights or movements).

C. Symptoms must be present in early childhood (but may become manifest only when social demands exceed limited capacities, or maybe subsequently masked by learned strategies).

D. Symptoms limit daily socio-professional functioning.

E. These symptoms are not better explained by an intellectual disability (ID, intellectual development disorder) or an overall delay in development. ID and ASD are frequently associated, and for social diagnosis of ASD and ID, social communication must be below the level expected for the overall level of development.

Specified further: with or without ID; with or without language disorder; associated with medical or genetic pathology, or environmental factors; associated with another neuro-developmental, mental, or behavioral disorder; with catatonia.

DSM V (American Psychiatric Association, 2013) also introduced a severity scale to describe the intensity of the disorder (Table I). Thus, the severity of ASD is rated from one to three, depending on the impact of symptoms on the functioning of the person: a “level 3” "ASD" subject requires a high level of support and aid.

Severity level for ASD	Social Communication	Restricted, Repetitive Behaviours
Level 3 Requiring very substantial support	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others.	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.
Level 2 Requiring substantial support	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others.	Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.
Level 1 Requiring support	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful responses to social overtures of others. May appear to have decreased interest in social interactions.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

Table I: Current severity specifiers for Autism Spectrum Disorder (DSM-V).

1.3 Evolution of the symptoms

Classically, ASD symptoms appear between 12 and 24 months. Nevertheless, they may be present during the first year of life if developmental delay is severe. Sometimes subtle symptoms are observed only after the age of 24 months. Many studies are focusing on earlier detection of autism. For example, lack of interest in social interactions can occur during the first 12 months of life. Recently, eye fixation (a marker of deficits in eye contact) has been shown to

be present in infants with ASD in the first few months and then declining between the second and sixth months (Bonnet-Brilhault, 2017; Jones and Klin, 2013).

Language delay is very often the first symptom alerting the entourage and the professionals. This delay in language is often accompanied by a series of associated symptoms: lack of social interest or atypical social interactions (such as taking the hand of others without trying to look at it), atypical playing behaviors (misuse of objects), unusual communication (like knowing the alphabet by heart but not answering his own name). During the second year, repetitive, atypical behaviors and the absence of pretend play are observed in ASD (playing "house" with a pretend kitchen and eating plastic food, turning a box into a fortress or a stuffed animal into a talking playmate). The distinction between typical ritualized behavior of early childhood and stereotyped behavior witnessed in ASD may be difficult before the age of five. Indeed, in young children, this developmental phase of typical pleasure of repetition exists (eating the same meals, watching the same cartoons...). The distinction can then be made on the type, frequency, and intensity of behaviors (such as aligning objects for hours, with intense distress when moving these objects).

Learning and compensation for ASD can continue throughout life. In general, there is an improvement in skills during childhood and adolescence for the majority of patients with ASD. Nevertheless, for a small proportion of subjects, there is a worsening of behavioral disorders during adolescence. Adults with ASD have more difficulty accessing independence. Patients who do not suffer from ID can use compensation and adaptation strategies to mask their difficulties in public. However, they present an increased risk of anxiety and depression compared to the general population. They often remain more naive and socially vulnerable, with difficulties organizing practical tasks (American Psychiatric Association, 2013).

1.3.1 Comorbidity

The two most important mental disorders associated with ASD are intellectual disability (ID) and developmental language disorders (APA, 2013). Comorbid psychiatric disorders with ASD are also common: about 70% of individuals have one comorbid disorder, and 40% have

two or more (Simonoff et al., 2008). The main psychiatric comorbidities of ASD are social anxiety disorder (29%), Attention Deficit Hyperactivity Disorder (ADHD, 28%), depressive disorders, oppositional defiant disorder (28%) (Simonoff et al., 2008).

ASD children, adolescents, and adults often do experience a number of comorbid medical conditions (see review by: Masi et al., 2017). The most common medical conditions of comorbidity are: epilepsy (see review by: Berg and Plioplys, 2012), sleep disorders, gastrointestinal disorders, hormonal dysfunctions, mitochondrial dysfunction (see review by: Bauman, 2010), allergies and immune system abnormalities (Zerbo et al., 2015). Early onset anorexia nervosa disorders are also common in ASD, and restricted feeding patterns can persist (American Psychiatric Association, 2013).

1.3.2 Epidemiology

The prevalence of ASD is currently estimated 1.46% in the general population (Table II), according to Centers for Disease Control and Prevention (CDC, Christensen et al., 2016). This makes ASD a frequent disorder, with a sharp increase since the seventies. It is unclear whether this increase in prevalence reflects an expansion of diagnostic criteria in DSM-V including mild forms of ASD (Maenner et al., 2014; Kim et al., 2014), an increase in awareness of this disease, differences in methodology (Thomas et al., 2012), or a real increase in the frequency of ASD, potentially linked to environmental toxins (Christensen et al., 2013; Sweeten et al., 2004; Chess et al., 1978).

ASD is reported to occur in all racial, ethnic, and socioeconomic groups. It is 4.5 times more common in boys (1 in 42) than in girls (1 in 189) (CDC, Christensen et al., 2016). According to twin studies, girls appear to be more protected from the risk of autism (Robinson et al., 2013), but the reasons for this difference remains unclear. The Extreme Male Brain theory discusses a huge role played by the testosterone in the etiology of ASD (Baron-Cohen, 2002). Interestingly, subsequent works have found increased androstenedion in ASD adults' serum (Ruta et al., 2011). It appears, among other studies, that the manifestations of social and communication difficulties are more subtle among girls. In the clinical population, girls are more

often diagnosed with ID associated with ASD suggesting that those who do not show ID or language delay would be misidentified (American Psychiatric Association, 2013).

Surveillance Year	Birth Year	Number of ADDM sites Reporting	Prevalence per 1000 Children Range	This is about 1 in X children...
2000	1992	6	6.7 4.5-9.9	1 in 150
2002	1994	14	6.6 3.3-9.8	1 in 150
2004	1996	8	8.0 4.6-9.8	1 in 125
2006	1998	11	9.0 4.2-12.1	1 in 110
2008	2000	14	11.3 4.8-21.2	1 in 88
2010	2002	11	14.7 5.7-21.9	<u>1 in 68</u>
2012	2004	11	14.6 8.2-24.6	<u>1 in 68</u>

Table II: Identified prevalence of ASD (CDC).

According to Autism and Developmental Disabilities Monitoring (ADDM) Network 2000- 2012; source Centers for Disease Control and Prevention CDC website (7 may 2017).

Despite current advances in ASD research, the underlying molecular, anatomical, and circuit underpinnings remains incompletely understood. ASD is broadly viewed as turmoil of network between various brain parts including the frontal cortex, striatum, hippocampus and the cerebellum (Di Martino et al., 2014; Fatemi et al., 2012; Bauman and Kemper, 1985).

1.4 Etiology of ASD

1.4.1 Environmental risk factors

The environmental risk factors considered for autism are particularly related to pregnancy, such as maternal infections or prenatal exposures to teratogenic agents or toxins. Some studies have shown that maternal infections such as rubella (Chess et al., 1978; Chess, 1971) or cytomegalovirus (Sweeten et al., 2004; Yamashita et al., 2003) are implicated in the etiology of autism. In addition, hypothyroidism in the untreated mother (Andersen et al., 2014) and alcohol consumption (Stevens et al., 2013) or the use of VPA antiepileptic treatment (Christensen et al., 2013) during pregnancy, are prenatal factors that may be involved in the

development of autism. Other environmental factors potentially associated with autism would be perinatal. In particular, the involvement of premature birth, fetal suffering or hyper-bilirubinemia are suspected (Guinchat et al., 2012). Some metals, particularly zinc and copper, may also produce autistic syndromes, influencing the functioning of the GABAergic system (Bjorklund, 2013). However, there is insufficient evidence to determine whether these risks are causal or simply play a secondary role in the development of autistic symptoms in patients with genetic vulnerability. Finally, the study of epigenetic factors potentially involved in autism is a field in full expansion. Epigenetics describes the factors that form to specific regions of DNA and impact genetic activity. Independent of DNA sequence variations, environmental factors could modulate the genetic factors responsible for the manifestation of ASD in childhood, basically through DNA methylation and alterations in chromatin structure (Henikoff and Matzke, 1997). Observations of the differences in methylation in monozygotic discordant twins' genomes for autism suggest that it may play a role in the etiology of the disease (Wong et al., 2014).

1.4.2 Genetic risk factors

Family studies and twin studies have demonstrated a broad genetic contribution and a high degree of heritability in ASD. In addition, the description of rare monogenic diseases with a typical presentation of autism in their symptoms, as well as forms related to chromosomal rearrangements have also highlighted the contribution of genetic factors in autism. Twin studies have reported for the first time the existence of a genetic contribution to ASD. Indeed, these studies showed that the concordance rate for monozygotic twins was about 90%; and between 10 and 20% for dizygotic twins (Hallmayer et al., 2011; Bailey et al., 1995). Since the concordance rate is theoretically 100% for monozygotic twins and 50% for dizygotic twins, a strong genetic contribution is suggested. Moreover, the discordance rate could be evidence that environmental factors would also influence the development of autism. Many family studies have subsequently been able to support the existence of this strong genetic influence. This is the case, for example, with the study by Ozonoff et al. (2011) which showed that the risk of developing autism was increased by 20 to 50 times for a child with an autistic sibling compared to the general

population. Another study conducted on 5000 families also revealed a genetic contribution by demonstrating that the recurrence risk of ASD among brothers and sisters of an affected child was twice as large as that of half-siblings (Constantino et al., 2013). More recently, screening and association studies, including exome sequencing of the father, mother and affected child, revealed that autism is often due to the appearance of inherited or de novo Copy Number Variants (CNV). These CNV include deletions or duplications of certain regions of genes. De novo CNVs may appear during early zygote development or in germ cells, particularly in elderly fetuses (Kong et al., 2012; Neale et al., 2012). In the case of CNVs, the hypothesis would be that autism is a result of a decrease or increase in expression of one or more susceptibility genes that would reside in the region affected by the CNV mutation. These studies have made it possible to delineate chromosomal regions of susceptibility participating in the development of ASD. CNVs may concern well-defined random zones or zones within certain loci. This is the case for duplications in region 15q11-13, responsible for 2% of cases of ASD and deletions or duplications of the 16p11 region, corresponding to approximately 1% of cases (Vorstman et al., 2006). Interestingly, deletion of the 16p11.2 region is independently associated with autism and macrocephaly, whereas 16p11.2 duplication is related to schizophrenia and microcephaly (Horev et al., 2011). In addition, CNVs in the 16p11.2 region are involved in brain anatomical development, intellectual disability, behavioral changes, hyperactivity, difficulty adapting to change, sleep problems and restrictive and repetitive behaviors. The work of Vorstman et al. (2006), summarized the chromosomal regions involved in ASD and defined "cytogenetic regions of interest" (CROIs) to facilitate the detection of candidate genes. But also revealed new additional loci such as 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3 and Xp22.2-p22.3.

Apart from these loci, CNVs may relate to a single specific gene. In this case, these CNVs lead to very penetrating syndromic developmental disorders, which involve about 7% of cases. This includes Fragile X syndrome (FXS), Tuberous sclerosis complex (TSC), Angelman

syndrome, Timothy Syndrome (TS), neurofibromatosis type 1 (NF1), Rett syndrome (RTT) and Phelan Mc-Dermid syndrome (PMS) (Zoghbi and Bear, 2012). Each mutation is generally responsible for less than 1% of the castotals (see Table III).

Gene	Disease	Prevalence in ASD	Gene function
FMR1	Fragile X Syndrome	2-5%	Repressor
TSC1/2	Tuberous sclerosis	1-4%	mTOR inhibitor
PTEN	Cowden disease and Lhermitte-Duclos disease	1%	P13K/mTOR pathway inhibitor
NF1	Neurofibromatosis Type 1	0-4%	Ras GAP
MECP2	Rett Syndrome	2%	Transcriptional repressor
UBE3A	Angelman Syndrome	1%	Ubiquitine ligaseE3A
CACNA1C	Timothy Syndrome	<1%	alcium channel, voltage-dependent, L type (Ca _v 1.2)
NLGN3/4	ASD	<1%	Synaptic adhesion
NRXN1	ASD	<1%	Synaptic adhesion
SHANK3	ASD and Phelan Mc-Dermid syndrome	>1%	Scaffold protein

Table III: The various monogenic forms of autism with intellectual disabilities.

The prevalence of each disease in ASDhas been reported (adapted from Kelleher III and Bear, 2008).

1.4.3 The role of the synapse in ASD

Despite strong genetic and clinical heterogeneity, genes involved in autism seem to be part of common functional networks. Thus, some common biological pathways have emerged, giving hope to a reduction of the apparent complexity of the mechanisms behind autism. O'Roak et al. (2012) have demonstrated that 40% of de novo mutations identified in their study converge to the protein/beta-catenin/chromatin remodeling interaction network that controls neuronal differentiation and synaptic formation. Iossifov et al. proposed the involvement of genes coding for the proteins associated with the fragile X mental retardation protein (FMRP), thus suggesting

the involvement of synaptic plasticity in autism (Iossifov et al., 2012). In accordance with other studies, Neale et al. also have shown the role of synaptic genes in autism (Neale et al., 2012). However, other genetic studies suggest the involvement of narrower pathways. This is the case of Pinto et al. who have demonstrated an enrichment of the genes involved in the development and function of synapses, the genes of the Ras signaling pathway and in particular the Rho GTPases involved in the regulation of dendrites and the plasticity of dendritic spines (Pinto et al., 2010). Glessner et al. (2009) described the involvement of neuronal cell adhesion networks and degradation pathways related to ubiquitin. Levy et al. (2011), using a novel method of analysis of the biological pathways NETWORK-BASED ANALYSIS OF GENETIC ASSOCIATIONS (NETBAG), applied to the genes affected by de novo CNVs, also showed a functional convergence, in particular towards pathways involved in the synaptogenesis, axonal guidance and neuronal motility (Gilman et al., 2011). Sanders et al. suggested a role of imbalance in excitation-inhibition (E/I) in ASD through the involvement of the SCN2A gene encoding a sodium-dependent voltage channel (Sanders et al., 2012). Moreover, the involvement of etiological factors in the embryonic differentiation of glutamatergic cortical pyramidal neurons has also been demonstrated (Parikshak et al., 2013; Willsey et al., 2013).

These studies therefore probably indicate that autism is due to different alterations in synaptic function and plasticity, connectivity or cellular fate. Overall, these studies emphasize the central role of glutamatergic neurotransmission in the etiology of autism. Two major biological factors appear to be particularly important: the mTOR / PI3K on the one hand, and the imbalance of the E/I balance due to mutations in synaptic genes on the other hand (Bourgeron, 2009). Although they are not currently considered as core features of ASD, motor impairments are of importance due to their high prevalence and important impacts on ASD patients. Reports suggest that motor troubles in ASD can occur early in development (Brian et al., 2008) and persist over life time (Fournier et al., 2010). The next chapter will be dedicated to describe the motor impairments in ASD.

Chapter 2: Motor impairments in ASD

The motor delays encountered in ASDs are the atypia and delays of early motor development, motor stereotypies, impaired coordination, tics, lateral disturbances, tip-toe walking, synkinesis (involuntary muscular movements), Catatonia (motor immobility) and oculo-motor disorders. In this chapter, we will discuss the issue of impairment of imitation in ASD. In this work, we have been particularly interested in dyspraxia as a disorder of complex motor sequences, and that will be dealt with through the chapter.

2.1 Motor Delay

At present, early diagnosis of ASD is rarely performed before the age of two, mainly because of the multiplicity of developmental trajectories in autism (Ozonoff et al., 2011) and the absence of specific symptoms. Nevertheless, the atypies of the movement and the retardation of motor development are interesting prodromal signs of autism (Bonnet-Brilhault, 2017; Yirmiya and Charman, 2010). Early studies in this field were retrospective: Analysis of family videos have shown that, within a year, movements and postures of children with ASD were asymmetric (Esposito et al., 2009). In addition, other authors demonstrated that between 9 and 12 months (Baranek, 1999), children with ASD were delayed in early motor skills and walking. Finally, Dewrang and Sandberg (2010) carried out retrospective family interviews on early motor development, finding before the age of two years a deficit of imitation, clumsiness and a lack of coordination.

Today, studies of babies at risk for ASD provide prospective data on early symptoms of this disorder. In a follow-up study of premature babies, Karmel et al. (2010) showed that a decrease in arm tone was present at the age of one month in those with ASD. Other studies carried out on high-risk babies with autism, i.e., having a brother or sister with ASD, make it possible to look for symptoms relevant to screening. At six months, it would appear that the symptom enabling to differentiate children who will develop ASD from healthy controls is atypical motor control (Brian et al., 2013). In a study in 20 siblings of an individual with ASD, Leonard et al. (2014) showed that a decrease in motor skills at 9 and 40 months in these children

was associated with an increase in symptoms of ASD at five and seven years, regardless of the intelligence quotient (IQ). These prospective studies reveal a series of potentially discriminatory symptoms: decreased activity level at 6 months (Zwaigenbaum et al., 2005), decreased postural maintenance time (Iverson & Wozniak, 2006), decreased fluidity and non-directed character of spontaneous motor activity (Brian et al., 2008). The Mullen Scale study assessed 235 children with or without high-risk of ASD, resumed a slowing down of fine and overall motor development at around 14 months. According to Landa et al. (2013), the presence or absence of fine motor delay at 14 months could predict the developmental trajectory of children who develop ASD. In the same line, Bhat et al. (2012) reported that 70% of high-risk ASD babies who showed early motor retardation will have a communication deficit.

In conclusion, pro- and retrospective studies describe a disruption of early motor development of toddlers with ASD, with delays in motor acquisitions. These symptoms have interesting screening potential and may be relevant predictors of the evolution of the pathophysiology of ASD. Nevertheless, it is not currently possible to identify an early motor development specific to the ASD (Yirmiya & Charman, 2010). In this regard, it is now well admitted that children with ASD make less eye contact than their age matched controls, and this marker is used to detect ASD after the age of 2 years. However, a study By Jones and Klin (2013) concluded that newborns by the age of 2 months can display decline in eye fixation, an earlier detection of ASD that offers the chance for early management and intervention (Jones and Klin, 2013).

2.2 Repetitive behavior

Motor stereotypes are one of the core symptoms of autism. These are repetitive motor behaviors, which seem uncontrollable, and are not directed towards a functional goal (APA, 2013). These may be hand and finger mannerisms, arm beating, or complex body movements (Lord et al., 1994). In typical development, they may appear at about one year of age, with a peak at about three years, followed by a decrease with a disappearance after four years. This

development sequence is related to neuro-motor maturation (Leekam et al., 2007). The prevalence of stereotypies in ASD is about 60 to 70% according to a study conducted on 277 four-year-old children (Goldman et al., 2009). The presence of motor stereotypes is more prevalent in children with ASD than in non-ASDs (MacDonald et al., 2007). The batteries conventionally used in the studies to measure motor atypies are Aberrant Behavior Checklist (ABC, Aman et al. 1985), Behavior Problem Inventory (Rojahn et al., 1989), Stereotypy Severity Scale (SSS, Miller et al., 2006), and the Repetitive Behavior Scale Revised (RBS-R, Bodfish et al., 1999).

2.3 Dyspraxia

Dyspraxia, a form of developmental coordination disorder (DCD), is a common disorder affecting gross and fine motor coordination in children and adults (Henderson and Henderson, 2003). It is currently accepted that ASD patients show a decline in motor performance. According to a meta-analysis of 83 studies, the motor skills of ASD patients are at -1.20 standard deviations from those of neuro-typical controls, for posture, walking, coordination and movement of the arms (Fournier et al., 2010). Five other literature reviews were conducted on this subject. Since, Fournier has included in his studies elements of sensory-motor aspects, which are not part of the dyspraxia criteria. Miyahara, (2013) examined specifically five studies, precisely aiming at patients with dyspraxia symptoms, and found higher scores in ASD patients. According to this meta-analysis, the motor performance of ASD patients is at -2.91 standard deviations from the performance of typical population. Gowen and Hamilton (2013) provided a computational look at motor skills in ASD, based on the Wolpert & Ghahramani model (Wolpert & Ghahramani, 2000). Finally, three other literature reviews (Downey & Rapport, 2012; Emck et al., 2009; Williams et al., 2004) focused on the cognitive aspects of motor skills in ASD namely imitation, perception of one's own skills, and dyspraxia. All these studies concluded that motor performance is impaired in ASD patients in three main areas: gross motor skills, fine motor skills and graphomotricity (the association between the visual and motor skills needed in the writing

process). Nevertheless, studies that use validated tests for dyspraxia (BOT or M-ABC batteries) show that some subjects with ASD do not meet the severity criteria of the dyspraxia (Dewey et al., 2007).

2.4 Motor coordination deficits

2.4.1 Motor planning

Difficulties of motor planning seem to be the most accompanying motor impairment in ASD. To perform fine motor demanding tasks, as returning a glass to a table, neurotypical people often move in an atypical position in order to end up in a comfortable position (Rosenbaum, 1991). However, patients with ASD do not seem to plan the posture of fine movements and thus would begin a fine motor planning demanding task with a comfortable posture that would end the gesture in an uncomfortable position (Hughes, 1996). Similarly, studies addressing preparation prior to movement in ASD patients show an increase in reaction time, which corresponds to shifts in the planning phase of the action (Nazarali et al., 2009; Glazebrook et al., 2008, 2006; Rinehart et al., 2006, 2001). However, this planning time was not variable in other studies that reported that motor planning was not changed in subjects with ASD when compared to healthy controls (Mari et al., 2003). Furthermore, ASD patients did not show motor planning deficit in a “grasp & turn fine movement task” (Van Swieten et al., 2010). In addition to motor planning, whether ASD patients suffer from motor performance deficits is still controversial: only few studies support an increase in the movement time for scoring, especially when the task is complex, i.e. when the task involves reprogramming (Nazarali et al., 2009; Glazebrook et al., 2008). According to other authors, ASD patients show a spared or even faster execution of the movement (Mari et al., 2003, Rinehart et al., 2001).

2.4.2 Dysgraphia

The term dysgraphia refers to deficits in the ability to write; pen movement alterations characterized by increased time on paper, increased time in the air, decreased pressure, decreased readability, decreased frequency, decreased spatial organization and increased number of letters erased are often present. Dysgraphia is more common in children with ASD than in typically

developing children. In general, there is a decrease in manual dexterity, diadochokinesis (difficulty in performing alternate rapid movements), dysgraphia, inadequacy in manual force, drop in speed during the finger tapping test, and slowness in dexterity task (Hardan et al., 2003). Motor performance control is mainly analyzed through the postural control in the ASD literature. Schmitz et al., (2003) proposed a lifting task (the coffee boy task) where children must lift a weight on a tray held by his other hand. She showed that the stabilization performances of children with ASD were not diminished, but that the strategy was not the same with more weight given to proprioceptive information (slow down load shedding) and less anticipation (pattern of contraction unstable) (Schmitz et al., 2003). Thus, in fine motor skills, alterations are found in the planning, execution and control of the action, but these results are not always replicated and remain controversial.

To conclude, there are alterations in motor coordination at the levels of both gross and fine motor skills in ASD patients. Yet the studies report very variable or even contradictory results. This may be mainly due to the evaluation methods that are very heterogeneous (non-validated batteries, or very specific tasks and therefore not replicated), with many confounding factors (comorbidity, psychotropic treatments, ASD subtypes, intellectual disability, and small cohorts mostly less than 20 patients).

2.4.3 Tone and posture

Global motor coordination deficits include deficits in tone, posture and locomotricity. According to a cohort of 154 ASD children aged 2 to 18 years, 51% had mild to moderate hypotonia throughout the body. None exhibited severe hypotonia or loss of muscle strength (Ming et al., 2007). From a posture perspective patients with ASD appear to have a lack of balance (Chang et al., 2010; Minshew et al., 2004). This difficulty has been recorded by varied methods: standing on one or two feet or in virtual reality with a moving visual environment. Many studies have used posturometry to study the one-legged standing with open eyes in patients with ASD. Travers et al. (2013) used a simplified wii balance board. For both conditions of open and closed eyes, the results were not uniform between studies. In the open eyes

condition, most studies fail to show balance deficits in ASD patients (Travers et al., 2013; Greffou et al., 2012; Fournier et al., 2010). Gepner et al. (2002) found even better postural stability in patients with ASD compared to controls. However, studies of large cohorts, 70 participants per group, show an increased sway in one-legged standing with open eyes (Minshew et al., 2004; Molloy et al., 2003). It appears that subjects with ASD have a different profile than healthy controls due to a suppression or alteration of sensory information. Several studies find postural hyporeactivity in ASD patients upon a modification of the visual information with a moving virtual reality video (Greffou et al., 2012; Gepner et al., 2002). In closed eyes condition, some studies found a decrease in adaptive postural response with an increased sway in the ASD versus control group (Minshew et al., 2004; Molloy et al., 2003). However, others did not find any significant difference (Travers et al., 2013; Kohen-Raz et al., 1992).

2.4.4 Locomotion

Being also impaired in ASD patients, locomotion studies have drawn interest in the ASD field in the recent years. Kinematic studies showed altered parameters of simple walking in ASD patients: reduced cadence, altered gait velocity and step length (Lim et al., 2016), and decreased stride length (Weiss et al., 2013). Another parameter that is important for the balance of the human while walking, the base of support, was found to be increased in patients with ASD (Nayate et al., 2012). Furthermore, many studies have suggested that patients with ASD almost always show a greater variability in stride length (Rinehart et al., 2006; Hallet et al., 1993, Vilensky et al., 1981), decreased step length (Vernazza et al., 2005), as well as deficits in upper body movements (Vernazza et al., 2005; Vilensky et al., 1981). Studies of locomotion in ASD assessed the neuroanatomic substrates of motor dysfunction with major differences in the brain areas that are suspected to be affected. While Vilensky et al. (1981) found a pseudo-parkinsonian step (smaller steps) that was attributed to a dysfunction of the central gray nuclei, Hallet et al. (1993) pointed at the variation in locomotion in the ASD group to cerebellar ataxia as the length of the steps were variable among this group. Vernazza et al. (2005) demonstrated altered walking trajectory (planning), suggesting shifts in prefrontal or parietal lobe. Such heterogeneous

conclusions can be explained by a number of factors, including differences in the characteristics of the participants in the studies: with or without ID, age groups, with or without treatment, with Asperger syndrome or High Functioning Autism or the use of small cohorts. It is therefore necessary to replicate the results by reducing the confounding factors. Nevertheless, in their review of the literature, Emck et al. (2009) conclude to a decline in gross motor performance in ASD patients.

2.5 Tics and Tourette syndrome

Tics are motor movements or involuntary, brief, repetitive and non-rhythmic vocalizations, occurring around the age of 7 years in patients (Leckman et al., 1998). Between 20% and 60% of ASD patients have tics at some point in their lives (Attwood, 2006). The prevalence of transient tics in the general population is about 15% (Gillberg, 2010). The prevalence of Tourette's syndrome is estimated to be 8%, whereas the prevalence of chronic tics is estimated to be 1% in the general population (Gillberg, 2010). Similarly, about 50% of patients with a Tourette's syndrome also have ASD (Clarke et al., 2012). Children with ASD who have a Tourette's have a higher risk of ADHD (State, 2010). Tics are therefore an important element in the diagnosis of ASD.

2.6 Lateralization

Unlike children with typical developments, the lateralization of children with ASD is delayed. The ability to use both right and left hands in adapted form, or Ambidexterity, is present in about 20% of children with ASD (Markoulakis et al., 2012). Lateralization is important for performing bimanual tasks. This lack of lateralization thus contributes to difficulties in bimanual coordination (Perrin et al., 2013). Lateral left lateralization is also more common in the ASD population: around 30% of left-handed people are 4 to 5 years old, half of which are left-handed after age 12, while left-handers are not more than 11% in the population (Markoulakis et al., 2012). Some authors link these lateral disorders to language disorders, and a defect in hemispheric lateralization to autism (Lindell et al., 2013).

2.7 Toe-walking

Toe-walking is common in 3 years old children. Yet if present after the age of 5, toe-walking is rather linked to delayed neurological development. Interestingly, toe-walking has been associated with language delays and ASD (Barrow et al., 2011). Moreover, according to a cohort in children with ASD, 19% had a tip-toe for at least 6 months during the child's life (Ming et al., 2007). Nevertheless, this was intermittent, and only 10% (of the 154 children studied in this cohort) had a reduction in ankle mobility and none needed surgery (Ming et al., 2007).

2.8 Conceptual and biological frames behind motor impairments in ASD

Many cognitive models were developed by the scientific community to link motor function disorders and ASD (see McCleery et al., 2013).

2.8.1 Defeated functional connections

ASD is characterized by hypofunctional connections of high-level processes. A lack of functioning in connections between the frontal lobe and the rest of the cortex, associated with an excess of functioning of the low-level process, has been suggested (Mostofsky et al., 2009). Deficits in cerebellar activation and motor execution networks were shown in functional imaging studies. In one imaging study in 13 children with ASD, the authors demonstrated a decrease in the connectivity of motor performance networks (primary motor areas, cerebellum, thalamus and additional motor areas) associated with decreased cerebellar activity during the finger tapping motor task. Authors linked the hypoconnectivity in the motor networks to a bad coordination of the circuits necessary to the automation of the movements. Reduced cerebellar activity may reflect a difficulty in moving motor performance from the cortical regions to regions associated with more usual and more automated execution, as suggested (Mostofsky et al., 2009) (Figure 1).

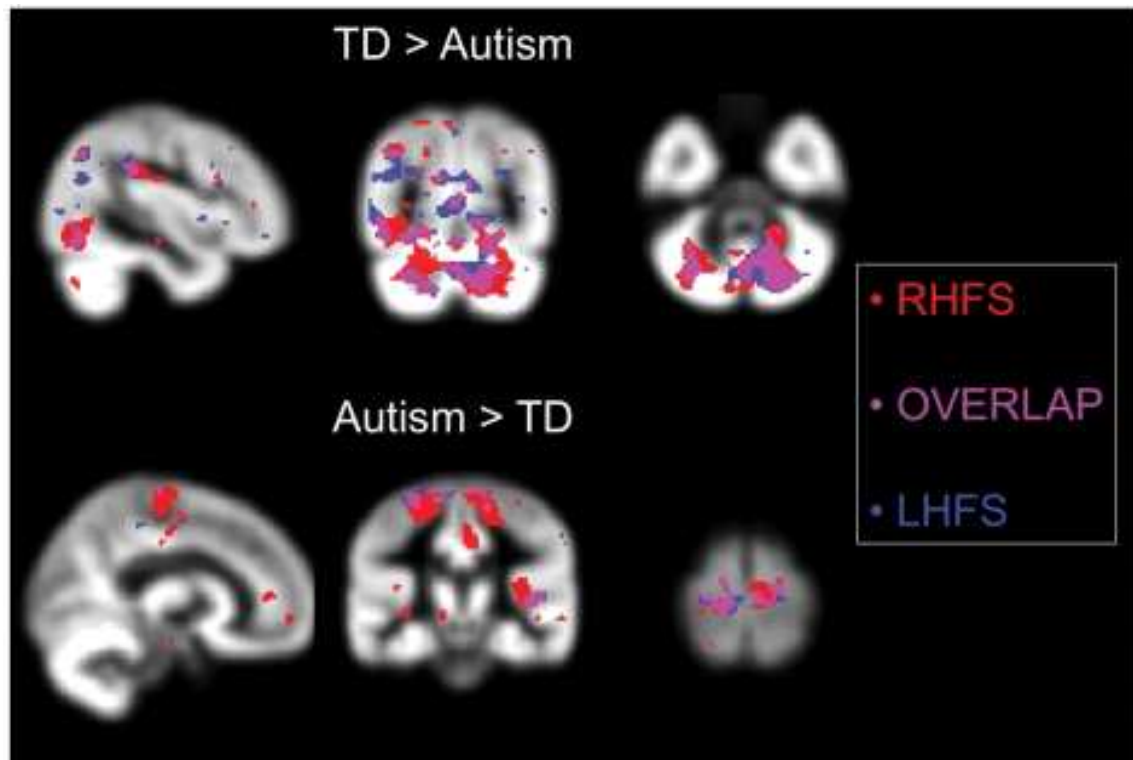


Figure 1: Differences in fMRI activation between children with autism and typically developing (TD) children.

Right-handed finger sequencing RHFS (red), left-handed finger sequencing LHFS (blue), and the overlap between RHFS and LHFS (pink). The upper maps show regions where TD children displayed greater activation than did those with autism; the lower maps show regions where children with autism showed greater activation than did TD children.

2.8.2 Sensory inputs, motor outputs

Bonnet-Brilhault (2012) defined autism and pervasive developmental disorders as "a group of heterogeneous neurodevelopmental pathologies that affect different sectors of brain functioning". This psychopathological disorder is characterized by disturbances during the major stages of brain development. Psychophysiological and genetic explanatory hypotheses and models have multiplied in recent years. The hypotheses of "enhanced perceptual functioning" and "weak central coherence" seem to be constructed within the same theoretical frame. As early as 1989, Frith identified perceptual specificities in patients with autism and formulated the theory of central lack of coherence. According to this author, autistic disorders result from a lack of central coherence that reduces the ability to process and integrate local information into a coherent whole, in a comprehensive manner with particular attention to the details of the environment. In non-autistic individuals, this ability to group perceptual information into a

coherent whole is innate. Mottron (2006) suggested a "bias for global and configurational aspects in relation to local aspects". This skill would be impaired in patients with autism who, on the contrary, would identify more local elements than the overall context. This phenomenon would be linked to reduced attention for global information and a difficulty in dealing with information in contextual terms. Indeed, it is now recognized that ASD patients show different features of information processing deficits. At the level of perceptions, the two main models that go in this direction are the "weak central coherence" (Happé & Frith, 2006) and the "enhanced perceptual functioning" (Mottron et al., 2006), describing a deficit in perceptual treatment of high level for the first model, and an over-functioning (especially visual) of low level for the second model. In motricity, Cattaneo puts forward a "deficit of actions chain of motor acts" (Cattaneo et al., 2007). Recent anatomical and functional studies (Bonnet-Brilhault, 2012; Courchesne et al., 2011; Dinstein et al., 2011) can be linked to these hypotheses. In particular, the use of functional magnetic resonance imaging (fMRI) has revealed abnormalities in brain connectivity and, more specifically, a predominance of local hyperconnectivity, in patients with autism, to the detriment of connectivity between distant cerebral areas. This lack of connectivity would lead to disturbances in the integration of sensory information processed in distinct and remote brain areas. This atypical neuronal functioning, demonstrated in ASD patients, may be the consequence of alterations in synaptic development (Gilman et al., 2011; Bourgeron, 2009) and / or an imbalance between excitation and inhibition of the central nervous system (Markram et al., 2007; Rubenstein and Merzenich, 2003). These findings emphasize the role of sensory dysfunctions in autism (Happé and Frith, 2006; Mottron et al., 2006) since researchers demonstrated that these brain abnormalities are involved in very early social responses of children with ASD (Courchesne and Pierce, 2005) and may impact on the overall brain development and induce major disrupted functions in autism (communication, social interactions, and restricted and repetitive behaviors and behaviors).

More recently, Gowen and Hamilton in (2013), inspired by the previously described models of Mottron and Frith (2006), hypothesized an increase in sensory inputs and motor outputs that they termed "Input/Output noise hypothesis" and that is associated with sensory-motor integration difficulties. The application of these models to the motor level would be expressed by (i) an increase in the variability of sensory inputs and motor executions, (ii) a hyper-dependence on certain sensory inputs for motor skills and (iii) a slower planning and motor performance and cognitive overhead to successfully regulate even the most basic movements (Gowen and Hamilton, 2013).

2.8.3 Executive functions deficits

In his 2010 meta-analysis, Fournier highlighted fronto-striatal and central gray-nuclei abnormalities in relation to motor difficulties seen in patients with ASD (Fournier et al., 2010), hence the fronto-striatal disorder framework. The locomotion in ASD patients would be impacted by presenting deficits in executive functions, with disturbances in motor planning (Glazebrook et al., 2008; Vernazza et al., 2005; Rinehart et al., 2001) and difficulties in shifting attention (Nazzarali, 2009). One of the earliest theories regarding motor function disorders in autism was that of a pseudo-parkinsonian disorder (Vilensky, 1981). Vilensky analyzed several parameters of gait and demonstrated a parkinsonian gait in children with autism. The increase in the volume of the caudate nucleus found in post-mortem studies resonates well with motor difficulties in ASD (Esposito and Paşca, 2013).

2.8.4 Cerebellar Ataxia

Another traditional theory involving motor function disorders in the ASD population is "cerebellar ataxia" (Hallett et al., 1993). This type of ataxia originates from lesions to the cerebellum. As discussed in details in the following chapter, the cerebellar cortex is composed of molecular layer, Purkinje cell layer and granular layer. At the neuroanatomical level, PC deficits have been studied and well described in the cerebellum of ASD patients (Fatemi et al., 2012; Whitney et al., 2008; Bailey et al., 1998; Bauman and Kemper, 1985). In one meta-analysis on

the neuroanatomy of autism, a reduction of cerebellar vermal lobules VI, VII, VIII and X was found in autism linking the disorganization of cerebro-cerebellar connections to the motor dysfunction present in ASD patients (Stanfield et al., 2008). In another meta-analysis, the authors found hypoplasia of cerebellar vermis in lobules VI-VII (Courchesne et al., 1988, 1994). Similarly, hypoplasia of cerebellar vermis was identified in patients with a 22q13 deletion, causing Phelan-MacDermid syndrome, which is commonly associated with autism (Aldinger et al., 2013).

In conclusion, there are many motor disturbances in ASD patients: atypical and delayed early motor development (Yirmiya and Charman, 2010), motor stereotypes, impaired coordination, imitative disorders, and tics (Bhat et al., 2012). However, there is no consensus on the underlying mechanism of these dysfunctions, which are probably linked to multiple neurodevelopmental motor, perceptual and cognitive functioning (McCleery et al., 2013). In ASD patients, studies report alterations in motor coordination at all levels: gross motor skills, fine motor skills, planning, execution and control (Gowen and Hamilton, 2013).

Chapter 3: Anatomic and functional organization of the cerebellum

Given that the cerebellum is the most consistently found brain area with abnormalities in ASD patients and given its role in movement coordination, we are dedicating a chapter to this brain region. We will describe the functional anatomy of the cerebellum; what are the different cell types, loops and pathways of the cerebellum?

3.1 Morphology and functional anatomy

The cerebellum is present in all vertebrates, from most of the primary prevertebral organisms up to the most advanced vertebrates (Larsell and Jansen, 1970; 1967). The shape and morphology of the cerebellum varies in vertebrates. It is blade smooth or folded in the dipneustes (fish primitive), amphibians and snakes, then it is furrowed and lamellous in Chondrichthyans (cartilagenous fish), crocodilians, birds and mammals (Voogd and Glickstein, 1998). We will concentrate our description on the cerebellum of mammals whose anatomy and organization is similar in different species, where most differences lie within the relative sizes of the different subparts (Figure 2 A-C).

3.1.1 Anatomical and functional subdivisions

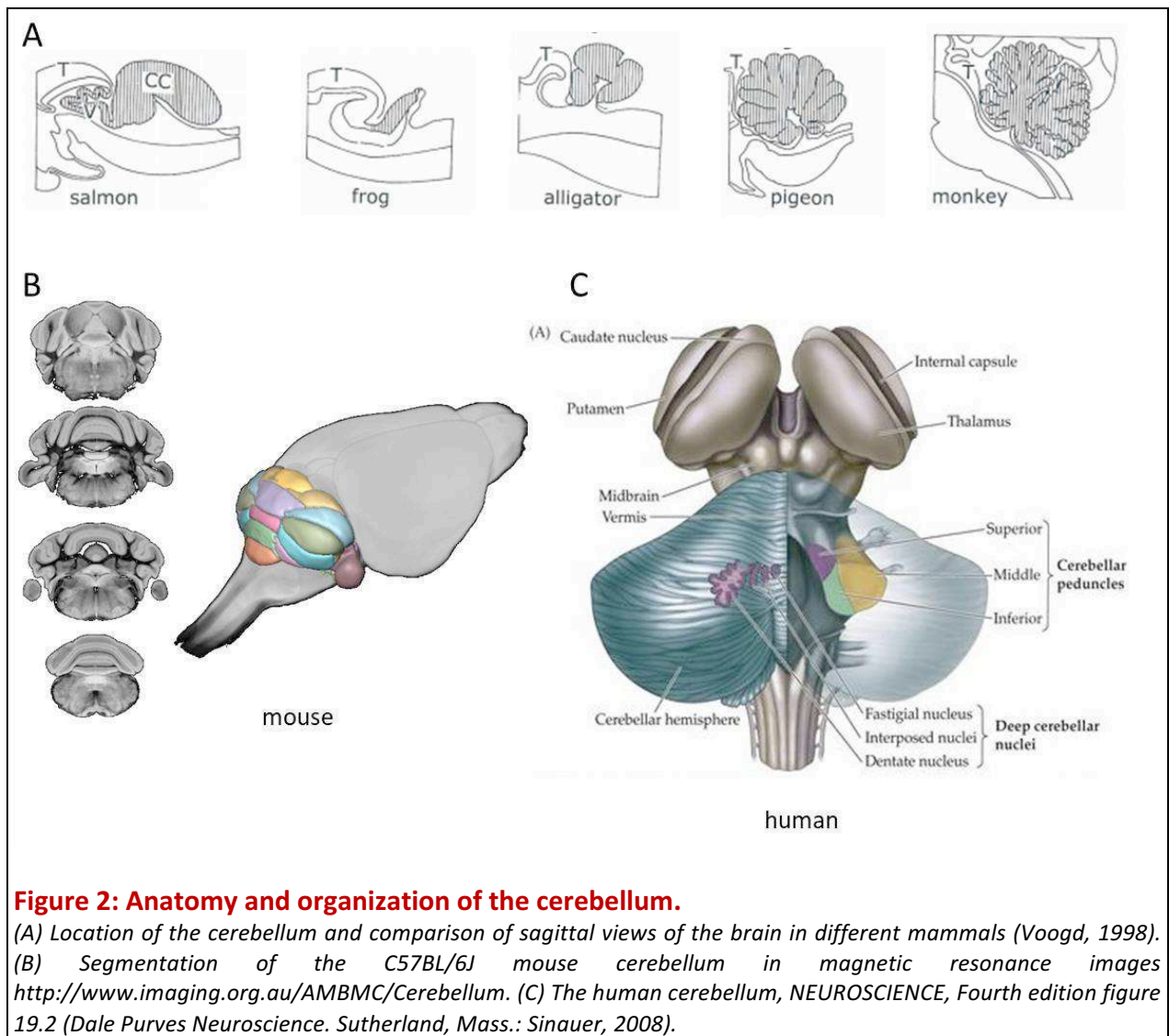
In spite of its Latin name meaning "small brain", in human the cerebellum contains more cells than the rest of the entire brain, for only 10 to 15% of its weight (Ito, 1984). It is a median and odd structure, in posterior derivation of the anterior rhombencephalon (Figure 2 A). During the development, the cerebellum is formed from the two cerebellar, ventricular and granular neuro-epithelium, and derives mostly from the metencephalon to form the caudal part and from the mesencephalon (midbrain) to form the rostral part (Martinez and Alvarado-Mallart, 1989). It is connected to other structures of the nervous system by three pairs of fiber bundles, called cerebellar peduncles (Figure 2 B, bottom). The phylogenetic evolution of the cerebellum was marked by a double organization, transversal and longitudinal (Figure 2 C).

In the transverse direction two deep fissures, the posterolateral fissure and the primary fissure, anatomically share the cerebellum in three successive parts (Figure 2 C, top):

- The anterior lobe, or paleocerebellum, which superimposes the preceding in amphibians, reptiles, and birds, and represents in the primates (and especially in human) only a small part of the cerebellar volume.
- The posterior lobe, or neo-cerebellum, which has attained its full development in the primates, where it forms the greater part of the cerebellar mass.
- The flocculonodular lobe, or archicerebellum, appeared for the first time in fish, and is located behind the posterolateral fissure.

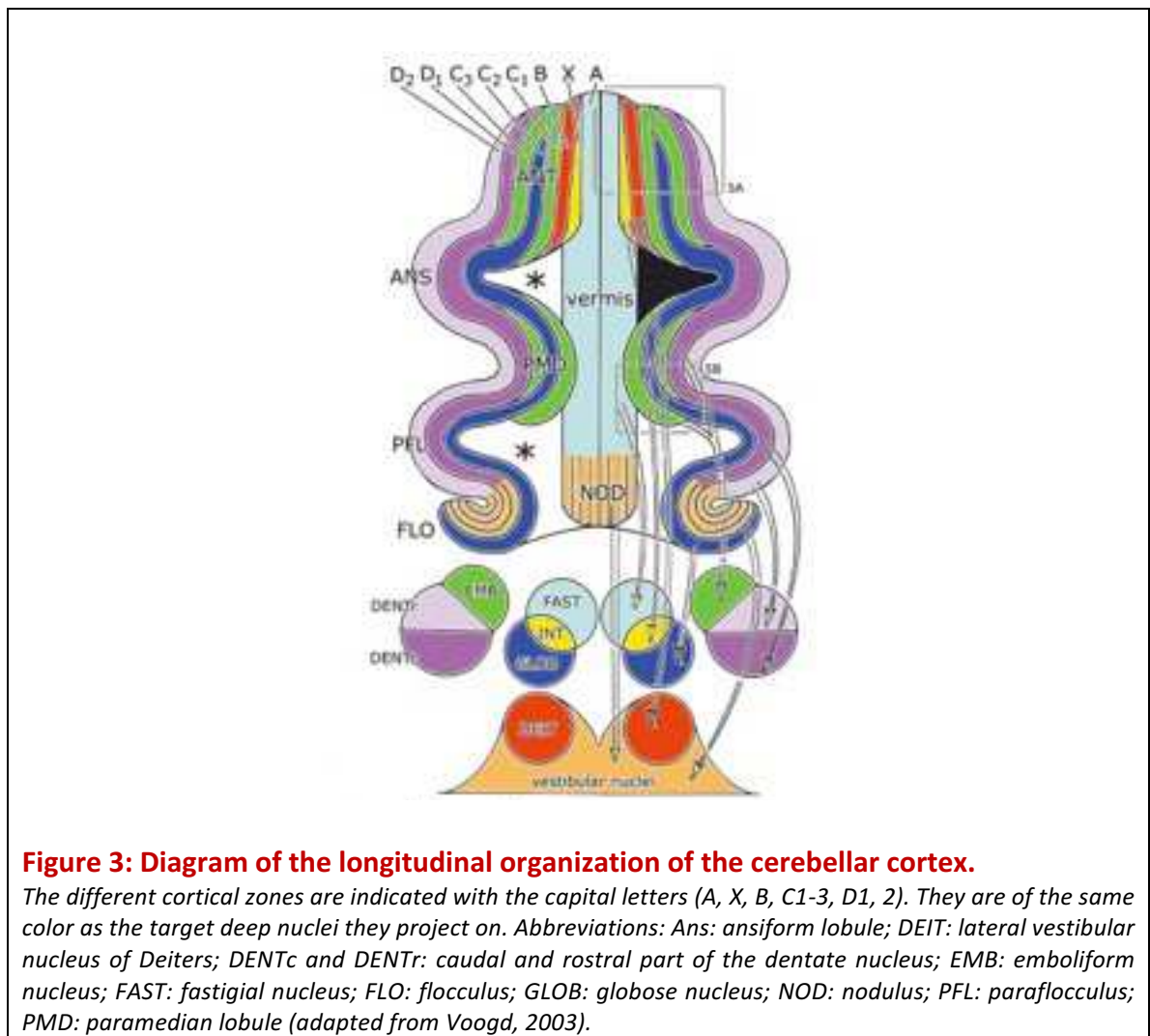
Parallel fissures, less deep, subdivide the anterior and posterior lobes into a series of transverse lobules, called folia. In mammals, there are ten lobules numbered from I to X (Larsell, 1952, 1937) (Figure 2 B, bottom). Until the late 1970s, the three anatomical transversal subdivisions that we have just described were also considered as three functional areas. Recent experimental data, including hodological data, have shown that functional subdivisions of the cerebellum are not transverse but longitudinal (Voogd and Glickstein, 1998). From a purely morphological and structural point of view, only two cerebellar hemispheres differentiated from a single median part, the vermis, can be distinguished in the longitudinal plane. Thus, the structure in itself may appear uniform and does not suggest a more precise longitudinal organization.

It was not until the work of Hawkes and Leclerc (1987) that immunological marking techniques were used to demonstrate a modular longitudinal organization of the cerebellar cortex. These subdivisions are reciprocally connected to different deep nuclei, situated in the cerebellar white matter: the dentate nucleus, the globular and emboliform nuclei (or anterior and posterior interposed nuclei in rodents) the fastigial nucleus (or medial nucleus in rodents); and vestibular nuclei located in the bulb (Larsell, 1937).



There are four main subdivisions (called zone A to zone D in the mediolateral direction), forming strips parallel to the longitudinal axis of the vermis and which are connected to the deep nuclei in the following manner (Figure 3):

- The medial part of the anterior vermis is projected on the medial vestibular nucleus, as well as on a region of fastigial nucleus, which in turn projects into the vestibular nuclei (the anterior part of zone A).
- The posterior vermis is projected on the fastigial nucleus at the caudal part (posterior part of zone A).
- The lateral part of the lower vermis and the adjacent regions of the single lobule project on the lateral vestibular nucleus (zone B).
- The intermediate hemisphere is projected onto the globular nucleus and the emboliform nucleus (zone C).
- The lateral hemisphere is projected onto the dentate nucleus (zone D).



These areas are associated by their connectivity with specific deep nuclei that are part of distinct anatomical loops (Voogd, 2003; Voogd and Glickstein, 1998). In the next section, we will take a closer look at the connections of the cerebellum with the spinal cord, the subcortical or cortical structures that make up these different anatomical loops.

3.1.2 Cellular organization of the cerebellum

The cerebellar cortex is a simple structure regularly organized in successive layers, parallel to the surface, that follow in shape the lobules and convolutions of the cerebellum. Ramon y Cajal, revealed cellular elements that constitute each layer by refining the silver impregnation technique developed by Golgi (Cajal, 1911).

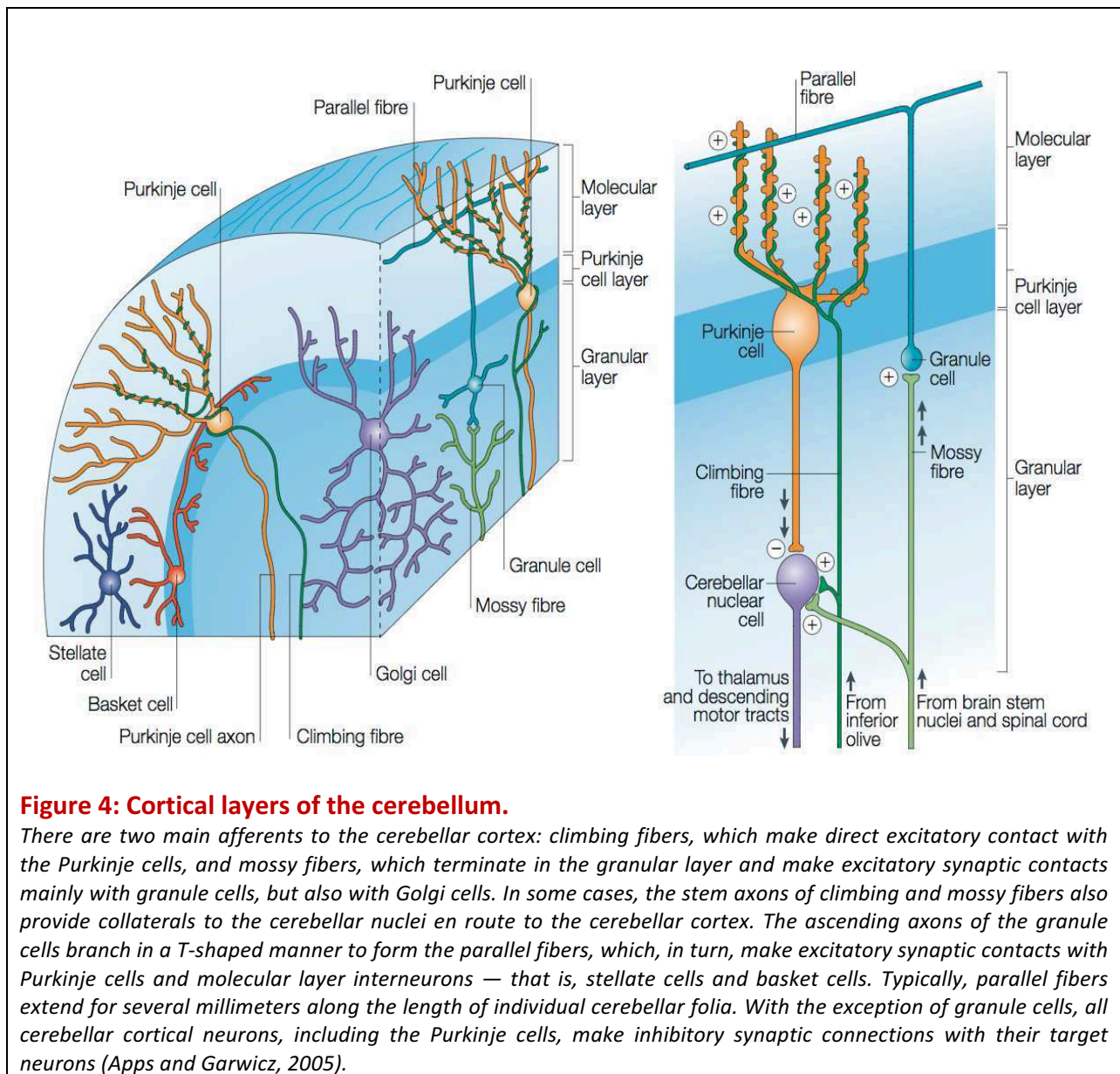


Figure 4: Cortical layers of the cerebellum.

There are two main afferents to the cerebellar cortex: climbing fibers, which make direct excitatory contact with the Purkinje cells, and mossy fibers, which terminate in the granular layer and make excitatory synaptic contacts mainly with granule cells, but also with Golgi cells. In some cases, the stem axons of climbing and mossy fibers also provide collaterals to the cerebellar nuclei en route to the cerebellar cortex. The ascending axons of the granule cells branch in a T-shaped manner to form the parallel fibers, which, in turn, make excitatory synaptic contacts with Purkinje cells and molecular layer interneurons — that is, stellate cells and basket cells. Typically, parallel fibers extend for several millimeters along the length of individual cerebellar folia. With the exception of granule cells, all cerebellar cortical neurons, including the Purkinje cells, make inhibitory synaptic connections with their target neurons (Apps and Garwicz, 2005).

According to modern histology atlas, the three layers of the cerebellar cortex -from the outside to the inside successively- are organized as follow (Figure 4):

- **The molecular layer** that contains basket cells, stellate cells, dendritic arborization of PC, and axon terminals of olivary neurons.
- **The Purkinje-cell layer** that contains the somas of the PC in a monolayer.
- The internal **granular layer** that contains the somas of granule cells, golgi cells and mossy fiber terminals.

The cerebellum contains as well an important amount of glial cells, these being found in both the grey and white matters. All the types of the glial cells are present; the astrocytes, microglia, and the oligodendrocytes. Thus, the cerebellar cortex contains five main cell types:

- Three inhibitory interneurons: stellate cells, basket cells and Golgi cells.
- One excitatory cell type: granule cells that are of excitatory cell type that relay afferences from the mossy fibers and synapse on the PC which constitute the one and only inhibitory exit pathway.

The **Purkinje cells**, originally described by Johannes Purkinje in 1837, is the central element of the cerebellar cortical synaptic network. All other cell types, previously listed, are projected onto Purkinje cells. PCs are GABAergic neurons, and are among the biggest cells of the CNS. The diameter of their soma is approximately 20-23 μm in the mouse and rats (Takács and Hámori, 1994; Caddy and Herrup, 1990). An organized dendritic tree arises, entirely contained in the molecular layer. It extends up to 400 μm wide in the sagittal plane, but its thickness in the transverse plane is only up to 20 μm . The main dendrite stems from the apical region of the cell and branches into secondary dendrites, which in turn branch into tertiary dendrites. Each PC, which receives only one climbing fiber, is also contacted by hundreds of thousands of parallel fibers. The PC terminal dendrites are roofed with thousands of spines that interact with the parallel fibers (Palay and Chan-Palay, 1974). The axon of PC arises from the basal region of the cell body. It is then myelinated, passes the granular layer, connects again to the white matter and ends in the deep nuclei or at the vestibular nuclei (outside the cerebellum). It is the only efferent pathway of the cerebellar cortex. From this axon can emerge collateral which bifurcate and recontact PCs, hence their name as recurrent collaterals.

The **granule cells**, have smaller somas of about 5 μm when compared to PC, are glutamatergic excitatory neurons that have a very high abundance (up to 30 million in mice) and may well be the most represented neurons of the brain with a proportion that is described as being of 50-70% (ref). This high density gives a granular look to this layer. The granule cells

have long axons known as the **parallel fibers**; these join the molecular layer by an ascending segment that produces up to 20% of all the synapses with the PC. When parallel fibers arrive at the surface of the cerebellar cortex, they split and spread parallel to the cortex to form extensions of 2 to 3 millimeters in length (Harvey and Napper, 1988). By crossing the dendrites of PC perpendicularly, parallel fibers establish maximum contacts with PC while innervating each cell only once (Harvey and Napper, 1988). The dendritic tree of the granule cells contains up to 5 dendrites that form excitatory synapse with the **mossy fibers**. The granule cells are therefore the relay of the information provided by the **mossy fibers**.

The **inhibitory interneurons** are Golgi, stellate and basket cells. Located in the granular layer, Golgi cells surround the mossy-granule cell excitatory synapses to participate in the modulation of the activity of this synaptic transmission. The basket and star cells are located in the molecular layer and project predominantly on PC.

3.1.3 The afferent and efferent pathways of the cerebellum

The mouse cerebellum receives two major entrance pathways; the climbing fibers and the mossy fibers. Other pathways include the monoaminergic and cholinergic fibers that are diffused in the all cerebellar layers (Barmack et al., 1992). The mossy fibers, that are predominantly excitatory, have granule cells as their target. This group of cortical afferents comprises fibers from five different origins: spinal cord, trigeminal system, vestibular system, reticulate nuclei, and pons nucleus (Cajal, 1911). The mossy termini come into contact with the dendrites of the granule cells that are contacted by the axons of the Golgi cells. The climbing fibers, axons of the lower olive divide in the molecular layer at the level of the primary dendrite in several ramifications. Then they leave the white matter and cross the internal granular layer without much branching, following what, mostly innervate the PC and, by collateral branches, innervate the entire cells found in cerebellar cortex (Palay and Chan-Palay, 1974). Monoaminergic fibers play a modulating role, mainly controlling synaptic transmission between cortical interneurons and PC (Oertel, 1993). Both noradrenergic and serotonergic fibers penetrate the cerebellum through the cerebellar peduncles. The noradrenergic fibers are derived from the locus coeruleus.

They are distributed in the white matter, the inner granular layer, surrounding the glomeruli and orient towards the molecular layer (Kimoto et al., 1981; Mugnaini and Schnapp, 1974) where they contact PC main dendrites. Serotonergic fibers, originates from the median raphe nucleus, are abundant in the deep nuclei, but some of them are located in the granular and molecular layers (Takeuchi et al., 1982). Finally, **cholinergic fibers** from the vestibular nucleus orient sparsely towards the flocco-nodular lobe (Altman and Bayer, 1977), and may participate in modulating the activity, in these nuclei, of the PC. We have just described how the afferent information from different structures of the central nervous system is distributed across the different pathways of the cerebellar cortex. These multiple afferents all converge at the level of the Purkinje cells which is the only way out of the cerebellar cortex. The majority of efferent cerebellar pathways (red; Figure 5) pass through the superior peduncle. The dentate nucleus projects its fibers on the ventro-lateral and ventro-anterior nuclei of the thalamus and then reaches the cerebral cortex. Efferent nerve fibers of the interposed nucleus reach the red nucleus and those of the fastigial nucleus reach the reticulate formation and the vestibular nuclei.

3.1.4 Cerebellar loops

The cerebellum is implicated in several, functionally distinct, anatomic loops. The cerebellar peduncles are distinguished by the type of fibers that constitute them; all the afferent or efferent fibers of the cerebellum pass through the cerebellar peduncles thus making it possible to ensure the connections between the cerebellum and other cerebral structures (Figure 5). The types of fibers that constitute the peduncles (afferent or efferent) are distributed as follows:

- The **superior cerebellar peduncles** unite the cerebellum to the mesencephalon (mid brain). They are almost exclusively composed of efferent fibers (cerebello-thalamic tract and the cerebello-rubral tract), which stem from the different deep nuclei and projects to the red nucleus (also known as nucleus ruber), the vestibular nuclei, the reticular formation and the thalamus.
- The **middle cerebellar peduncles** connect the cerebellum to the pons. Most of the fibers constituting these peduncles are afferent centripetal fibers and originate from the pontine

nuclei, which convey information from various areas of the cerebral cortex and the superior colliculus.

- The **inferior cerebellar peduncles** connect the cerebellum to the bulb. They contain afferent fibers from the vestibular nuclei, the spinal cord and several regions of the mesencephalic tegmentum. It also contains efferent fibers towards the vestibular nuclei and the reticular formation.

The origins of the afferent nerve fibers that can reach the different parts of the cerebellum, the projections from these parts to the deep nuclei, and the projections of these nuclei to different structures of the brain, all allow the identification of distinct anatomical loops characterized by specific functions.

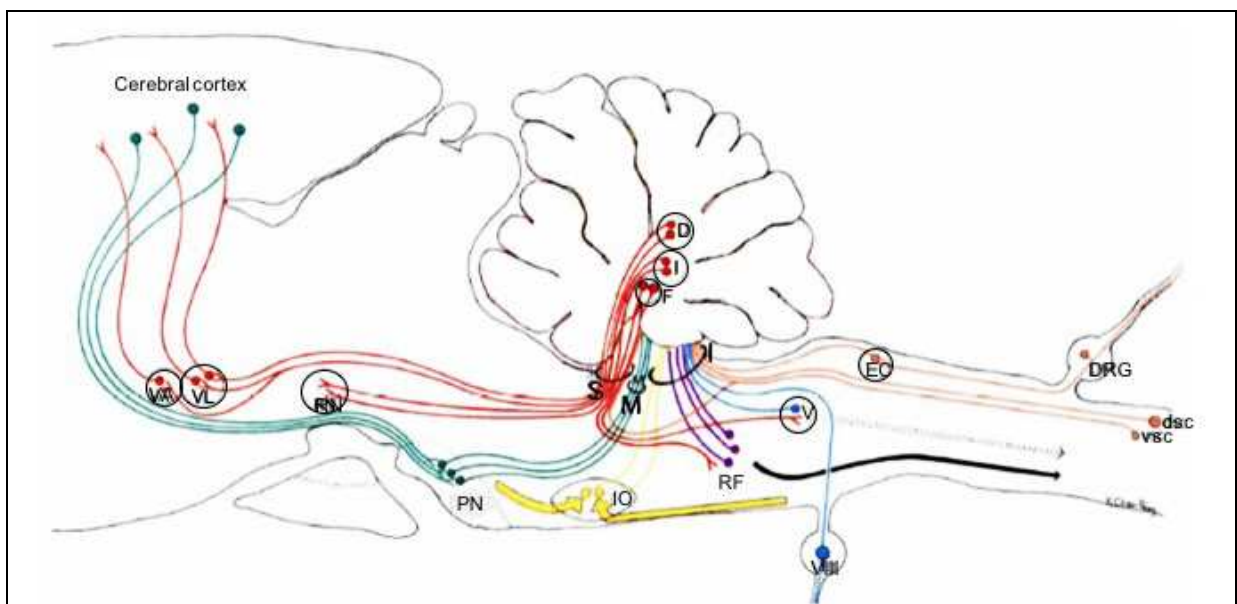


Figure 5: Diagram of the afferent and efferent nerve fibers of the cerebellum.

The afferent pathways of the spinal cord (orange) reach the cerebellum via the ventral and dorsal Spinocerebellar (vsc and dsc) pathways via the inferior (I) and upper (S) cerebellar peduncles. A dorsal ganglion (DRG) which projects to the nucleus of the external cuneate (EC) is indicated. The vestibular afferent pathways (blue) include fibers of the vestibular nucleus (V) and primary vestibular fibers (VIII). These fibers project towards the fastigial nucleus (F) and the flocculonodular lobe. Afferent pathways from the reticular formation (RF, in violet) are distributed through the vermis. The fibers of the inferior olive (IO, in yellow) step through the inferior peduncle and project over the entire cerebellar cortex. The afferent nerve fibers of the pontine nuclei (PN, in dark green) originate from the cerebral cortex; they project over the entire cerebellar cortex via the middle peduncle (M). The majority of efferent cerebellar pathways (red) pass through the superior peduncle (S). The dentate nucleus (D) projects its fibers on the ventro-lateral (VL) and ventro-anterior (VA) nuclei of the thalamus and then reaches the cerebral cortex. Efferent nerve fibers of the interposed nucleus reach the red nucleus (RN) and those of the fastigial nucleus reach the reticulate formation and the vestibular nuclei (Palay and Chan-Palay, 1974).

3.1.4.1 The loops involving spinal, vestibular and reticular formation

The **anterior part of the zone A** (middle part of the anterior vermis) receives somesthetic information (olivary nuclei, dorsal columns and spinal trigeminus), proprioceptive (posterior spinocerebellar tract), exteroceptive (reticulo-cerebellar fibers), as well as a copy of the motor commands (ponto-cerebellar fibers and anterior spinocerebellar tract). In turn, the median anterior vermis is projected onto the medial vestibular nucleus and the motor reticulate formation (via the fastigial nucleus). Thus, this regulation loop will modulate the reticulo-spinal and vestibulo-spinal pathways involved in the control of walking (Botterell and Fulton, 1938).

The **posterior part of zone A** (posterior vermis) receives information from oculomotor commands resulting from oculomotor reticular formation, visual information from the upper colliculus, pons fibers, vestibular information transmitted by vestibulo-cerebellar fibers, and proprioceptive and motor information transmitted by the spino-cerebellar tract. The posterior vermis then projects, via the fastigial nucleus, predominantly on the superior colliculus and the oculomotor reticular formation. This loop will therefore be mainly involved in the production and control of eye movements (Supple and Leaton, 1990).

Zone B (lateral part of the anterior vermis) receives the same types of somesthetic, proprioceptive and motor information as zone A, but these come exclusively from axial areas. It then projects to the vestibular nuclei which constitute the lateral (then median) vestibulo-spinal tract involved in the control of balance and posture (Andersson and Oscarsson, 1978).

The **flocculo-nodular lobe** receives vestibular and visual information from the pons and reticular formation. It then projects directly, or via the fastigial nuclei, onto the vestibular nuclei. It is thus, via this loop, also involved in the eye movements (Yamada and Noda, 1987).

3.1.4.2 Cerebello-thalamo-cortical loops

The **zones C and D** are involved in two large **cerebello-thalamo-cortical loops** that we will explain in details because these subsystems are likely to be involved in more complex functions of regulation and motor adaptation, or even cognitive functions. In these two loops, the red nucleus constitutes an important relay between the cerebellum, the cerebral cortex and the

thalamus. Its evolution was parallel and interdependent to that of the cerebellum. Its magnocellular part appeared with the **intermediate cerebellar hemisphere** (Zone C, spinocerebellum) and projected onto the bulb and the spinal cord. Its parvocellular part appeared with the **lateral cerebellar hemisphere** (Zone D, pontocerebellum) and projects on the ipsilateral olivary body (Rispol-Padel, 1993). **Zone C** (intermediate hemisphere or spinocerebellum) receives pons and spinal cord afferents that provide somesthetic, motor and proprioceptive information of the same type as zone A, but this time from limbs (schema of loops). The C-zone of the cerebellar cortex is then projected onto the interposed nuclei, which project in turn on the posterior part of the ventro-lateral nucleus of the thalamus (VPL) and the magnocellular part of the red nucleus (MRN). MRN inhibitory efferent will then contact the accessory olivary nucleus forming a feedback loop that is at the basis of theories regarding motor control. Indeed, the MRN jointly receives the efferent nerve fibers from the cerebellum and connections from the spinal cord and the primary cortex. This loop would thus make it possible to compare the copies of the motor control and the make movement actually executed. **Zone D** (lateral hemisphere or pontocerebellum) receives afferent nerve fibers from the upper colliculus and the oculomotor reticular formation via the main olive nucleus as well as afferent nerve fibers from the pons nuclei. All these afferents converge visual, oculomotor, somesthetic and auditory information. The D-zone of the cerebellar cortex then projects on the dentate nucleus, which gives some descending projections (brain stem, reticular formation, primary olivary nucleus) and predominantly upward projections towards the parvocellular part of the red nucleus (PRN) and the VPL in the thalamus. VPL efferent nerve fibers connects to many areas of the cerebral cortex, namely deep layers of the primary and premotor cortex, upper layers of the primary motor cortex, supplementary motor area, prefrontal cortex, posterior parietal cortex, and basal ganglia (Clower et al., 2005; Kelly and Strick 2003; Middleton and Strick 2001). In the cerebral cortex, cerebellar information is integrated with other types of information (motor, associative) and will be redirected to the PRN nucleus, that will project itself to the pons nuclei and the

olivary nuclei, thus completing another cerebello-thalamo-cortical loop. This loop allows direct control of the motor system via corticospinal pathways, but also acts on intracortical and cortico-cortical circuits of the upper layers of the cerebral cortex; thus, being able to intervene in the central processes preceding the initiation of the movement (Rispoli-Padel, 1993). Interestingly, a connection between the dentate nucleus and striatum via the thalamus has been recently demonstrated (Hoshi et al., 2005). This discovery brings to light a new cerebello-thalamo-striatal loop that could anatomically shape the functional relationship already observed between the cerebellum and the basal ganglia in associative motor learning (Laforte Jr. and Doyon, 2002, 2001). The anatomical loops we have just described thus show that information of different natures (visual, premotor, etc.) converge towards the cerebellum. In turn, the cerebellum projects on a large number of structures of the central nervous system with distinct functions (motor, premotor, associative, etc.). This particular connectivity allows the cerebellum to integrate information of various origins. However, it seems that depending on their origins, information is treated in anatomically different cerebellar circuits. In order to understand how these different fluxes of information are processed, we need to gaze at the cerebellar internal architecture that we describe in the next section.

3.2 Role of the cerebellum in motor and cognitive functions

The present understanding of the role played by the cerebellum originates from more than a hundred years of research that aimed to elucidate the contribution of this structure to the CNS function. It was noted, back to the 18th century, that the total ablation of the cerebellum provokes disturbances in posture and in the control of voluntary movements (Rolando et al., 1809). Theories that followed suggested a cerebellar role in spontaneous and target-directed movements coordination, balance and posture regulation (Dow et al., 1958). These theoretical approaches have been questioned by many studies concluding that the cerebellum has a more precise role in adapting motor performance to environmental constraints (Lisberger et al., 1988). The development of these theories has concentrated on the role that the cerebellum could play in

the regulation of reflex movements, while considering its particular anatomy and determining its underlying neural networks and computational centers. Finally, emerged the theories of the implication of the cerebellum in motor learning (Ito et al., 1984). These approaches have led to the development of simple protocols to study eye blink conditioning and other reflex movements, where anatomical circuits associated with the stimuli and the motor responses are well identified (Ito et al., 2006).

Finally, numerous human and animal models studies have shown that the cerebellum may be involved in non-motor cognitive mechanisms (Thach et al., 1997). The interest of comparing the studies -that employed simple motor learning protocols- and the behavioral observations of cognitive tasks helped in determining the fundamental operations performed in the cerebellum.

3.2.1 Implication of cerebellum in motor skills

In the early 19th century, Ivan Pavlov studied the dogs' digestive system and in specific the role of saliva in digestion. His studies led to the understanding that a natural reflex can be modified by learning. All the interest of his observations resides in conditioning, that is, the relationship between two initially distinct stimuli during learning (Pavlov et al., 1927). In this section, we will focus on an experimental paradigm whose underlying neural networks involve the cerebellum; the eye blink conditioning. In this paradigm, conditioned response (CR) is a motor adaptation that takes place after a learning process. In general, this adaptation is called motor learning.

The cerebellum controls motor skills: a voluntary motor command causes the arrival to the cerebellum, on one hand, vestibular information, direct or indirect somatosensory through the neocortex and, secondly, a copy of the motor order from motor cortex and an execution program of the motor order from the spinal cord. The cerebellum modulates the genesis of movement and control on the level of primary motor cortex through the thalamus. It thus gives the motor programs of the movement a chronological, somatotopic and spatio-temporal organization (Figure 6). In this context, the role of the cerebellum would be to generate an internal model of

the motor apparatus using sensory information from the external environment (Therrien and Bastian, 2015). The cerebellum also participates in motor learning such as the adaptation of the vestibulo-ocular reflex. It would also allow us to anticipate a predictive adaptation of the movement in relation to sensory information (Schlerf et al., 2012; Bastian, 2006). The damage of the cerebellar circuit leads to motor deficits resulting in clumsy gestures and a difficulty of the patients to perform fine and precise gestures. Writing becomes irregular in the horizontal and vertical direction. Dysmetria appears, accompanied by: hypermetria, asynergy (poor motor coordination, movement is no longer linked but it is decomposed) and poor distribution of tone in muscles. Cerebellar patients also display: (i) a delay in initiating and stopping the movement (dyschronometry), (ii) reduced rapid associated movements (adiadocokinesis) and (iii) tremors. We speak of cerebellar ataxia in reference to the poor coordination of movements. Cerebellar lesions also cause oculomotor deficits such as dissymmetric abrupt and irregular movement and nystagmus that can indirectly affect visual perception. It is thought that the cerebellum recalibrates the visual perception of the dynamics of visual stimulation (Therrien and Bastian, 2015).

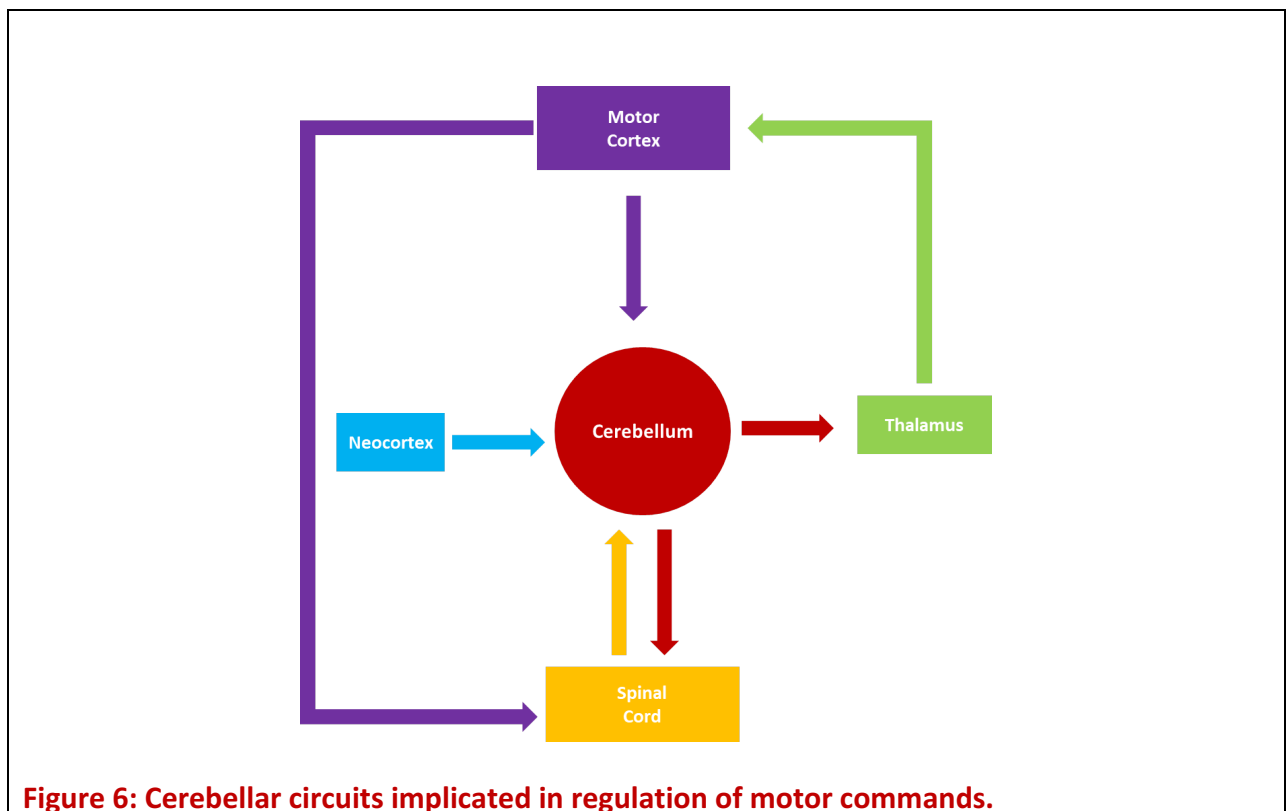


Figure 6: Cerebellar circuits implicated in regulation of motor commands.

3.2.2 Cerebellar participation in cognitive functions

We have previously discussed the fact that the cerebellum is anatomically connected to the zones of the associative and prefrontal cortex by its projections from the dentate nucleus (Middleton and Strick, 2001). In turn, the parietal, temporal and frontal associative cortices project on the lateral part of the cerebellar cortex (Massion et al., 1993). Thus, the cerebellum receives and projects information to these associative areas involved in non-motor functions. In addition, it has been shown that the lateral parts of the cerebellar and ventrolateral hemispheres of the dentate nucleus have developed considerably in relation to the rest of the cerebellum during evolution (Leiner et al., 1993). New functional imaging techniques have been used to study the activation of the cerebellum during cognitive tasks (Desmond et al., 1998). Patients with ASD present deficits of language and social communication; of interest, the cerebellum, seems to play crucial roles in many non-motor behaviors. We will present some cognitive functions such as language, reading, and emotional processes that are disturbed in ASD and for which the activation of the cerebellum has been detected; and associated pathological cases when cerebellar damage were present.

Recent studies of afferent and efferent connections of the cerebellum (Figure 7) using neurotropic viruses as trans-neuronal tracers as well as electrophysiological approaches (Bostal et al., 2013) have contributed to modify this purely motor vision of the function of the cerebellum and implicate it in other various complex functions as perceptual processes (Baumann et al., 2015). Indeed, the cerebellar nuclei project on many regions of the neocortex such as the pre-motor cortex, prefrontal cortex, and posterior parietal through multiple thalamic nuclei. The cerebellum is also connected to the basal ganglia that play not only a role in the programming and control of movements but also in the motivation of behaviors, in the treatment of cognitive processes and the regulation of mood.

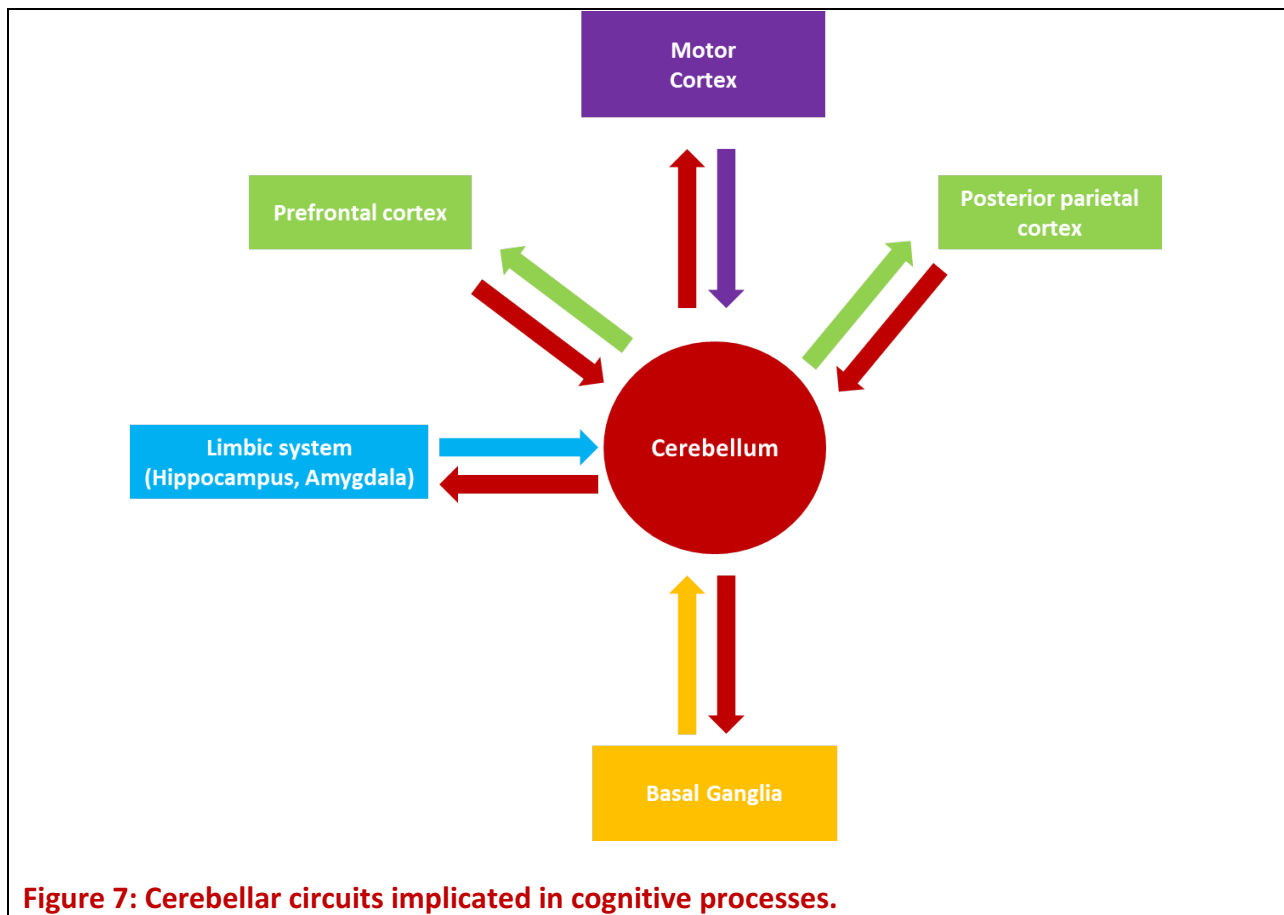


Figure 7: Cerebellar circuits implicated in cognitive processes.

3.2.2.1 Role of the cerebellum in language and reading

Children operated with a cerebellar tumor show a language deficit characterized by short answers, lack of elaboration and enthusiasm to enter the conversation, long latencies of answers and difficulties to find the words (Riva et al., 2000a). In a second study, the authors observed cases of mutism related to lesions in the vermis (Riva et al., 2000b). The process of speech production requires motor (articulations of words) and non-motors procedures (semantic search). In one of the first studies on functional imaging language, the subjects were asked to associate a verb with a common noun presented and to pronounce it verbally (e.g. when the word "bike" is presented, the subjects had to say "ride") (Petersen et al., 1990). Unexpectedly, an area of the right cerebellar hemisphere has been activated for this task. Interestingly, it was not activated for the simple reading of the same names. The cerebellum could thus participate in developing, as in the case of motor learning, a new association between the recognition of a word (bike) and the motor response associated with it (ride). In addition, after 15 minutes of presentation of the same names, the subjects increased the accuracy of their response. In addition to the improve in

performance, decreased activation of the right cerebellar hemisphere lead to a similar activation triggered by the simple reading of the names (Raichle et al., 1994). Thus, when the new association is successful, activation of the cerebellum decreases, which could indicate that the learning is complete and cellular activation can return to baseline levels.

Understanding words during reading involves the identification of letters, phonological assembly, and its association with its meaning in the mental lexicon (Pugh et al., 1997). These three functions were studied separately by asking subjects to identify word pairs that were identically written (letter identification), rhyme pairs (phonological assembly), or if these pairs of words belong to the same category (semantic processing) (Fulbright et al., 1999). Functional imaging demonstrated that the cerebellum is implicated in reading and is differentially activated in response to the different tasks. An activation of the lateral region of the cerebellar cortex was observed in response to phonological tasks. A similar important activation of the deep nuclei was seen in response to semantics processing.

3.2.2.2 Role of the cerebellum in emotions

Pathologies such as ASD or "cerebellar affective syndrome" are characterized in patients with attention and emotional disorders (Schmahmann and Sherman, 1997). In these pathologies, it has been proposed that the cerebellum could be one of the anatomical bases responsible for some of the complex behavior that has been reported. In humans, cerebellar lobules VI and VII are associated with the anterior cingulate cortex that plays an important role in emotion (Buckner et al., 2011). Crus I and II are cerebellar regions known to be engaged in emotional facial processing (Deeley et al., 2007). These regions are connected to cortical regions implicated in emotions and social cognition which are hypoactive in ASD patients (Pantelis et al., 2015).

3.2.3 ASD symptoms in patients with cerebellar disorders

In recent years, numerous studies have shown that one of the major causes of this disease are developmental abnormalities in the central nervous system, mainly the cerebellum and the prefrontal cortex. Postmortem studies have shown that in 95% of autopsied autism cases, anatomical cerebellar abnormalities were observed (Townsend et al., 2001). Studies show

bimodal distribution of the cerebellar vermis area with 87% hypoplasia and 13% hyperplasia in autism (Courchesne et al., 1994). Subsequent studies have demonstrated, by quantitative MRI, an inverse proportionality between the size of lobules VI and VII and that of the frontal cortex in autistics, whereas this is not the case in controls (Carper et al., 2000). A decrease in the number of PC in the cerebellum of autistic patients was observed in the early eighties (Ritvo et al., 1986). The areas where the PC would be in a smaller counts project to the fastigial nucleus of the cerebellum, which would lead to insufficient inhibition. In turn, the fastigial nucleus projects to the reticulated formation which in this case could be hyper activated. These functional connections may be at the basis of the sensory overload theory:

- Hyperacusis, which is a debilitating hearing disorder that accounts for increased sensibility to certain tone frequencies.
- Hyper-reactivity to light.
- Axial hypertonia, which accounts for the tip-toe walking.
- Hyper vestibular excitation, which accounts for the swinging frequently seen in certain subgroups of ASD patients.

The "cerebellar affective syndrome" described by Schmahmann (1997) is characterized in patients with localized lesions of the cerebellum with the following symptoms: motor performance deficits, verbal fluidity, spatial cognition disorders, personality disorders, changes in moods, and language problems. The emotional cognitive deficits observed in patients with cerebellar affective syndrome are attributed to cerebellar deficits that are similar to “pathological laughter and crying” (Parvizi et al., 2001). The uncontrolled episodes of emotions were linked to lesions in the cortico-ponto-cerebellar pathway. The cerebellum controls emotions according to the contextual recognition of emotions, and an absence of its control triggers an inappropriate behavior.

Taken all together, some of the cognitive deficits observed in cerebellar patients attempted to characterize cerebellar function in these cognitive processes. The role of the

cerebellum in these functions that involve prefrontal and associative structures is more complex to determine than in motor learning protocols whose neural circuitry is well identified. However, in each of the cognitive tasks presented, the literature suggests that the cerebellum may have a role similar to that identified in the motor adaptation functions. Its role as an association between contextual information and a motor or non-motor response could be the common operation performed by the cerebellum in motor and cognitive functions (Ito, 1993). During a cognitive task, the operation performed within the cerebellum would be the same as in a motor learning, but the anatomo-functional loops would be distinct.

Chapter 4: Mouse models of ASD

As previously mentioned, ASD is a set of spectrum of neurodevelopmental disorder characterized by social and communication impairments, as well as repetitive and restrictive behaviors. The heterogeneity of ASD phenotypes has made it hard to elucidate the etiology and underlying pathophysiology of the disorder. The syndromic profile of ASD is often conveyed by deficits such as seizures, hyperactivity, and sensorimotor abnormalities. To our benefit, the production of rodent models of ASD permitted the scientific community to evaluate and test diverse factors increasing the risk of ASD. Both genetic and environmental factors have been assessed for their involvement in the manifestation of ASD symptoms. At a broader scale, mouse and rat models of ASD have helped establish a better understanding of the underlying molecular pathways and physiological mechanisms in each one of the different etiologies. This approach should enable a better understanding of ASD on different levels including the (i) clinical, (ii) molecular, (iii) anatomical, and (iv) behavioral aspects of the disorder, with a focus towards translational benefits. It is hereafter mandatory to identify a common ground of behavioral phenotypes in relevance to ASD. In this section, we will cluster the ASD animal models into: genetic and non-genetic. We will also summarize major behavioral and anatomical findings for each model with their clinical relevance to ASD.

4.1 Genetic mouse models of ASD

Based on the identified risk factors possibly implicated in the etiology of ASD, animal models were generated mimicking the underlying pathophysiological mechanisms in order to understand the behavioral deficits of the disorder. Thus, the genetic and environmental models stood out and became apparent in the ASD research. The environmental models include prenatal infection and prenatal exposure to VPA. Genetic models comprise knockout mice of several identified genes that involve the pathology of syndromic and non-syndromic forms of ASD such as Fragile X syndrome, Tuberous sclerosis, Rett syndrome, Reelin, SHANK, Vasopressin, Dishevelled-1, Sert (serotonin transporter), PTEN and Neuroligins (Table IV). Although the number of animal models of ASD is rapidly increasing, more efforts are needed to validate ASD

phenotypes and elucidate the underlying pathologic relevance. Genetically modified mice are useful tools to discover the function of a gene and better understand the pathophysiology of the disease of interest. There are animal models for the study of rare monogenic forms of autism, associated with a given mutation, which show the disease symptoms. For example, mice lines with mutations of either PTEN, TSC, FMR1, MECP2, SHANK, NLGN or NRXN genes were generated. The characterization of these models helped evaluate defects in social interaction, communication, and repetitive behaviors that confirmed the implication of these genes in ASD. Mouse models such as FMR1 or SHANK2 gene knock-outs also exhibit hyperactive behaviors, symptoms present in individuals with ASD. At the cellular and molecular level, these animal models allowed to identify defects allowing to better determine the causes and consequences of this disease. Decreases or increases in synaptic density can be observed; a reduction in dendrites is found in the models of Rett syndrome, SHANK3 and tuberous sclerosis model, whereas the reverse effect is seen in models of fragile-X syndrome. Concerning neuronal activity, the same paradoxical observations have been found. Some models of autism present an increase and others a decrease in their excitatory or inhibitory currents.

4.1.1 Fragile X mental retardation 1 (FMR1)

The *FMR1* gene encodes for fragile X mental retardation protein (FMRP) (Verheij et al., 1993). FMRP is an RNA-binding protein that is found in brain, and genital glands. FMRP plays a role in local protein synthesis regulation in dendrites, as well as mRNA transport from nucleus to the cytoplasm (Garber et al., 2008). FMRP plays also an essential role in synapse development (Weiler et al., 1997), which is essential for normal neurotransmission, learning and memory, and plasticity of the synapses. Mutations in FMR1 due to expanded CGG repeats in the 5' gene untranslated region (5'UTR) blocks the production of FMRP and leads to a developmental disorder called Fragile X Syndrome (FXS). FXS is depicted by ID, developmental delays, congenital defects, seizures and autism-like behaviors and symptoms (Garber et al., 2008). About 10 to 30% of FXS patients are also diagnosed with autism (Hatton *et al.*, 2006). *FMR1* knockout mice have been generated to study the behavioral defects and underlying physiological

mechanisms in FXS. Interestingly, *FMR1* KO mice exhibit comorbidities with autistic behaviors including reduced sociability, augmented repetitive behaviors, anxiety, hyperactivity (Bernardet and Crusio, 2006), reduced spatial learning, and decreased object recognition ability (Brennan et al., 2006; Mineur et al., 2006). Nonetheless, some of these outcomes are questionable, as some researchers reported that *FMR1* KO mice show increased sociability and anxiety (McNaughton et al., 2008; Spencer et al., 2005), whereas others reported reduced social approach and anxiety (Liu and Smith, 2009; Mineur et al., 2006). Physiologically, *FMR1* knockout mice display increased dendritic spine length (Irwin et al., 2000), decreased mGluR5 expression (Bear et al., 2004) and increased mGluR-dependent Long-term depression (LTD) (Nosyreva and Huber, 2006), decreased Long-term potentiation (LTP) (Zhang et al., 2009), and an imbalance between excitation and inhibition (Silva and Ehninger, 2009). Nevertheless, based on the behavioral and physiological findings, *FMR1* KO mouse model offers helpful understandings of the *FMR1* gene in the etiology of ASD.

4.1.2 Methyl CpG binding protein 2 (MeCP2)

MeCP2, is a transcriptional repressor that binds to methyl groups in CpG islands of DNA (Yasui et al., 2007) and activates gene transcription (Chahrour et al., 2008). In mammals, regulation of transcription by *Mecp2* is essential for chromatin modulation. In humans, MeCP2 mutations leads to Rett syndrome, a neurodevelopmental disorder that causes mental retardation and developmental delays in females (Rett, 1966). Rett syndrome was originally considered a subtype of autism, yet recent DSM-V criteria distinguish Rett syndrome and ASD (American Psychiatric Association, 2013). Rett was then modeled into mice to help understand the pathophysiology of the disorder; MeCP2 knockout mice present: (i) increased anxiety (Chahrour and Zoghbi, 2007), (ii) decreased motor coordination (Guy et al., 2001), impaired sociability and social memory, (iii) reduced ability of nest-building, and (iv) impaired learning (Chahrour and Zoghbi, 2007; Moretti et al., 2006). Collectively, this highlights the potential pathophysiological role of epigenetic dysregulation on genetic determinants of ASD.

4.1.3 Neuroligins

NLGN genes encode for neuroligin proteins, cell adhesion molecules essential for normal synaptic functions (Sudhof, 2008). NLGN is found in the postsynaptic membrane and facilitates synapse transmission between neurons (Jamain et al., 2008). Mutations in NLGN3 are associated with intellectual disability, seizures, and ASD (Jamain et al., 2003). Although NLGN mutations might provoke ASD in rare case (Ylisaukko-oja et al., 2005), the functions of some subunits were studied in mouse models. NLGN1 knockout mice exhibit: (i) impaired spatial memory and (ii) increased repetitive grooming (Blundell et al., 2010), two core features of ASD. Since mutation of NLGN3 is associated with ASD in humans (Comoletti et al., 2004), NLGN3 mutant mice were generated as a model of ASD. These mice display (i) impaired social interactions, and (ii) enhanced spatial learning (Etherton et al., 2011). Although NLGN mutant mice display reduced sociability, more research is needed to link the reduced social behavior to ASD.

4.1.4 Neurexin

NRXN1 is a presynaptic cell adhesion molecule that mediates synaptic interaction through interactions with NLGN (Li et al., 2006). Individuals with NRXN1 deletion display (i) mental retardation, (ii) language delay, (iii) schizophrenia (Need et al., 2009; Walsh et al., 2008), and ASD (Ching et al., 2010). Although they do not show obvious social defects, NRXN1 α deficient mice display: (i) pre-pulse inhibition, (ii) reduced ability of nest-building, (iii) increased repetitive grooming, and (iv) improved motor learning (Etherton et al., 2009). Physiologically, these mice exhibit variations in excitatory synaptic transmission. In brief, the observed phenotypes in NRXN1 α deficient mouse could be associated to disorders other than ASD, especially that they show normal social behavior.

4.1.5 Phosphate and tensin homolog

PTEN is a tumor suppressor protein that plays a role in cell cycle and apoptosis (Chu and Tarnawski, 2004). Generation of PTEN mouse models was encouraged by the presence of many genetic mutations of PTEN in patients with ASD (Butler et al., 2005). PTEN mutant mice show (i) reduced social interactions, (ii) hyperactivity, and (iii) reduced prepulse inhibition (Ogawa et

al., 2007). In addition, many studies have shown an increased cell proliferation due of PTEN deletion (Bonaguidi et al., 2011; Gregorian et al., 2009), suggesting that PTEN regulates proliferation of neural stem cells (Zhou and Parada, 2012). Finally, PTEN interacts with PI3K/AKT and TSC/mTORC1 signaling pathways that are known to contribute in the physiopathology of ASD (Zhou and Parada, 2012).

4.1.6 SH3 and multiple ankyrin repeat domains protein (SHANK)

SHANK are scaffold proteins that interact with receptors, channels, and membrane proteins on the postsynaptic side. During CNS development SHANK play an important role in the formation of the synapse as well as the maturation of the dendritic spine. Given the fact that SHANK genes dysregulation is involved in ASD in humans (Jiang and Ehlers, 2013), SHANK knock-out mice were generated to study the behavioral and physiological alterations. Many studies have reported mutations in the SHANK2 gene in ASD patients (Leblond et al., 2012; Berkel et al., 2010). ProSAP1/Shank2^{-/-} mutant mice display ASD-related behaviors as: (i) repetitive grooming, (ii) hyperactivity, and (iii) reduced sociability (Schmeisser et al., 2012). The mutant mice show less dendritic spines and decreased synaptic transmission (Schmeisser et al., 2012). Microdeletions of 22q13, are observed in SHANK3 mutations. In human patients with ASD SHANK3 genes mutations were found to be associated with developmental deficits, and ASD-related behaviors (Manning et al., 2004). SHANK3B^{-/-} mice exhibit: (i) increased repetitive behavior and (ii) reduced sociability (Peca et al., 2011). However, it is important to note that the behavioral phenotypes observed in Shank3 mutant mice require careful analysis because similar mutations are associated with schizophrenia (Gauthier et al., 2010).

4.1.7 Tuberous sclerosis complex protein (TSC)

Mutations of TSC1 or TSC2 are associated with the Tuberous sclerosis complex (TSC) disorder. TSC1/TSC2 act as tumor growth suppressors and play important roles in cell proliferation. TSC patients have a high prevalence of ASD (20 to 60%) (Bolton et al., 2002; Curatolo et al., 2004). These mutations are also associated with cognitive dysfunction and epilepsy (Goorden et al., 2007; DiMario, 2004). Loss of neuronal TSC1, in mice, induced

cortical hyperexcitability and seizure susceptibility (Meikle et al., 2007). TSC1 conditional knockout in mice provoked increased brain size and mTORC1 signaling (Carson et al., 2011). Interestingly, TSC loss in mice provoked: (i) reduced sociability, (ii) increased repetitive behaviors, and (iii) impaired ultrasonic vocalizations, with (iv) reduced PC cell count (Tsai et al., 2012). Animal models of TSC could help unpin pathways associated with ASD.

In Brief, every specific etiologic condition being modeled by genetic modification holds a uniquely different set of ASD-like behavioral and neuropathological profiles. Thus, genetic models tend to be more related to synaptopathies, especially that most of the behavior they induce can be linked to other psychiatric disorders as schizophrenia, OCD and ADHD.

Table IV: ASD behavioral and pathological features in genetic models in relevance to clinical symptoms and neurobiology.

Mouse model of ASD	ASD behavior	Other symptoms	Neurobiological deficits	Clinical symptoms and neurobiology	references
FMR1 KO	<ul style="list-style-type: none"> • Repetitive behaviors. • Reduced sociability. 	<ul style="list-style-type: none"> • Anxiety. • Seizures. • Reduced spatial learning. • Impaired object recognition. 	<ul style="list-style-type: none"> • Increased dendritic spine length. • Increased mGluR-dependent LTD. • 50% reduced mGluR5 expression. • Imbalance between excitation and inhibition in the brain circuitry. • Increased dendritic spine length. • Cortical LTP decrement. • Delayed GABA polarity developmental switch (i.e. from depolarizing to hyperpolarizing) and dysregulated intracellular chloride levels. 	<ul style="list-style-type: none"> • Main cause of FXS, from FMR1 mutation, i.e. expanded CGG trinucleotide repeats (55-230) in the 5' untranslated region (5'UTR) & halts the production of FMRP. • 10-30% of FXS are diagnosed with autism. 	Garber et al., 2008; Hatton et al., 2006; Bernardet and Crusio, 2006; Silva and Ehninger, 2009; Brennan et al., 2006; Spencer et al., 2005; McNaughton et al., 2008; Mineur et al., 2006; Irwin et al., 2000; Nosyreva and Huber, 2006; Bear et al., 2004; Zhang et al., 2009;
MeCP2 KO	<ul style="list-style-type: none"> • Impaired sociability. • Impaired nest building behavior. • Impaired social memory. 	<ul style="list-style-type: none"> • Anxiety. • Motor coordination deficits. • Impaired learning. • Impaired memory. 	<ul style="list-style-type: none"> • Increased neuronal transcription through enhanced histone acetylation. • Neurotoxicity due to excessive glutamate release from microglia. • Abnormal dendrites and axon development. • Reduced inhibitory quantal size of GABAergic neurons. 	<ul style="list-style-type: none"> • Main cause of x-linked, female –prevalent Rett syndrome, an MeCP2 mutation disorder. 	Rett, 1966; Chahrour and Zoghbi, 2007; Guy et al., 2001; Moretti et al., 2006; Guy et al., 2011;
NLGN1 KO	<ul style="list-style-type: none"> • Repetitive/stereotype grooming 	<ul style="list-style-type: none"> • Impaired spatial memory 	<ul style="list-style-type: none"> • Decreased NMDA/AMPA ratios in cortico-striatal synapses. • Decreased hippocampal LTP. 	<ul style="list-style-type: none"> • NLGN mutations may cause autism in rare occasions. 	Blundell et al., 2010
NLGN3 mutant mice	<ul style="list-style-type: none"> • Impaired sociability. • Deficits in USV. • Deficits in preference for social novelty. 	<ul style="list-style-type: none"> • Enhanced spatial learning. • Olfactory deficits. • No change in PPI 	<ul style="list-style-type: none"> • Increased inhibitory synaptic transmission with no apparent effect in excitatory synapses in the somatosensory cortex. 	<ul style="list-style-type: none"> • Arg451Cys (R451C) mutation of NLGN3 is associated with autism. 	Comoletti et al., 2004; Etherton et al., 2011;
NRXN1-	<ul style="list-style-type: none"> • Impaired nest building. • Increased grooming 	<ul style="list-style-type: none"> • Impaired PPI. • Enhanced motor 	<ul style="list-style-type: none"> • Defects in excitatory synaptic transmission. 	<ul style="list-style-type: none"> • NRXN1 deletion was implicated with ASD 	Chiang et al., 2010; Etherton et al., 2009

Mouse model of ASD	ASD behavior	Other symptoms	Neurobiological deficits	Clinical symptoms and neurobiology	references
alpha-deficient mice	<ul style="list-style-type: none"> repetitive behavior. No social deficits. 	<ul style="list-style-type: none"> learning. 		<ul style="list-style-type: none"> along with other disorders 	
PTEN mutant mice	<ul style="list-style-type: none"> Impaired sociability. 	<ul style="list-style-type: none"> Excessive responses to stimuli. Hyperactivity. Decreased PPI. 	<ul style="list-style-type: none"> Macrocephaly and neuronal hypertrophy. Increased cell proliferation. 	<ul style="list-style-type: none"> PTEN variations were observed in ASD with macrocephaly phenotypes. 	<ul style="list-style-type: none"> Butler et al., 2005; Ogawa et al., 2007; Gregorian et al., 2009; Bonguidi et al., 2011
SHANK2 -/-	<ul style="list-style-type: none"> Repetitive grooming and jumping. Impaired SUVs and social interaction behaviors. 	<ul style="list-style-type: none"> Hyperactivity. 	<ul style="list-style-type: none"> Fewer dendritic spines and lower basal synaptic transmission with increased excitatory currents by NMDA receptors. Decreased NMDA receptor function (another study). 	<ul style="list-style-type: none"> Mutations in the SHANK2 gene were reported in ASD patients. Defective dendritic branching and decreased postsynaptic clustering. 	<ul style="list-style-type: none"> Berkel et al., 2010; Leblond et al., 2012; Berkel et al., 2012; Schmeisser et al., 2012;
SHANK3 mutant mice	<ul style="list-style-type: none"> Deficits in social interaction. Mild social deficits in juvenile. Repetitive grooming (controversial). Decreased USVs (controversial). 	<ul style="list-style-type: none"> Decreased reversal learning (controversial). Impaired novel object recognition and deficits in motor coordination. 	<ul style="list-style-type: none"> Cortico-striatal circuit and striatal synaptic defects. Decreased EPSCs in the pyramidal neurons of hippocampal CA1. Reduced GluR1-immunoreactive puncta of the stratum radiatum. 	<ul style="list-style-type: none"> Mutations of the SHANK3 genes, such as the microdeletions of 22q13, have been implicated in ASD. link to schizophrenia due to similar findings regarding SHANK3 mutations. 	<ul style="list-style-type: none"> Manning et al., 2004; Peca et al., 2011; Gauthier et al., 2010;
Conditional TSC1 KO	<ul style="list-style-type: none"> Deficits in social interaction. Repetitive behaviors. Impaired Vocalizations. 	<ul style="list-style-type: none"> Increased seizure susceptibility. 	<ul style="list-style-type: none"> Significant brain pathology. Cortical excitability. Enhanced AMPAR and NMDAR-mediated EPSCs. Increased brain size and elevated mTORC1 signaling but declined mTORC2 signaling. Reduced PC counts. 	<ul style="list-style-type: none"> Mutations of TSC1 or TSC2 cause Tuberous sclerosis (TSC). 20- 60% of TSC patients have ASD. TSC1/TSC2 mutations associated with cognitive dysfunction. 	<ul style="list-style-type: none"> Bolton et al., 2002; Curatolo et al., 2004; DiMario, 2004; Goorden et al., 2007; Meikle et al., 2007; Carson et al., 2012; Tsai et al., 2012;

Abbreviations: USVs: ultrasonic vocalizations; PPI: Prepulse inhibition

4.2 Environmental mouse model of ASD

4.2.1 VPA mouse model of ASD

VPA is a commonly prescribed pharmaceutical agent to manage seizures, migraine and bipolar disorder manic episodes. Several studies have shown that VPA use during pregnancy leads to many defects in children, comprising neural tube defects (Ornoy, 2009), intellectual (Moore et al., 2000), and behavioral and cognitive deficits (Moore et al., 2000), many of which are connected to the core behaviors and symptoms described in ASD. Indeed, the increased incidence of autism in children exposed to medication prior to birth has encouraged the development of animal models that reproduce prenatal exposure to VPA in order to study this pathophysiological hypothesis. The idea of this animal model to study autism was born from the observation of cases of autism that may have been induced in children during pregnancy (Christensen et al., 2013; Christianson et al., 1994).

Indeed, prenatal exposure to VPA, an antiepileptic treatment, seems to be at the origin of an increased number of births of autistic children. This model, thus, is of special interest to understand a possible etiology of autism, and to mimic the development of pathology. Studies of children exposed to VPA during pregnancy suggest that VPA is likely to result in autism during the first trimester of pregnancy (see: Ornoy, 2009; Arndt et al., 2005; Rodier, 2004, 2002; Kemper and Bauman, 1998). Some autistic children exposed to VPA before birth had malformations of the limbs indicating an abnormal development occurring at the onset of embryogenesis. In addition, animal models' studies have shown that single exposure to VPA at an early stage of embryogenesis, during the period of neural tube closure in mice E12,5, which corresponds to the 28th day window in humans (O'Rahilly and Müller, 2002). This critical time point, at which VPA exposure provokes autism, led to the creation of rat-VPA model (Kim et al., 2011; Rodier et al., 1997; Arndt et al., 2005; Kemper and Bauman, 1998). The clinical manifestations observed in the VPA prenatally exposed rat offspring on around E12,5 validated the model for presenting both ASD behavioral and anatomical characteristics as those seen in human patients.

For instance, VPA prenatally exposed rats present: (i) Reduced social interaction, (ii) increased repetitive behavior, (iii) impaired eye blink conditioning, (iv) anxiety (Zhang et al., 2012; Kim et al., 2011; Markram et al., 2008; Stanton et al., 2007; Schneider et al., 2007, 2006, 2001; Schneider and Przewlocki, 2005), (v) reduced motor cell counts in the brain stem (Rodier, 2002; Rodier et al., 1996) and reduced PC counts in cerebellum (Ingram et al., 2000; Rodier et al., 1997).

Animal models are generated in order to (i) test hypotheses about the etiology underlying a disease and (ii) assess the translational benefit of a medication (Chadman et al., 2009; van der Staay, 2006). To achieve these goals animal models must meet certain validity criteria: construct, face, and predictive validity (Nestler and Hayman, 2010). Thus, animal models' strength can be assessed based on the common features between the animal model and human disease. The animal model should also show replicability (van der Staay et al., 2006). Construct validity is based on the degree of resemblance of the mechanisms underlying the disease modeled in animals and the human disease. Thus, the behavioral and physiological factors present in human patients must be shared by the animal model (Bourin et al., 2007; Epstein et al., 2006). Bearing in mind human evidence of ASD followed by an early exposure to VPA, Rodier et al. (1996) first generated the VPA animal model of autism by prenatally exposing rats to VPA through the intrauterine route (Rodier et al., 1996). This model stood on the data showing the increased risk of ASD in children prenatally exposed to VPA (Bescoby-Chambers et al., 2001; Williams et al., 2001). While autism can be described as an umbrella of "spectrum of disorder" the VPA animal model perfectly mimics the clinical features of the disease seen in ASD patients (Figure 8). With regards to construct validity, the pathogenesis of autism via VPA induction can be traced in a number of intersecting pathways involving: (i) oxidative stress, (ii) histone deacetylase inhibition, (iii) Excitatory/Inhibitory imbalance, and (iv) hyperserotonemia. It has been suggested that VPA may create specific changes in fetal brains in animals that become

more sensitive to increases in reactive oxygen species (ROS) than any other fetal organs (Ornoy, 2009).

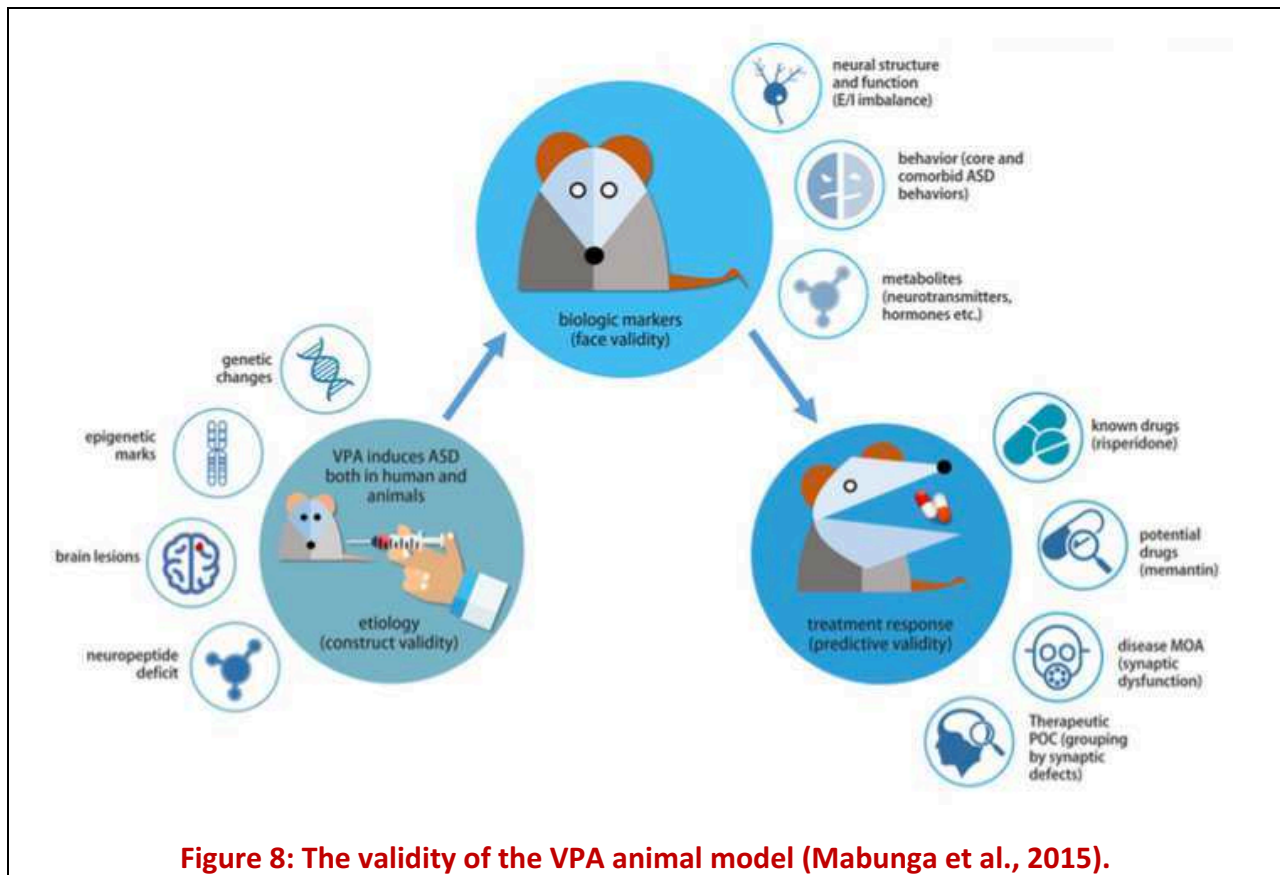


Figure 8: The validity of the VPA animal model (Mabunga et al., 2015).

This vulnerability to oxidative stress added to immature embryonic antioxidant defense system may contribute to the resulting teratogenic effect of VPA. It has been also shown that VPA exposure provokes hyper acetylation in the fetal brain causing increased apoptotic cell death and reduced cell proliferation in certain brain regions (Kataoka et al., 2013; Phiel et al., 2001). In addition, VPA induces deficits in the neural development leading to E/I imbalance (Kim et al., 2014; Fukuchi et al., 2009).

Face validity requires the presence of disease endophenotypes in animals comprising Behavioral symptoms, neurophysiological, pathological, and neurochemical alterations (Chadman et al., 2009). The occurrence of these endophenotypes is the first step in identifying an animal model of a psychiatric disorder (Geyer and Moghaddam, 2002). ASD is diagnosed by two criteria: (i) persistent social deficits and (ii) occurrence of restricted repetitive behaviors (APA, 2013); along the presence of other traits. It was suggested that an animal model should

demonstrate at least two core features of the ASD human symptoms (Belzung and Lemoine, 2011). Of interest, VPA treatment at E12.5 induced reduced social interaction and repetitive behavior postnatally in offspring (Kim et al., 2011; Schneider and Przewłocki, 2005).

Finally, predictive validity, in general, indicates the extrapolation of human behavior through laboratory-manipulated animal behavior (Epstein et al., 2006). This means that the animal model must demonstrate the same response human demonstrates when exposed to certain drugs (Chadman et al., 2009). Furthermore, the drug development processes the animal model should be able to identify drugs that might be of benefit to human patients (Sarter and Bruno, 2002; Geyer and Markou, 1995). Donepezil increased social behavior and decreased repetitive and hyperactive behavior in VPA-treated mice (Kim et al., 2014). The same effect was also found in another study using the H3-Antagonist Ciproxifan in VPA mice (Barinio et al., 2015). Evidence of environmental enrichment boosting the performance of rats in various learning tasks and social behavior has led to the hypothesis that it can also be beneficial in VPA animal model of ASD (Schneider et al., 2006). In humans, it was lately suggested that sensorimotor enrichment seems to be a cost-effective tool in managing autism (Woo et al., 2015; Woo and Leon, 2013).

In view of the remarkable symptoms parallels seen in both VPA animal model and ASD patients, the VPA model is widely used to study ASD. Aiming to understand the neurobiology of ASD, VPA was also modeled in mice showing the two core signs of ASD: (i) reduced social interaction, and (ii) increased repetitive behavior. An overview of the VPA mouse model studies in the literature is summarized in Table V.

Mouse Strain	VPA Dose	Exposure window	outcome	Ref
ICR (CD-1)	300 mg/kg; single s.c. injection	E 10	lack of sociability ↓ social interactions ↓ nest building ↑ repetitive digging behavior	Kim et al., 2014b
C57BL/6 J	600 mg/kg; single i.p. injection	E 12,5	lack of sociability ↓ social interactions ↓ number of pup vocalizations ↓ number of complex call types ↑ number of Flat call type ↑ repetitive behavior	Moldrich et al., 2013
CD1	500 mg/kg;	E 9 E 12,5 E 14,5	↓ social interactions ↑ anxiety ↓ locomotor activity ↓ general exploratory activity memory deficits in E12.5 VPA-treated mice	Kataoka et al., 2013
C57BL/6Hsd	600 mg/kg; single s.c. injection	E 13	↑ repetitive self-grooming and digging behaviors ↑ anxiety	Mehta et al., 2011
Hybrid of (C57BL/6, CF-1, Swiss Webster, DBA-2)	800 mg/kg; 1.5 g of peanut butter mixed VPA	E 11	Lack of sociability ↑ latency to find home bedding ↓ general exploration	Roulet et al., 2010
C57BL/6Hsd	600 mg/kg single s.c. injection	E 13	↓ pup distress calls ↓ adult vocalizations ↓ social preference ↓ prepulse inhibition ↑ repetitive self-grooming ↑ anxiety	Gandal et al., 2010
Balb/C	200 mg/kg	E 12–17	↑ latency to right at P10 in VPA-treated	Wagner et al., 2006
	600 mg/kg	E 13	Impaired postnatal righting reflex reduced grip strength on hanging wire test	
	200 mg/kg 400 mg/kg	P 14 P 14	Impaired spatial learning Impairments in mid-air righting ↑ latency to find visible platform (400 mg/kg) in Morris Water maze Impaired spatial learning (both doses) in VPA-treated	

Table V: Dose, Exposure window and outcomes in VPA mouse model of ASD.

Abbreviations: E — embryonic day, P — postnatal day, i.p. = intraperitoneal; s.c. = subcutaneous, VPA — valproic acid.

Several studies in the literature have assessed cellular and molecular alteration in VPA prenatally treated mice and have concluded that VPA prenatal treatment negatively altered different molecular players as well as the anatomical networks of CNS (Table VI).

Mouse Strain	VPA Dose	Exposure window	Outcome	Ref
CD1	500 mg/kg; single i.p. injection	E 9 E 12,5 E 14,5	↓ numbers of cortical neurons ↓ embryonic neurogenesis ↑ cortical embryonic apoptosis and ↓ cell proliferation	Kataoka et al., 2013
ICR (CD-1)	500 mg/kg; single i.p. injection	E 12,5	↓ Nissl-positive neurons in layers II-III and V of the prefrontal cortex of female mice	Hara et al., 2012
Hybrid of (C57BL/6, CF-1, Swiss Webster, DBA-2)	800 mg/kg; 1.5 g of peanut butter mixed with VPA	E 11	↓ BDNF mRNA in the somatosensory cortex	Roulet et al., 2010
C57BL/6Hsd	600 mg/kg; single s.c. injection	E 13	↓ gamma-phase locking and evoked gamma-power	Gandal et al., 2010
Hybrid of (C57BL/6, CF-1, Swiss Webster, DBA-2)	800 mg/kg; 1.5 g of peanut butter mixed VPA	E 11	↓ neuroligin 3 mRNA in hippocampal subregions (CA1, CA3, DG) and in the somatosensory cortex	Kolozsi et al., 2009
C57BL/6	500 mg/kg; single i.p. injection	E 10,5	↓ parvalbumin-positive inhibitory neurons in parietal and occipital cortices	Gogolla et al., 2009

Table VI: Cellular and molecular abnormalities induced by VPA prenatal treatment.

Abbreviations: E = embryonic day, i.p. = intraperitoneal; s.c. = subcutaneous, VPA = valproic acid.

4.2.2 Maternal Immune Activation models of ASD

Maternal immune activation (MIA) is a risk factor for abnormal brain development provoking neurodevelopmental disorders such as ASD (Mazina et al., 2015). It has been demonstrated that MIA during gestation, in animal models, leads to a disruption in the immune system in offspring resulting eventually in autism-like behaviors that persist until adulthood (Patterson, 2011). Furthermore, it has been shown that interleukin-6 (IL-6) plays a role in the transcriptional and behavioral abnormalities of MIA in offspring (Smith et al., 2007), and was lately shown to provoke ASD in children (Daniels et al., 2008). The fact that IL-6 has been found to be boosted in brains (Benvenuto et al., 2009) and plasma of ASD patients (Ashwood et al., 2011), suggests that it is of relevance to study IL-6 and its contribution in ASD etiology.

The association between prenatal infections and psychiatric and neurological disorders such as autism, schizophrenia and cerebral palsy has been shown repeatedly (Atladottir et al., 2012;

Hagberg et al., 2012; Limperopoulos et al., 2008; Edwards and Tan, 2006; Dammann et al., 2002; Nelson and Willoughby, 2000). Numerous studies have been conducted on the effects of an infection on the development of ASD. Thus, an initial study estimated that 10% of children with congenital rubella suffer from autism (Chess, 1977). Exposure to rubella virus during pregnancy, particularly before maturation of fetal immune system functions, would seem to be responsible for the development of autism (Lotspeich and Ciaranello, 1993). The meta-analysis of Fombonne (2003) showed that 0.3% of cases of autism may be related to congenital rubella. Currently, vaccination can greatly reduce the incidence of this disease in pregnant women. Three main components of pathogens, or molecules that mimic them, have been used in MIA models, either the Lipopolysaccharide (LPS) of *E. coli*, the influenza virus or poly I:C, a synthetic compound (double stranded RNA) that mimics viral nucleic acids attack. The bacterial membrane LPS is recognized by the immune system Toll Like Receptor-4 (TLR-4). However, viral mimetics such as Poly I:C and double stranded RNAs are recognized by the TLR3 (Figure 9). Although TLR4 and TLR3 activation provokes different signaling pathways, activation of these receptors ends by the transcription and translation of inflammatory cytokines (IL-1 β ; IL6; TNF α). Because of the crucial role played by these different immune molecules in normal CNS development, many have hypothesized that immune activation leading to abnormal expression of these molecules can harm the CNS development (Bilbo and Schwarz, 2017, 2012, 2009; Deverman and Patterson, 2009). Interestingly, the cytokine levels, namely IL-6, increases in the placenta and amniotic fluid of pregnant mice that received influenza virus, and provokes abnormal development of the pups that persists in postnatal life as behavioral and anatomical changes (Shi et al., 2003), as does the double stranded RNA poly I:C (Shi et al., 2009).

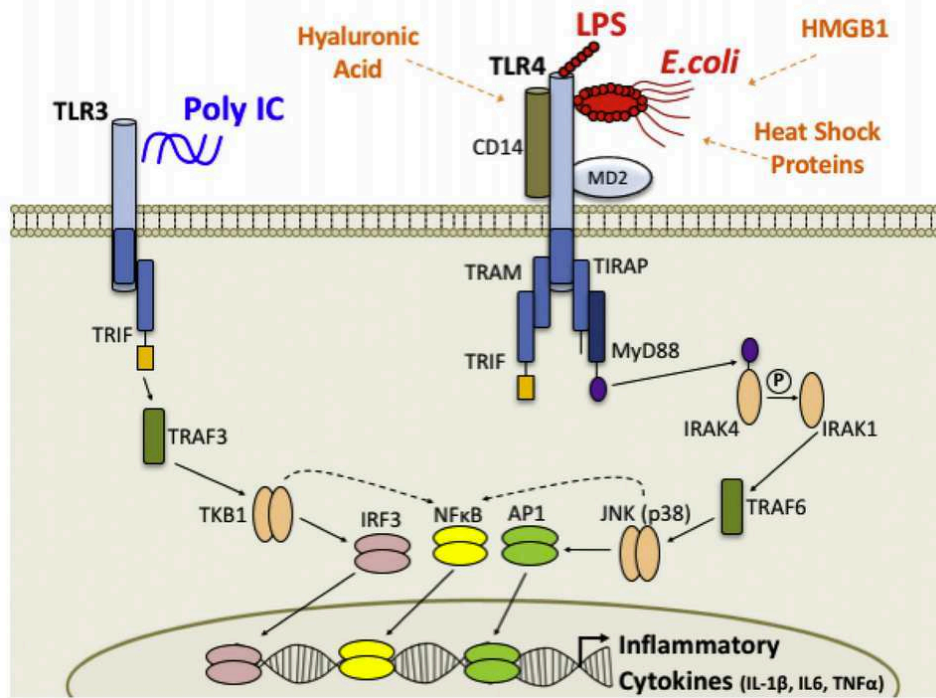


Figure 9: TLR of the innate immune system recognizes Poly I:C and LPS.

HMGB: high mobility group box. CD14: cluster of differentiation 14. TRIF: toll-like receptor adaptor molecule. TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein. MyD88: Myeloid differentiation primary response gene (88). IRAK: interleukin-1 receptor-associated kinase. TRAF: TNF receptor-associated factor. JNK: c-Jun N-terminal kinase. TKB1: a tyrosine kinase. AP1: activator protein 1 transcription factor. NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor. IRF3: interferon regulatory factor 3 transcription factor (adapted from Schwarz and Bilbo, 2011).

Poly I:C, the dsRNA lacking a pathogen, is used to model ASD in mouse by maternal immune activation. Exposure to poly I:C induces anti-viral inflammatory immune response in the host pregnant dams. As previously mentioned poly I:C is recognized by toll-like receptors TLR-3, and exposure to this dsRNA during specific time window is enough to provoke behavioral and anatomical deficits in the pups similar to those seen in influenza infected mothers offspring (Shi et al., 2008, 2003; Winter et al., 2008). Advancement in animal models of MIA has presented promising outcomes, yet further research is needed to validate these models and to connect immune dysregulation with ASD (Hsiao et al., 2012).

As previously described, Poly I:C is a ds RNA that mimics viral infection that can induce MIA. Offspring of rats that have been prenatally exposed to Poly I:C during gestation display autism-like behaviors, including augmented anxiety, increased repetitive behaviors (Meyer et al., 2011; Patterson, 2009), reduced social behavior and communication, and decreased sensorimotor

coordination (Naviaux et al., 2013) and decreased prepulse inhibition (Meyer et al., 2011; Patterson, 2009). Rat and mice brains prenatally injected with poly I:C display a local deficit in PC in the lobule VII of the cerebellum, in addition to delayed migration of these cells in lobules VI and VII (Naviaux et al., 2013).

Furthermore, synaptosome aberrations were also spotted, comprising postsynaptic density (PSD) malformation, down-regulation of purinergic receptors and reduced phosphorylation of ERK1/2 and CAMKII (Naviaux et al., 2013). However, poly I:C prenatal exposure in rodents have also been used to model other psychiatric disorders as schizophrenia (Kumamaru et al., 2014). Overall, MIA rodent models of ASD could be interesting tools in studying the infection etiology involved in ASD. Although the type of microbes and pathogens used in these models may differ from those in the human cases with ASD, the autism-like behaviors induced by MIA are quite similar to phenotypes involved in human ASD (Patterson, 2011). However, behavior present in the poly I:C mouse model compels careful examination especially that these mice were used to model other psychiatric disorders, namely schizophrenia (Meyer et al., 2008). The poly I:C mouse model has been used, and many studies reported deficits in social interaction, memory and novel object exploration, altered eye-blink conditioning, and pre-pulse inhibition (Table VII).

Strain	Poly I:C Dose	Exposure window	outcome	Ref
C57BL6/N	5 mg/kg; i.v. single injection	E 9	deficits in social approach behavior abnormal stereotyped digging impaired acquisition and expression of tone-cued fear conditioning in juveniles	Vuillermot et al., 2017
BTBR C57BL/6J	20 mg/kg; i.p. single injection	E 12,5	↓ social approach ↑ ultrasonic vocalization ↑ repetitive marble burying	Schwartz et al., 2013
C57BL/6J	2 mg/kg; i.p. single injection	E 12,5	↓ social preference in males ↓ sensorimotor coordination in males (rotarod)	Naviaux et al., 2013
C57BL/6J	20 mg/kg; i.p. single injection	E 12,5	↓ % PPI ↑ % Freezing ↓ center entries in open field	Hsiao and Patterson, 2011
C57BL/6J	5 mg/kg i.v. 20 mg/kg i.p.	E 12,5	↑ latency to find platform in morris water maze (20 mg/kg dose) ↓ novel object recognition (20 mg/kg dose)	Ito et al., 2010
C57BL6/J	5 mg/kg; i.p. single injection	E 9 E 17	E 9: significantly impaired sensorimotor gating and reduced prefrontal dopamine D1 receptors in adulthood E 17: impaired working memory	Meyer et al., 2008
mouse	20 mg/kg; single i.p. injection	E 12	Impaired extinction of conditioned eye-blink conditioning	Lee et al., 2007

Table VII: Dose, Exposure window and outcomes in Poly I:C mouse model of ASD.

Abbreviations: E = embryonic day, i.p. = intraperitoneal; i.v. = intravenous.

In addition, adult offspring prenatally exposed to poly I:C also show several cellular and molecular deficits. A core feature of the cellular consequences is the PC loss in lobule VII (Naviaux et al., 2013; Shi et al., 2009) in line with clinical reports in human patients of ASD. Other neuropathological deficits observed in the poly I:C model, including reduced reelin and parvalbumin- positive cells in PFC (Meyer et al., 2008), and reduced PC count (Shi et al., 2009) are summarized in Table VIII.

Strain	Poly I:C Dose	Exposure window	outcome	Ref
C57BL/6J	2 mg/ kg; i.p. single injection	E 12,5	malformed, hypomorphic PSD abnormal accumulation of electron-dense matrix material PC loss in lobule VII	Naviaux et al., 2013
C57BL/6J	5 mg/kg i.v. 20 mg/kg i.p.	E 12,5	abnormal c-Fos expression in area CA1 pyramidal neurons following novel object exposure (5 mg/kg dose)	Ito et al., 2010
C57BL/6J	20 mg/kg; single i.p. injection	E 12,5	↓ 20% PC in lobule VII in adult offspring	Shi et al., 2009
C57BL6/J	5 mg/kg; i.p. single injection	E 9 E 17	E 9: reduced prefrontal dopamine D1 receptors in adulthood E 17: potentiated the locomotor reaction to the NMDA-receptor antagonist dizocilpine, and reduced hippocampal NMDA- receptor subunit 1 expression. Reduced reelin+ and parvalbumin+ cells in PFC	Meyer et al., 2008

Table VIII: Cellular and molecular abnormalities induced by Poly I:C prenatal treatment.

Abbreviations: E = embryonic day, i.p. = intraperitoneal; i.v. = intravenous.

In this work, we will use the VPA and poly I:C mouse models of ASD. These two models are in line with two etiologies of ASD: (i) the prenatal exposure to antiepileptic treatments and (ii) maternal immune activation. These previously established models have been validated and well characterized for social and cognitive impairments. However, motor behaviors were under looked a gap that if filled opens the door on early diagnosis and better therapeutic management of ASD.

Part II. MATERIALS and METHODS

1. Animals, housing and treatment

Housing of the animals and all animal experimental procedures were performed in accordance with the guidelines of the French Agriculture and Forestry Ministry (decree 87849) and the European Communities Council Directive (2010/63/EU). All efforts were made to reduce the number of animals used and their suffering. All mice were purchased from Charles River Laboratories-France. Mice were housed in ventilated plexiglass cages (Techniplast, www.techniplast.fr 39,1 x 19,9 x 16,0 cm) prefilled with bedding and nesting material, and animals had access to food and water *ad libitum*. Male mice were housed individually, and 4 females were housed per cage. Room's temperature was maintained at 23 °C on a 12h-light/ 12-h dark cycle (light cycle starts at 8h00).

For time mating, 3 females C57BL/6J (age, 12 to 24 weeks) were placed with a single male C57BL/6J mouse (age, 12 weeks to 10 months) and left over night, the presence of a vaginal plug, was checked the next day 3 times a day (7h00, 12h00, and 19h00), and was designated as gestational day zero (G0). After the detection of a copulation plug, dams were returned to home cages and weighed daily to confirm the pregnancy, (non-pregnant females were returned to the home cage). Pregnant mice (gestational day 12,5/ E12,5) received a single intraperitoneal injection of either VPA (450 mg/kg) or poly I:C (20 mg/kg). Treated females were then given nesting material and left undisturbed. On E17 treated females were housed individually until they gave birth. Soon after birth, we performed an initial assessment of developmental milestones to assess the impact of early prenatal insult. Later at P21 sex and age matched litters from the same group of treatment were weaned and mixed to avoid littermate effect. Pups were then mixed, at P23, into six groups: VPA Males (n=34), Poly I:C Males (n=11), Saline Males (n=27), VPA Females (n=25), Poly I:C Females (n=10) and Saline Females (n=30).

2. Behavioral experimental plan

Behavioral tests are major tools in the study of impairment of motor functions following prenatal treatment of VPA and poly I:C In order to evaluate the social deficits caused by these

insults and to better characterize the early motor phenotype, a battery of behavioral tests was performed. We carried out for this study a set of tests combining both the overall assessment of the motor deficit and the analysis of fine movements. In addition, the combination of these tests will allow the study of different motor parameters such as motor coordination, balance, or gait. (Table IX).

Behavioral test	Parameters studied	Age
Developmental milestones	General development Righting reflex Open eyes	P6- P19
Locomotor activity	Horizontal locomotor activity	P28- 29
SHIRPA primary screen	General health (well-being) General motor abilities General sensorimotor functions Exploratory behaviors	P30
Cylinder test	Rearing exploratory behavior Grooming repetitive behavior	P30
Transversal of «challenging beam»	Motor coordination Equilibrium	P33
Gait analysis	Motor coordination Gait parameters Synchronization of the walk	P34
Three chambers test	Sociability	P35- 45

Table IX: Behavioral tests.

Abbreviations: P = Postnatal day

We performed an initial assessment of developmental milestones (P6- P19) in Saline and ASD mice. Next, we performed comprehensive screening of qualitatively abnormal phenotypes using standardized set of experimental procedures (SHIRPA). Then we performed state of the art precise exploration of vast innate and learned behaviors (P28- P45) including: sociability, spontaneous activity, motor control, coordination, development (Figure 10). Most of these tests were monitored by automated behavioral apparatus: Three chambers test of sociability, gait analysis (CatWalk system), and locomotor activity. Other tests were assessed by semi-automated apparatus: challenging Beam test of motor coordination, cylinder test. The battery of tests was performed in the least stressing order; animals were tested on their light cycle from 9 am till 6

pm. Both males and females were included into the study. All mice were age and sex matched among the wildtype and ASD groups; mice from different litters were mixed to avoid littermate effect.

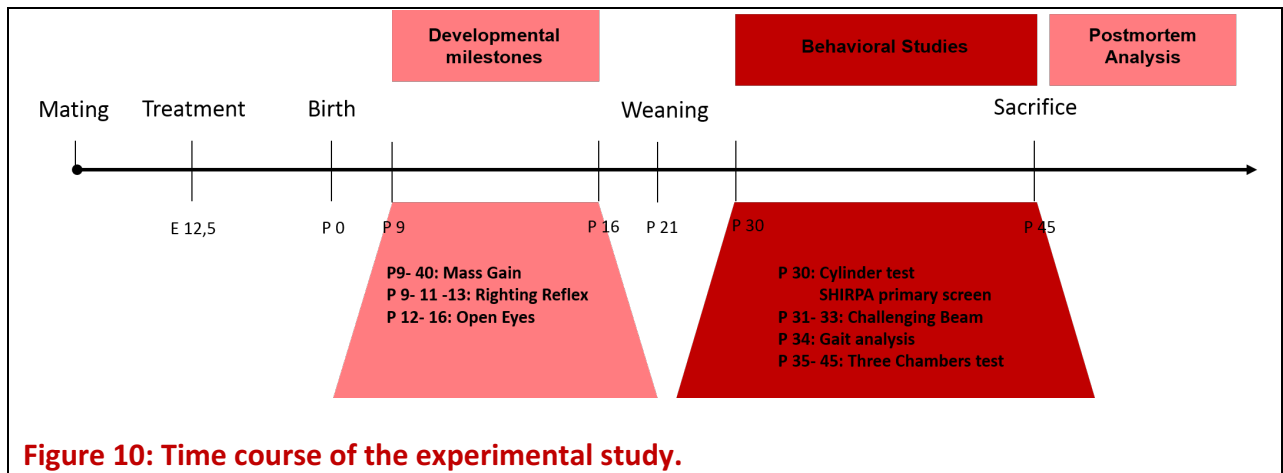


Figure 10: Time course of the experimental study.

2.1 Assessment of developmental milestones (P6- P19)

During their early postnatal life (infantile period 1-4 weeks), mice pups were kept with dams and their general developmental trajectories, mass gain, and motor reflexes were examined. We assessed the mass gain from P9 till P40, righting reflex on P9, P11, P13 and delayed eye opening from P12 till P16. To assess righting reflex, mice were placed in the supine position and their ability to right themselves was observed (Figure 11). Each mouse was observed three times with a five-minute inter-trial.



Figure 11: Mouse pups while righting after being put in the supine position.

2.2 Assessment of exploratory locomotor activity (P28- P29)

Infrared photocell-based detection actimeter (Imetronic Neurosciences, Pessac, France) was used to assess spontaneous locomotor activity. Before each session, we checked the actimeters by interrupting the infrared beams and verifying the corresponding number of beam breaks recorded by the computer. On two consecutive days, animals were placed into single

plexiglass cages (19×11×14 cm) and allowed to freely explore the environment for 60 minutes. Each cage is equipped with two infrared photocell units to record the horizontal activity. Horizontal infrared beam crossings were recorded in 5-minute blocks for 60 minutes.

2.3 SHIRPA primary Screen (P30)

The primary SHIRPA screen serves to identify global disturbances in gait, posture and muscle tone deficits, as well as motor control and coordination abnormalities. The SHIRPA apparatus comprise: a plexiglass arena, a metal wire, a cylinder and a grid. The mice were first placed in weighing container and then were moved to the transparent plexiglass arena (55x33x22 cm) to observe and evaluate certain cognitive and motor behavior. A grid paper 11x11 cm square was put under the arena to be able to evaluate exploration and locomotion. Finally, a 3-mm metal wire diagonally crossing the top was used to assess muscle functions and motor coordination (Figure 12).

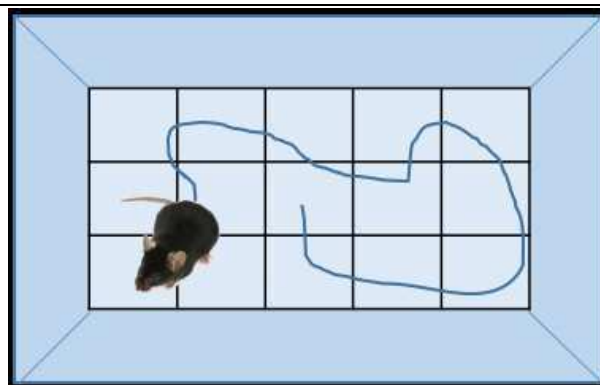


Figure 12: The SHIRPA arena.

A grid paper (11x11 cm squares) was put under the SHIRPA arena to assess Locomotor Activity the first 30 seconds after the transfer to the SHIRPA arena.

Subsequently tail suspension manipulations were performed to assess limb grasping and clasping in addition to grip strength, wire maneuver (Figure 13), and negative geotaxis. For the wire maneuver test, the mice were held by the base of the tail and approached the metal wire and left after being attached to the wire parallel to the ground of the arena (Figure 13 A). The ability to stay attached to the wire (Figure 13 B) and the time spent climbing the wire (Figure 13 C) were assessed.

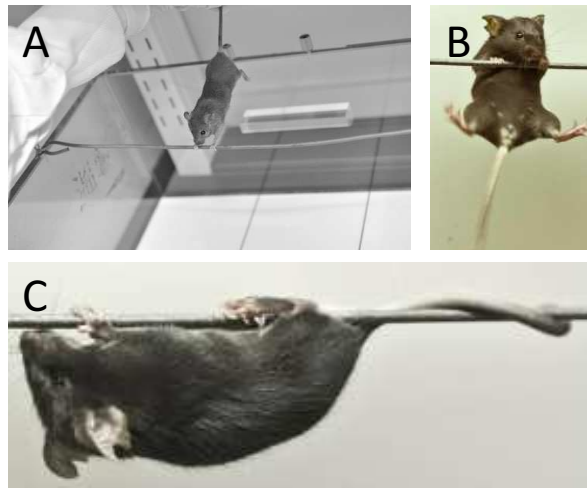


Figure 13: The wire maneuver test.

(A) The mice are presented and left to hang to the wire. (B) the ability to stay attached to the wire (time to fall) and (C) time spent climbing were assessed.

2.4 Spontaneous activity in the cylinder (P30)

Two clean cages were inverted on the top of a table, next to each other. A transparent piece of glass was placed on the top of the 2 cages and a transparent plexiglass, with a diameter of 12 cm, was put on it. Beneath the glass, at an angle of 45°, a mirror was put so that its view included the full diameter of the cylinder (from below). Finally, a video camera was set up in front of the mirror. Mice were then placed in the cylinder and were allowed to freely explore the inner walls of the cylinder (Figure 14). The activity was videotaped for 3 minutes. An observer blind to the genotype then rated the videos and quantified the number of rears (vertical exploration) and the time spent grooming (repetitive behaviour).

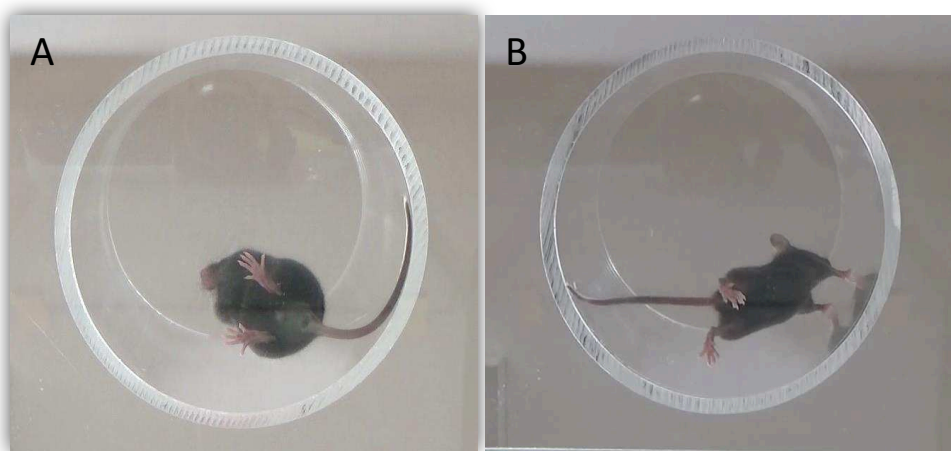


Figure 14: The cylinder test.

(A) Repetitive grooming behavior. (B) Vertical exploratory rearing.

2.5 Assessment of motor coordination on the challenging beam (P33)

The "Challenging beam test" is an adaptation of the "beam walking test" introduced by Drucker-Colin and Garcia-Hernandez for the study of motor deficits in the rat (Drucker-Colin and Garcia-Hernandez., 1991). The animals must walk along an inclined beam to reach a platform and the number of slips during the crossing is then counted. This test was subsequently adapted in mice for the study of balance and motor coordination (Figure 15). In our lab, we have used a device adopted from Carter and his collaborators (Carter et al.,1999), 1 meter long plexiglass beam is assembled with four sections of 25 cm each of decreasing width (3.5, 2.5, 1.5, 0.5 cm).

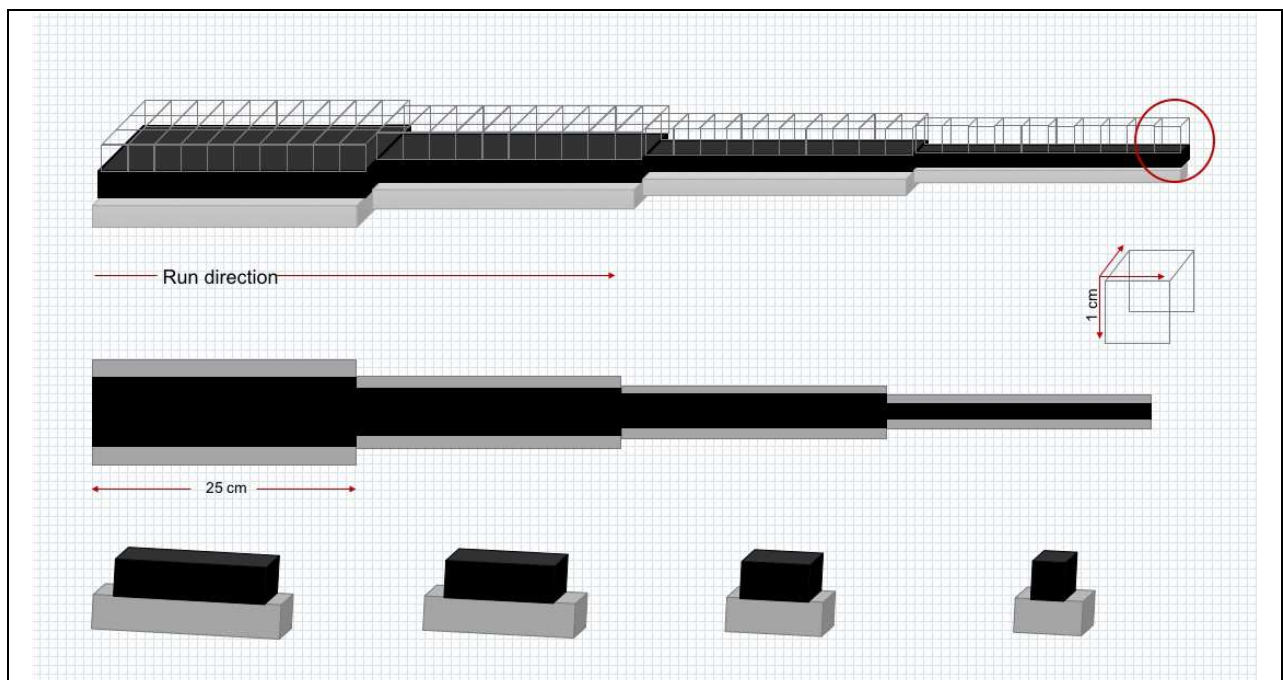
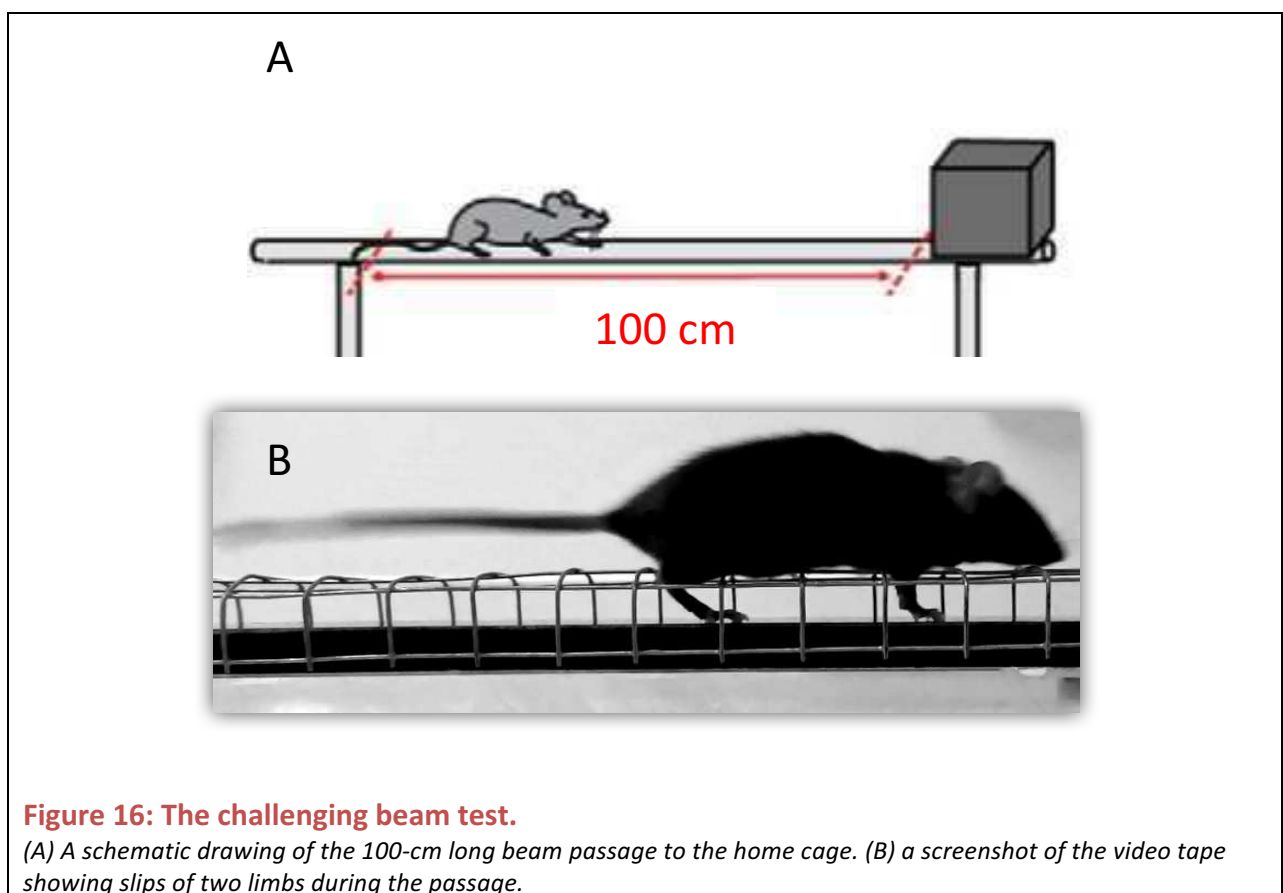


Figure 15: Schematic diagram of the challenging beam motor coordination test.

The Plexiglas beam consists of four sections (25 cm each), each section having a different width, starting at a width of 3.5 cm and gradually narrowed to 0.5 × 1 cm increments. The grid, on the top of the beam, is a 1 x 1cm regular metal grid.

The challenging beam was performed as described previously (Fleming et al., 2013, 2004). Animals were first trained to traverse the length of the beam starting at the widest section and ending at the narrowest section that leads directly into the animals' home cage. Animals received 2 days of training before testing. On the day of the test, and to increase the difficulty of the crossing, a mesh grid (1 cm squares) of corresponding width was placed over the beam

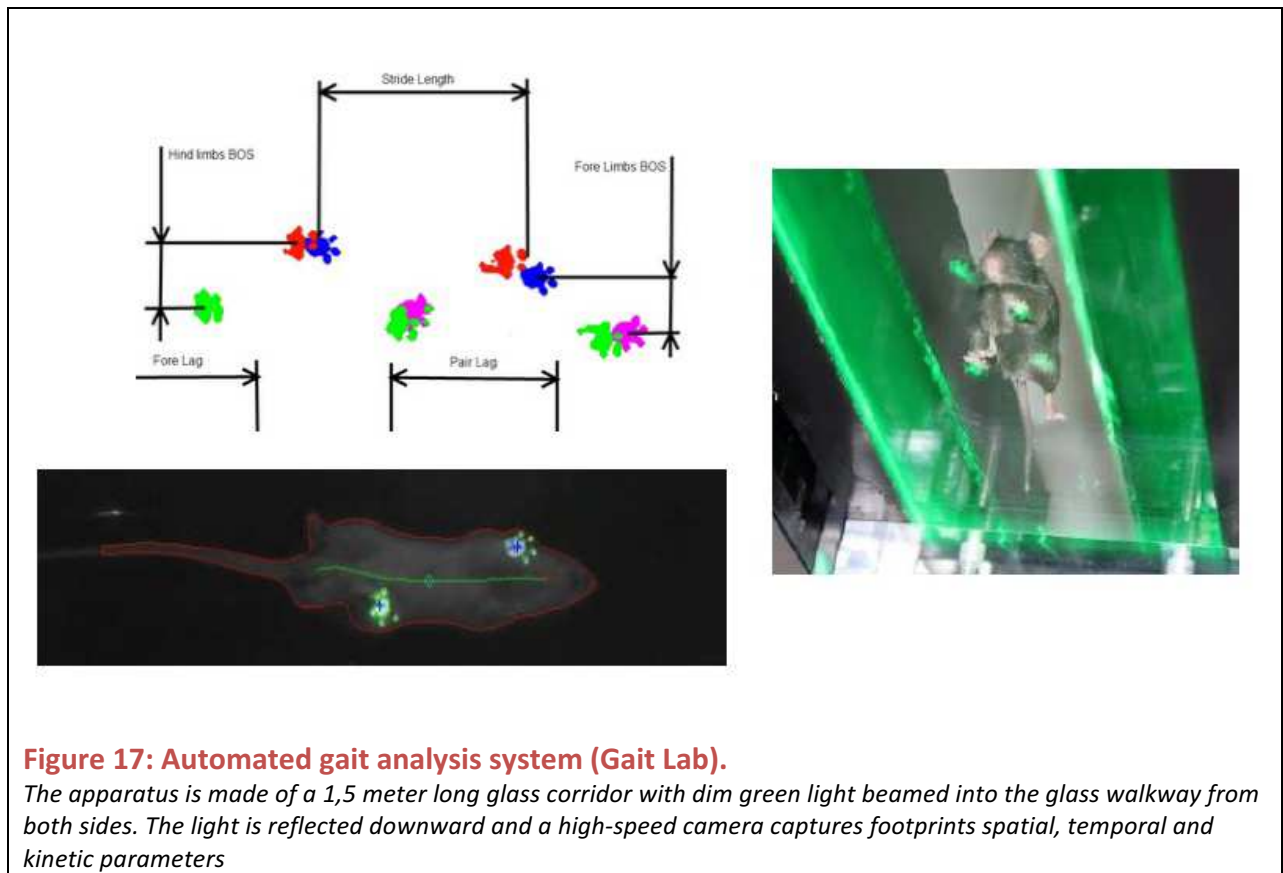
surface leaving a ~1 cm space between the grid and the beam surface. On the testing day, mice received a pretest session of 5 crossings to adapt to the grid. The animals were put back to home cages to rest to receive then a final 5 trials of beam crossing. Animals were then videotaped while traversing the grid-surfaced beam for a total of five trials. Errors, number of steps made by each animal, and time to traverse across five trials were then rated by an investigator blind to the mouse treatment group (by viewing and rating the videos in slow motion for errors). An error was counted when, during a forward movement, a limb (forelimb or hind limb) slipped through the grid and was visible between the grid and the beam surface (Figure 16 B). An individual animal could make a maximum of four slips per step.



Slips were not counted if the animal is not making a forward movement or when the animal's head was oriented to the left or right of the beam. Error per step scores, time to traverse, and number of steps were then calculated for both wild type and ASD mice across all five trials and averaged (results are shown as a mean of 5 trials).

2.6 Spatial, temporal, and kinetic parameters of gait (P34)

Mice gait was analyzed during their spontaneous walk through the catwalk automated gait analysis system (Viewpoint behavior analysis technology, France) (Figure 17).



At P34, one day after the motor coordination assessment, mouse where subjected to gait analysis. Briefly, each mouse was assessed individually for 3 consecutive regular runs for a comprehensive analysis of gait impairments. After each run the computer assisted system automatically analyses the run and generates a wide range of gait data including: 1) spatial parameters of each single paw (stride length, base of support, and area) 2) temporal parameters (walk speed, and regularity), and other parameters as paw placement detailed in the following table (Table X). The speed threshold was set to a minimum of 15cm/sec, and the regularity was set to 98%.

Parameters	Description
Mean Speed	The distance the animals cross per second (minimal speed setting is set to 15 cm/sec) expressed in cm/sec.
Regularity	The index for the rhythmic run based on the acceleration of the center of gravity of the mouse during the run (regularity index setting is set to 97 % to assure that all the mice run at the same regular pattern regardless the treatment group).
Stride Frequency	The rate at which the support changes from one paw to the next.
Stride Length	The distance between two consecutive paw placements of the same paw and is expressed in centimeters.
Left / Right Pair Gap	Distance separating front and rear limbs on the left or right side (cm).
Area	Area of the paw print (length x width of the paw print) (cm ²).
Left / Right Limbs Base of Support	Distance between front and rear legs on left / right side (cm).

Table X: Set of walking parameters and their descriptions provided by Gait Lab.

2.7 Assessment of sociability in the three chambers test (P35-P45)

A three-chamber social interaction assay was performed to determine the social interaction behavior according to Moy et al., 2004. Briefly, a plexiglas box 60×45×22 cm partitioned into three chambers with retractable doorways allowing for access to side chambers was used. The first phase (Phase I) comprises two identical nonsocial stimuli (inverted wire-cups). The second phase (Phase II) comprises a nonsocial stimulus and a social stimulus (mouse of the same strain). Social stimuli were naïve mice of matched age and sex with no previous contact with the tested animal. For 10 minutes in each phase, time spent at each chamber was recorded (Figure 18). To analyze the behavior automatically we employed a video analyzing program that we developed previously in the lab in collaboration with XLIM-Poitiers.

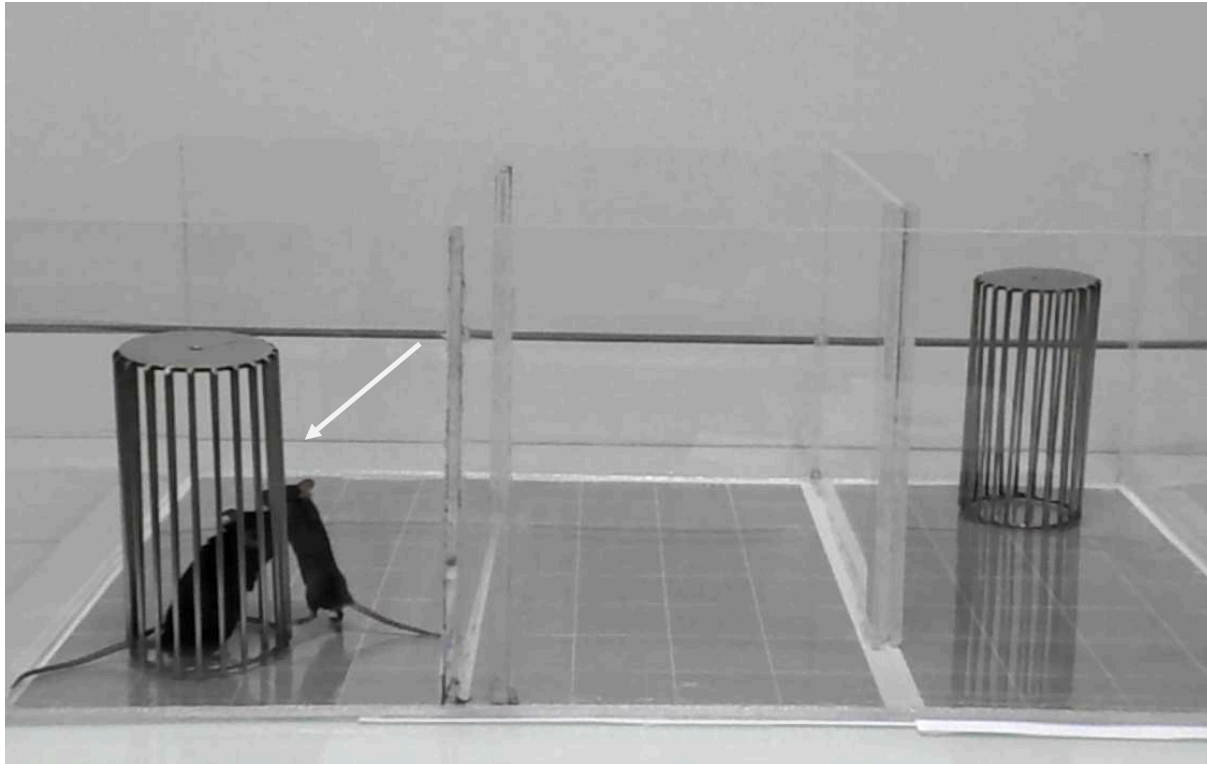


Figure 18: Crawley's three chambers test.

White arrow showing the social sniffing behavior in the social chamber (SC) during PHASE II of the test.

Videos were then rated offline and the time spent at each chamber in each phase of the test was counted. To allow the comparison of the social behavior Sociability Index (SI) was calculated as follows:

$$SI = \frac{\text{time exploring Social Chamber} - \text{time exploring Non Social Chamber}}{\text{time exploring Social Chamber} + \text{time exploring Non Social Chamber}}$$

3. Statistical analysis

Data were analyzed using the GraphPad Prism7 software (San Diego, USA). Data were expressed in mean \pm Standard Error to the MEAN (SEM). Statistical significance was evaluated using one-way and two-way analysis of variance (ANOVAs) followed by multiple comparisons using Tukey's and Sidak's when appropriate. $P < 0,05$ was considered as significant. We further performed correlation studies to know whether there is a relationship between the screened shifts behavior and the anatomical changes.

Part III. EXPERIMENTAL RESULTS

Chapter 1: Behavioral screening results in VPA mice

1.1 VPA mice displayed delayed post-natal development

Clinical findings have revealed that children born to women exposed to VPA had a significantly higher rate of birth defects (Bath and Scharfman, 2013). In-utero exposure to antiepileptic drugs provokes fetal developmental delays and early physical malformations (Meador et al., 2006). To examine the effect of prenatal exposure to VPA on general development and on central nervous system development, we investigated the delay in offspring eye opening and righting reflex. VPA prenatal exposure did not alter the general development of mice that kept gaining mass normally till P40 (Figure 19A). As shown in Figure 19, prenatal exposure to VPA altered several postnatal behaviors in both males and females. Two-way ANOVA showed a significant effect of the treatment [$F(3, 336) = 31.05, p < 0.0001$], age [$F(2, 336) = 323.3, p < 0.0001$] and interaction [$F(6, 336) = 18.64, p < 0.0001$] (Figure 19B). Tuckey's Post hoc revealed a significant latency in righting in VPA males at P9 and P11 compared to Saline littermates ($p < 0.0001$). VPA females displayed an increase in the latency only at P9 ($p < 0.0001$). This is indicative that the developmental delays are more pronounced in males than in females (Figure 19B). In addition, we found a significant effect of the treatment [Two-way ANOVA $F(3, 560) = 22.60, p < 0.0001$], age [$F(4, 560) = 830.9, p < 0.0001$] and interaction between the treatment and age [$F(12, 560) = 6.623, p < 0.0001$]. Tuckey's Post hoc analysis revealed a significant eye-opening delay in VPA males at P13, P14 ($p < 0.001$) and P15 ($p < 0.01$). The delay was observed in VPA females only at P14 ($p < 0.001$) suggesting that the delay in eye opening appears earlier and persists in more males (Figure 19C).

Taken together these results indicate that VPA prenatal exposure provokes early developmental delays and motor abilities.

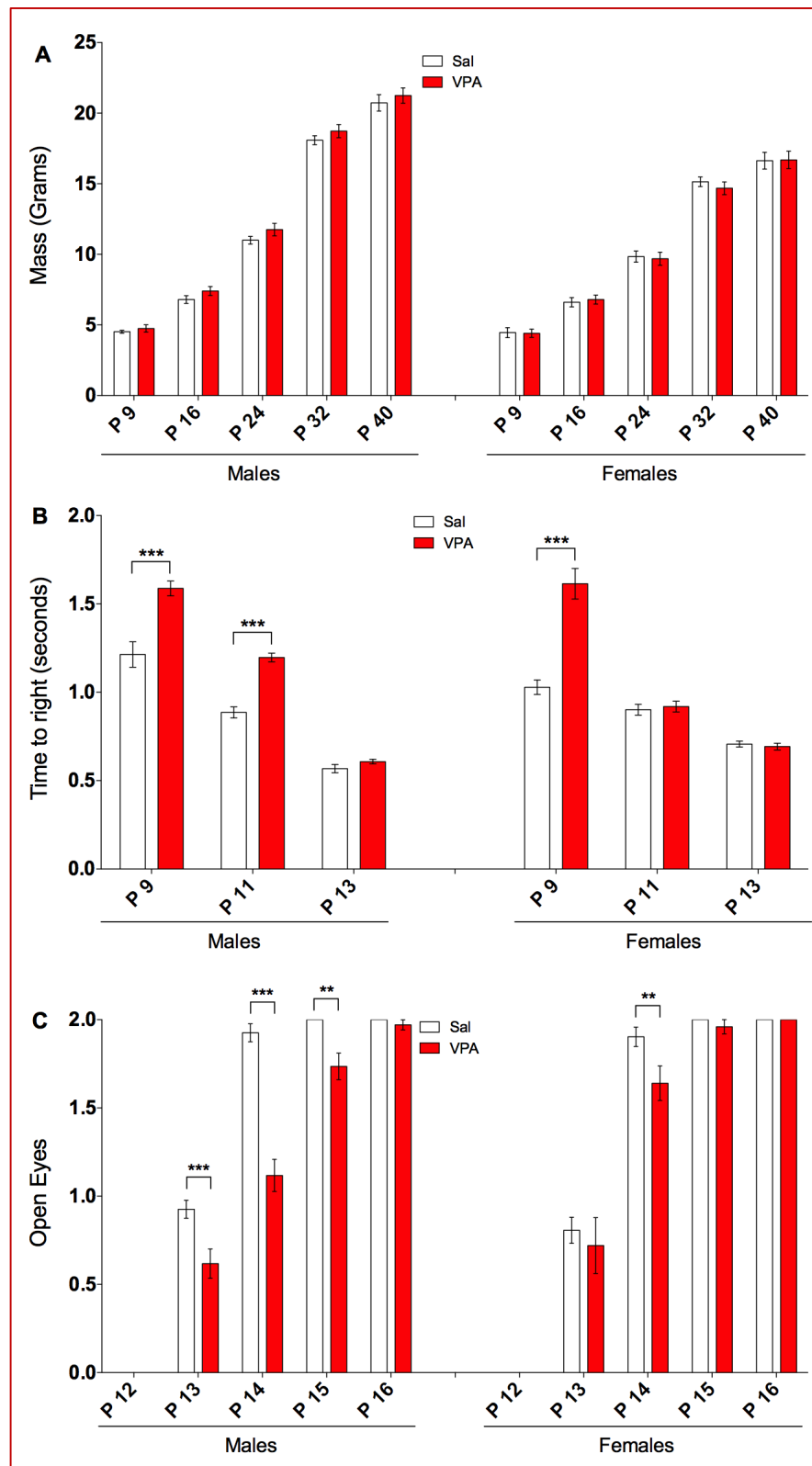


Figure 19: VPA prenatal treatment altered developmental milestones.

(A) VPA prenatal treatment did not alter mass gain between P9 and P40 in both males (Left) and females (Right). (B) VPA prenatal treatment affected neonatal righting reflex; VPA males (Left) show a significant increased latency to righting on P9 in comparison to the Saline littermates. The significant increased latency to righting persisted until P11. Similar to males, VPA females (Right) displayed an increase in the latency to righting on P9. (C) VPA prenatal treatment provoked a delayed eye opening. On P13 VPA males (Left) exhibited delayed eye opening that persisted until P15. VPA females (Right) displayed a significant delay in eye opening only on P14 in comparison to Saline littermates. VPA males (n=34/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as mean \pm SEM * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ two-way ANOVA.

1.2 VPA males displayed faster habituation

One week after weaning, when mice were 4 weeks old, we tested the locomotor activity (LA) in Infrared photocell-based detection actimeters activity chambers for 60 minutes on two consecutive days. The computer recorded beam crossings during 60 minutes by 5 minutes intervals. In comparison to Saline, only VPA prenatally treated males show significant decrease in exploratory locomotor activity on Day 2 compared to Day 1 suggesting a reduced interest in the new environment, two-way ANOVA (Figure 20A). We found a significant effect of the group [Two-way ANOVA $F(3, 1356) = 6.832, p=0.0001$], time [$F(11, 1356) = 53.47, p<0.0001$] and interaction between the group and time [$F(33, 1356) = 1.521, p=0.0303$] on Day-1. Tuckey's post-hoc analysis revealed lower LA in VPA males at $t=50$ mins ($p<0.05$). However, higher LA was observed in VPA females at $t=55$ minutes ($p<0.001$) (Figure 20B). Similarly, on Day 2, we found a significant effect of group [Two-way ANOVA $F(3, 1356) = 16.29, p<0.0001$], and time [$F(11, 1356) = 42.23, p<0.0001$]. Tuckey's Post hoc analysis revealed a significant decrease in LA in VPA males at $t=25$ mins ($p<0.001$). However, a significant increase in LA was observed in VPA females at $t=45$ and 50 minutes ($p<0.05$) (Figure 20C). We also analyzed the differences of LA in the last 30 minutes of each day, where most of the change happens. Comparing the LA of VPA males on Day 1 to Day 2, we found a significant effect of the day [$F(1, 34) = 13.25, p<0.001$], the time [$F(5, 170) = 13.80, p<0.0001$] and interaction: Day x Time [$F(5, 170) = 3.087, p<0.05$]. Sidak's post-hoc analysis revealed a significant decrease in LA in VPA males on day 2 at $t=35$ minutes ($p<0.05$), $t=40$ mins ($p<0.001$), and $t=50$ ($p<0.0001$) minutes (figure 21D).

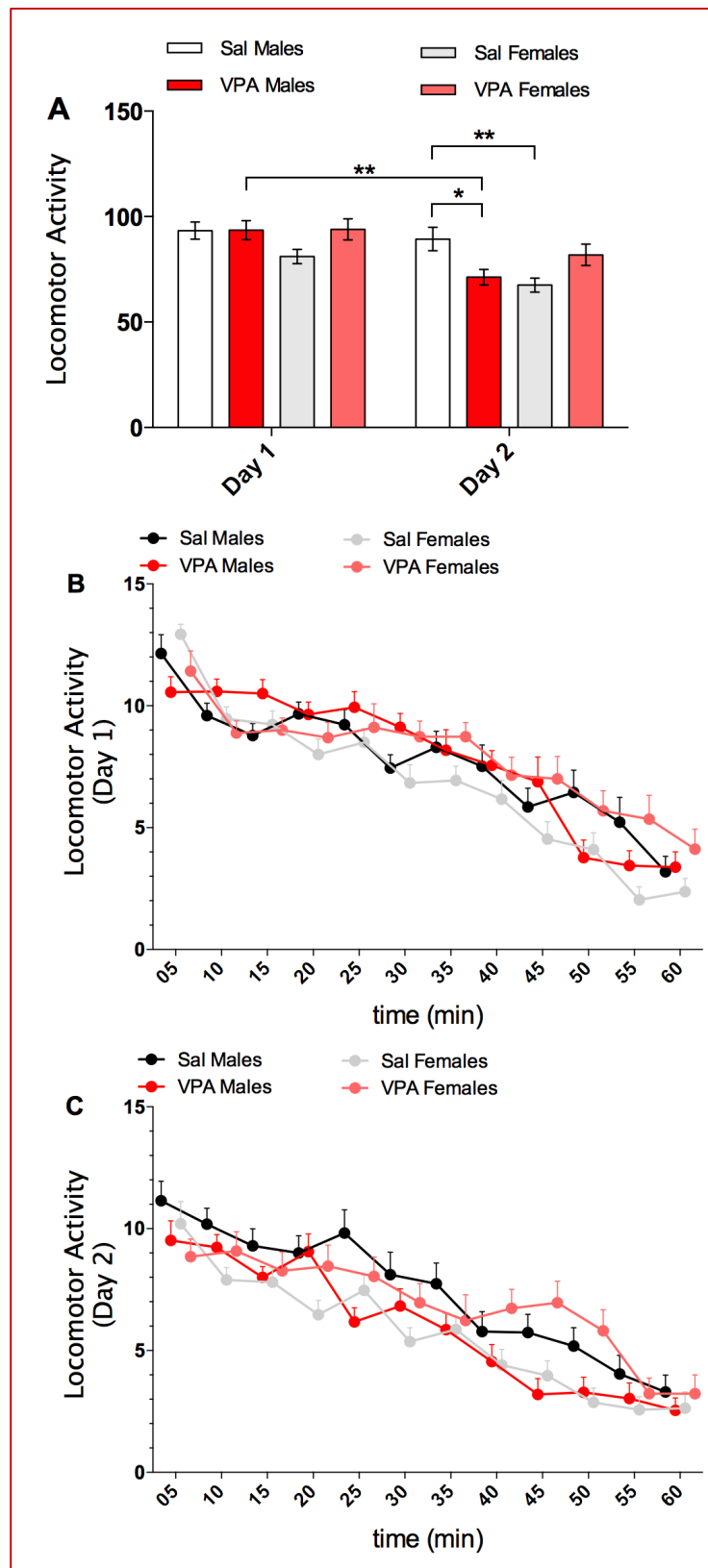


Figure 20: VPA prenatal treatment altered exploratory locomotor activity (LA).

(A) On Day-2 VPA males shows reduced Locomotor Activity (LA; assessed by number of beam crosses: y-axis) in comparison to the activity in the same group on Day-1. On Day-2 VPA males shows reduced LA in comparison to Saline males. On Day-2 Saline females shows reduced LA relative to Saline males. (B) VPA males had a reduced LA at the end of Day 1 as shown by the 5 minutes block analysis of time. VPA had an inverse effect on females that were more active at the end of Day 1. (C) VPA males had a reduced LA at t=25 Day 2. Females had increased LA at t=45 and t=50 minutes Day 2. VPA males (n=34/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as mean \pm SEM * $P < 0.05$, ** $P < 0.001$, two-way ANOVA.

However, in VPA females we found a significant effect of day [F (1, 324) = 15.86, $p < 0.0001$], and time [F (5, 5324) = 7.394, $p < 0.0001$]. Sidak's post-hoc analysis revealed a significant increase in LA in VPA females, in comparison to Saline females, on Day 2 at $t=45$ minutes ($p < 0.05$), $t=50$ minutes ($p < 0.05$) (Figure 21F). Furthermore, comparing LA of VPA females on Day-1 to Day-2, we found a significant effect of time [F (5, 125) = 8.293, $p < 0.0001$]. Sidak's Post hoc analysis revealed a significant decrease in LA in VPA females on Day 2 at $t=35$ minutes ($p < 0.05$) (Figure 21H). Taken together these results indicate that VPA treatment altered LA, and VPA treated mice display reduced interest in new environment.

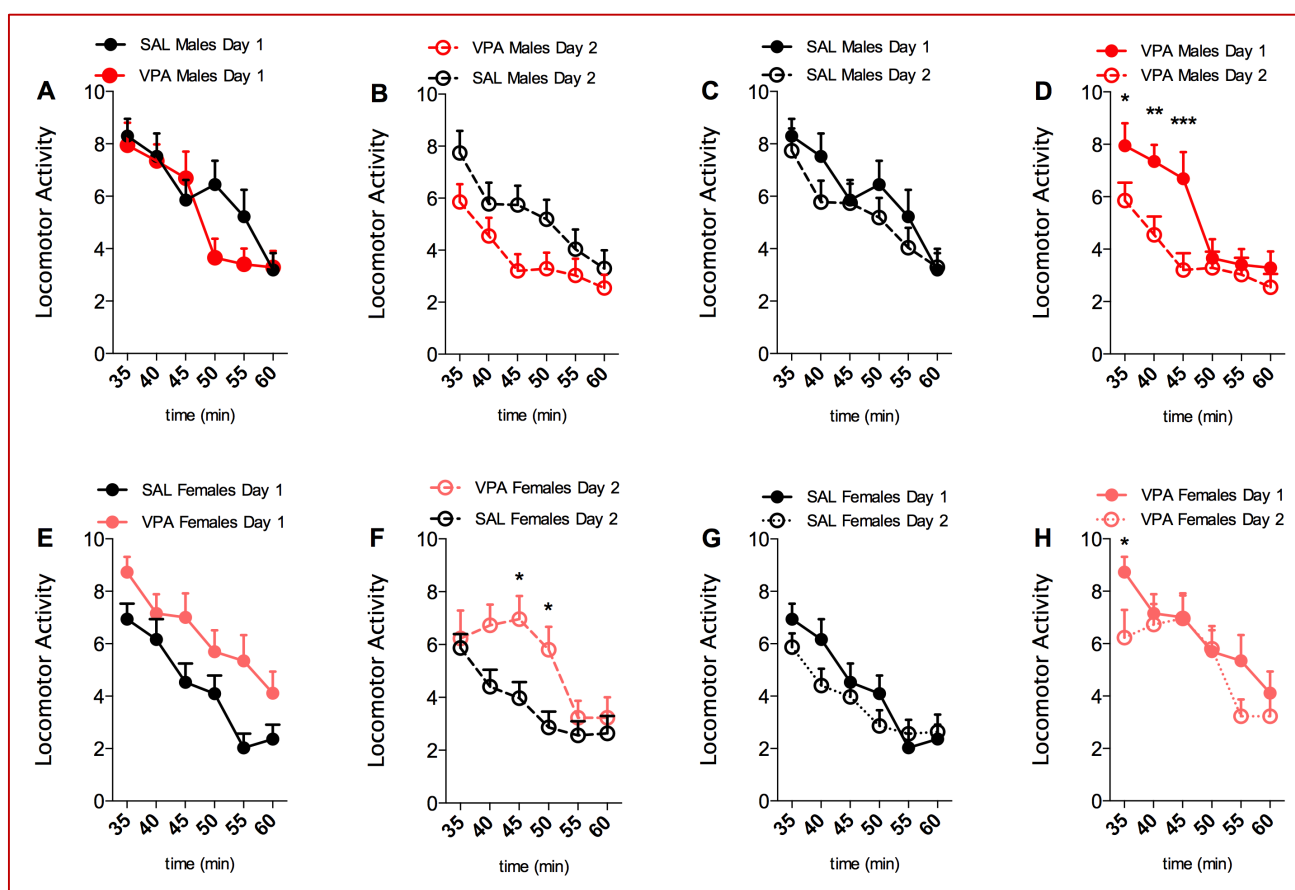


Figure 21: LA analysis of the last 30 minutes on Day 1 and Day 2 in VPA and Saline.

(A) Graph showing no significant difference in LA between VPA males and Saline littermates on Day-1. (B) Similarly, no significant difference was found between both groups on Day-2. (C) No significant difference in LA in Saline males between Day-1 and Day-2. (D) VPA prenatally treated males show significant decrease in exploratory LA on Day-2 compared to Day 1 at $t= 35, 40,$ and 45 minutes. (E) No significant difference in LA between VPA females and Saline littermates on Day 1. (F) A significant increased activity in VPA females on Day 2, in comparison to Saline females on the same day. (G) No significant difference in LA in Saline females between Day 1 and Day 2. (H) VPA prenatally treated females show a significant decrease in locomotor activity after 35 minutes on Day-2. VPA males ($n=34/34$), Saline Males ($n=27/27$), VPA females ($n=25/25$), and Saline females ($n=30/30$). Data expressed as mean \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA.

1.3 VPA mice displayed altered behavior in SHIRPA primary Screen

Time spent immobile following transfer to SHIRPA arena was assessed and two-way ANOVA revealed a significant effect of treatment [$F(1, 112) = 214.2, p < 0.0001$], the sex [$F(1, 112) = 136.7, p < 0.0001$], and a treatment-sex interaction [$F(1, 112) = 155.6, p < 0.0001$] in VPA mice (Figure 22A). Tuckey's post-hoc analysis revealed that only VPA males showed three folds increase in immobility ($p < 0.0001$). Directly after the transfer to the SHIRPA arena, locomotor activity was assessed (Figure 22B). An effect of treatment [$F(1, 112) = 129.4, p < 0.0001$], sex [$F(1, 112) = 14.64, p = 0.0002$], and treatment-sex interaction [$F(1, 112) = 90.78, p < 0.0001$] on locomotor activity were revealed by Two-way ANOVA. Tuckey's post-hoc analysis revealed a significant (-50%) decrease in locomotor activity in VPA males versus Saline males ($p < 0.0001$) while no such difference was observed in females. Here, we tested postural stability and coordination in space using negative geotaxis measured as the time needed to turn and time needed to climb a 45° inclined grid. We found an effect of treatment [Two-way ANOVA, $F(1, 112) = 16.62, p < 0.0001$] on time needed to turn on the grid. Tuckey's post-hoc analysis a significant increase in time to turn in VPA females ($p < 0.001$) (Figure 22C). In addition, both treatment [$F(1, 112) = 117.0, p < 0.0001$] and sex [$F(1, 112) = 6.557, p = 0.0118$] had an effect on the time needed to climb the grid (Figure 22D). Indeed, both VPA males and females spent significantly more time than Saline littermates, to climb the grid (+78%) and (+59%) respectively ($p < 0.0001$) (Tuckey's post-hoc analysis). Finally, we implemented the wire maneuver test to assess coordination and muscle function. Two-way ANOVA revealed a significant effect of the treatment [$F(1, 112) = 21.40, p < 0.0001$] (Figure 22E). Tuckey's post-hoc analysis that both VPA males and females were statistically different from Saline littermates as they spent (+15%) ($p < 0.05$) and (+25%) ($p < 0.001$) more time to climb the wire respectively. Taken together, these results indicate that VPA prenatal exposure provoked increased immobility, reduced exploration, and reduced motor abilities in males. In females, VPA prenatal exposure induced early motor deficits. These results are in line with our previous results in reduced motor reflexes and LA.

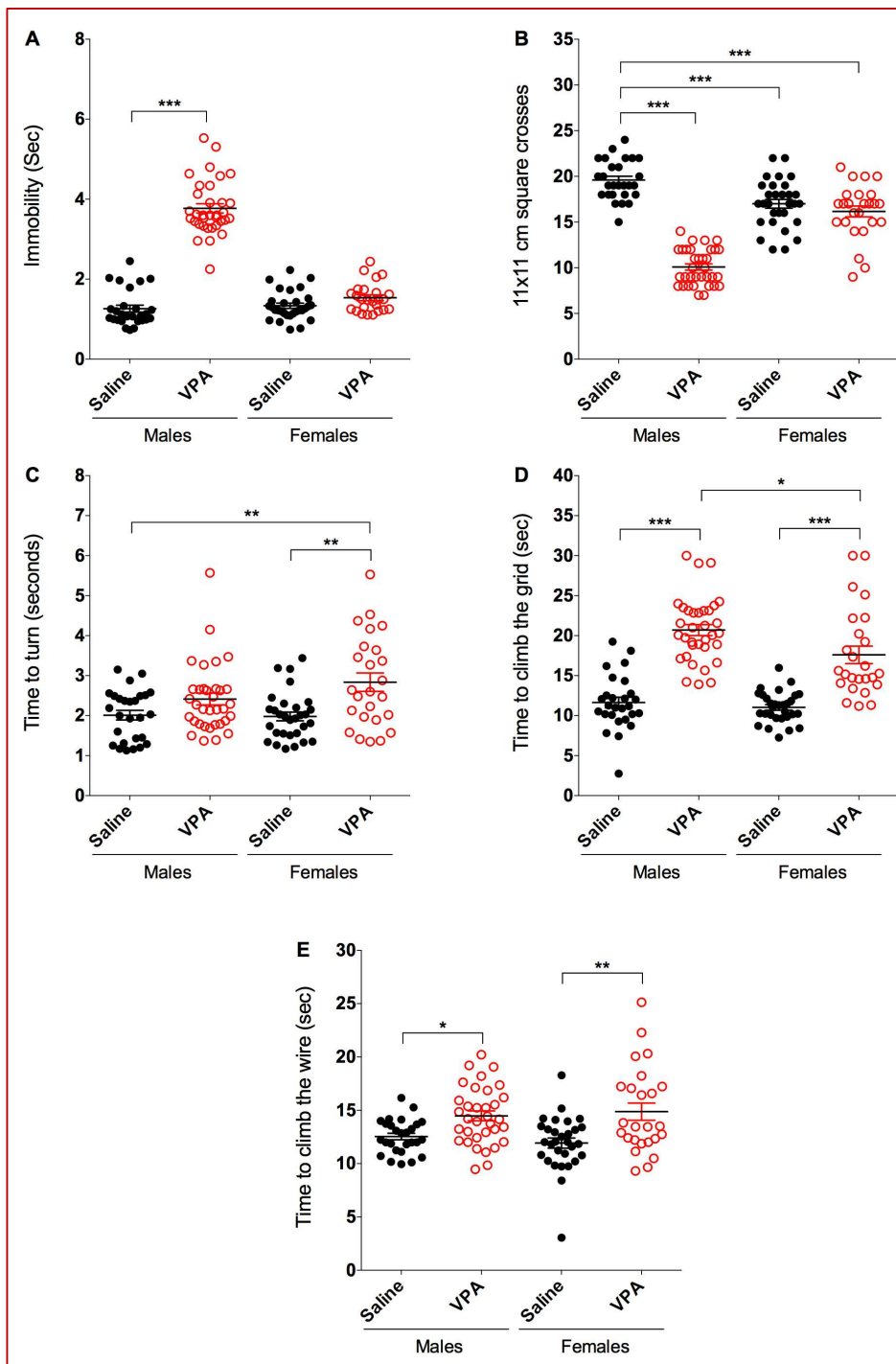


Figure 22: VPA treatment altered several SHIRPA parameters.

(A) scatterplot of time spent immobile when transferred to the SHIRPA arena. VPA males show a significant increase in time spent freezing, in comparison to Saline males (Left). When transferred to the SHIRPA arena, VPA females show no significant differences in immobility (Right). (B) Scatterplot showing LA assessed by number of 11x11cm squares crossed, thirty seconds after the transfer to the SHIRPA arena. VPA males show reduced LA in comparison to Saline males. Both Saline females and VPA females show reduced LA in comparison to Saline males. (C) scatterplot of time to turn on the grid in the wire in the negative geotaxis test. VPA males show no change in time spent turning on the grid, in comparison to Saline males. VPA females show a significant increase in time to turn on the grid, in comparison to Saline females and Saline males. (D) VPA males show a significant increase in time to climb on the grid, in comparison to Saline males. VPA females show a significant increase in time to climb the grid, in comparison to Saline females and Saline males. (E) VPA males show a significant increase in time spent climbing the wire, in comparison to Saline males. VPA females show a significant increase in time spent climbing the wire, in comparison to Saline females. VPA males (n=34/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as scatterplot and mean \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA.

1.4 VPA mice displayed core ASD symptoms (P30; P35- 45)

One of the hallmarks of ASD are deficits in social interactions (APA, 2013). Here, we implemented the three chambers test, a commonly used approach to assess the social behavior in 5-6 weeks old male and female mice prenatally exposed to VPA or Saline. None of the treatment groups, regardless the sex, showed a preference to any of the chambers during the 10 minutes habituation (PHASE I) (Figure 23A). However, we detected a significant effect of chamber side on duration [Two-way ANOVA $F(1, 128) = 243.8, p < 0.0001$] in phase II, when a naïve mouse was introduced to one of the chambers (social chamber). Indeed, while Saline mice spent more time in the social chamber than in the empty chamber (69% versus 31%, $p < 0.0001$), VPA males spent similar time in both chambers (Figure 23B). This treatment effect was not observed in females that spent more time in the social chamber than in the empty chamber, regardless of the treatment. The differences were even more striking when expressed as sociability index (SI), as VPA males showed a 7 folds decrease in sociability ($p < 0.0001$) when compared to Saline males, while females had similar SI regardless of the treatment. Indeed, we found a significant effect of the treatment [Two-way ANOVA $F(1, 64) = 6.285, p = 0.0147$], sex [$F(1, 64) = 5.453, p = 0.0227$] and treatment-sex interaction [$F(1, 64) = 25.75, p < 0.0001$] on sociability index (Figure 23C). These results show significant impairments in sociability, a core feature in ASD, only in VPA males.

We then assessed repetitive grooming and rearing behaviors in a new environment, using the Plexiglas cylinder test for 3 minutes. Time spent grooming was affected by treatment [Two-way ANOVA, $F(1, 112) = 26.47, p < 0.0001$] (Figure 23D). Tukey's post-hoc analysis revealed a significant increase in the time spent grooming in both VPA males (+69%) ($p < 0.001$) and females (+41%) ($p < 0.05$). Additionally, there was an effect of treatment [Two-way ANOVA $F(1, 112) = 11.99, p < 0.001$] and sex [$F(1, 112) = 14.57, p < 0.001$] on rearing behavior (Figure 23E). Indeed, VPA males, but not females, showed significant reduction in rearing behavior compared to Saline (-41%) ($p < 0.01$) and to VPA females (-50%) ($p < 0.001$). This is reminiscent of the previous results also showing reduced exploratory behavior in the same group.

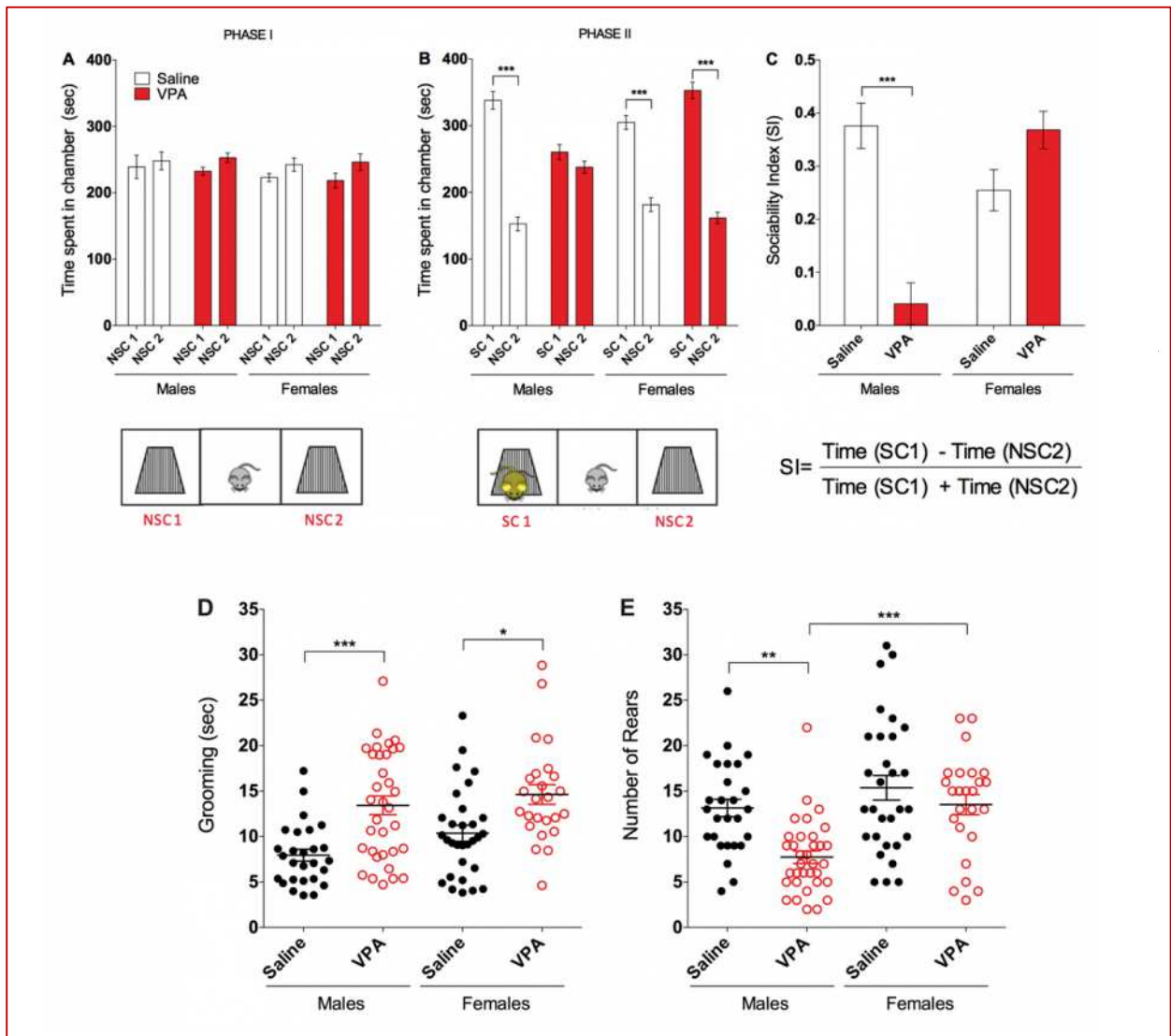


Figure 23: VPA males show core ASD features.

(A) Histogram showing no change in the time spent in nonsocial chamber-1 (NSC1) versus nonsocial chamber-2 (NSC2) regardless the treatment in both males (Left) and females (Right) in PHASE I of the three chambers test. (B) VPA prenatal treatment induced reduced sociability only in males (Red bars- Left); no significant difference in the time spent in the social chamber (SC1) versus the nonsocial chamber-2 (NSC2) In PHASE II. (C) VPA prenatally treated males show a significantly decreased sociability. (D) Scatterplot of time spent grooming. VPA males show a significant increase in the time spent grooming, in comparison to Saline males (Left). VPA females show a significant increase in time spent grooming, in comparison to Saline females (Right). (E) VPA males show a significant decrease in number of vertical rears, in comparison to Saline males. VPA females show a significant increase in number of rears, in comparison to VPA males. For three chambers test: VPA males (n=27/34), Saline Males (n=10/27), VPA females (n=16/25), and Saline females (n=15/30). For cylinder test: VPA males (n=34/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as scatterplots and mean \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA.

1.5 VPA mice displayed significant deficits in motor coordination and gait (P33- 34)

Motor coordination was assessed using the challenging beam test following 2 days of training (Fleming et al., 2004). The “Challenging beam test” is an adaptation of the “beam walking test” developed by Drucker-Colin and Garcia-Hernandez (1991) to study motor deficits in aged rats. On the testing day, each animal (P-33) traversed the beam for 5 trials. Time needed to traverse the full length of the beam, errors per step, and errors at each section of the beam were counted and results were expressed as the mean of 5 trials. The time needed to traverse the beam sections was not affected by the treatment [Two-way ANOVA, $F(1, 112) = 0.0045$, $p = 0.9462$] (Figure 24A). However, the treatment affected errors per step, [$F(1, 112) = 67.04$, $p < 0.0001$] (Figure 24B). Tuckey’s post-hoc analysis revealed a significant increase in the errors per steps (+125%) in VPA males ($p < 0.0001$) in comparison to Saline males. Interestingly a similar increase of (+110%) was also seen in VPA females ($p < 0.0001$) when compared to Saline females. We have also found a significant effect of beam section [two-way ANOVA, $F(3, 448) = 101.0$, $p < 0.0001$], group [$F(3, 448) = 60.61$, $p < 0.0001$] and beam section x group interaction [$F(9, 448) = 3.135$, $p = 0.0011$] (Figure 24C). Indeed, male and female VPA treated mice showed a significant increase in the number of errors per beam section. Tuckey’s post-hoc analysis revealed significant differences on the second, third, and fourth beam sections ($p < 0.0001$) in comparison to Saline males and females.

Of interest, VPA females that displayed normal social behavior, displayed reduced motor coordination and ataxic gait. These results indicate that significant alterations in motor coordination are present in both male and female VPA mice.

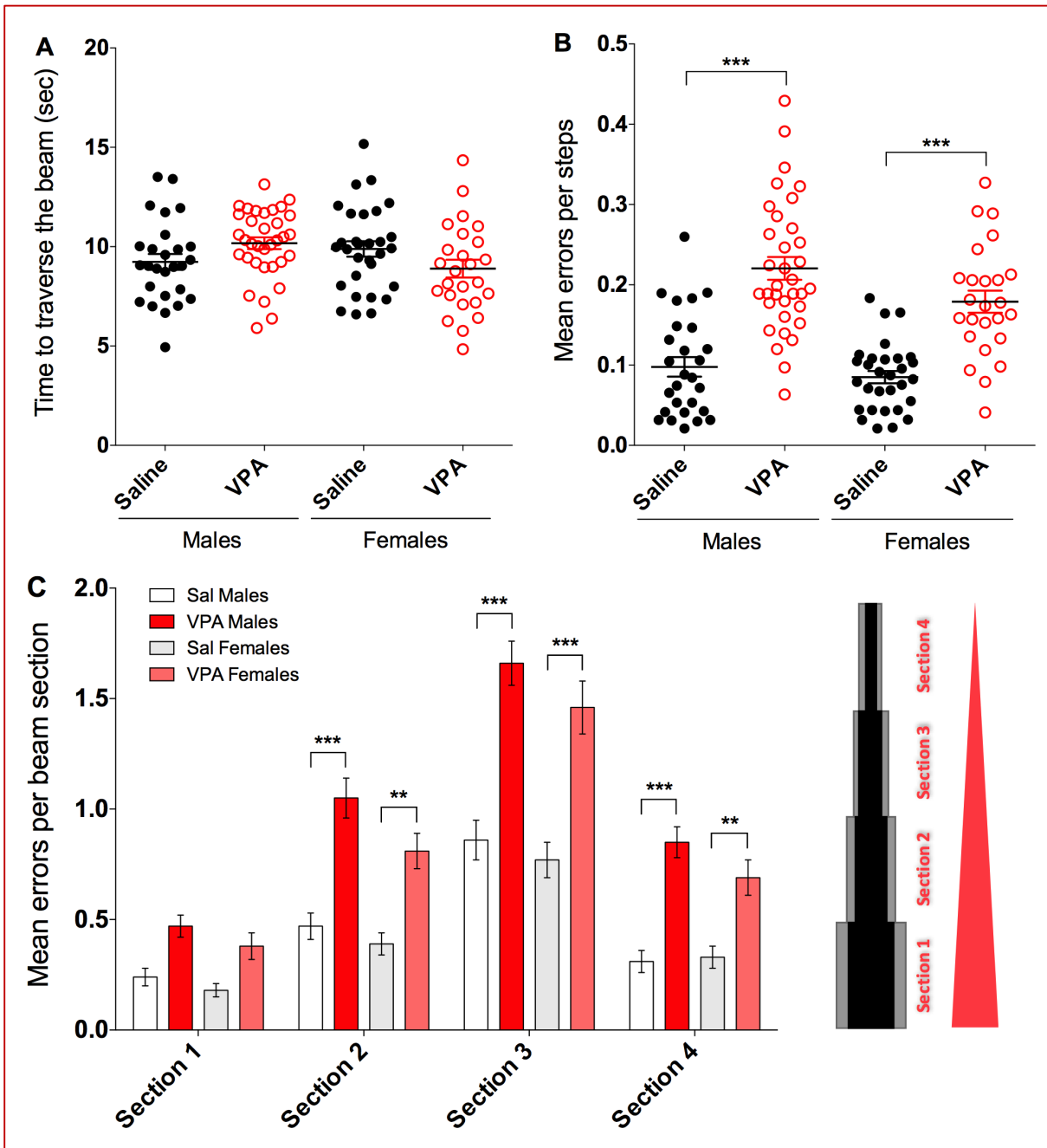


Figure 24: VPA prenatal treatment altered motor coordination on the challenging beam in both males and females.

A) VPA mice needed the same amount of time to traverse the beam. (B) VPA mice show significant increase in the number of errors per steps. VPA prenatally treated males (Red bars) show significant increase in the number of errors per steps on the second, third, and fourth sections of the beam, in comparison to Saline males (White bars). Similar to the males, VPA females (Light Red bars) show significant increase in the number of errors per steps on the second, third, and fourth sections of the beam, in comparison to Saline females (Gray bars). VPA males (n=34/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as scatterplots and mean \pm SEM *** P<0.001, two-way ANOVA.

As several findings in ASD patients clearly pointed to gait disturbances, including reduced stride length and increased base of support (Vilensky et al., 1981; Weiss et al., 2013), we analyzed gait in our mouse models with a special focus on stride length, pair base of support and pair gap.

We found a significant effect of group [Two-way ANOVA $F(3, 220) = 49.86, p < 0.0001$] on the increased pair gap (Figure 25A). Indeed, VPA males showed significant increase ($\approx +2.3$ folds) in both right ($p < 0.0001$) and left pair gap ($p < 0.0001$) versus Saline males. Similarly, VPA females showed significant increase ($\approx +1.62$ folds) in both right ($p < 0.0001$) and left ($p < 0.0001$) pair gap (Tuckey's Post hoc). In addition, two-way ANOVA revealed a significant effect of the group [$F(3, 220) = 33.74, p < 0.0001$] on pair base of support (Figure 25B). Indeed, post-hoc analysis showed that both VPA males and females have a significant increase of ($\approx +29$ to 40) in both right and left pair base of support when compared to Saline littermates ($p < 0.0001$). Similarly, we found an effect of treatment [$F(1, 109) = 46.39, p < 0.0001$] on stride length (Figure 25C). Tuckey's post-hoc analysis revealed a significant decrease ($\approx -6.5\%$) in stride length in both VPA males and females ($p < 0.0001$) in comparison to Saline controls. We report here that VPA treatment did not affect males or females paw area, nor speed or regularity of the run (Figure 25D-F), suggesting that the significant changes in gait parameters are not due to morphological factors (Paw area) nor to test parameters (Speed and Regularity of the run).

These results indicate that a significant ataxic gait is present in both VPA males and females, in line with our results obtained in fine motor coordination tests and with observations in ASD patients.

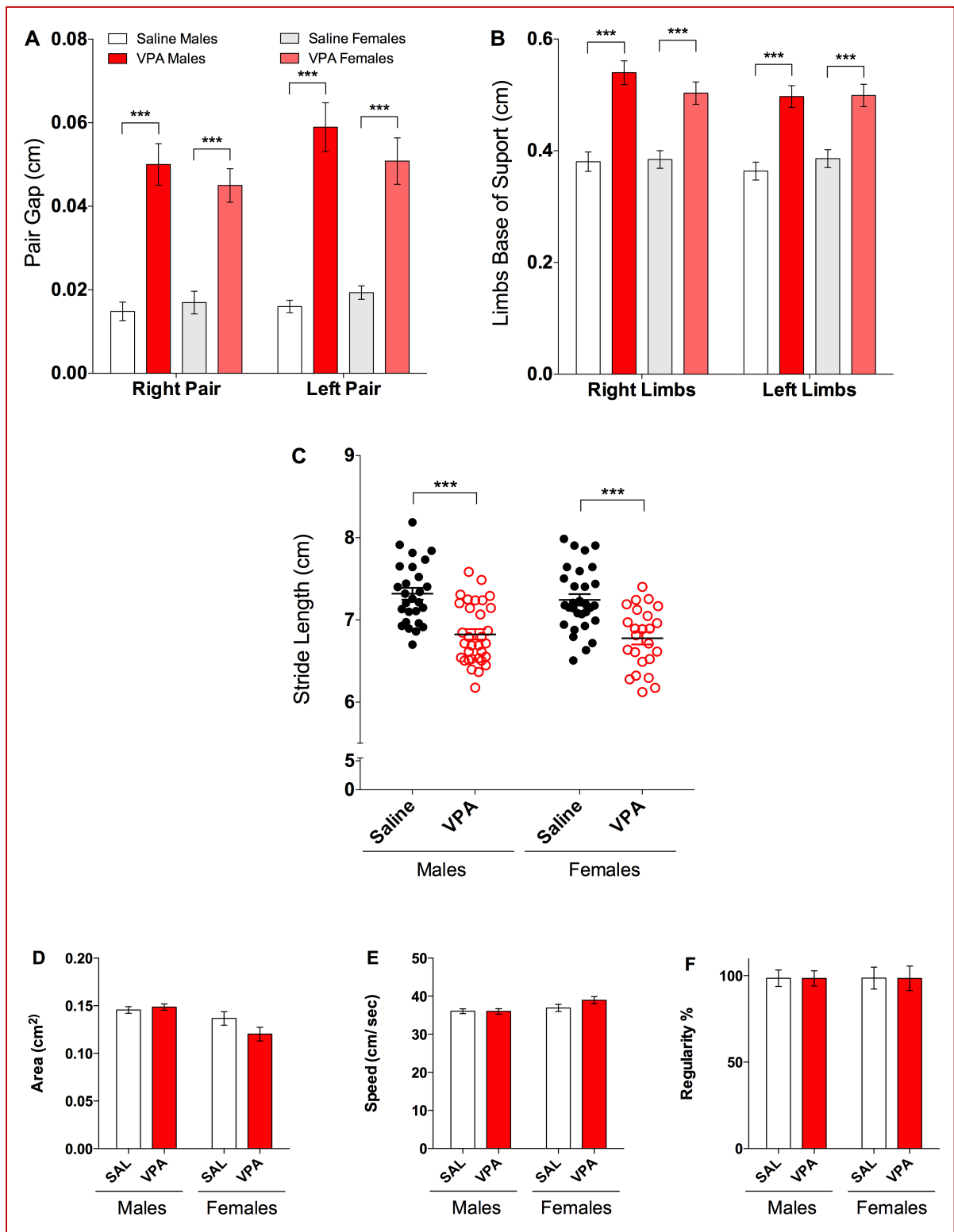


Figure 25: VPA prenatal treatment provoked ataxic spatial parameters of gait.

Gait analysis was assessed using the Viewpoint Gait-Lab. (A) VPA mice show significant increase in both right and left pair gap during the run on the Gait-Lab Plexiglas corridor and an increase in both right and left limb base of support (B) and a decrease in stride length (C). VPA mice show no significant change in (D) Area, (E) Speed, and (F) Regularity of the run. VPA males (n=32/34), Saline Males (n=27/27), VPA females (n=25/25), and Saline females (n=30/30). Data expressed as scatterplots and mean \pm SEM *** $P < 0.001$, one-way ANOVA, two-way ANOVA.

Chapter 2: Behavioral screening results in Poly I:C mice

2.1 Poly I:C mice displayed delayed post-natal development

To examine the effect of prenatal exposure to poly I:C on general postnatal development, we investigated the delay in offspring eye opening and righting reflex at P6-P19.

Two-way ANOVA revealed a significant effect of group [$F(3, 370) = 15.33, p < 0.0001$], age [$F(4, 370) = 642.8, p < 0.0001$], and group x age interaction [$F(12, 370) = 3.164, p = 0.0003$] on the mass gain. Tukey's multiple comparison test showed a significant increase in mass gains in poly I:C females at P32 and P40 ($P < 0.05$). Poly I:C males kept gaining mass normally till P40 (Figure 26A). Regarding the righting reflex, two-way ANOVA showed a significant effect of treatment [$F(3, 222) = 15.30, p < 0.0001$], age [$F(2, 222) = 83.37, p < 0.0001$] and treatment x age interaction [$F(6, 222) = 3.421, p = 0.0030$] (Figure 26B). Tukey's post-hoc revealed a significant latency in righting in poly I:C males at P9 ($p < 0.0001$) and P11 ($p < 0.05$) compared to Saline littermates. Poly I:C females displayed an increase in the latency only at P9 ($p < 0.01$). This is indicative of more pronounced developmental delays in poly I:C males compared to poly I:C females. Regarding the eye opening (Figure 26C), we found a significant effect of treatment [Two-way ANOVA $F(3, 370) = 9.348, p < 0.0001$], age [$F(4, 370) = 736.9, p < 0.0001$] and interaction between the treatment and age [$F(12, 370) = 2.891, p = 0.0008$]. Tukey's post-hoc analysis revealed a significant eye-opening delay in poly I:C males at P13 ($p < 0.001$) and P14 ($p < 0.01$). The delay was observed in poly I:C females only at P14 ($p < 0.0001$) indicating that the delay in eye opening appears earlier in poly I:C males (Figure 26C).

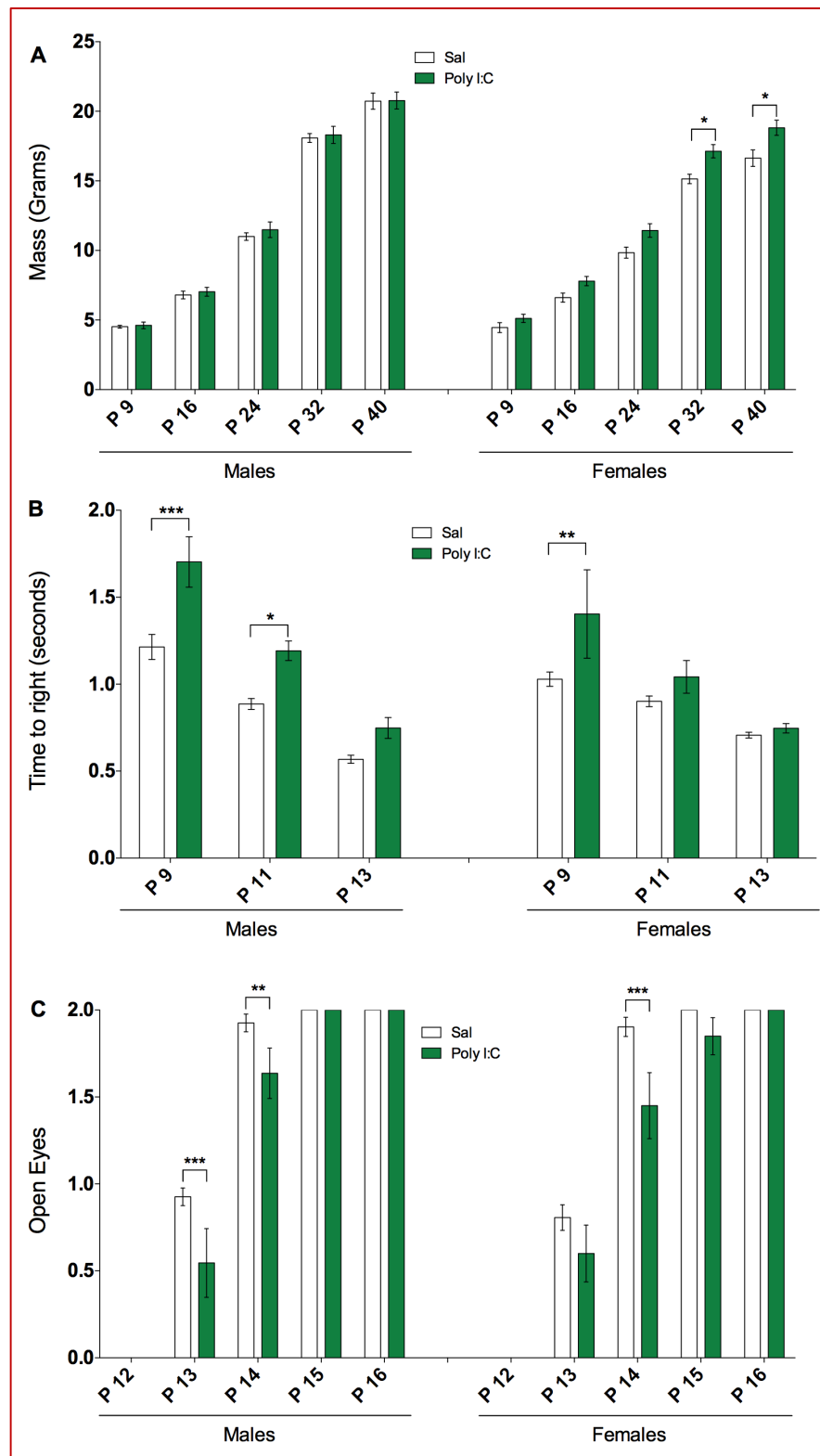


Figure 26: Poly I:C prenatal treatment altered developmental milestones mostly in males.

(A) Poly I:C prenatal treatment did not alter mass gain between P9 and P40 in males (Left), females (Right) showed normal mass gain until P32 where a significant increase in mass persisted till P40. (B) Poly I:C prenatal treatment affected neonatal righting reflex; Poly I:C males show a significant increased latency to righting at P9 in comparison to the Saline littermates. The significant increased latency to righting persisted until P 11. Poly I:C females displayed a significant increase in the latency to righting at P9. (C) Poly I:C prenatal treatment provoked a delayed eye opening. At P13 Poly I:C males exhibited delayed eye opening that persisted until P14. Poly I:C females displayed a significant delay in eye opening only on P 14 in comparison to Saline littermates. Poly I:C Males $n=11/11$; Saline Males $n=27/27$; Poly I:C Females $n=10/10$; Saline Females $n=30/30$. Data expressed as mean \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ two-way ANOVA.

2.2 Poly I:C males displayed faster habituation

One week after weaning, when mice were 4 weeks old, we tested the locomotor activity in infrared photocell-based detection actimeters activity chambers for 60 minutes on two consecutive days. The computer recorded beam crossings during 60 minutes by 5 minutes intervals. We found a significant effect of the group [Two-way ANOVA, $F(3, 148) = 5.054$, $p = 0.0023$], and day of test [$F(1, 148) = 7.726$, $p = 0.0062$]. Tukey's post-hoc analysis revealed a significant decrease of 27% in LA in poly I:C males on day 2 ($p < 0.05$). On the same day, Saline females had a significant decrease of 24% in LA in comparison to Saline males ($p < 0.01$) (Figure 27A). To examine the effect of prenatal exposure to poly I:C on exploratory behavior, we investigated the locomotor activity over the course of the 60 minutes session. On Day 1, we found a significant effect of the group [Two-way ANOVA $F(3, 888) = 4.659$, $p = 0.0031$], and time [$F(11, 888) = 39.20$, $p < 0.0001$]. Tukey's post-hoc analysis revealed a significant decrease in LA in Saline Females at $t = 50$ minutes ($p < 0.01$) (Figure 27B). Similarly, on Day 2 we found a significant effect of the group [Two-way ANOVA $F(3, 888) = 11.03$, $p < 0.0001$] and time [$F(11, 888) = 30.93$, $p < 0.0001$]. Tukey's post-hoc analysis revealed a significant decrease in LA in Saline females in comparison to Saline males at $t = 20$ and $t = 30$ minutes ($p < 0.05$) (Figure 27C). We further analyzed the differences of LA in the last 30 minutes for each day, where most of the changes happen. Comparing the LA of poly I:C males on Day 1 to Day 2, we found a significant effect of the day [$F(1, 10) = 6.126$, $p < 0.05$], and the time [$F(5, 50) = 6.505$, $p < 0.0001$]. Sidak's Post hoc analysis revealed no significant difference in the decrease in LA in poly I:C males on day 2 (Figure 28D). Taken together these results indicate that poly I:C provoked reduced exploratory in males.

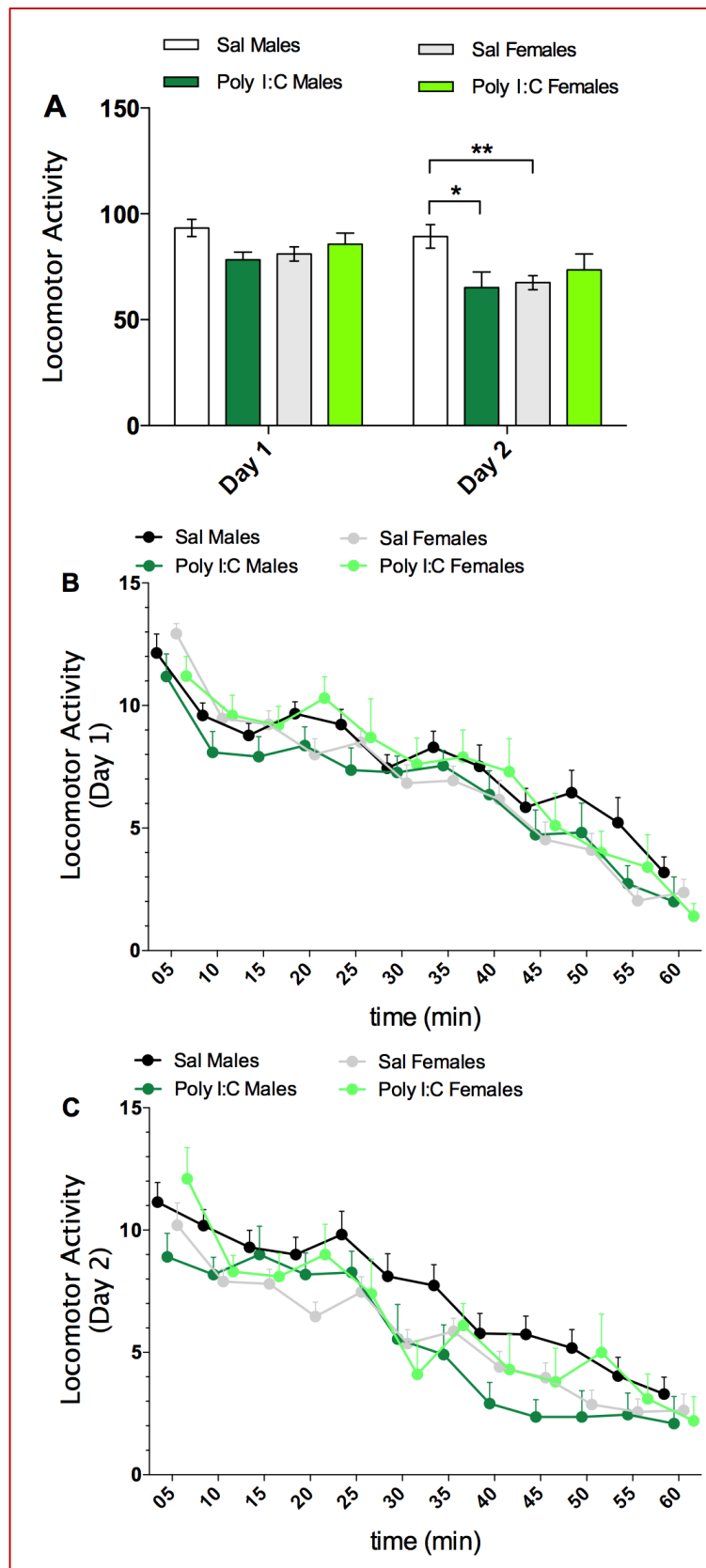


Figure 27: POLY I:C prenatal treatment altered locomotor activity (LA) in activity cages.

(A) On Day-2 Poly I:C males shows reduced Locomotor Activity (LA; assessed by number of beam crosses: y-axis) in comparison to the activity in Saline males. On Day-2 Saline females shows reduced LA relative to Saline males. (B) Saline females had a reduced LA at t=50 mins on Day-1 in comparison to Saline males. (C) Saline females had a reduced LA at t=20 and t=30 mins in comparison to Saline males on Day-2. Poly I:C Males n=11/11; Saline Males n=27/27; Poly I:C Females n=10/10; Saline Females n=30/30. Data expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ two-way ANOVA.

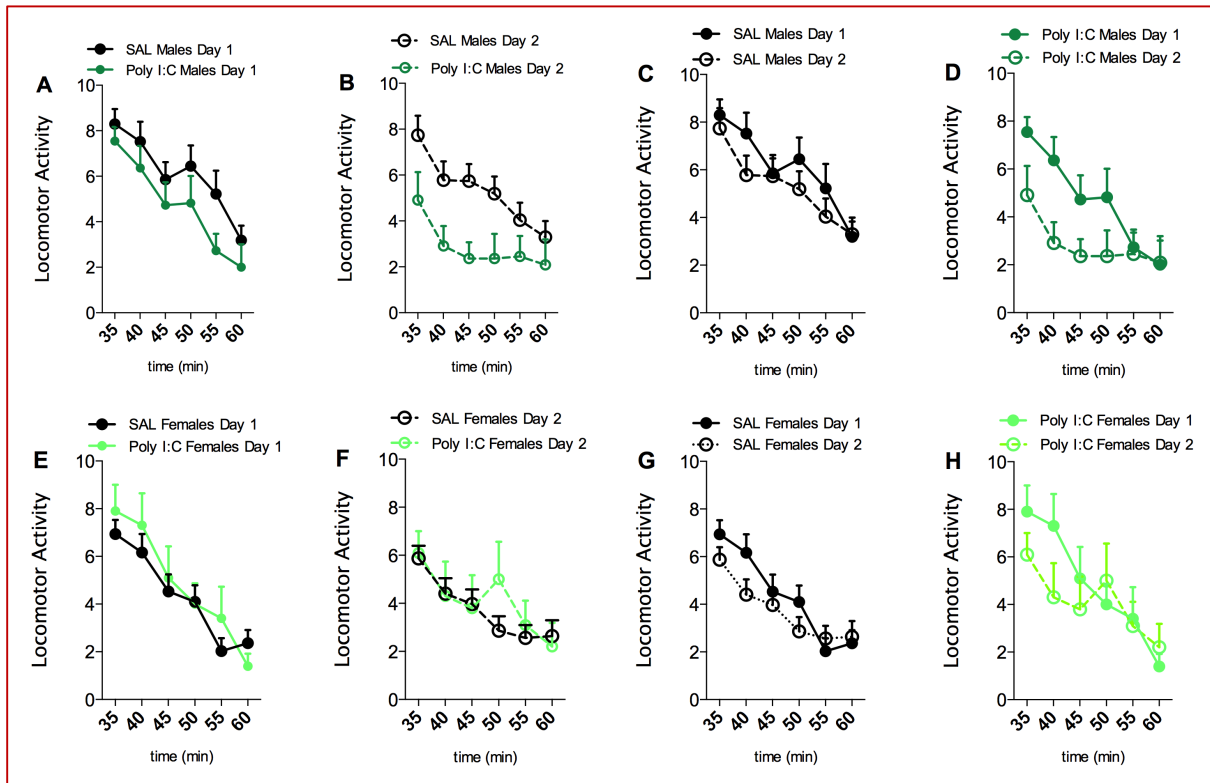


Figure 28: LA analysis of the last 30 minutes on Day-1 and Day-2 in POLY I:C and Saline.

(A) Graph showing no significant difference in LA between Poly I:C males and Saline littermates on Day-1. (B) Similarly, no significant difference was found between both groups on Day-2. (C) No significant difference in LA in Saline males between Day-1 and Day-2. (D) Poly I:C prenatally treated males shows no significant decrease in exploratory LA on Day-2 compared to Day-1. (E) No significant difference in LA between Poly I:C females and Saline littermates on Day-1. (F) No significant change in activity in Poly I:C females, in comparison to Saline females on Day-2. (G) No significant difference in LA in Saline females between Day-1 and Day-2. (H) Poly I:C prenatally treated females show no significant change in locomotor activity on Day-2. Poly I:C Males n=11/11; Saline Males n=27/27; Poly I:C Females n=10/10; Saline Females n=30/30 Data expressed as mean \pm SEM two-way ANOVA.

2.3 Poly I:C mice displayed altered behavior in SHIRPA primary Screen

Time spent immobile following transfer to SHIRPA arena was assessed and two-way ANOVA revealed a significant effect of the treatment [$F(1, 47) = 25.50, p < 0.0001$] (Figure 29A). Post-hoc analysis revealed that only poly I:C males showed 65% increase in immobility ($p < 0.0001$). Directly after the transfer to the SHIRPA arena, locomotor activity was assessed. An effect of Treatment [$F(1, 74) = 18.72, p < 0.0001$], and treatment x sex interaction [$F(1, 74) = 6.124, p = 0.0156$] on locomotor activity was revealed by two-way ANOVA (Figure 29B). Tukey's post-hoc analysis revealed a significant (-24%) decrease in locomotor activity in poly I:C males versus Saline males ($p < 0.0001$) while no such difference was observed in females. In comparison with Saline males, both Saline and poly I:C females showed significant reduced activity of 13% and 20% respectively ($p < 0.01$). Here we tested postural stability and coordination in space using negative geotaxis measured as the time needed to turn and the time needed to climb a 45° inclined grid. We found an effect of treatment [Two-way ANOVA, $F(1, 74) = 9.794, p = 0.0025$] on time needed to turn on the grid. However, Tukey's post-hoc analysis revealed no significant change in time to turn in poly I:C mice (Figure 29C). In addition, only sex [$F(1, 74) = 4.486, p = 0.0375$] had an effect on the time needed to climb the grid. Indeed, poly I:C males spent significantly more time, than Saline females, to climb the grid (+18%) ($p < 0.01$) (Figure 29D). Finally, we have implemented the wire maneuver test to assess coordination and muscle function. Two-way ANOVA revealed a significant effect of the treatment [$F(1, 47) = 7.509, p = 0.0077$], and sex [$F(1, 47) = 6.267, p = 0.0145$] on the time spent climbing the wire (Figure 29E). Indeed, Tukey's post-hoc analysis showed that both poly I:C males were statistically different from Saline littermates as they spent (+18%) ($p < 0.05$) more time to climb the wire. Furthermore, when compared to Saline females, poly I:C males showed a significant increase, of 19%, in time spent climbing the wire ($p < 0.01$).

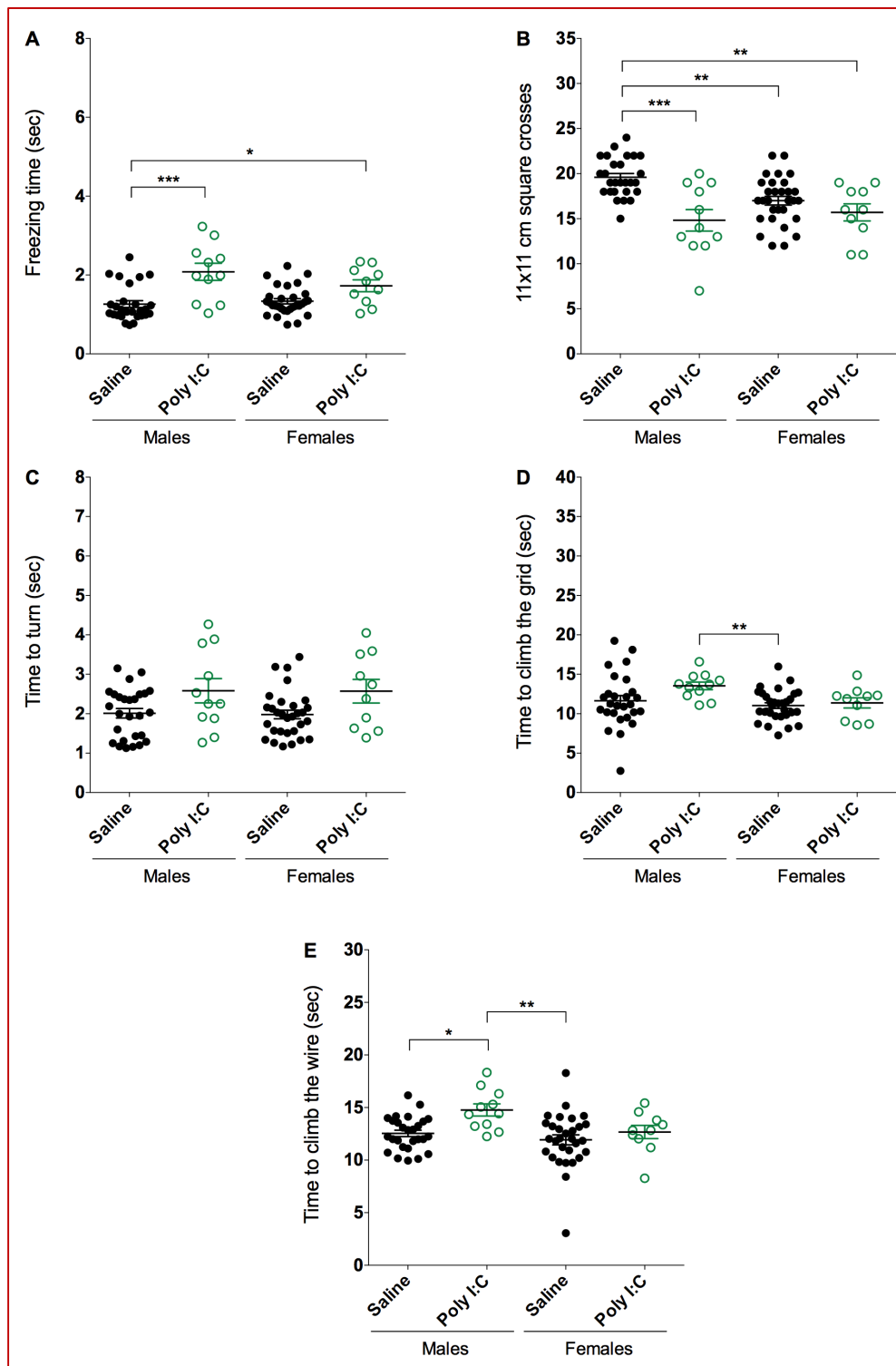


Figure 29: Poly I:C treatment altered several SHIRPA parameters in males.

(A) scatterplot of time spent immobile when transferred to the SHIRPA arena. Poly I:C males show a significant increase in time spent freezing, in comparison to Saline males (Left). When transferred to the SHIRPA arena, Poly I:C females (Right) show a significant increase in immobility compared to Saline males (Left). (B) Poly I:C males show reduced LA in comparison to Saline males. Both Saline females and Poly I:C females show reduced LA in comparison to Saline males. (C) scatterplot of time to turn on the grid in the wire in the negative geotaxis test. Poly I:C mice show no change in time spent turning on the grid, in comparison to Saline littermates. (D) Poly I:C males show a significant increase in time to climb on the grid, in comparison to Saline females. (E) Poly I:C males show a significant increase in time spent climbing the wire, in comparison to Saline males (Left) and females. Poly I:C Males n=11/11; Saline Males n=27/27; Poly I:C Females n=10/10; Saline Females n=30/30. Data expressed as scatterplot and mean \pm SEM * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ two-way ANOVA Tukey's post-hoc.

2.4 Poly I:C males displayed reduced sociability, but normal repetitive behaviors (P30; P35- 45)

We assessed sociability in 5-6 weeks old mice prenatally exposed to poly I:C or Saline. None of the treatment groups, regardless the sex, showed a preference to any of the chambers during the 10 minutes habituation (PHASE I) (Figure 30A). However, we detected a significant effect of chamber [Two-way ANOVA $F(1, 32) = 76.40, p < 0.0001$] and treatment x chamber interaction [$F(1, 32) = 11.03, p = 0.0022$] in phase II. Indeed, while Saline males spent more time in the social chamber than in the empty chamber (69% versus 31%, $p < 0.0001$), poly I:C males spent 58% in the social chamber versus 42% in the nonsocial chamber ($p < 0.01$) (Figure 30B). Thus, poly I:C males presented a significant increase of 39% in the time spent in the non-social chamber when compared to Saline littermates ($p < 0.05$). In females, we detected a significant effect of chamber [Two-way ANOVA $F(1, 36) = 69.60, p < 0.0001$] and treatment x chamber interaction [$F(1, 36) = 4.241, p = 0.0468$] in phase II. Indeed, females spent more time in the social chamber than in the non-social chamber, regardless of the treatment.

The effect of poly I:C on sociability was then expressed as sociability index. Indeed, although the two-way ANOVA failed to show a significant effect of the treatment [$F(1, 34) = 0.3266, p = 0.5714$], and sex [$F(1, 34) = 0.7873, p = 0.3812$], there was an effect of treatment x sex interaction [$F(1, 34) = 7.502, p = 0.0097$] on sociability index. Tukey's post-hoc analysis showed no significant difference in the SI, were all the groups were compared to each other. However, when male and female groups were compared separately, Sidak's post-hoc analysis showed that poly I:C males have a 57% decrease in sociability ($p < 0.05$), while females had similar SI regardless of the treatment (Figure 30C).

Then, repetitive and exploratory behaviors were assessed in Plexiglas cylinder. Time spent grooming was not affected by treatment [Two-way ANOVA, $F(1, 47) = 2.240, p = 0.1387$] (Figure 30D). However, there was an effect of treatment [Two-way ANOVA $F(1, 47) = 16.87, p < 0.001$] on rearing behavior (Figure 30E). Indeed, both male and female poly I:C mice showed

significant reduction in rearing behavior compared to Saline littermates (-47%) ($p < 0.05$) and (-38%) ($p < 0.05$) respectively.

All together, these results indicate that poly I:C induced reduced sociability in sex dependent manner. In addition, poly I:C prenatal exposure provoked normal grooming repetitive behavior, but reduced vertical rearing, a spontaneous exploratory behavior, which is in line with our previous results in automated LA and SHIRPA.

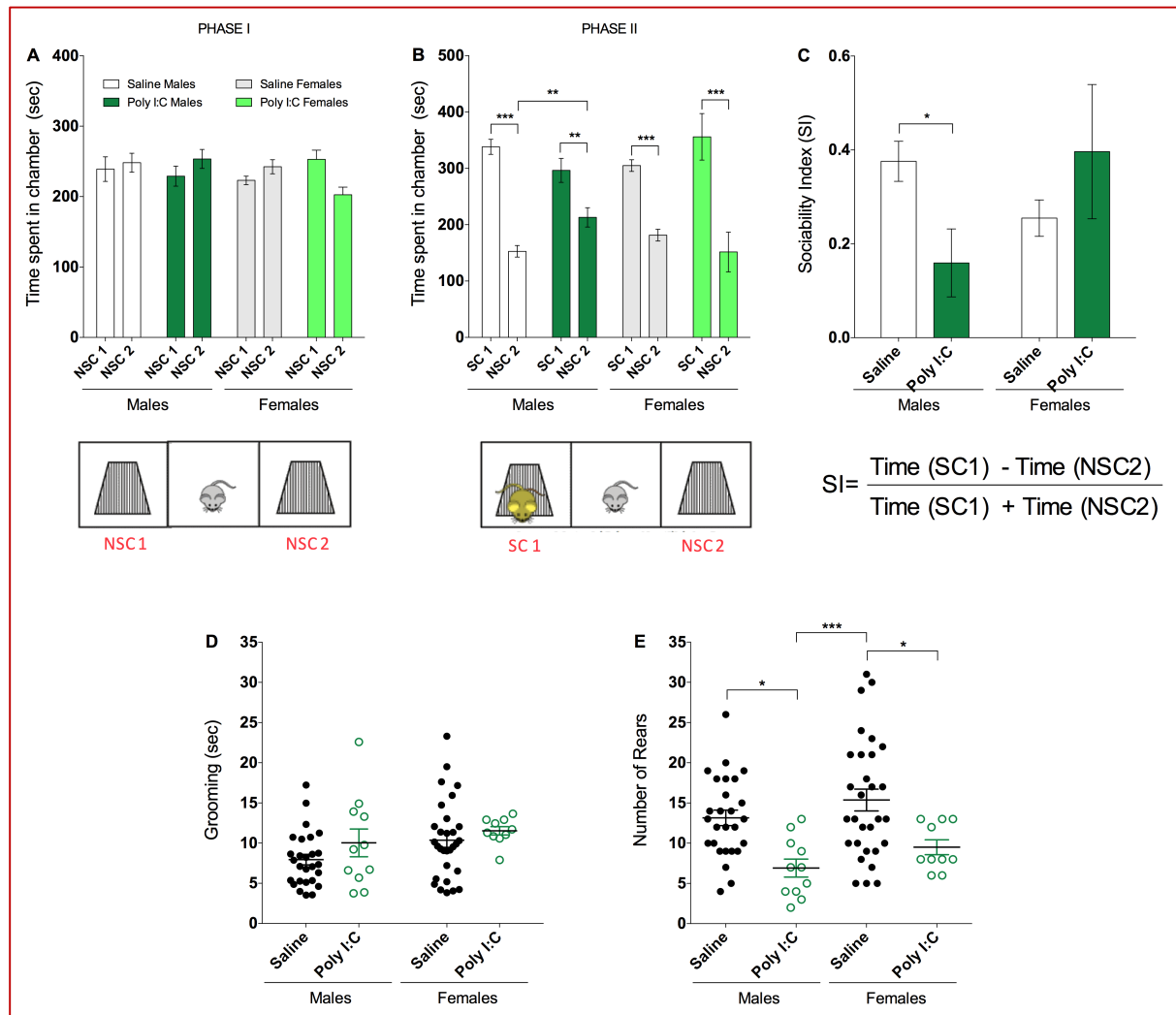


Figure 30: Poly I:C males show reduced social behavior.

(A) Histogram showing no change in the time spent in nonsocial chamber-1 (NSC1) versus nonsocial chamber-2 (NSC2) regardless the treatment in both males (Left) and females (Right) in PHASE I of the three chambers test. (B) Poly I:C prenatal treatment induced reduced sociability only in males (green bars- Left). (C) Poly I:C prenatally treated males show a significantly decreased sociability as shown in the Sociability index (SI). (D) Scatterplot of time spent grooming. Poly I:C mice show no significant change in the time spent grooming, in comparison to Saline littermates. (E) Poly I:C males show a significant decrease in number of vertical rears, in comparison to Saline males and Saline females. Poly I:C females show a significant decrease in number of rears, in comparison to Saline females. For the three chambers test of sociability: Poly I:C Males $n=8/11$; Saline Males $n=10/27$; Poly I:C Females $n=5/10$; Saline Females $n=15/30$; For the cylinder test: Poly I:C Males $n=11/11$; Saline Males $n=27/27$; Poly I:C Females $n=10/10$; Saline Females $n=30/30$. Data expressed as scatterplots and mean \pm SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA.

2.5 Poly I:C mice displayed normal motor coordination and gait (P33- 34)

Motor coordination was assessed using the challenging beam test. The time needed to traverse the beam sections was not affected by the treatment [Two-way ANOVA, $F(1, 47) = 0.3317, p = 0.5664$] (Figure 31A). Moreover, errors per step was not affected by the treatment [$F(1, 47) = 0.4130, p = 0.5224$] as both poly I:C males and females showed no change in the errors per steps compared to Saline (Figure 31B). We have found a significant effect of beam section on all treatment groups [two-way ANOVA, $F(3, 296) = 34.19, p < 0.0001$] (Figure 31C). However, male and female poly I:C pretreated mice showed no significant change in the number of errors per beam section. Tukey's post-hoc analysis revealed no significant differences on the first, second, third, and fourth beam sections in comparison to Saline males and females.

We analyzed gait in our mouse models with a special focus on stride length, pair base of support and pair gap. However, we found a significant effect of group [Two-way ANOVA $F(3, 148) = 49.86, p < 0.0001$] on the pair gap (Figure 32A). Indeed, when compared to Saline, poly I:C males showed significant increase (+1.97 folds and +1.76 folds) in both right ($p < 0.001$) and left pair gap ($p < 0.05$) respectively, (Tukey's post-hoc). This effect was not observed in poly I:C females. In addition, two-way ANOVA revealed no significant effect of the group [$F(3, 148) = 0.7706, p = 0.5122$] on pair base of support (Figure 32B). Similarly, we found no effect of treatment [$F(1, 47) = 0.1638, p = 0.6868$] on stride length (Figure 32C). The two-way ANOVA showed an effect of sex [$F(1, 47) = 4.801, p = 0.0316$] on stride length. We report here that poly I:C treatment did not affect poly I:C males or females paw area, nor speed or regularity of the run (Figure 32D-F).

In brief these results indicate that poly I:C prenatal exposure did not alter motor coordination in both males and females.

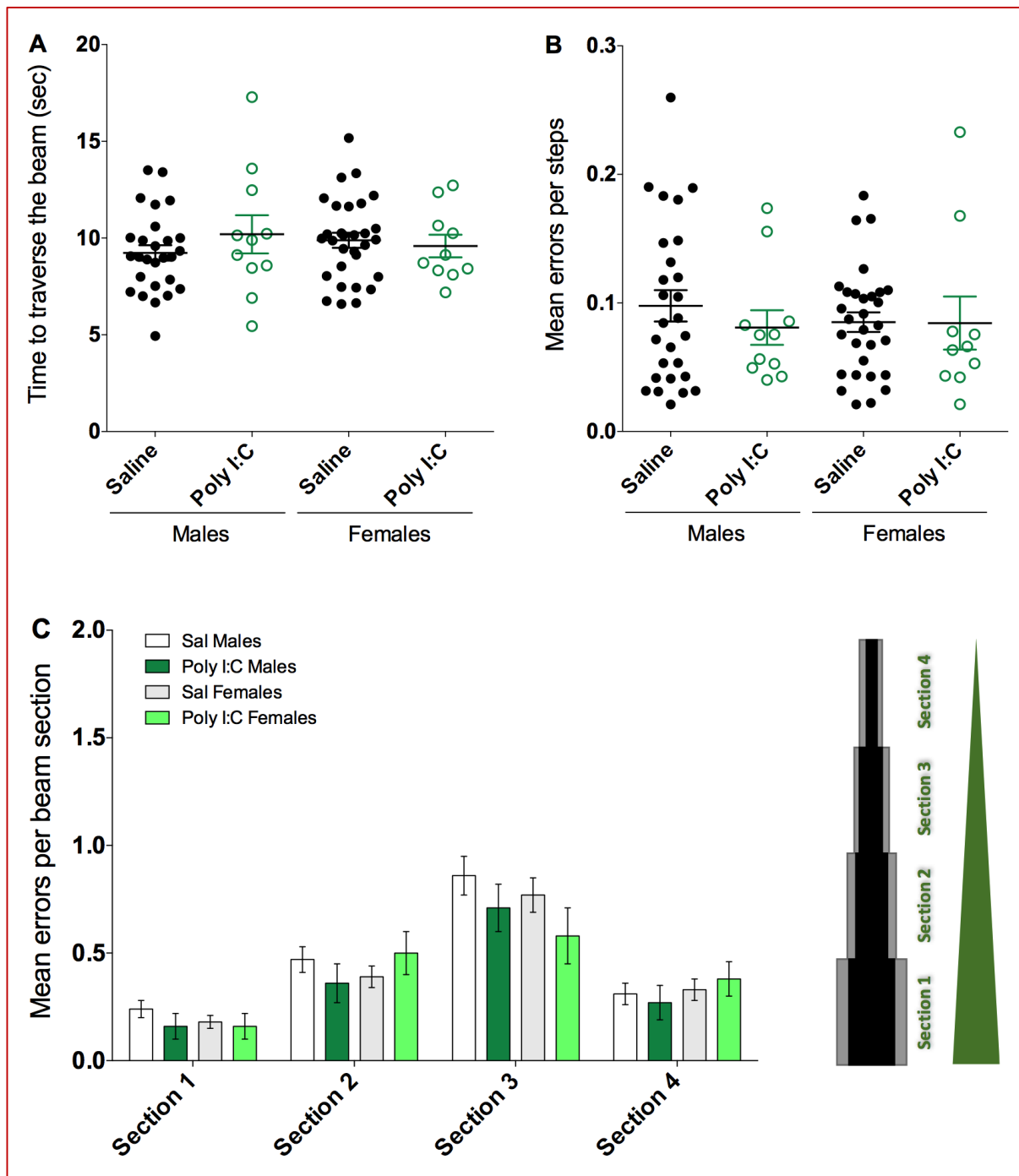


Figure 31: Poly I:C had no effect on motor coordination on the challenging beam test.

(A) Poly I:C mice needed the same amount of time to traverse the beam. (B) Poly I:C mice show no significant change in the number of errors per steps. (C) Poly I:C prenatally treated males (Green bars) show no change in the number of errors per steps on the all beam sections, in comparison to Saline males (White bars). Similar to the males, Poly I:C females (Light Green bars) show no change in the number of errors per steps on all the sections, in comparison to Saline females (Gray bars). Poly I:C Males $n=11/11$; Saline Males $n=27/27$; Poly I:C Females $n=10/10$; Saline Females $n=30/30$. Data expressed as scatterplot and mean \pm SEM two-way ANOVA.

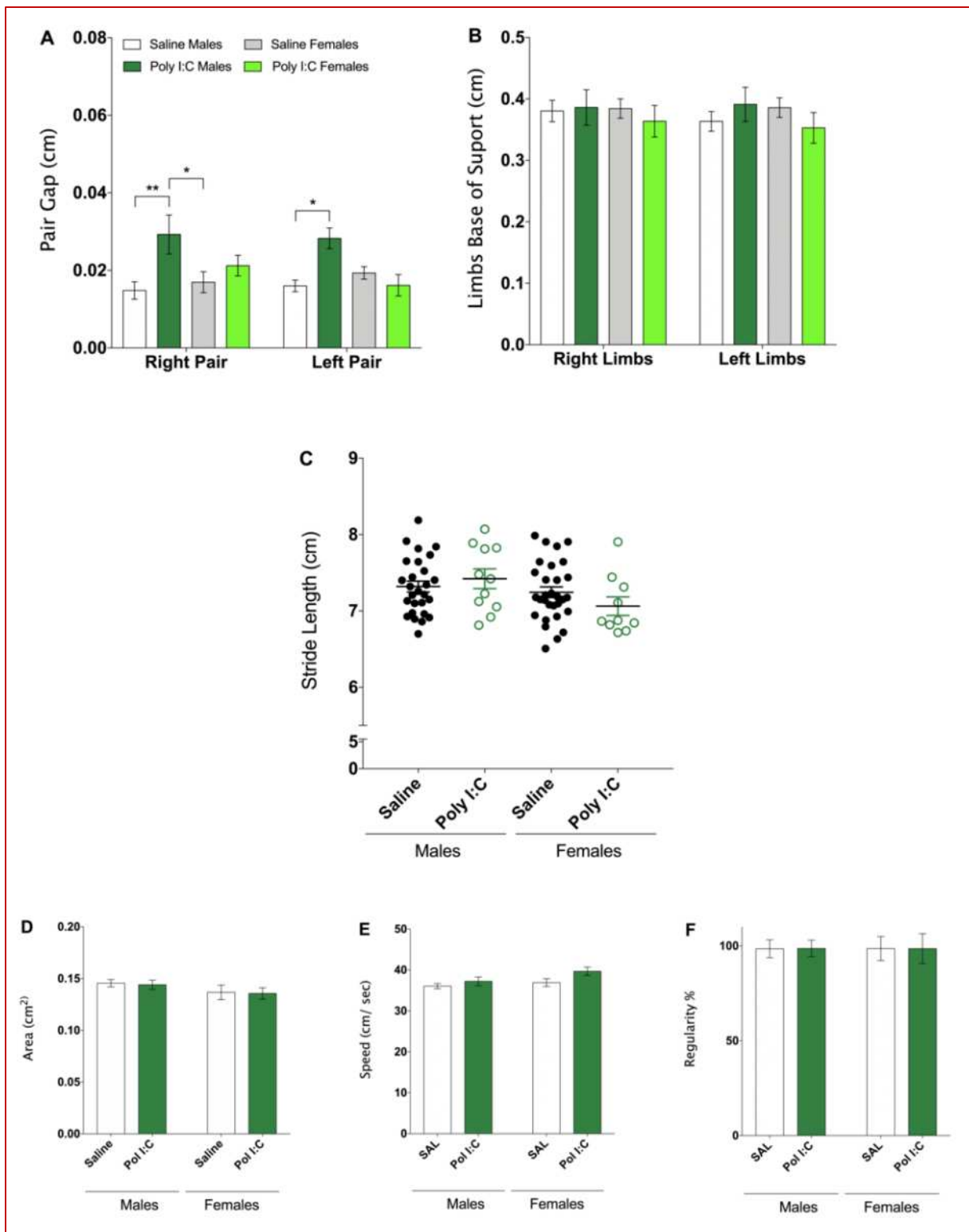


Figure 32: Poly I:C prenatal treatment did not provoke ataxic gait.

Gait analysis was assessed using the Viewpoint Gait-Lab. (A) Poly I:C males show significant increase in both right and left pair gap during the run on the Gait-Lab Plexiglas corridor. (B) Poly I:C mice show no significant change in both right and left limb base of support and (C) stride length. Poly I:C mice show no significant change in (D) Area, (E) Speed, and (F) Regularity of the run. Poly I:C Males n=11/11; Saline Males n=27/27; Poly I:C Females n=10/10; Saline Females n=30/30. Data shows mean \pm SEM * P<0.05, ** P<0.01, one-way ANOVA/ two-way ANOVA.

Part IV. DISCUSSION and PERSPECTIVE

Chapter 1: Discussion

1.1 General Discussion

We characterized sociability deficits, repetitive behavior, and motor coordination and gait in VPA and Poly I:C mouse models, in line with two main etiologies in environmental factors inducing ASD: 1) prenatal exposure to antiepileptic treatment and 2) MIA. Regarding the two diagnostic criteria of ASD, (i) social deficits and (ii) restricted interest and stereotyped behavior, the VPA mouse model presents a sex-dependent reduced sociability and behaviors resembling stereotypes in both males and females. In addition, early developmental trajectories and mid-postnatal motor deficits were observed in both sexes in the VPA model. The Poly I:C mouse model presented minor reduction in sociability in males and no changes in motor behaviors in both sexes. Our work indicates that the behavioral phenotype of VPA mouse model shares, in a sex dependent manner, ASD-like traits observed in patients with ASD. In both models, we have characterized differences between males and females, in line with our initial hypothesis aimed at unpinning sex differences in behavior after prenatal exposure to VPA or Poly I:C. We have demonstrated in this work that in utero environmental insults can induce early developmental delays suggesting alterations in brain development of the fetus. Significant and consistent epidemiologic evidence has already indicated an increased risk of ASD with (i) exposure to the antiepileptic treatment VPA and (ii) viral infection during pregnancy. However, to date, there was little or no experimental data to support the presence of early motor delays, reduced motor coordination, and gait troubles in these two models of ASD. Today, the model of prenatal exposure to VPA provides the first experimental data indicating the relevance of examining motor behavior, not only for being precise, quantitative and reproducible, but also because of being expressed equally by both males and females. The results associated with this preclinical model may also indicate that the high prevalence of ASD in males might be due to the focus on the social behavior, thus underestimating other behavioral deficits in females.

1.2 Relevance of behavioral protocols used

We have chosen to develop a series of specific behavioral tests for the observation of ASD behaviors in mice. We relied on the diagnostic criteria and behavioral traits frequently associated with ASD in the literature to test the behaviors in our the VPA and Poly I:C mouse models. We also developed specific tests to assess motor behavior, fine motor skills, motor coordination and gait. Subsequently, based on the literature review of neurobehavioral alteration models, and based on our hypothesis regarding motor deficits in ASD, we selected relevant tests for our study. Each test was therefore chosen based on the behaviors analyzed in relevance to ASD behaviors observed in humans (Table XI).

To test sociability in our mice we adopted the **3 chambers test**, the most widely used test for characterization of social behavior in rodents (Moy et al., 2004). **The cylinder test** was employed to assess repetitive behavior and vertical exploration in new environment (Fleming et al., 2013). In addition, “restricted interests” behaviors were assessed in the **locomotor activity** and **SHIRPA** battery (Brooks and Dunnett, 2009), where cognitive and motor functions were assessed. We were also inspired by the protocols validated and used elsewhere in the domains of motor behavior deficits including the **“challenging beam test”** of motor coordination (Fleming et al., 2004), **Gait lab** analysis of walk (Tsai et al., 2013). Most of these tests were developed, tuned and/ or validated in our laboratory specifically for this project. Finally, we reproduced the mouse model exposed to VPA and Poly I:C in the process of developing and/or validate the selected tests.

	Human Behavior	Behavioral test used	Behavior tested in mouse
ASD Diagnostic criteria	Social deficits	Three chambers test	Social interaction
	Repetitive behavior	Cylinder test	Stereotypic movements (Self-Grooming)
	Restricted interests	Locomotor activity	Exploration/ habituation
		SHIRPA	Spontaneous activity in new environment (freezing, exploration...)
		Cylinder test	Vertical exploration (Rearing)
Motor Behavior	Motor coordination Ataxia	Challenging beam test	Motor coordination
		Gait Lab	Ataxia
		SHIRPA	General motor abilities Motor coordination

Table XI: Behavioral tests selected to study ASD in mouse models in relevance with diagnostic criteria symptoms of ASD in humans.

With dark shading, tests previously developed and used in ASD research; with light shading validates tests previously developed and used in behavioral characterization of animal models of other disorders.

1.3 Stress affects the behavior of the animals

Many factors can induce short, medium, or long-term stress in animals. It is therefore essential to take this into account when planning, carrying out and analyzing the experiments. It has been shown that the so called "routine" procedures, such as animal transport, can cause significant stress. After a 3 hours road transport, changes in corticosterone levels, heart rate, and blood pressure were observed in the rat, which lasted up to 16 days after the event. Behavioral changes were also observed with an increase in grooming behaviors and a decrease in social interaction and motor activity (Arts et al., 2012). Behavioral changes or physiological parameters related to stress caused by daily or routine procedures have also been demonstrated during cage changes, gavage procedures, blood sampling (see Balcombe et al., 2004). It is therefore essential to minimize the disturbances of animals when unnecessary and to take into

consideration that every manipulation or external event can influence the behavior of the animals. Since stress can alter physiological parameters and behavior of animals with "simple" routine procedures, it is therefore important to question the stress intrinsically caused by the environment of the tests we administer to animals. Many tests use specific devices and therefore represent a new environment for the animal that can be stressful, and therefore the animal's priority will be to carefully explore the new environment in order to ensure the absence of threats (Suckow et al., 2005).

We have undergone the following procedures to minimize the stress caused by standard manipulations: (i) performing biweekly cage changes after administering the tests or at least 2 days before the test day, (ii) one person is assigned to daily care, from the arrival of mice, there timed mating, intra uterine treatment, until birth and the administration of all the behavioral tests in order to increase the familiarity of the animals with the experimenter, and (iii) use a single experimentation room to which animals are accustomed very early.

In locomotor activity test, cylinder test, and certain parameters of SHIRPA battery, the test is based on instinctive reaction and this is precisely the behavior we aimed to analyze. However, in a test such as that of social interactions, this stress component is to be taken into consideration. Pretest familiarization and habituation periods are very useful in this regard but very little documented, which can lead to arbitrary decisions as to their implementation. From one laboratory to another, these procedures vary enormously in form and duration, while they can have a considerable impact on the interpretation and reproducibility of the data. Other forms of stress can occur when performing a test. This is the case, for example, of the maternal odor recognition test, where the pups are separated from their mothers, albeit for a short time, thus causing distress behavior, which can have long term consequences (Marais et al., 2008). Moreover, it is known that the restraint of the animal creates an important stress. It is one of the methods frequently used to study the effects of stress in rodents (Campos et al., 2013). Any subsequent test would then prove unusable due to animal fatigue. The time required for the

animal to "recover" from a test is also very poorly documented. We therefore chose to leave a period of minimum 2 days between some manipulations of the animal in order to allow them time to rest, based on pilot studies that preceded this work. It would therefore be beneficial for the reliability and reproducibility of the results to continue investigating the consequences of the daily manipulations of laboratory animals.

1.4 VPA provokes ASD in human and rodents

There are many examples of drugs that have major teratogenic effects on in utero exposure. This is the case, for example, of thalidomide, a sedative and anti-nausea drug that was used mainly by pregnant women in the 1950s and was withdrawn from the market in 1961 because of its teratogenic effects. Indeed, several reports indicated that up to 30% of the children who were exposed between 21st and 24th gestation days developed ASD (Strömmland et al., 1994). This period corresponds to a very critical developmental stage, *the neural tube closure* (Lemire, 1988). VPA, or valproate, or 2-propylpentanoic acid, has been discovered for its anticonvulsant properties (Meunier et al., 1963), and was since then a widely used antiepileptic drug. It is prescribed primarily for the treatment of epilepsy, manic episodes, bipolar disorders, and migraine (Christensen et al., 2013). The first behavioral study of a rodent model exposed to VPA was published in 2001 (Schneider and Przewłocki, 2001). Four years later, a more detailed phenotypic behavioral characterization was published by the same team (Schneider and Przewłocki, 2005). Because of the behavioral resemblance between the rodent model and the symptoms observed in individuals with ASD, this rodent model rapidly became a *“reference model”* used in experimental research on ASD.

The scientific consensus of this model stands on the data associated in line with the concordant clinical consequences in humans with ASD. In utero exposure to VPA is associated with neural tube defects, spina bifida, facial anomalies, major organ abnormalities including respiratory, cardiovascular, gastrointestinal, genitourinary, and skeletal (Iwata et al., 2010). As early as 1980, the teratogenic effects of this drug were demonstrated (Brown et al., 1980), and as

early as 1984, they were termed as fetal valproate syndrome (DiLiberti et al., 1984). The first epidemiological report of a possible association between VPA and ASD dates back to 1994 (Christianson et al., 1994). The authors described in this study four children exposed in utero to the VPA all of which showed a developmental delay and one of these children has severe ASD. Since then, many studies have come to support this association (for review: Chomiak et al., 2013, Rouillet et al., 2013). According to Christensen et al. (2013), the risk of developing ASD is multiplied by 5 in children who have been exposed in utero to VPA. It is interesting to note that this increase in the risk of ASD is specific to VPA and not to in utero exposure to antiepileptic treatments in general (Wood et al., 2015).

All VPA rodent prenatal studies proposed an exposure between the 11th and 13th embryonic days (E11 to E13) that corresponds to the period of the *neural tube closure* in rodents (Morriss-Kay et al., 1993). One study compared this exposure period with two earlier exposures at E7 and E9.5 and a late exposure at E15 (Kim et al., 2011). In addition, three studies used exposure just prior to tube closure period (Dufour-Rainfray et al., 2010; Narita et al., 2010; Nakasato et al., 2008). Thus, it is widely admitted that exposure at E12,5 induced the most solid ASD symptoms in rodents. The vast majority of studies used a single dose of 600 mg/kg per intraperitoneal injection, as described at the outset by Schneider et al. (2005, 2001), but the doses used ranged from 350 mg/kg (Cohen et al., 2013) to 800 mg/kg (Raza et al., 2015; Nakasato et al. 2008). It should be noted that some studies also presented an after birth VPA exposure as a model of ASD. This is a much less well characterized model, and post-natal exposure to VPA varies greatly from study to study: between P6 and P20, with doses ranging from a single exposure of 200 mg/kg (Wang et al., 2015) to a daily exposure of 150 mg/kg for two weeks (Chomiak et al., 2010). We are mainly interested in prenatal treatment paradigm, so we will not develop on these models in more detail in the next sections. A single injection of 450 mg/kg VPA at E12.5 is widely recognized in the literature to be sufficient to induce ASD-like features in offspring.

In the next sections of the discussion, we will go through studies presenting behavioral characterization of this VPA model. Developmental data will be presented first; then the behavioral observations related to the main symptoms used for the diagnosis of ASD: sociability, communication, stereotypies, and sensory sensitivity; finally, the behavioral observations associated with frequent comorbidities of ASD.

1.4.1 Characterization of VPA model in rodents: Developmental observations (teratogenicity markers)

Most studies show at least one teratogenic effect following prenatal exposure to VPA, such as developmental delays or malformations (Olde Loohuis et al., 2015; Banerjee et al., 2014; Foley et al., 2014; Wellmann et al., 2014; Favre et al., 2013; Dendrinis et al., 2011; Dufour-Rainfray et al., 2010; Markram et al., 2008; Schneider & Przewłocki, 2005). Some studies have shown no difference in development (Kumar and Sharma, 2016; Raza et al., 2015; Nakasato et al., 2008), and many studies do not provide information on litter development (Zhao et al. 2015; Olexová et al., 2013; Wang et al., 2013; Schneider et al., 2006). Few data are available on the acquisition of neonatal reflexes. Some have shown a standard acquisition of the reversal reflex in the negative geotaxis test between P7 and P12 (Dufour-Rainfray et al., 2010; Nakasato et al., 2008; Schneider and Przewłocki, 2005). However, two studies have found motor function acquisition deficits evaluated in a forced swimming test (Sandhya et al., 2012; Schneider and Przewłocki, 2005). In summary, there is little data available on neonatal development, but the teratogenic effect of VPA at the time of neural tube closure is very common.

1.4.2 Characterization of VPA model in rodents: Behavioral observations and tests previously used

Although some studies do not follow the general trend (Cohen et al., 2013; Dendrinis et al., 2011), it appears that the dose and the window exposure are important determinants of the changes in sociability. Sociability is the most studied behavior in the VPA models with very consistent results. The study of rat sociability is most often carried out according to one of the following tests of social interaction: (i) the three chambers test and (ii) the test in pairs. For the

pair test, two age and sex matched mice are allowed to freely interact in a cage and all social interactions are assessed manually or by computer software. There are also some variations: use of rectangular or circular cage, adding a partition in the middle to make two rooms with enough space for one mouse to freely move between the two chambers, with the presence of a tube to hide from the other congener. Tests that are directly related to communication are more rarely used because they are more difficult to implement and the results are more difficult to interpret. Two studies have focused on communication in VPA mice, especially during a social interactions test (Raza et al., 2015; Wellmann et al., 2014). Microphones placed around and above the cages allowed to record the ultrasonic vocalizations of animals. The first study showed a decrease in social interactions in the VPA group, that was also associated with a communication deficit, with a greater latency to initiate the first ultrasonic call and fewer calls exchanged between the animals. The latter did not show any such social and communication differences in VPA, but it should be noted that VPA animals studied were all females.

Tests related to motor skills and symptoms such as repetitive activities and stereotypies, novelty (exploration), as well as common comorbidities such as hyperactivity are present in the behavioral characterizations of the VPA rodent model. The paradigms set up in the tests varies, the age of the animal at the time of the test can have a strong influence on the results, and the behaviors studied can vary in the same test. However, there is a trend, namely an increase in motor activity characterized in previous VPA rat studies (Zhao et al., 2015; Sandhya et al., 2012; Schneider et al., 2008; Schneider and Przewłocki, 2005), accompanied by a decrease in constant exploration behavior (Kumar and Sharma, 2016; Favre et al., 2015; Olexová et al., 2013; Markram et al., 2008; Schneider et al., 2006; Schneider and Przewłocki, 2005). One study reported a decrease in motor activity (Olexová et al., 2013) and in three studies of the team that originally developed this model, stereotypical behaviors were observed in these VPA rats (Schneider et al., 2008, 2006; Schneider & Przewłocki, 2005).

The open field test is a simple test widely used in behavioral studies since it allows to inspect many parameters including motor activity, distance traveled and exploratory activity. However, when used alone, results must be interpreted with caution as animal activity includes locomotor activity, exploration, anxiety, and stereotypy (Tanaka et al., 2012); all factors that can influence the interpretation of results. For this test, the mouse is placed in a cage and a camera records all the trajectories of the mouse during the test, which can vary from 1 to 60 minutes. This open field test allows to quantify locomotor activity and spontaneous exploration in a new environment. Many variations of this test exist, for example, the use of cage with ground infrared beams or **actimeters**, that count beam crosses. This makes it possible to study habituation with time course. Other less frequently used tests were also employed to characterize the motor and exploratory abilities of mice exposed to VPA. The test of the **rotarod** consists of a wheel on which the mouse is deposited and which rotates faster and faster. This test normally evaluates the animal's forced motor skills, motor coordination and endurance, but it can also impose a rhythm and might have effects on plasticity if training paradigms were involved. There are also tests of motor reflexes such as the neonatal walking test, where a circle is drawn on a paper and the pup is placed over and the time needed to walk outside the drawn region is recorded. In the same category, the gripping test where a pup is suspended and the time needed to grip is recorded. We did not use these two tests as they oblige the isolation of pups from their mothers. The Y-maze test is a test that presents a very interesting paradigm in the study of ASD. This test makes it possible to study whether the animal prefers to explore new territories (classical behavior of animals), or if they prefer a certain routine, associated with repeated behaviors and a need for immutability characteristic of ASD.

To evaluate repetitive behavior, we adopted the cylinder test (Fleming et al., 2013). This approach makes it possible to record the spontaneous and exploratory activity in novel environment in addition to repetitive behavior.

1.5 Relevance of VPA behavioral data in comparison to previous studies

We have evaluated the developmental, social, cognitive and motor behavior in mice that received a single injection of 450mg/kg to VPA on day 12.5 of gestation. Our study is the first to examine gait impairments and motor coordination deficits, in addition to autistic-like traits, in both male and female mice prenatally treated with VPA. Our results show that prenatal exposure to VPA substantially alters early developmental trajectories, social cognitive and motor behaviors in offspring in a sex dependent manner. VPA offspring showed early developmental delays in open eyes and righting reflex. In addition, VPA males expressed reduced locomotor activity early adolescence. Furthermore, VPA males expressed reduced sociability and increased repetitive behavior, two core features of ASD. However, the sociability was intact in VPA females that expressed repetitive behavior. Interestingly, early motor coordination deficits and ataxic gait were observed alike in both male and female VPA offspring (Recap. Table XII).

The VPA animal model has been extensively used with a major interest that VPA is already known to raise 5-folds the risk of ASD in children (Christensen et al., 2013). Moreover, many human ASD-relevant behaviors have now been portrayed for this model (Nicolini and Fahnestock, 2017; Rouillet et al., 2013). Thus, using this model in our study allows us to unpin motor behavior in relevance to ASD. We used a dose of VPA generally and widely accepted in the literature as being appropriate to model ASD-like behavior in mice, asingle i.p. injection of 450mg/kg at E12.5. This dose sufficiently induces ASD behavior in mouse offspring, and also alters their embryonic development. Upon VPA exposure, ASD-like behaviors can be observed in early development (Reynolds et al., 2012). Indeed, in the current study, developmental aberrations were observed as delays in general development. In agreement with previous results, VPA prenatally exposed males and females displayed a delay in eye opening (Rouillet et al., 2010) and an increased latency to righting (Podgorac et al., 2016; Iijima et al., 2016). These results suggest that VPA prenatal exposure affects postnatal development and motor reflexes during the first weeks.

Cluster	Day	Test	Behavior tested	VPA		Poly I:C	
Teratogenic features	P9 to P16	Righting reflex Open eyes	Developmental trajectories	↓↓♂ ↓↓♂	↓♀ ↓♀	↓♂ ↓↓♂	= ♀ ↓♀
Motor behavior	P 30	Wire maneuver	Motor coordination	↓↓♂	↓↓♀	↓♂	= ♀
	P30	Negative Geotaxis	Posture/ coordination in space	↓↓♂	↓♀	= ♂	= ♀
	P33	Challenging beam test	Motor coordination	↓↓♂	↓↓♀	= ♂	= ♀
	P34	Gait analysis	Gait	↓↓♂	↓↓♀	= ♂	= ♀
Stereotypes	P30	Repetitive Grooming	Repetitive behavior *	↑↑♂	↑↑♀	↑↑♂	= ♀
Sociability	P 35 to P45	Three chambers test	Sociability *	↓↓♂	= ♀	↓♂	= ♀
Cognitive functions	P28- P29	Locomotor activity	Exploration/ habituation	↓↓♂	= ♀	= ♂	= ♀
	P30	Immobility (TR- SHIRPA)	Spontaneous activity in new environment	↑↑♂	= ♀	↑♂	= ♀
	P30	Locomotor activity (SHIRPA)	Spontaneous activity in new environment	↓↓♂	= ♀	↓♂	= ♀
	P30	Rearing	Vertical exploration	↓↓♂	= ♀	↓♂	↓♀

Table XII: Recapitulative summary of the behavioral phenotypes observed in each model.

* Reduced sociability and repetitive behavior are ASD-core features according to the DSM-V (APA)

↓: Reduced; ↑: Increased; = No change.

In addition, the current study shows that mice prenatally exposed to VPA exhibit decreased locomotor activity one week after weaning. Of note, Kataoka et al. (2013) found that VPA-exposed mice displayed reduced locomotor activity at the age of 4 weeks. VPA rats showed decreased locomotor activity in puberty and adulthood (Olexova et al., 2013). In addition, it was recently shown that CD1 mice males prenatally exposed to 500 mg/kg (E12.5)

display reduced locomotor activity on P28 (Yamagushi et al., 2017). These results might be indicative of reduced interest of new environment. Reminiscent to the reduced locomotor activity, VPA males displayed reduced rearing in the cylinder, increased immobility, and reduced locomotor activity in the SHIRPA arena (P30). Of interest, Yamagushi et al., (2017) reported reducing rearing exploratory behavior in VPA mice at 4 weeks of age. This is in line with the clinical settings where it has been shown that Children with ASD spend less time by active exploration (Pierce and Courchesne, 2001).

Motor stereotypes are one of the core symptoms of ASD. These are repetitive motor behaviors, which seem uncontrollable, and are not directed towards a functional goal (APA, 2013). These may be hand and finger mannerisms, arm beating, or complex body movements (Lord et al., 1994). Grooming behavior in rodents is often suggested to reflect repetitive/stereotypic behavior and anxiety (Kalueff et al., 2016; Lewis et al., 2007). In our study VPA mice show increased self-grooming, a finding similar to previous reports (Kang and Kim 2015; Gandal et al., 2010). It is important to note here that repetitive behavior have been also assessed by marble burying test and it has been shown that VPA males prenatally treated with 600 mg/kg (E11) buried more marble and expressed increased self-grooming (Castro et al., 2016).

Furthermore, the present study shows a sex difference in the effect of VPA exposure on the social behavior. Indeed, deficits in social interaction are among the core symptoms in patients with ASD (Kim et al., 2011; Nadel and POSS, 2007; Adrien et al., 1992). Several studies have demonstrated deficits in social behavior in rat (kim et al., 2014a; kim et al., 2011; Markram et al., 2008; Schneider and Prezewlocki, 2005) and mouse VPA models of ASD (Kim et al 2014b; Kataoka et al., 2013; Moldrich et al., 2013; Rouillet et al., 2010). The present study showed the presence of decreased sociability in male mice prenatally exposed to VPA. In contrast, the VPA-exposed females showed normal sociability at age of 5-6 weeks. This is in relevance to recently published reports in VPA mouse (Hara et al., 2017b; de Theije et al., 2014)

and rat models (Kim et al., 2013). This finding may be in agreement with the higher prevalence of ASD in males. Our hypothesis to explain the sex dependent difference observed in our behavioral results rely on the fact that CNS networks involved in sex-dependent behavior depend on uterine testosterone and estradiol balance (Peper and Koolschijn, 2012). In neural and glial cells, testosterone is transformed to estradiol by aromatase (Montelli et al., 2012). Aromatase activity and expression appears to be modulated by the brain in a sexual dimorphic manner in specific brain regions and at sensitive times during development (Vizziano-Cantonnet et al., 2011). Fetal testis produce major amounts of testosterone that peaks around the second trimester. In male rat fetus, this peak of testosterone production starts at E15 (Kalat, 2015). In mouse fetus, gonad differentiates sexually between E11 to E12 (Munger et al., 2013). This peak of testosterone production is crucial for the development of male typical behaviors rather referred as “brain masculinization”. With minor testosterone production, the development of the brain in female fetus depends less on this testosterone/aromatase pathway (Schwarz & McCarthy, 2008). Thus, VPA exposure at sensitive time point in early embryonic development might have the potential to alter testosterone levels in CNS cells during brain development, and consequently induce male-specific behavioral alterations.

In the SHIRPA primary screen, our results show significant impairments in fine motor skills in both males and females treated with VPA. Motor coordination deficits were observed as early as P30, both VPA males and females spent more time climbing the wire. This is indicative of reduced motor coordination in the wire maneuver test. One might argue that the extra time spent might be due to reduced grip or muscle functions. However, it is not the case here as the VPA mice had normal growth (mass gain), normal grip, and muscle functions assessed by the SHIRPA primary screen.

Interestingly, the current study demonstrated for the first time the effect of prenatal exposure to VPA on motor coordination using a static beam. The challenging beam test was useful to show precise motor performance and coordination. VPA prenatal treatment altered

motor coordination in both males and females highlighting the importance of evaluating the behavior in both sex groups. In addition to motor coordination deficits, VPA mice had shorter stride length and an increased pair gap and pair base-of-support. These results are in line to previously published results in VPA repeated prenatal exposure rat model of ASD (Main and Kulesza, 2017), where an oral administration of 800 mg/kg VPA at E10 and E12 induced motor deficits. Ataxic gait with reduced stride length and increased stride width was previously shown in *Tsc1* mutant mice, where sociability and Purkinje cells counts were reduced in the same group (Tsai et al., 2012).

Currently there is no drug approved to treat social deficits in ASD patients. Of interest, recent preclinical data suggests that the VPA rodent model displays predictive validity. Much attention has been recently paid to ameliorate the ASD behaviors in VPA rodent models. For instance, chronic administration of Risperidone and Aripiprazole improved the VPA induced sociability deficits and memory impairments in mouse (Hara et al., 2017c). In another study, single intranasal administration of oxytocin corrected social interaction deficits in VPA males (Hara et al., 2017). In addition, increased grooming and marble burying was reversed with MPEP, mGluR antagonist, in mice prenatally exposed to 600 mg/kg VPA at E11. Donepezil hydrochloride monohydrate corrected social interaction deficits and repetitive behavior in VPA rat and mouse (Foley et al., 2014). Similar encouraging results were also obtained in another VPA rat study, where prenatal administration of resveratrol (RSV) prevented the VPA- induced social deficits (Bambini-junior et al., 2014).

In conclusion, the results of our behavioral studies demonstrated an experimental evidence of the consequences of VPA exposure on early postnatal development and motor behavior. In spite of the sex-dependent effect of the treatment on sociability, the delayed central nervous system development and immature motor system was present in VPA males and females. Considering the early detection of disturbed motor behavior, the fact that these deficits

are quantitative and present in both sexes, and their correlation with social deficits suggest the valuable contribution that these parameters, having a better understanding the physiopathology underlying ASD, with potential earlier detection, as well as better treatment strategies.

1.6 Prenatal infections and psychiatric disorders

Prenatal exposure to viral infections is a recognized risk factor for developmental psychiatric disorders (Vuillermot et al., 2017). Birth in spring or winter seasons is a risk factor for schizophrenia (Tochigi et al., 2004). Many studies have examined schizophrenia incidence following influenza epidemics, and the majority have discovered an increased incidence of schizophrenia amid exposed offspring. Moreover, Brown et al., (2006, 2004, 2002) analyzed the medical records of 12,000 pregnant women and concluded that respiratory infection in the 2nd trimester of pregnancy increases the risk for schizophrenia, 3 to 7 folds, in newborns. They suggest that 14 to 21% of the schizophrenia cases analyzed were induced by maternal infection, i.e. influenza infection. These outcomes are also reinforced by an association between elevated anti-influenza antibodies in the maternal serum and schizophrenia in the offspring (Brown et al., 2004).

A link has also been associated between maternal infection and the increased risk of ASD in children. Rubella epidemics were associated with increased risk for autism in offspring with prior in utero exposure to rubella (Chess, 1977; Desmond et al., 1967). Scarcer cases of rubella infection were seen after the rubella vaccine was developed. Since then, evidence has gathered to support the fact that prenatal exposure to viral infection is a risk factor for ASD in the offspring (Patterson, 2011; Meyer et al., 2011). For instance, a 3-fold increase in ASD diagnosis was reported in children born to mothers hospitalized, during pregnancy, for viral infections (Atladdottir et al., 2010).

1.6.1 Poly I:C model of MIA

The variety of infections associated as risk factors for psychiatric developmental disorders have no direct effects on the fetus, but rather the maternal immune system is

accountable for the increased incidence of these disorders in offspring (Patterson, 2005, 2002). Animal models of prenatal exposure to infections were generated and have shown that MIA is capable of inducing various behavioral and histological alterations in the brains of the offspring.

The innate immune system recognizes RNA as a viral infection. Poly I:C, a double stranded RNA, is potent against TLR3 that induces inflammatory cascade that ends in the production of cytokines. Thus, injection of poly I:C in pregnant mice mimics a flu infection. Offspring prenatally exposed to poly I:C show deficits in sensory-motor gating, latent inhibition, sociability, and explorations in open field (Smith et al., 2007; Shi et al., 2003; Zuckerman et al., 2003). Interestingly, poly I:C causes PC deficits in cerebellar lobule VII (Naviaux et al., 2013; Shi et al., 2009), in line to anatomical findings in ASD patients.

MIA by Poly I:C is known to provoke various defects in the offspring's behavior and neuroanatomy. This treatment mimics maternal infection; upon injection of Poly I:C, it binds to the toll-like receptor 3 (Alexopoulou et al., 2001) stimulating the production of the inflammatory cytokines like IL-1 β , IL-6, and TNF- α as well as interferons like IFN- α and IFN- β (Bilbo et al., 2017). This mimics the acute phase response to a viral infection (Traynor et al., 2004). In our experiments, Poly I:C treated pregnant females were reported to lose body mass 24 hours post-injection, confirming that the treatment had an anticipated influence. This is in relevance to previously shown results demonstrating that Poly I:C treatment provokes short-term sickness behaviors in the treated pregnant dams that are easy to monitor and include reduced activity, reduced food intake, and mass loss (Ballendine et al. 2015). In addition, poly I:C treatment induced a reduction in the litter size which is in line with the previously published evidence in both rat (Ballendine et al. 2015; Yee et al. 2011) and mouse models of poly I:C (Naviaux et al., 2013). Despite these acute effects induced by poly I:C treatment, adult mice prenatally exposed to poly I:C were indistinguishable from Saline controls in terms of mass gain and, gross behavior, and general appearance.

1.7 Relevance of Poly I:C behavioral data in relevance to previous studies

The current study reports previously demonstrated changes in poly I:C mice in many behaviors relevant to ASD. Indeed, our results show that a single poly I:C i.p. injection at E 12.5 can induce reduced sociability only in males. In addition, in developmental trajectories poly I:C males exhibit increased time in righting reflex and delays in eye opening, a feature that was less prominent in the poly I:C females. Interestingly, the behavioral deficits observed after MIA in poly I:C males included restricted behaviors as evidenced by increased immobility in new environment, reduced locomotor activity, two parameters assessed in the SHIRPA arena, and reduced horizontal exploration in the cylinder. However, our study is the first to explore fine motor skills, coordination, and gait in this model.

Previous studies in poly I:C mouse models has underscored the presence of ASD-like behaviors in these mice (for example see: Malkova et al. 2012). Many of the behavioral results presented by the poly I:C mice in the current study are in line with previously reported outcomes, including decreased social interaction. For instance, Schwartz et al., (2013) reported reduced social preference and normal grooming behavior of mice offspring born to females treated with 20 mg/kg poly I:C at E12.5. In their study Saline mice displayed a strong preference for the social chamber. In contrast, poly I:C mice showed decreased preference for the social chamber (Schwartz et al., 2013). However, similar to our results, the preference for the social chamber remained significantly greater than the preference for the non-social chamber in poly I:C mice (Schwartz et al., 2013). In addition, normal repetitive grooming was reported, similar to our findings. However, increased marble burying was reported, that might be indicative of increased stereotypic behavior (Schwartz et al., 2013). It is important to note that the mice were assessed for social interaction at P70, and one week later marble burying tests and repetitive grooming were assessed. In contrast, we assessed social interaction at P45 and repetitive grooming at P30.

Interestingly, prior research using similar dose and time of exposure of poly I:C have found that poly I:C mice show decreased exploration in open-field 10-minutes session as well as reduced preference for the social chamber in three chambers test (Smith et al., 2007). Our results are in line with these findings where poly I:C mice displayed: (i) increased freezing time and decreased locomotor activity in the SHIRPA, (ii) decreased rearing in the cylinder, and (iii) reduced sociability.

The finding that gestational MIA can induce postnatal behavior deficits underlines the roles played by the prenatal environment in shaping brain development and in provoking lasting complex behaviors. Gestation is a critical and sensitive developmental period where environmental insults can alter neuronal migration and maturation through the CNS (Rice and Barone, 2000). The differential effect induced by these environmental insults on the brain, depends on the window of time of exposure. Indeed, it has been previously shown that MIA with poly I:C prompt distinct behaviors in offspring depending on early or late embryonic exposure. For example, poly I:C exposure on E9 produced offspring exhibiting reduced sensorimotor gating, whereas pregnant mice injected with poly I:C on E17 produced offspring displaying impairments in working memory (Meyer et al., 2008). These behavioral changes caused by MIA, early or late in gestation, suggest: (i) the presence of sensitive period of development and (ii) highlight the various behavioral results that can be induced with environmental insults. Poly I:C mouse model was adjusted in severity by modulating the dose of poly I:C used, and the window of time of exposure. For instance, Naviaux et al (2013) used either single (2 mg/kg E12.5) or double (3 mg/kg on E12.5 and 1.5 mg/kg on E17.5) i.p. injections of poly I:C. they reported that the later administration of double injection resulted in reduced fecundity of 40%. However, the single exposure paradigm of poly I:C at E12.5 induced biochemical abnormalities, but less behavioral deficits. The double exposure at E12.5 and E17.5 augmented these outcomes and induced ASD-like behaviors. Poly I:C males showed 54% reduction in social preference and a 28% decrease in sensorimotor coordination as measured by latency to fall on rotarod test. In

another study, pregnant mice were exposed to 5 mg/kg by means of i.v. injection at E9 (Vuillermot et al., 2017). Similar to our results in social interaction, this study demonstrated that poly I:C males show a significant decrease in sociability. Interestingly, and similar to our protocol the behavioral approaches were applied in pre-pubertal offspring (Vuillermot et al., 2017).

It is important to note strains can react differently to poly I:C induced MIA, as normal social behavior was observed in poly I:C mice born to C57BL/6NcrJ dams that were exposed to 20 mg i.p. poly I:C at E12.5 (Ehninger et al., 2012). In our pilot study, we used mice of C57BL/6NRj genetic background and observed deficits in motor coordination in the challenging beam test (data not shown), In contrast to normal motor coordination in poly I:C C57BL/6 mice.

Some of the behavioral and cognitive deficits reported in MIA offspring may also be in relevance to other psychiatric disorders such as schizophrenia. However, the fact that poly I:C provoked reduced sociability at periadolescent to pre-pubertal period, suggests the more translationally relevant age to ASD. In addition, the presence of behavioral deficits usually not associated with schizophrenia such as increased repetitive behavior and reduced exploration suggest that this MIA model may have a better relevance to ASD.

In this study, we have performed a detailed exploration of motor behaviors in male and female offspring prenatally exposed to poly I:C which has not been explored before. Our results demonstrate the presence of early developmental delays and motor abilities. However, normal motor coordination and gait were observed in these mice. Unlike the poly I:C mice, the VPA mice displayed motor coordination deficits and ataxic gait, suggesting that prenatal exposure to different environmental insults yields different postnatal behaviors. This may be in relevance to the heterogeneity of the ASD that comprises spectrum of disorders and severity.

In summary, our results demonstrate that MIA, by exposure to poly I:C on E12.5, induces: (i) early developmental delays, (ii) cognitive deficits, and (iii) reduced sociability in offspring in a sex-dependent manner. These data further designate the need to study how gender

may protect against the effects of MIA in ASD and emphasize the importance of assessing behavioral phenotypes in both sexes in animal models relevant to neurodevelopmental disorders as the ASD.

1.8 Critical discussion of the Poly I:C and VPA models

Mouse pretreatment with prenatal VPA and Poly I:C involves two different mechanisms that contribute to the underlying etiology of ASD in humans. Therefore, studying these two environmental factors has an advantage of achieving a better understanding of the pathophysiology of ASD and the motor and non-motor behaviors associated to the disorder.

The behavioral characterization of both prenatal exposure to VPA and MIA-inducing poly I:C also served the goal of inducing robust and various changes, which is valuable in understanding phenotypically heterogenic disorders such as ASD. Indeed, much of the research published in animal models of ASD propose single model characterization approaches, often only in males, which leads to a “superficiality” in addressing ASD that is associated with complex behaviors. Thus, we suggest that designing multi-model studies in ASD research may be valuable approach that requires vast exploration of several environmental (and or genetic) factors that are most commonly associated with the etiology of ASD in humans. While our outcomes may argue for the added value of the two-model approach, our work also exposed some limitations. An important factor that has to be addressed critically is the sample size needed to sufficiently power the statistical analysis. This in turn is dependent on the magnitude of the deleterious effects generated by the VPA and Poly I:C treatments. For instance, it has been previously reported that Poly I:C treated females commonly deliver reduced litter size (Naviaux et al., 2013), and in some cases strong sickness provokes fetal resorption and abortion. Others reported the death of pregnant dams due to VPA treatment (Baronio et al., 2015). Here we report that VPA treatment induced 25% frank abortion and 36.3% fetal resorption (Figure 33). These outcomes are in line with negative pregnancy effects observed in the clinical settings following

VPA therapy in pregnancy. Abortion rate, as high as 25%, has been reported following a VPA monotherapy (Pittschieler et al., 2008). Furthermore, fetal malformations due to VPA in utero exposure were observed at a high rate of 16% in comparison to lower rates (2.38%) with other antiepileptic treatments (Vajda et al., 2004).

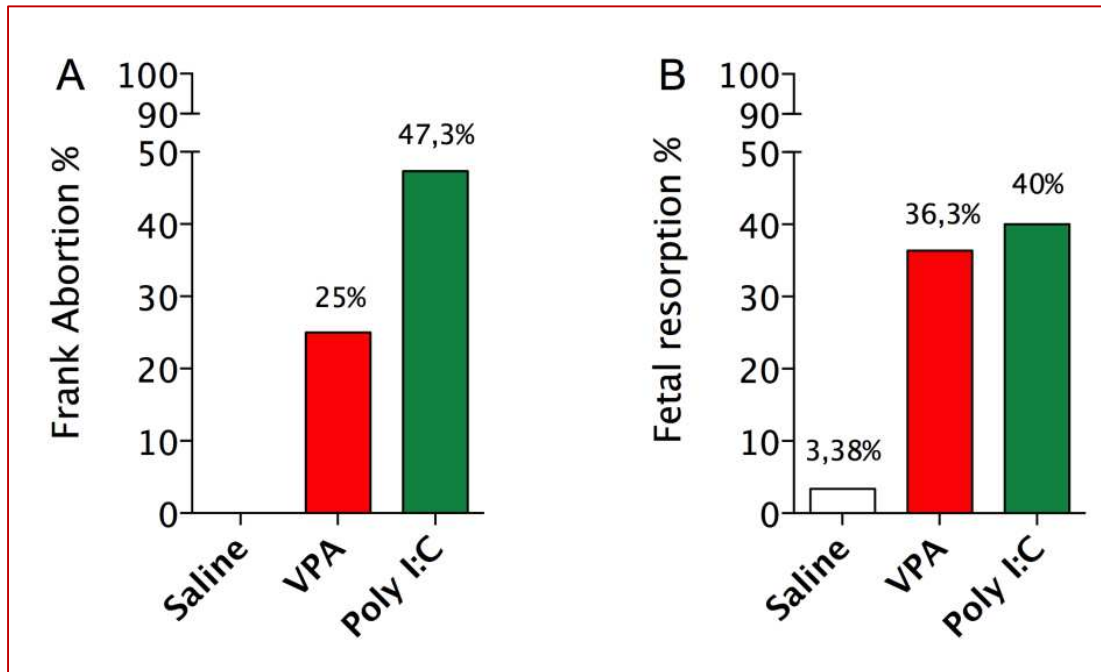


Figure 33: Effects of VPA and Poly I:C treatments on embryonic development.

(A) Percentage of frank abortions. (B) Percentage of fetal resorption.

Poly I:C induced higher rates of abortion (47.3%), and 40% fetal resorption. Indeed, routine study of neurodevelopmental models implies designing studies where the generation of a lot of pregnant females is a must to empower the study with a desired count of offspring. Required sample sizes are expected to be high due to a relatively low effect size of such models. This underscores the heavy effect induced by prenatal exposure that should be critically discussed from the animal welfare perspective. To avoid huge loss of animals, one could use animals with a genetic predisposition to ASD that can enhance certain aspects of poly I:C and VPA treatment. In addition, one can also manage to test the effect of the prenatal treatment on different lineage of mice; this is highly recommended as we have previously observed lower abortion rate due to poly I:C treatments when C57BL/6NRj – (JANVIER LABS- France) were used (Data not shown- pilot study).

Aiming to trace the developmental trajectories in VPA and poly I:C mouse models of ASD, we focused on longitudinal aspects of the models; this has in turn served the goal of increasing the models' robustness. On one hand, developmental animal models necessitate functional assessments to be made recurrently and at different time points through development in order to inspect the trajectories of the pathology and to unpin and propose novel therapeutic interventions. This discussion underscores the need for behavioral tests that are reproducible, accurate, and do not impose practice effects. This argument is essential when the behaviors of interest are clustered to "motor behaviors" of motor coordination and gait. An example of such a test is the challenging beam test, as demonstrated in this thesis, as it enables inspecting motor coordination in freely walking mice without imposing a rhythm or training effect as it is the case with the rotarod, the commonly used instrument in assessing motor coordination. On the other hand, and to avoid any potential biases, the following approaches were applied:

- Littermate effect was avoided and thus one pup was selected, at P21, from each litter and mixed to 3 other pups (matched by sex and treatment).
- Different apparatus, when applicable, were employed in testing behavior of males and females; if not males were tested first and then females were tested.
- Test apparatus were cleaned between cages.
- Test apparatus were washed with detergent and then cleaned with 70% ethanol at the end of the testing day.
- Mice were habituated to the testing room at least one hour before the behavioral tests.
- Mice were handled starting early postnatal life.
- Ear tagging, tail tattoos were avoided; instead tail color were applied to identify the mice.
- The experimenter was blind to the treatment.
- The experimenter changed the cages.
- The behavioral tests were applied in the least stressing order.
- The 3 chambers test was employed at the end of the battery.
- The testing environment was not stressing.
- Behavioral tests were replicated 3 times and applied to the different treatments on the same time points.

These are important practices that enable a "truly" prospective study. We have applied correlation analyses, to parallel behavioral deficits to anatomical cell loss affected by the environmental insults. The observed behavioral abnormalities resonate well with findings obtained in our group showing decreased cell counts in sob-lobules of the cerebellum. This is the first study showing, that developmental milestones, sociability, motor coordination, and gait

deficits were strongly correlated to reduced cell counts in Crus-I. In addition, motor coordination and gait impairments were correlated to the reduced social interaction in VPA males (Table XIII; Annex 1).

Exposure to prior developmental insults during early embryonic life induce a time-course progressing “disease-state” in animals’ offspring. While long durations between sickness-stimulating environmental insults and the behavioral assessments are likely to impose negative influence on the robustness of the neurodevelopmental animal model, this time window is very valuable as it allows the generation of novel therapeutic approaches. For instance, in adolescence, vast changes in the brain systems characterize this developmental stage (Brenhouse and Andersen 2011). Thus, one might propose that intervention before this stage can be of interest as it might stop further development of neurodevelopmental disorders and reduce associated behavioral deficits observed at later stages. Although, we are still far from a rigid framework of early diagnosis of ASD, there is an urging need to develop interventions for patients at risk or in the “prodromal phase” of ASD. To this regard, our study in environmental animal models of ASD provide initial proof for the possibility to earlier “discovery” of ASD by focusing on motor behaviors. Hopefully, this might pave the road to better “prevent” the emergence of the spectrum of behavioral deficits observed in ASD.

According to our findings, both the VPA and the Poly I:C mouse models of ASD are appropriate to study motor behavior deficits in ASD. These developmental models have been used in the “cognitive approach” of studying ASD and are shown here to be beneficial in understanding the impact of environmental factors on “motor signatures” implicated in the pathophysiology of ASD. This approach would enrich the research of early behavioral markers and the development of novel therapeutic approaches.

Chapter 2: Perspective

The etiology of ASD still remains not well identified, however a number of specific genetic and environmental factors have been associated with the risk of developing the disorder. The core features of ASD include delayed and disturbed language skills, impaired social interaction, restricted interests, and an insistence on sameness. Individuals with ASD also exhibit fine and gross motor skills dysfunctions. In that regard, the cerebellum and connected brain areas including the prefrontal cortex, and striatum have been consistently reported to show abnormal structure in ASD, and may be associated with motor skills dysfunctions observed in this disease. For instance, the majority of postmortem studies on individuals diagnosed with ASD, regardless of age, sex, and cognitive ability, have reported a significant decrease in the number of PC in the cerebellum. There is a need to better understand the motor phenotype present in patients with ASD. Which motor abnormalities can be predictive of social difficulties? How loss of PC occurs, and what are the behavioral and cellular consequences of this loss? What are the genes and environmental factors that can alter the symptoms? Can we compensate for PC cell loss and will that have an effect on behavior? Answering these questions, we will be able to identify and develop new diagnostic and therapeutic strategies in ASD.

2.1 Therapeutic interventions in ASD

There is no current consensus on ASD treatment, except in rare cases where autism results from an identified pathology (eg, metabolic disease, cerebellar lesion...). After psychoanalysis or psychotherapy, researchers turned to drug trials targeting ASD behaviors. Although it is possible to alleviate some of the symptoms, for example by improving behavior, or by treating the associated syndromes, there are no specific medications for ASD. In addition, and under the influence of parents, educational methods have finally appeared. These include: (i) Sensory Integration Therapy (Ayres, 1972), (ii) Therapy of Exchange and Development (Thérapie d'Échange et de Développement: TED (Bonnet-Brilhault and Barthélémy, 2012; Barthélémy, et al., 1995; Lelord et al., 1978), and (iii) Cognitive-behavioral therapies CBT (Myers and Johnson, 2007).

2.2 Non-invasive Brain stimulation

Encouraging outcomes of non-invasive brain stimulation (NIBS) in adult psychiatric disorders are appealing for the application of transcranial stimulations techniques in children. In transcranial magnetic stimulation (TMS), electrical currents are conducted to the cortex by extra-cranial magnetic fields source. However, in transcranial direct current stimulation (tDCS) electrical currents are induced constantly by contact electrodes that are attached to the head. Being tolerated by adults, adolescents and children, both tools have the ability to adjust local excitability in cortex (Palm et al., 2016; Rajapakse and kitron, 2013). TMS has investigational, analytic and therapeutic potential. This technique offers the experimenter the ability to either quantify or regulate cortical excitability. This is highlighted by the fact that TMS has gained the FDA approval for motor and language mapping (Eldaief et al., 2013), and treatment of major depression (Eldaief et al., 2013), pain (Klein et al., 2015) and migraine (Schwedt and Vargas, 2015). TMS is a valuable tool that is rapidly gaining major interest, in clinical conditions, for its efficiency and safety. Repetitive transcranial magnetic stimulation (rTMS) is a very efficient technique that can be applied, on children, to either enhance or depress the excitability of deficient localized networks (Rajapakse and kitron, 2013; Frye et al., 2008). Thus, cortical excitability may be manipulated by chains of TMS or rTMS. rTMS modulates cortical excitability, by altering GABAergic activity and induction of LTP or LTD-like changes in excitatory synapse, according to the protocol used (usually protocols differ by altering location of stimulation and its frequency and intensity) (Oberman et al., 2016; Trippe et al., 2009; Ahmed et al., 2006). Although to date none of the tDCS instruments has yet been approved by the FDA, the devices are undergoing examination for possible satisfying applications that can be applied in various psychiatric disorders (Palm et al., 2016). Of major interest, many studies have targeted, by rTMS, different brain regions in patients with ASD. It has been lately shown that 6 sessions of low frequency rTMS resulted in suppressing the cortical excitability when applied over the left dorsolateral prefrontal cortex (L-DLPFC), as well as reducing: (i) repetitive behaviors (checked by RBR-R); (ii) Aberrant Behavior Checklist (ABC); (iii) autonomic

measures (Oberman et al., 2016). In another controlled, randomized, and partial cross over trial, 24 ASD children received twenty sessions of Non-invasive brain stimulation (NIBS) over the L-DLPFC (Gómez et al., 2017). Children aged 11 to 13 received rTMS, while tDCS was used with patients aged less than 11. Interestingly, a significant decrease in the ABC, Autism Treatment Evaluation Checklist (ATEC), and the Autism Diagnostic Interview-Revised (ADI-R) was observed one month after the treatment and persisted six months.

Cerebellar Transcranial Direct Current Stimulation (ctDCS) have been recently developed to manipulate the cerebellar excitability. For instance, this technique is used to assess connectivity measures between the cerebellum and the primary motor cortex. Importantly ctDCS can ameliorate motor and cognitive behavior as reported in studies of processing emotional facial expression (Ferrucci et al., 2012) and cerebellar ataxia (Grimaldi and Manto, 2013).

In conclusion, the field of NIBS is gaining more attention due to its efficiency and safety. The flexibility of protocols used upon application makes this technique a promising therapeutic approach, especially for motor troubles in ASD, to disorders lacking other treatment options. While significant improvement has been achieved in the search for non-invasive stimulation treatments, intensive early behavioral interventions seem to be effective for some children with ASD (Warren et al., 2011). The Agency for Health Care Research and Quality (US department of Health & Human Services) evaluated the data supporting both applied behavior analysis and the Denver Model, and concluded that "... improvements occur in some aspects of language, cognitive ability, adaptive behavior, challenging behaviors, and potentially, educational attainment, for some children" (Warren et al., 2011). The fact that these approaches are: (i) efficient on a group of ASD children, and (ii) efficient only when applied early, shed the light on other cost-effective therapeutic approaches. The enriched environment stands out as an efficient low-cost ASD management tool, ameliorating ASD symptoms, and promising to be effective at a wider age window.

2.3 Enriched Environment

After its beneficial effect in animal models of ASD, EE was suggested as a probable treatment for children with ASD (Schneider et al., 2006; Schneider and Prezewlocki, 2005). In this section, we will discuss the enriched environment EE clinical trials in children with ASD.

Fascinated by the animal studies results and expecting that sensorimotor enrichment will enhance the behavioral functions in ASD children, the group of *Michael Leon* (University of California Irvine- the Center for Autism Research and Treatment) translated the EE approach applied in animal models to ASD children. In a randomized controlled trial, 28 male children with autism were assigned to either sensorimotor enrichment group or to standard care group (Woo and Leon, 2013). The EE group were exposed daily to different sensorimotor stimulations, regularly throughout the day. These sensorimotor enrichment exercises involved different aspects of cognitive and motor stimulations; with increasing challenges during the course of the 6 months the EE exercises and that involved motor, balance, tactile, auditory, visual, thermal, language, and cognitive activities. As determined by the Childhood Autism Rating Scale (CARS), 42% of the EE group and 7% of standard care group showed an improvement of 5 points on that scale. Maladaptive behavior ameliorated in the EE group especially in behavioral responses examined in this scale (emotional response, body use, general impressions, adaptation to change, etc...). In addition, EE resulted in enhancements in cognitive domains in autism children when assessed by the Leiter score (Leiter international Performance Scale-revised). In an attempt to replicate the results and extend the conclusion of the previously mentioned study, the group of Michael Leon studied the effect of sensorimotor enrichment in a second cohort of children with ASD by applying objective assessment of the symptoms (Woo et al., 2015). After six months, the 28 ASD-children exposed to sensorimotor enrichment displayed enhancements in their: (i) IQ scores, (ii) a significant decrease in atypical sensory response, and (iii) improvements in receptive language performances. Furthermore, 21% of enriched children who were classified with autism, using the ADOS scale, no longer met the criteria for classic autism (Woo et al., 2015). Therefore, EE by means of sensorimotor enrichment, seems to stand out in

the treatment of children with ASD. The approach is cost-effective and efficient in treating a wide range of symptoms by engaging ASD children, on a daily basis, to an umbrella of sensorimotor enrichment exercises. It is important to determine whether the effects of EE are long lasting and if continued therapy can offer any progress in the management of the symptoms. It is also of interest to determine if this approach can ameliorate gait and motor coordination in ASD- children of both sexes. Finally, it is also important to understand how EE changes plasticity and how EE beneficial effects are obtained.

2.4 The translation of data to clinical research

The goal of a work such as the one presented here is to be able to bring data that may be of relevance to the clinical field, especially for the purpose of earlier and better management of ASD. The first difficulty in addressing data portability concerns the comparability of rodent prenatal and neonatal brain development with humans. Rodents are the species most commonly used in experimental models of neurodevelopment disorders (Romijn et al.,1991). However, there are recognized differences in cerebral maturation of rodents and humans. By determining points of equivalence in the development of the brain, we will be able to translate into clinical settings the experimental data obtained in animal studies. Rodents are born at a relatively early stage of maturation, making it very useful for studying the direct effects of early experimental manipulations on cerebral development (Pressler and Auvin, 2013). Different criteria are used to compare the degree of cerebral maturation between the two species, such as, cerebral growth, neurogenesis, synaptogenesis. For example, neural tube closes in mouse at 12 days of gestation which corresponds to that of 4 to 5 weeks of gestation in humans (Bayer et al., 1993). Thus, early environmental insults at this very specific period of time seem to have a direct effect on the development of the nervous system.

Other parameters are to be considered such as functional measures of postnatal development, as is the case for example for the acquisition of the walk. Rat locomotion skills develop during the first few weeks of life. The rat pup begins to be able to crawl through a traction of its front

which allows it to raise its torso and its head starting the 3rd or 4th day of life, with a peak towards the 7th day. It is only from days 8-10 that the rat pup is able to completely clear its abdomen from the ground, and around the days P12-13, that the rat will be able to walk with all its weight on its limbs so that finally the action of crawling disappears completely in favor of walking towards the 15th day of life (Wood et al., 2003). Locomotion abilities develop in mice around P7 as well, that can walk before they even have their eyes open. The acquisition of walking in children develops at a much later period, around the age of 13 months. A child is able to support his weight standing on his feet around the age of 24-28 weeks if someone holds his hands, and can move, standing on furniture for example at about 48 weeks (Wood et al., 2003). Using these types of criteria, and many others as acquisition of reflexes, it is now commonly accepted that the brain development of a rat pup at P12 corresponds approximately to that of a human newborn child (Romijn et al., 1991). In the case of our experiments with VPA and Poly I:C, at E=12.5, this would correspond to an exposure of the first trimester of pregnancy in humans. All these points of discussion highlight the need of the reproducibility of the data from one study to another, in particular in the field of neurodevelopment and behavior. It is important to develop a discussion of good laboratory practice in the study of animal behavioral models. It would also be interesting to have more detailed descriptions of the tests and standardized protocols set up in the studies (habituation, lighting, frequency of animal handling) in order to be aware of the limitations of each study. In addition, it is essential to always adapt the analysis of the data to the nature of the pathology studied. Indeed, all studies employing behavioral tests do not have the same goal nor do they concern the same pathologies, therefore, a test is validated in a field of study, its interpretation or analysis will not necessarily be valid in another field. In addition, the absence of standard data and protocols on habituation periods, test age, housing conditions, and test illumination conditions can have a very strong influence on the data and limit their reproducibility. However, in the field of science, the reproducibility of the results is indispensable for obtaining scientific consensus. A scientific consensus is reached when we have

the necessary level of evidence, and this level of evidence will be achieved through reproducibility of the results. Only once this scientific consensus has been reached, can adequate translational measures be taken.

The work presented here is a part of a major work including a cohort of clinical study in children with ASD (Figure 34). The aim of the project is to associate animal experiments and patient research in order to characterize the motor behavior, which has not been done much before, and to determine the neural networks involved in this disorder at the anatomical, cellular, molecular and neurochemical levels with particular attention to motor structures such as cerebellum, striatum and motor cortex. We hypothesize that this approach can open a new way in the early and quantitative diagnosis of these disorders, potentially identifying the common neural networks that underlie the cognitive and motor impairments characteristic of autism and opening up new therapeutic pathways targeting specifically the cerebellum. The specific aims of the team's general project are: (i) to characterize motor dysfunctions in a cohort of patients with ASD, (ii) to determine which motor abnormalities can be predictive of social difficulties, (iii) to decipher physiological disturbances underlying motor symptoms in animal models of ASD. To achieve these aims some tasks needs to be addressed. The main task of this project, that is to characterize the motor dysfunction in ASD patients following social and diagnostic criteria application. To assess different motor components related to the cerebellum, isolated sub-tasks will be performed to evaluate: (i) integration of sensorial information, (ii) programing of motor sequence, (iii) psychomotor function evaluation (Gross motor; balance; rhythm following; manual abilities), and (iv) kinematics movement analysis. The study will propose new methodology of evaluation in order to better characterize and identify components of motor phenotypes disrupted at early stages of ASD.

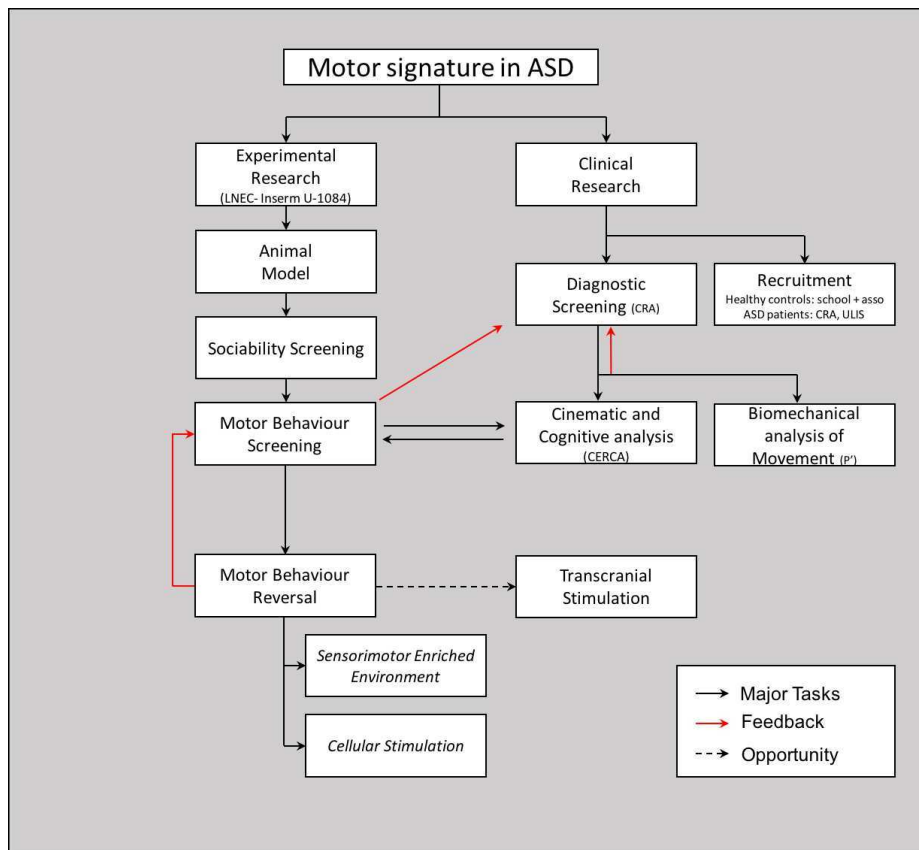


Figure 34: The bigger project

This project is in collaboration with researchers and institutions from different backgrounds and cultures but perfectly complementary in a unique translational approach that addresses the ASD in these mechanistic aspects:

- In mechanical physics (PP '': Institute for research and engineering for transport and the environment, CNRS UPR 3346).
- In cognitive sciences (Center for research of cognition and learning, CERCA, CNRS UMR 7295).
- In activity and research Clinic (Autism Resource Center Vienne of the psychiatric hospital of Poitiers).
- In neurosciences (Laboratory of Experimental and Clinical Neurosciences, Inserm UMR_S 1084).
- Associations of patients such as Association Autisme Vienne.

2.5 Implications of this work in the understanding of ASD

As presented previously, it is important to bear in mind that ASD have a very high phenotypic variability of symptoms and severity which is surely indicative of a great etiological variability since it is not a unique pathology but a spectrum. From one end of the autistic spectrum to the other, it is therefore probable that there is also an etiological spectrum where

different factors have to be combined to obtain a multitude of possible combinations: environmental factors, windows of impaired developmental susceptibility, the genetic susceptibilities, the mechanisms involved. This work made it possible to highlight different environmental exposures in utero which led to the development of autistic traits in mice, with particular characteristics specific to each model and sex. ***Our results are consistent with a number of scientific (epidemiological and preclinical) evidence. This supports a number of biologically plausible etiological hypotheses and reinforces scientific advocacy to address the etiologic research of ASD by approaching different categories of behaviors.***

This project contributes to the identification of ***motor behavior*** induced by environmental risk factors in the development of ASD and opens up prospects for significant societal benefits. ***By supporting a level of mainly epidemiological scientific evidence, we bring for the first-time experimental data that support the presence of early motor endophenotypes often under seen in the diagnosis of ASD.*** The causal links being verified and admitted by scientific consensus, this project may help in influencing future research initiatives and hopefully public health policies aimed at early diagnosis of ASD and introducing new therapeutic avenues.

ANNEX 1

		DVPMT		MOTOR											SOCIAL	HISTO		
		OE	RR	TR	LA	WM	NG1	NG2	EPS	SL	LPG	RPG	LLBS	RLBS	SI	Crus I	Crus II	M1/M2
DVPMT	OE	coeff. .414	.596**	-.530	.066	.148	.468	.370	-.167	.456	.289	.179	.350	-.474	-.535	.269	-.649**	
		p = .070	.006	.016	.781	.534	.037	.108	.482	.044	.216	.450	.131	.197	.018	.266	.002	
	RR	.143		.643**	-.586**	-.212	.042	.423	.340	-.030	.270	.624**	.152	.406	-.840**	-.254	-.144	-.569**
		.558		.002	.007	.368	.859	.063	.143	.901	.249	.003	.522	.076	.005	.294	.556	.009
MOTOR	TR	.321	.357		-.776**	.217	.209	.685**	.713**	-.285	.480**	.401	.566**	.386	-.851**	-.602**	-.247	-.634**
		.181	.134		.000	.358	.377	.001	.000	.223	.032	.080	.009	.093	.004	.006	.308	.003
	LA	-.068	.050	.040		-.312	-.125	-.795**	-.521	.050	-.560	-.555	-.394	-.487	.690	.444	.189	.468
		.781	.837	.871		.180	.600	.000	.018	.834	.010	.011	.086	.029	.040	.057	.438	.038
	WM	.257	.270	.020	-.482		-.384	.207	-.145	.219	.233	.173	.392	.001	-.397	-.020	.044	-.021
		.289	.263	.937	.036		.095	.381	.543	.353	.322	.465	.088	.997	.290	.937	.858	.930
	NG1	-.250	.379	-.167	-.421	.533		.288	.617**	-.315	-.356	-.392	.106	-.077	-.724	-.241	.102	.025
		.302	.109	.495	.073	.019		.218	.004	.176	.123	.087	.657	.746	.027	.320	.678	.916
	NG2	.021	.179	-.148	-.362	.738**	.777**		.635	-.337	.411	.342	.469	.489	-.705	-.635	-.120	-.542
		.932	.464	.546	.127	.000	.000		.003	.147	.072	.141	.037	.029	.034	.004	.623	.013
	EPS	-.307	.336	.344	.219	-.223	-.006	-.022		-.566**	.158	.046	.388	.150	-.902	-.704	-.218	-.339
		.202	.160	.149	.367	.359	.980	.930		.009	.506	.846	.091	.529	.001	.001	.370	.144
	SL	-.077	-.327	-.124	-.200	-.184	.052	-.264	-.127		-.060	.347	-.296	-.270	.368	.847**	.044	.264
		.754	.172	.613	.412	.450	.832	.275	.605		.803	.134	.206	.250	.330	.000	.858	.261
	LPG	-.125	.042	.510	.041	-.044	-.022	-.237	.161	.186		.480	.178	.523	-.590	-.340	-.123	-.534
		.611	.863	.026	.866	.857	.930	.328	.511	.445		.032	.453	.018	.094	.154	.616	.015
RPG	-.012	.300	.296	-.090	.322	.329	.412	.443	-.187	.274		.076	.279	-.697	-.002	-.246	-.470	
	.962	.212	.219	.713	.179	.169	.080	.058	.444	.255		.752	.234	.037	.993	.311	.037	
LLBS	-.184	.362	-.007	-.064	.430	.167	.197	.191	-.072	-.025	.349		.017	-.217	-.427	.022	-.278	
	.450	.128	.977	.796	.066	.495	.418	.434	.769	.918	.144		.943	.575	.068	.927	.235	
RLBS	-.028	.361	.063	.102	.310	.531	.591**	.177	-.286	-.109	.482	-.012		-.515	-.489	-.305	-.420	
	.908	.128	.798	.679	.196	.019	.008	.468	.235	.657	.036	.960		.156	.034	.205	.066	
SOCIAL	SI	.341	.868**	.293	-.585	.759	.627	.920**	.178	-.073	.171	.465	.487	.011		.681	-.219	.708
		.409	.005	.482	.128	.029	.096	.001	.673	.686	.245	.221	.980		.044	.570	.033	
HISTO	Crus I	-.249	-.557	-.138	-.120	-.110	-.028	-.179	-.295	.510	.268	-.064	-.346	.062	-.849**		.021	.443
		.319	.016	.585	.635	.664	.912	.478	.234	.031	.281	.802	.160	.806	.008		.935	.057
	Crus II	-.191	-.317	-.325	.228	-.484	-.050	-.425	-.380	.554	.089	-.374	-.411	-.133	-.397	.455		.067
		.448	.200	.188	.363	.042	.843	.079	.120	.017	.724	.126	.090	.600	.331	.058		.785
M1/M2	-.162	.444	.202	.076	-.137	-.149	-.264	.585**	-.187	.232	.168	.185	-.126	.558	-.467	-.235		
		.508	.057	.408	.758	.577	.542	.276	.009	.444	.340	.493	.449	.607	.151	.051	.348	

M A L E S

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Table XIII: correlation between behavioral deficits and anatomical changes.

Developmental milestones: OE= open eyes; RR= mean of time of the righting reflex on P9 and P11; **Motor parameters:** TR= transfer reaction (time of immobility); LA= locomotor activity (11x11 cm crossed in SHIRPA); WM= wire maneuver time to climb; NG1= negative geotaxis time to turn on the grid; NG2: time to climb the grid; EPS= errors per step made in the challenging beam test; SL= stride length; LPG= left pair gap; RPG= right pair gap; LLBS= left limbs base of support; RLBS= right limbs base of support; **Sociability:** SI= social index: calculated from the three-chambers test; **Anatomical findings:** Crus I, CrusII: PC counts in each sub-lobule of the cerebellum; M1/M2: number of neurons in the motor cortex. Blue filled cells present significant correlations in VPA males, and pink in VPA females. Strong correlations in the developmental milestones, motor coordination, gait and social interaction deficits and neuronal loss in males. *: p<0.05, **: p<0.01; Coeff: correlation coefficient r. Pearson's correlation.

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