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Présentée par :

Alexandre Maïsterrena
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Modélisation environnementale et génétique des troubles du spectre autistique : aspects comportementaux et moléculaires
Environmental and genetic modelization of Autism Spectrum Disorder: behavioural and molecular aspects

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Directeur de Thèse :
Mohamed Jaber
****

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****

JURY

Pr Jean-René Cazalets	INCIA – UMR 5287 Université de Bordeaux	Rapporteur
Pr Bruno Giros	CNRS UMR 8002 Université de Paris Descartes	Rapporteur
Dr Inserm Clément Lena	CNRS UMS 8197 Ecole Normale Supérieure	Examinateur
Pr Jean Xavier	CNRS UMR 6295 Université de Poitiers	Examinateur
Pr Mohamed Jaber	Inserm UMR-S 1084 Université de Poitiers	Directeur de thèse

Résumé

Les troubles du spectre autistique (TSA) sont un ensemble de troubles neurodéveloppementaux caractérisés par deux symptômes principaux : des déficits d'interaction sociale et descomportements stéréotypés et répétitifs. Les TSA ont un impact sociétal important avec une prévalence d'un enfant sur 100. Il n'existe aujourd'hui aucun traitement curatif des TSA, leur prise en charge passant principalement par des thérapies comportementales. Ces thérapies sont plus efficaces si implémentées tôt chez l'enfant. Or, le diagnostic des TSA est difficile et long, reposantprincipalement sur une évaluation des capacités sociales conduisant à une grande variabilité de laprévalence selon les méthodes diagnostics utilisés. Ainsi, le premier objectif de ma thèse aété d'étudier la présence de troubles moteurs et de la marche dans un modèle génétique avec une mutation du gène shank3 (SH3) des TSA et d'explorer les mécanismes cellulaires et moléculaires sous-jacents. Ceci est d'intérêt afin de proposer d'implémenter en clinique ces mesures pour contribuer à un diagnostic objectif et précoce.

La relative simplicité des techniques utilisées actuellement pour caractériser les interactions sociales chez les modèles animaux de TSA ne permet pas une étude prolongée dans le temps de ces déficits ni de déterminer leur nature dans un groupe d'animaux. Ainsi, le second objectif de ma thèse a été la mise en place d'une nouvelle méthode d'étude des interactions sociales, le Live Mouse Tracker (LMT), pour déterminer le comportement social dans un groupe de 4 animaux surune durée pouvant aller jusqu'à 3 jours. Ceci a été réalisé dans modèle des TSA éprouvé, celui de préexposition prénatale à l'acide valproïque (Dépakine) (VPA). Avec cette procédure, j'ai pu montrer la présence de déficits sociaux chez les femelles VPA qui n'étaient pas détectables auparavant avec la technique du test à 3 chambres (3-CT). J'ai aussi pu caractériser de manière détaillée ces troubles en montrant que les souris VPA présentent principalement des difficultés à établir et renforcer les contacts sociaux, mais une fois que ceux-ci ont été mis en place, les souris VPA présentent des interactions sociales similaires aux souris contrôles. De plus, j'ai pu montrer que la présence de souris contrôles avaient un impact positif sur les capacités sociales des souris VPA, mais que la présence de souris VPA avait-elle un impact négatif sur les capacités sociales des souris contrôles.

Enfin, j'ai contribué à un travail visant à déterminer l'état de la transmission glutamatergique et dopaminergique dans ce modèle VPA de TSA. Ce faisant, j'ai pu montrer que ces deux systèmes majeurs de transmission étaient faiblement impactés par la pathologie.

En conclusion, au cours de ma thèse, j'ai pu dans un premier temps montrer la présence de troubles significatifs moteurs et de la marche dans un modèle génétique des TSA. Puis dans un second temps, j'ai caractérisé le profil social détaillé d'un modèle environnemental des TSA, ainsi que mis en évidence l'importance de l'environnement social sur les capacités sociales des souris.

Summary

Autism spectrum Disorder (ASD) is a group of neurodevelopmental disorders defined by two main symptoms: deficits in social interaction and stereotyped and repetitive behaviour. ASD has an important impact on society due to its prevalence of around 1 for every 100 children. To date thereare no curative treatment for ASD. Patient care for ASD revolves around behavioural therapy forthe main symptoms. These approaches are most effective when implemented early in childhood. ASD is currently diagnosed based mainly on the evaluation of the social capacities of the child leading to a great variability of prevalence depending on the methods used. Thus, the first aim of my thesis was to study the presence of motor and gait disorders in a genetic model of ASD with amutation of the shank3 (SH3) gene and to explore potential underlying mechanisms. The goal wasto determine whether motor and gait deficits are of relevance to be implemented in clinical settingsas early and quantitative diagnostic criteria.

The relative simplicity of the methods currently used to study social interactions in animal models of ASD does not allow long term investigations or study of complex interactions in a group of animals. Thus, the second aim of my thesis was the implementation of a new method to study social interactions, the Live Mouse Tracker (LMT). The LMT allows the study of complex social interactions in a group of 4 mice for a duration up to 3 days. This study was performed using a robust model of ASD, the valproic acid (VPA) model based on the prenatal exposition to the drug. With this procedure, I was able to detectsocial deficits in females VPA mice which were not detectable previously with the 3-CT technique. I was also able to characterise in a detailed mannerthose deficits, showing that VPA mice mainly have difficulty to establish and reinforce social interactions but that once those were in place, socialinteractions became similar to control mice. Finally, by using groups of mice with mixed treatment within the LMT, I was able to prove that on one hand the presence of control mice had a positive impacton the social capacity of the VPAmice, but on another hand the presence of VPA mice had a negative impact on the social capacityof the control mice.

Last, I also contributed to a work aimed at determining the state of glutamatergic and dopaminergic transmission in this VPA model of ASD. In doing so, I was able to show that these two major transmission systems were weakly impacted by the pathology.

In conclusion, during my thesis, I was first able to show the presence of significative motor and gait deficits in a genetic model of ASD. Then I was able to create a detailed social profile of an environmental model of ASD, which revealed the importance of the social environment on the social capacity of the mice.

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Liste des abréviations

0-9

3-CT : Three Chamber Test

A-B

AC5 : Adenylate Cyclase 5
AMPAR : Récepteur glutamatergique AMPA
APA : American Pyschiatric Association
BPD : Bipolar Disorder
BTBR : Black and Tan Brachyury

C

CAMKIIα : Calmoduline-Dépendantes Kinases
CIM-10/CIM-11 : Classification internationale des maladies 10ème édition/11ème édition
CNTNAP2 : Protéine associées aux contactines 2
CNV : Copy number variation
CPU : Caudate Putamen (striatum)

D

D1/D2 : Dopamine receptor 1/2
DA : Dopamine
DAT : Dopamine Transporter
DHPG : 3,5-dihydroxyphénylglycine
DI : Déficience Intellectuelle
DSM-5 : Diagnostical and statistical Manual 5ème édition
DOPAC : 3,4-Dihydroxyphenylacetic acid

E-F

E12 .5 : Embryonic day 12.5
EAAT1/EAAT2 : Excitatory Amino Acid Transporter 1/2
ERK1/ERK2 : Extracellular signal-regulated Kinase 1/2
FMR1 : Fragile X Messenger Ribonucleoprotéine 1

G

GABA : Acide Gamma Amino Butyrique
GABA-A/GABA-B : récepteur Gabaergique de type A/B
GAD65/ GAD 67 : Glutamate Decarboxylase 65 /67

H-I

HDAC : Histones Déacétylase
HOMER1 : Homer protein homolog 1
HPLC : High Precision Liquid Chromatography
Il-6 : Interleukine 6

K-L

KO : Knock-Out
LMT : Live Mouse Tracker
LTD : Long-Term Depression

M

mGluR5 : Récepteur métabotropique au glutamate de type 5
MIA : Maternal Immune Activation
mIPSC : Miniature Inhibitory Post-Synaptic Current
MSN : Medium Spiny Neurons

N

Nac : Nucleus Accumbens
Nlgn1/Nlgn3 : Neuroligine 1/3
NMDAR : N-Méthyl-D-Aspartate receptor
NST : Noyaux Sous-Thalamique

O-P

OMS : Organisation Mondiale de la Santé
PC : Purkinje Cells
Poly I :C : Acide polyinosinique-polycytidyllique
PSD : Post-synaptic Density

R-S

RFID : Radio Frequency Identification
SCZ : Schizophrénie
SH2/SH3 : Shank2/3
sIPSC : Spontaneous Inhibitory Post-Synaptic Current
SNC : Substancia nigra pars compacta

T

TDAH : Trouble du Déficit de l'Attention avec ou sans Hyperactivité
TEACCH : Treatment and Education of Autistic and related Communication Handicapped Children
TH : Tyrosine Hydroxylase
TLR-3 : Toll-Like receptor 3
TRIF : TIR-domain-containing adapter-inducing interferon-B
TSA : Troubles du Spectre Autistique

V

VBS : Visible Burrow system
Vglut1 : Vesicular glutamate Transporter 1
VTA : Aire Tegmentale Ventrale
VPA : Acide Valproïque

Introduction

Chapitre 1 : Généralités sur les troubles du spectre autistique (TSA)

1.1 Définition

Les Troubles du Spectre Autistique (TSA) sont des troubles neurodéveloppementaux caractérisés par des déficits des capacités sociales et des comportements répétitifs avec des intérêts restreints (American Psychiatric Association & American Psychiatric Association, 2013). Les symptômes apparaissent durant la très jeune enfance, avant trois ans, mais de par la difficulté à poser un diagnostic à un âge précoce, les TSA sont généralement détectés plus tard (American Psychiatric Association & American Psychiatric Association, 2013).

Les TSA ont été décrits au milieu des années 40 par Kanner (1943) et Asperger (1944) de manière quasiment concomitante mais indépendante. Aujourd'hui encore l'étiologie des TSA reste mal comprise du fait de la complexité des atteintes physiopathologiques. Néanmoins, des facteurs de risques génétiques et environnementaux ont pu être isolés (Chaste & Leboyer, 2012).

Il n'existe pas de traitement curatif des TSA. La prise en charge à ce jour se contente d'atténuer les effets des symptômes principaux avec une thérapie comportementale et de traiter avec une thérapie pharmacologique les comorbidités associées (troubles de sommeil, anxiété, dépression etc.) quand cela est possible. Il est intéressant de noter que les thérapies comportementales, sont plus efficaces si elles débutent tôt dans le parcours du patient.

Dans cette première partie, je décrirai de manière générale les TSA avec les symptômes et comorbidités associés, puis j'aborderai les données épidémiologiques des TSA avec l'impact de ces troubles sur l'individu et la société. Enfin, je m'intéresserai à l'étiologie de la maladie avec les facteurs de risques associés et à sa physiopathologie.

1.2 Symptômes des TSA

1.2.1 Troubles de l'interaction sociale, comportements stéréotypés et répétitifs

Pendant longtemps, différentes nomenclatures et classification des TSA et de leurs symptômes ont existé. Par exemple, la Classifications Internationale des Maladies – dixième édition (CIM- 10), éditée par l'Organisation Mondiale de la Santé (OMS), et le Diagnostic and Statistical Manual of Mental Disorders – cinquième édition (DSM-5), éditée par l'American Psychiatric Association (APA), n'utilisent toujours pas la même classification pour les TSA (APA, 2013; ICD-10, 2016). Le CIM-10 distinguant 6 catégories de TSA tandis que le DSM 5 ne distingue que 3 niveaux de sévérité (Tableau I), précisant ensuite la présence ou non de déficits du langage ou de déficits intellectuels.

La dernière version du CIM-11 (ICD-11, 2020) s'est alignée sur la nomenclature et lesystème de classification du DSM-5. Ainsi, seuls les critères définis par le DSM-5 seront utilisés pour la suite.

Le DSM-5 définit les TSA de la manière suivante :

A. Déficits persistants dans les communications et interactions sociales dans plusieurs contextes se manifestant par :

1- *Déficits dans la réciprocité émotionnelle*
2- *Déficits dans les comportements de communication non verbaux utilisés pour les interactions sociales : contact visuel anormal, difficultés à comprendre le langage corporel et les gestes expressif, absence d'expression faciales.*
3- *Déficits dans le développement, maintien et compréhension des relations : difficultés à adapter le comportement selon le contexte social, difficultés à participer aux jeux imaginatifs et absence d'intérêts pour ses pairs.*

B. Répertoire de comportements, d'intérêts et activités restreints et répétitifs :

1- *Mouvements moteur, utilisation d'objets stéréotypés ou répétitifs*
2- *Adhérence inflexible aux routines, modèles ritualisés de comportements verbaux et non-verbaux : grande détresse en cas de changement, système de réflexion rigide.*
3- *Intérêt hautement restreint et focalisé*
4- *Hyper ou hyporéactivité aux stimuli sensoriels ou intérêt inhabituel pour des aspects sensoriels de l'environnement : indifférence aux changements de température, fascination visuelle par la lumière ou les mouvements.*

C. les symptômes doivent être présents durant la période précoce du développement (mais il est possible qu'ils ne se manifestent que de manière ultérieure quand les exigences sociales surpassent les capacités de l'individu, ou ils peuvent être masqués par des stratégies d'adaptation).

D. les symptômes causent des difficultés significatives pour le fonctionnement socio-professionnel quotidien.

E. ces symptômes ne doivent pas pouvoir être mieux expliqués par des déficiences intellectuelles (DI) ou des retards du développement.

Le DSM 5 se sert des critères A et B pour établir le diagnostic. En plus de ces symptômes, le DSM 5 inclut aussi une notion de sévérité aux TSA pour classifier l'intensité des symptômes (Tableau I). Le DSM 5 note 3 niveaux de gravité des TSA allant de faible à sévère.

Niveau de sévérité	Communication sociale	Comportements répétés, restreints
Niveau 1 : nécessite une assistance	Sans aide la communication sociale est un problème réel. Difficultés à initier des interactions et à répondre de manière adaptée. Peut apparaitre comme un manque d'intérêt pour autrui	Le manque de flexibilité comportementale cause des interférences dans le fonctionnement social dans plusieurs contextes. Difficultés à changerd'activité. Les problèmes d'organisation et de planification entravent l'indépendance.
Niveau 2 : nécessite une assistance importante	Difficultés notables en communication sociale verbale et non verbale. Cesdifficultés persistent mêmeavec la présence d'une aide. L'initiation des interactions sociales est limitée et les réponses inadaptées ou réduites. La personne s'exprime avec des phrases simples et n'interagit que sur certains sujets précis	Inflexibilité comportementale, difficultésà accepter le changement. Les comportements répétés/restreints sont notables et interfèrent avec le fonctionnement dans de nombreux contextes sociaux. Le changement d'intérêt ou d'action est difficile et angoissant.
Niveau 3 : nécessite une assistance très importante	Difficultés importantes en communication sociale verbales et non verbales, causant de grandes difficultés pour la compréhension des interactions sociales. La personne parle peu, initie rarement le contact, et ne répond qu'aux interactions directes	Inflexibilité comportementale, importantes difficultés à accepter le changement. Les comportements répétés/restreints ont un impact important sur le fonctionnement social dans toutes les situations. Le changement d'intérêt est très difficile et cause une angoisse importante.

Tableau I: Niveaux de sévérité des troubles du spectre autistique (American Psychiatric Association, 2013)

1.2.2 Comorbidités des TSA

En plus des deux symptômes principaux (troubles de la communication sociale, comportements répétés et restreints), il est noté que les TSA sont accompagnés de comorbidités dans 70 % des cas (Simonoff et al., 2008) (Tableau II). Ces comorbidités incluent des troubles neurologiques et psychiatriques, comme l'anxiété, l'épilepsie, la dépression, des troubles du sommeil, des DI et des troubles de l'attention avec ou sans hyperactivité (TDAH). On note aussi d'autres comorbidités qui ne sont pas reliées au système nerveux comme des troubles digestifs et des dérégulations du système immunitaire (McElhanon et al., 2014; Zerbo et al., 2015).

Principaux symptômes des TSA	**Comorbidité neurologiques**	**Comorbidité comportementales**	**Comorbidités systémiques**
- Déficits desinteractions sociales - Comportements répétitifs et restreints	- Déficits de l'attention - Hyperactivité - Anxiété - Troubles de l'humeur - Troubles du sommeil	- Troubles de l'attention avec hyperactivité - Troubles obsessionnels els compulsifs	- Désordres gastro-intestinaux Déficiences immunitaires

Tableau II: Symptômes et comorbidités des TSA. *En plus des symptômes principaux des TSA les patients peuvent présenter des comorbidités de type neurologique, comportementale ou systémique.*

1.2.3 Les troubles moteurs dans les TSA

En plus des symptômes et comorbidités précédemment décrits, Kanner avait noté une maladresse motrice chez les enfants atteints de TSA : « plusieurs des enfants étaient un peu maladroits dans leur démarche et capacité motrice » (Kanner, 1943). Depuis l'étude de Kanner,il a été montré qu'une majorité des patients atteints de TSA présentait des troubles moteurs variés (Provost et al., 2007). Néanmoins, en dehors des mouvements répétés, ces troubles ne sont pas pour l'instant pris en considération dans le diagnostic des TSA. De nombreuses études ont été dédiées à caractériser ces troubles moteurs qui peuvent apparaître dès les premiers mois avec une asymétrie de l'allongement ventral chez le nourrisson et un retard dans le développement de la motricité (Teitelbaum et al., 1998). Ces troubles moteurs incluent notamment, chez les enfants de 2 à 6 ans, une apraxie (difficulté à réaliser des mouvements volontaires), une hypotonie, une marche sur la pointe des pieds et une motricité globaleperturbée (Tableau III). Enfin, à l'instar de Kanner, des études ont montré des anomalies de la démarche avec notamment un raccourcissement de la longueur des pas et une phase d'appui plus longue (Weiss et al., 2013).

En raison de la difficulté de poser un diagnostic pour les TSA, ces troubles moteurs pourraient permettre de poser un diagnostic précoce et quantitatif basé sur des éléments objectifs physiques et permettre ainsi de mettre en place une thérapie comportementale et motrice adaptées (Ozonoff et al., 2008).

Motricité basique	Motricité spécifique (dyspraxie)
- Contrôle postural anormal - Marche irrégulière - Problème de coordination bilatérale - Difficulté au contrôle de la motricité fine : dextérité manuelle, écriture, contrôle d'objet, activité oculomotrice	- Difficultés à réaliser correctement une séquence de mouvements demandée età imiter des gestes - Problème dans la commande verbale - Difficultés à l'utilisation d'objet - Déficits dans l'organisation spatiale : positionnement du corps, utilisation des parties du corps requises - Déficits dans l'organisation temporelles : vitesse et synchronisationdes mouvements, retard à l'initiation du mouvement

Tableau III : Troubles moteurs dans le cadre des TSA

1.3 Epidémiologie des TSA

1.3.1 Les TSA en chiffres

La prévalence des TSA au niveau mondial est évaluée à 1 enfant sur 160 d'après l'OMS (Elsabbagh et al., 2012). Il faut noter que ce chiffre est une moyenne, la prévalence pouvant varier fortement selon les pays (Elsabbagh et al., 2012). Cela est expliqué en grande partie par les différentes méthodes de diagnostic utilisées ainsi que la reconnaissance de ces troubles selon le pays concerné. Il est aussi intéressant de noter que l'origine ethnique ainsi que le niveau socio-économique des patients semblent être des facteurs probablement dû aux différences d'accès aux soins (Christensen et al., 2016).

En France, le rapport du plan autisme de 2018 évalue la prévalence des TSA à 1 enfant sur 100 (Delobel-Ayoub et al., 2020). Aux Etats-Unis, la prévalence serait de 1 enfant sur 44 (Maenner et al., 2021).

On note que la prévalence des TSA a augmenté depuis les années 70 (Fombonne, 2009). Cela est probablement dû à plusieurs facteurs et notamment (i) la reconnaissance de plus en plus étendue des TSA en tant que maladie et non comme simple problème de comportement, mais aussi (ii) l'évolution des moyens de diagnostic avec des méthodes plus efficaces et standardisées capables de détecter des formes légères de TSA (Fombonne, 2009).

Cependant, il est notable que cette prévalence ait tendance à augmenter récemment (Maenner et al., 2021). Cela peut s'expliquer par une meilleure prise en charge de la maladie avec des outilsde criblage et de diagnostiques, plus efficaces ainsi qu'à une meilleure reconnaissance de la maladie. On pourra dans ce cas citer le pays d'Oman ou la prévalence de TSA a été multipliée par15 dans une étude en 2018 comparée à une étude précédente de 2011(Salari et al., 2022).

1.3.2 Répartitions des TSA dans la population

Le point le plus marquant sur la prévalence des TSA est la répartition inégale entre les hommes et les femmes. En effet le ratio homme/femme serait de 3:1 (Loomes et al., 2017). A l'heure actuelle, il n'a pas pu être déterminé si ce ratio était explicable par une plus grande vulnérabilité chez les hommes, ou par la présence de facteurs protecteurs chez les femmes. Plusieurs hypothèses pour expliquer ce dimorphisme sexuel ont été avancées. Parmi celles-ci, on peut noter le fait que les patients de sexe féminin disposeraient d'une meilleure capacité à s'adapter et à gérer leurs interactions sociales (Tierney et al., 2016). Ainsi, il est possible que les filles atteintes de TSA mettent en place des stratégies permettant de compenser les symptômes sociaux des TSA rendant le diagnostic plus difficile à poser, avec comme conséquence un sous-diagnostic de patientes TSA. De plus, il a été noté que certaines mutations impliquées dans les TSA ne sont pas réparties de manière égale entre les hommes et les femmes, comme c'est le caspour la mutation du gène Shank, où les hommes sont plus porteurs de ces mutations que les femmes (Sato et al., 2012).

1.3.3 Impact personnel et socio-économique des TSA

En raison des déficits d'interaction sociale des patients atteints de TSA, ces troubles ont un impact socio-économique important. Le rapport de la Cour des comptes de 2018 estime que 6,7milliards d'euros de finance publique sont dépensés chaque année pour la prise en charge des TSA (https://www.ccomptes.fr/sites/default/files/2018-01/20180124-rapport-autisme.pdf).Ce coût regroupe la prise en charge médico-sociale, psychiatrique, le suivi de longue durée ainsique les

allocations et engagement de l'éducation nationale pour les aménagements des patientsatteints de TSA.

Il prend aussi en compte la perte induite par l'incapacité professionnelle : selon ce rapport seul 0.7 % des patients TSA travaillent en milieu ordinaire ou en entreprise adaptée, la majorité étant accueillis en foyer occupationnel (50.8%) ou travaille dans des établissements de services d'aide par le travail (43.3%). Le rapport de la Cour des comptes note aussi que le coût pour les familles est également importantavec un impact financier moyen de 3000 € par an et un impact moral important pouvant avoir des conséquences familiales notoires.

Enfin, un coût difficilement estimable est celui de la mauvaise prise en charge des patients TSA, représentée principalement par les défauts d'accompagnement qui ralentissent l'évolution vers l'autonomie du patient. Ainsi, un quart des enfants atteints de TSA sont scolarisés dans des établissements standards sans aide. Le rapport de la Cour des comptes estime que seulement 44.4 % des patients TSA exercent une activité professionnelle, ou sont en étude.

En raison des coûts pour la société, pour la famille et des coûts dus à une mauvaise prise en charge, les TSA sont une pathologie ayant un impact socio-économique important soulignant ainsi la nécessité d'explorer de nouvelles pistes afin de développer de nouveaux outils diagnostiques et thérapeutiques.

1.4 Facteurs de risques des TSA

1.4.1 Facteurs de risques génétiques

1.4.1.1 Quelques exemples

Les TSA ont une étiologie complexe avec des facteurs de risques génétiques et environnementaux (Chaste & Leboyer, 2012). Parmi ces facteurs de risques, on retrouve l'hérédité. En effet, plusieurs études ont montré que la prévalence des TSA était plus importante chez les enfants dont l'un des frères ou sœurs est atteint (Muhle et al., 2004). De plus, il est notable que les jumeaux homozygotes présentent de fortes probabilités d'être tous deux atteints de TSA (Bailey et al., 1995; Bohm & Stewart, 2009). En tout, plus de 1200 gènes ont été identifiés comme cause potentielle des TSA (Yin & Schaaf, 2017; voir aussi https://www.sfari.org/resource/sfari-gene/).

Un élément remarquable dans les facteurs de risque est le fait que beaucoup de ces gènes codentpour des protéines impliquées dans le fonctionnement de la synapse, et plus particulièrement dans la formation et le fonctionnement des épines dendritiques. Les épines dendritiques sont des excroissances retrouvées au niveau des dendrites des neurones et jouent un rôle importantdans la communication et la plasticité synaptique (Lepeta et al., 2016). Cela explique pourquoi les TSA sont fréquemment considérés comme une synaptopathie (Lepeta et al., 2016).

Dans ce cadre, on peut citer CNTNAP2, une protéine de la famille des neurexines jouant un rôle important dans l'interaction entre la glie et les neurones. La mutation du gène correspondant està l'origine de symptômes de type des TSA et des troubles du langage (Alarcón et al., 2008; Arking et al., 2008).

Certains syndromes génétiques peuvent causer des symptômes de type TSA comme le syndromede l'X fragile, la première cause de retard mental génétique (Hunter et al., 2014). Chez les garçons, ce syndrome cause des TDAH avec des troubles de la communication et une anxiété sociale de manière similaire à l'autisme (Reiss & Hall, 2007).

Il est causé par une répétition du codon CGC au niveau du gène FMR1 entrainant ainsi une méthylation anormale du chromosome X. Cette mutation provoque un déficit de la protéine codée par ce gène FRMP, qui est essentielle au fonctionnement des épines dendritiques, ce qui affecte de manière significative le développement cognitif.

1.4.1.2 Le point sur la mutation de la protéine Shank3

Parmi les facteurs de risques génétiques, celui sur lequel j'ai travaillé est la mutation du gène codant pour la protéine Shank3 (SH3). SH3 est une protéine de la famille des protéines d'échafaudage Shank qui regroupe Shank1, Shank2 (SH2) et SH3. Toutes ces protéines sont retrouvées au niveau de la densité postsynaptique (PSD) des synapses excitatrices glutamatergiques (figure 1A) (Durand et al., 2007).

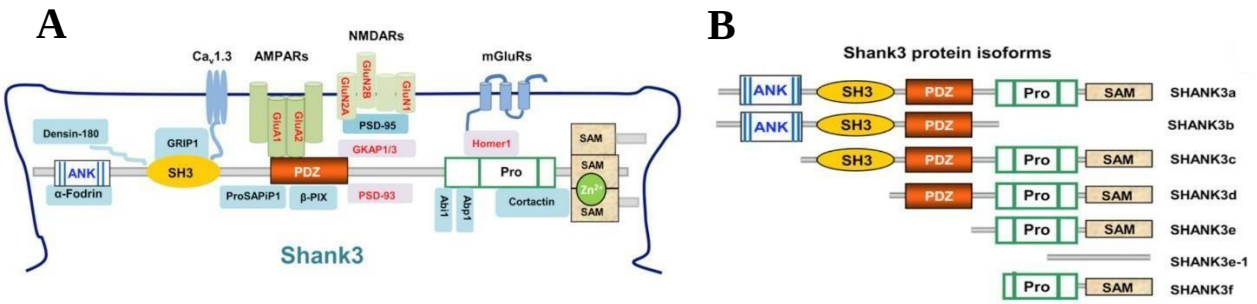


Figure 1 : (A) Schéma représentatif de la synapse glutamatergique et de la densité post synaptique (Jiang and Ehlers, 2013), (B) les différentes isoformes de la protéines SH3. *On notera la place centrale de la protéines SH3 qui interagit avec les récepteurs transmembranaires (AMPARs/NMDARs) et le cytosquelette (cortactin)*

La synapse glutamatergique est l'une des principales voies d'activation dans le système nerveux central. Le glutamate joue un rôle important au cours du développement cérébral en régulant, entre autres, la survie et la croissance neuronale ainsi que la synaptogénèse (Fatemi et al., 2012).SH3 ne possède pas de domaine catalytique ou enzymatique, mais joue un rôle important dans l'organisation de la PSD. En effet, SH3 possède de nombreux domaines lui permettant de se lier à d'autres protéines de la PSD. SH3 est capable d'interagir avec des récepteurs post synaptiques comme les mGluRs par l'intermédiaire de HOMER et avec les récepteurs NMDA et AMPA en liant son domaine PDZ à GKAP interagissant ainsi avec PSD 95 (figure 1B) (Roussignol et al., 2005). Enfin, le domaine PRO de la protéines SH3 avec le domaine SAM interagissent avec la cortactin qui régule la multimérisation du cytosquelette d'actine. SH3 possède aussi un rôle important dans la formation et la maturation des épines dendritiques (Roussignol et al., 2005). Du fait de sa position et de sa capacité à se lier à la fois aux récepteurs transmembranaires et au cytosquelette d'actine, SH3 joue un rôle important dans l'organisation de la PSD, dans le bon adressage à la membrane des différents récepteurs et dans la bonne transmission du signal. Il faut aussi noter que la protéine SH3 possède la capacité de se lier aux neuroligines, qui sont desprotéines jouant un rôle important dans l'adhésion cellulaire (G. Meyer et al., 2004). Dans des études précédentes, ces neuroligines ont été associées aux TSA (Paris Autism Research International Sibpair Study et al., 2003).

En 2007, Durand et al ont publié une étude montrant que plusieurs patients atteints de TSA présentaient des mutations hétérozygotes de la protéine SH3 (figure 2) (Durand et al., 2007). Les mutations observées pouvaient causer, en plus des symptômes caractéristiques des TSA, des déficits de langage et des déficits intellectuels. De plus, la désorganisation des épines dendritiques est en lien avec des difficultés d'apprentissage (Carlisle & Kennedy, 2005).

Globalement, SH3 est l'une des mutations associées aux TSA ayant la plus forte pénétrance (de la Torre-Ubieta et al., 2016). De ce fait, différents modèles des TSA reposant sur la mutation de la protéine SH3 ont été créés, ces modèles seront détaillés dans la partie des modèles animaux.

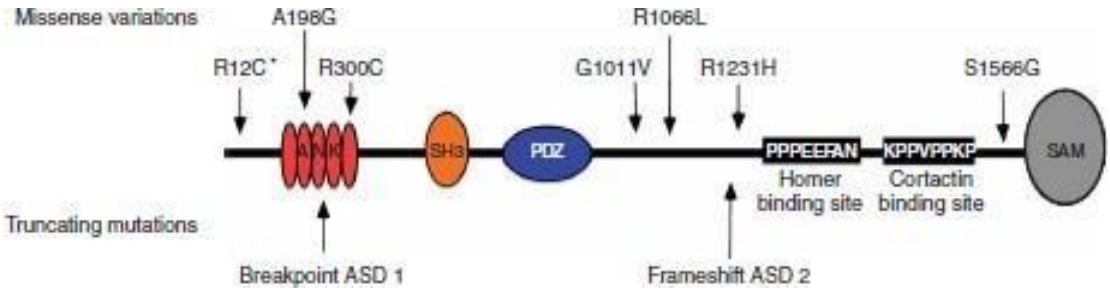


Figure 2 : Schéma des mutations de SH3 chez l'homme associé au TSA (Durand et al., 2007). *On notera la présence de mutations causant la formation d'une protéine tronquée (Truncating mutation) et de mutation de non-sens (missense mutation) pouvant rendre la protéine non fonctionnelle.*

1.4.2 Facteur de risques environnementaux

1.4.2.1 MIA (maternal immune activation)

En plus des facteurs de risques génétiques, il existe aussi des facteurs de risques environnementaux. La plupart de ces facteurs environnementaux interviennent durant la grossesse.

Par exemple, il a été montré que l'activation du système immunitaire maternel (ou maternal immune activation : MIA), durant la grossesse, augmente les risques pour l'enfant de développerdes TSA (Atladóttir et al., 2010). En effet, une forte activation du système immunitaire maternelentraînera une forte production de cytokines pro-inflammatoires comme l'interleukine 6 (IL-6) et altèrera le développement cérébral avec une diminution de l'épaisseur des couches corticalesI à VI et une diminution de l'épaisseur de l'hippocampe. Au niveau cellulaire, on notera une atrophie des cellules pyramidales de l'hippocampe (Fatemi et al., 2002). Au niveau moléculaire, on observe une dérégulation de plusieurs gènes impliqués dans la communication cellulaire, la transduction des signaux comme avec la mutation de SH3 et son rôle d'organisatrice de la réponse post-synaptique (Matas et al., 2021), la croissance et la préservation cellulaire avec par exemple la mutation de CHD8 qui agit sur la voie Wnt/B-catenine (Baranova et al., 2021), et le métabolisme protéique (Fatemi et al., 2005). Prises toutes ensemble, ces altérations du développement cérébral contribuent certainement à l'apparition de la symptomatologie des TSA (Smith et al., 2007).

1.4.2.2 *Toxicité médicamenteuse*

1.4.2.2.1 Utilisation du VPA

Il a été montré par Meunier et al (1963) que l'acide valproïque (VPA) possédait des propriétés d'anti-convulsion quand il était injecté à des modèles animaux atteints d'épilepsie (Meunier et al., 1963). Ce médicament a été utilisé depuis dans le cadre du traitement de l'épilepsie et commestabilisateur de l'humeur dans le traitement des troubles bipolaires (BPD) et est commercialisé en France sous le nom de Dépakine (Chiu et al., 2013).

1.4.2.2.2 Action du VPA

Le VPA va principalement agir sur la transmission gabaergique à plusieurs niveaux et favorise l'activité inhibitrice du GABA. Celle-ci sera obtenue en agissant en pré- et post-synaptique avec une augmentation de la disponibilité synaptique du GABA par augmentation de sa synthèse et son recyclage, et en retardant l'extinction de la réponse post-synaptique inhibitrice (Rogawski & Löscher, 2004). Le VPA augmente aussi la sensibilité aux stimuli des récepteurs GABA-A et GABA-B (figure 3). De par ses effets stimulants sur la transmission gabaergique, le VPA cause une inhibition de la transmission glutamatergique excitatrice, notamment en rétablissant l'équilibre NR2A/NR2B (Romoli et al., 2019).

Enfin, le VPA agit au niveau de l'ADN en inhibant les histones désacétylase (HDAC) (Figure 3). Cela aura pour conséquence d'inhiber les voies favorisant l'apoptose tout en activant celles favorisantla neuroprotection (figure 3).

L'inhibition des HDAC peut avoir un fort impact néfaste si le système nerveux est toujours en cours de développement. En effet, il a été montré que le VPA inhibait l'activité de HDAC1 et HDAC2 (Sun et al., 2020), qui jouent un rôle important dans la différentiation des cellules-souches neuronales en neurones et dont la perte cause une mort cellulaire excessive (D'Mello, 2019).

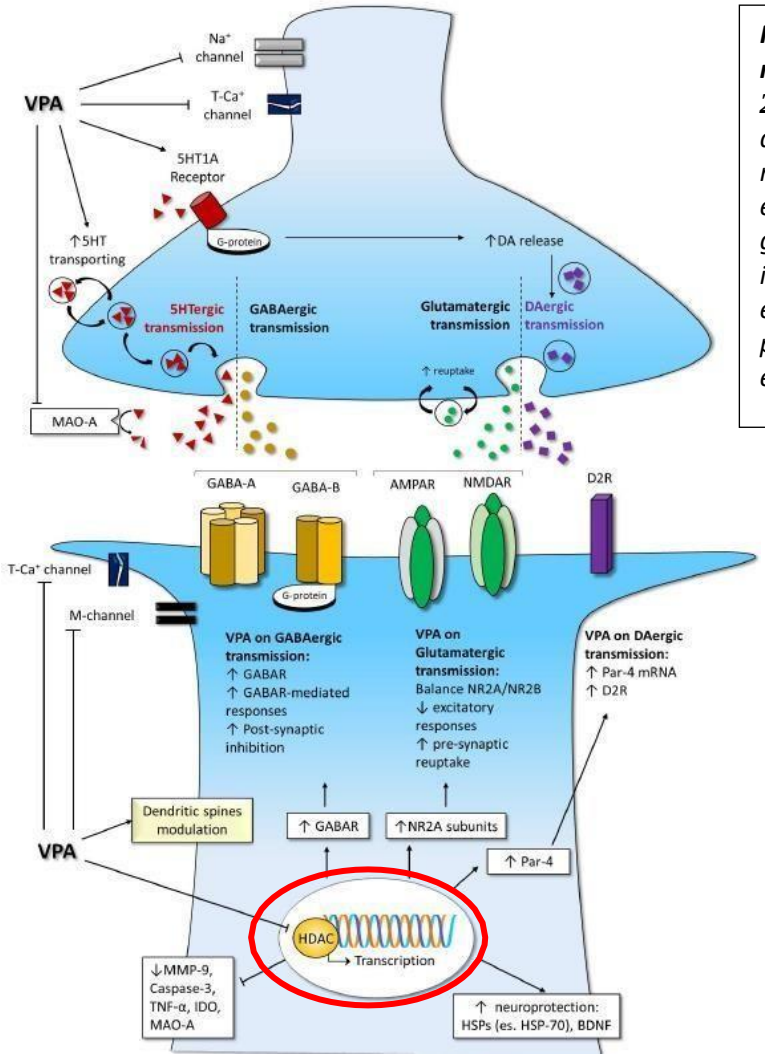


Figure 3 : Mécanisme d'action du VPA au niveau de la synapse (Romoli et al., 2019). *On notera l'inhibition de l'histone désacétylase (HDAC) par le VPA, modifiant ainsi l'acétylation des histones et donc le mécanisme d'expression des gènes. On notera de plus une action inhibitrice du VPA sur les canaux Na^{+}, Ca^{++} et sur la MAO-A ainsi qu'une action promotrice sur les auto-récepteurs 5HT1A et le transport de la 5-HT.*

1.4.2.2.3 VPA et autisme

Parmi les médicaments antiépileptiques, le VPA a été identifié comme ayant la plus forte tératogénicité (Werler et al., 2011). L'administration de VPA chez la femme enceinte est liée à de nombreuses malformations (Werler et al., 2011). L'exposition au VPA durant le premier trimestre de grossesse cause des malformations du tube neural chez l'embryon.

Cela serait à l'origine des déficits cognitifs ainsi que des déficits de la communication verbale et non-verbale (Meador et al., 2009, 2011). En plus de ces déformations, l'expositionau VPA a été associée à une augmentation du risque de 4 à 5 fois de développer des TSA chez l'enfant (Coste et al., 2020).

Le mode d'action du VPA en tant que facteur de risque des TSA n'est pas encore entièrement compris. Mais plusieurs études ont montré que les hyperacétylations des histones H3 et H4 étaient associées à des comportements de type autistique chez les souris exposées in utero au VPA. Cette hyperacétylation des histones serait due à l'inhibition de l'HDAC durant le développement embryonnaire (figure 3), indiquant ainsi que l'inhibition de HDAC peut jouer un rôle important dans l'étiologie des TSA. Le VPA aurait aussi un impact sur la plasticité neuronaleen cours de développement, probablement via l'inhibition indirecte de GSK3B, promouvant ainsila voie de signalisation des Wnt (Hall et al., 2002). Les Wnt étant une famille de protéines régulant la différenciation des progéniteurs corticaux en neurones, toute altération du fonctionnement de la voie des Wnt aurait un impact important sur le développement du cerveau chez l'embryon.

Etant donné les nombreux effets tératogènes du VPA, plusieurs modèles animaux ont été développés, avec entre autres des modèles de schizophrénie (SCZ) et des modèles de TSA. Ceux-ci seront développés plus en détail dans la partie traitant des modèles animaux des TSA.

1.5 Physiopathologie des TSA

Le cervelet est une structure proéminente du mésencéphale, il joue un rôle important dans la coordination motrice, la posture et l'équilibre (Stoodley et al., 2012). Des études récentes ont montré que le cervelet jouait aussi un rôle important dans le contrôle des émotions et la cognition ainsi que dans le langage (De Smet et al., 2013; Schmahmann, 2019).

Le cervelet est composé d'un cortex à trois couches et de quatre noyaux sous corticaux, il est attaché au tronc cérébral par les pédoncules cérébraux (Roostaei et al., 2014). Le cervelet est une région possédant une connectivité importante (figure 4A), souvent à travers le thalamus, avec certaines régions jouant un rôle essentiel dans la motricité comme le cortex moteur, le système nigro-strié (striatum (CPU), la substance noire pars Compacta (SNc) et le noyau accumbens (Nac)) (Bostan et al., 2013). De par son rôle d'intégrateur des informations, le cervelet reçoit des informations sensorielles de l'appareil vestibulaire pour assurer l'équilibre et de la moelle épinière pour aider au maintien de la posture (Ioffe, 2013). Il reçoit aussi des informations des aires motrices afin de réguler les mouvements volontaires (Daskalakis et al., 2004).

L'un des types cellulaires jouant un rôle central dans le fonctionnement du cortex cérébelleux sont les cellules de Purkinje (PC) (figure 4B). Les PC sont des neurones possédant une importante arborisation dendritique. Ces cellules jouent un rôle central dans l'intégration des informations arrivant au cervelet et constituent la seule voie de sortie du cortex cérébelleux.

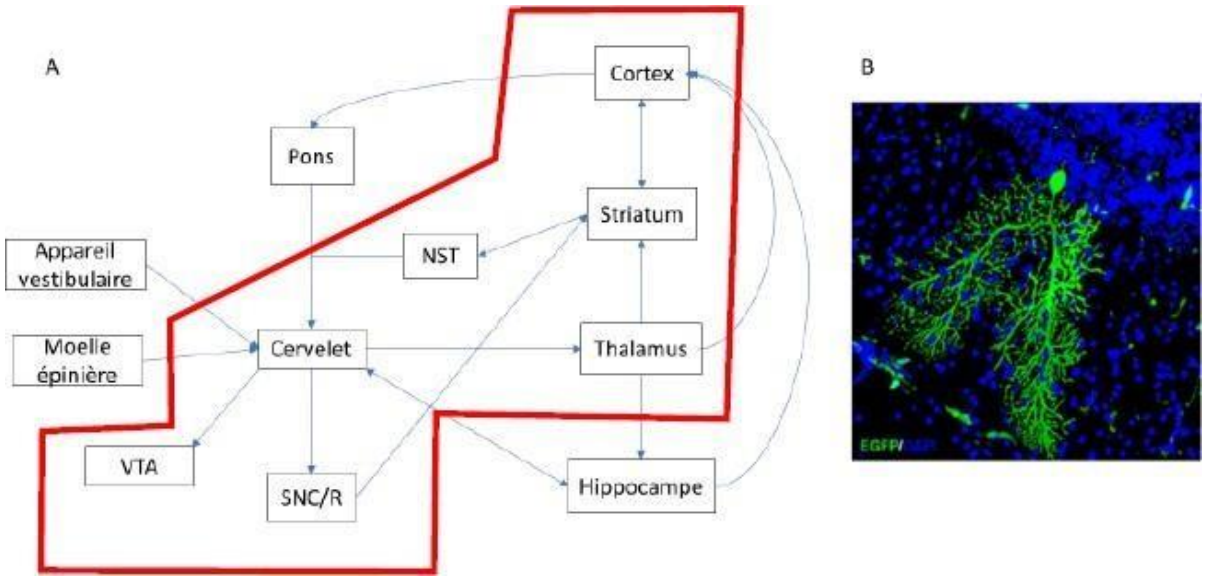


Figure 4 : (A) Schéma de la connectivité du cervelet (noyau sous-thalamique (NST)) ; Aire tegmentale Ventrale (VTA) ; substance noire pars compacta/reticulata (SNc/r) (B) représentation d'une cellule de Purkinje (PC) *(Kemp et al., 2018)* ***.*** *On notera l'importante arborisation dendritique de la PC et la forte connectivité du cervelet.*

Le cervelet est une région d'intérêt dans l'étude des TSA, car c'est la région la plus fréquemment affectée dans cette pathologie (Bauman & Kemper, 2005). Parmi les altérations les plusfréquentes, on retrouve une diminution du volume du cervelet et de la matière grise, avec une forte diminution du nombre de PC (D'Mello et al., 2015; Wegiel et al., 2014).

Etant donné son rôle dans la motricité fine et le contrôle des émotions et du langage, toute altération du cervelet peut avoir des conséquences importantes sur les fonctions régulées : motricité, langage et émotion, toute caractéristiques des TSA.

Le système nigro-strié est un système composé du striatum et de la SNc. Celui-ci joue un rôle capital dans la régulation des mouvements moteurs volontaires. Par exemple dans le cadre de la maladie de Parkinson, les neurones dopaminergiques de la SNc dégénèrent, la perte de cette innervation dopaminergique nigro-striée est à l'origine des symptômes moteurs observés dans le cadre de cette pathologie (Alexander, 2004).

Le CPU joue aussi un rôle central dans l'exécution des mouvements volontaires. Par exemple, dans le cadre de la maladie de Huntington, la perte des neurones striataux provoque des troubles moteurs tels que des troubles de la posture et de l'équilibre avec une démarche anormale, pouvant éventuellement rappeler les troubles moteurs observés dans le cadre des TSA (Ho et al., 2003).

Il est important de noter que des études chez l'homme ont montré que les TSA causent des altérations du volume et de la fonctionnalité du striatum. Ces altérations sont corrélées au comportement répétitif observé chez les patients atteints de TSA (Langen et al., 2014).

En plus du système nigro-strié, une autre région jouant un rôle capital dans la motricité est le cortex moteur qui est central pour l'apprentissage et la planification des mouvements, mais pas dans leur exécution (Kawai et al., 2015). Cette région n'a été que peu étudiée dans le cadre des TSA, mais de récentes études ont montré des altérations de cette région dans cette pathologie avec entre autres de l'inhibition gabaergique (Masuda et al., 2019; Nebel et al., 2014). Celles-ci seront décrites plus en détail dans la section des modèles animaux des TSA.

1.6 Traitement des TSA

Aujourd'hui, le traitement des symptômes principaux des TSA passe principalement par les thérapies comportementales pour stimuler les facultés cognitives et l'acquisition de l'autonomie. Ainsi, plusieurs de ces stratégies ont été mises en place comme le Treatment and Education of Autistic and related Communication Handicapped Children (TEACCH) pour s'adapter à chaque cas selon les capacités propres de l'individu. Le principe étant de mettre en place une prise en charge personnalisée pour chaque patient afin de faciliter leur intégration sociale.

Ces thérapies s'appuient sur des méthodes d'apprentissage favorisant l'acquisition et le renforcement de comportements adaptés avec l'élimination progressive des comportements inadaptés. La synthèse de Reichow a montré que la plupart de ces stratégies sont efficaces et particulièrement si elles sont implantées de manière précoce (Reichow, 2012).

En plus des thérapies comportementales, des traitements pharmacologiques sont fréquemment utilisés dans les TSA. Ces traitements sont généralement utilisés contre les comorbidités associées au TSA et ne ciblent pas directement la pathologie. Par exemple, le méthylphénidate est utilisé pour pallier les déficits de l'attention avec ou sans hyperactivité (Storebø et al., 2018).

Un point positif du traitement de cette comorbidité peut être un effet renforcé de la thérapie comportementale grâce à une meilleure attention de l'enfant. Les antipsychotiques atypiques sont utilisés pour traiter l'irritabilité et les stéréotypies. Les antidépresseurs en cas de dépression ou d'anxiété et la mélatonine sont utilisées pour traiter les troubles du sommeil (LeClerc & Easley, 2015).

Chapitre 2 : Modèles des TSA

2.1 Modèles génétiques

2.1.1 Modèles génétiques CNTNAP2, SH2, FRMX

Selon la base de données sur les gènes de la Simmons Foundation Autism Research Initiative (SFARI), les avancées récentes dans les techniques de tests génétiques ont permis d'identifier plus de 1200 gènes candidats et locus de variantes du nombre de copies (CNV) associés aux TSA (https://www.sfari.org/resource/sfari-gene/). Une cause génétique des TSA, y compris des réarrangements chromosomiques (englobant les CNV rares et de novo) et des mutations de séquences codantes, est identifiée jusqu'à 25% des cas de TSA (Huguet et al., 2013; Ziats & Rennert, 2016).

De multiples études ont montré que des mutations dans des gènes tels que NRXN, NLGN, SHANK, TSC1/2, FMR1 et MECP2 convergent sur des voies cellulaires communes au niveau dela synapse, suggérant que la pathogenèse des TSA, au moins en partie, peut être attribuée à un dysfonctionnement synaptique, qui peut entraîner des troubles fonctionnels et cognitifs (Stessman et al., 2017; Wang et al., 2016).

Bien que les rares mutations génétiques non-syndromiques ne représentent qu'une petite fraction des cas de TSA, elles fournissent une excellente fenêtre pour mieux comprendre les anomalies synaptiques sous-jacentes au TSA. La caractérisation de modèles animaux génétiquement modifiés créés sur la base de ces découvertes génétiques a conduit à l'identification de voies biochimiques susceptibles d'être pertinentes pour une population plus large que les seuls patients TSA et a fourni des résultats prometteurs en ce qui concerne le développement de nouveaux médicaments contre le TSA ou la proposition de nouvelles voies diagnostiques.

Du fait que l'aspect génétique joue un rôle important dans l'étiologie des TSA, plusieurs modèles animaux des TSA ont été créés en se basant sur des mutations observées chez l'homme et associées au TSA. Parmi ces modèles, on peut citer le modèle Cntnap2 KO caractérisé par Peñagarikano et al. (2011) qui montrent que les souris Cntnap2 KO présentaient des comportementsrépétitifs, une diminution de l'intérêt pour les interactions sociales, des troubles épileptiques etune hyperactivité ; ces deux derniers étant des comorbidités associées au TSA (Peñagarikano et al., 2011).

Au niveau cellulaire, il a été montré que ces souris avaient une connectivité neuronale anormale, élément retrouvé chez les patients TSA avec une mutation de CNTNAP2 (Scott-Van Zeeland et al., 2010).

Des mutations SHANK ont été trouvées chez des personnes atteintes de divers troubles neuropsychiatriques se chevauchant partiellement, y compris les TSA, la DI, la SCZ, le BPD et les TDAH (Guilmatre et al., 2014; Kleijer et al., 2014). Des mutations hétérozygotes dans l'un des gènes SHANK peuvent être détectées chez environ 1% des patients atteints de TSA en général (Leblond et al., 2014).

De fait, plusieurs lignées de souris mutantes Shank ont été générées indépendamment à traversle monde et sont utilisées comme modèles in vivo dans des études de traitement translationnel (Bozdagi et al., 2010; Hung et al., 2008; Kouser et al., 2013; Peça et al., 2011; Schmeisser et al., 2012; Wang et al., 2011; Won et al., 2012). La perturbation ciblée d'un des gènes Shank chez lasouris entraîne des phénotypes comportementaux qui rappellent les symptômes observés chez les personnes atteintes de TSA (Jiang & Ehlers, 2013).

C'est le cas de souris KO du gène SH2 qui est une protéine jouant un rôle similaire à SH3, mais est exprimée dans d'autres régions cérébrales tels que le cortex, le thalamus, et l'hippocampe (CA1, CA3) (Monteiro & Feng, 2017). Dans ce modèle, il a été observé des troubles comportementaux communs aux TSA, avec des comportements répétitifs et des déficits d'interaction sociale (Schmeisser et al., 2012). Il a été également noté une surexpression de SH3 à la synapse, avec une augmentation de l'expression des récepteurs AMPA et NMDA, montrant une fois encore le lien entre TSA et fonctionnement anormal de la synapse avec un déséquilibre de la balance excitation-inhibition (Schmeisser et al., 2012).

De plus, des modèles basés sur des mutations causant des troubles proches, mais différents des TSA ont été créés, comme celui se basant sur le syndrome de l'X fragile. Les souris modèles du syndrome de l'X fragile présentent, au niveau comportemental, des déficits de l'interaction sociale, des déficits de l'attention et des troubles épileptiques (de Esch et al., 2015). Au niveau cellulaire, on retrouvera des malformations des épines dendritiques ainsi qu'un fonctionnementsynaptique anormal (de Vrij et al., 2008). Ainsi, bien que le syndrome de l'X fragile soit différent des TSA, les nombreuses similitudes observées entre ces deux troubles peuvent se montrer utiles pour mieux comprendre la physiopathologie des TSA.

2.1.2 Modèles Shank3

2.1.2.1 Rôle de la protéine SH3

Le modèle sur lequel j'ai travaillé est celui se basant sur la mutation de la protéine SH3. Comme décrit précédemment, SH3 est une protéine d'échafaudage jouant un rôle important dans le développement cérébral, la croissance neuronale et la synaptogénèse (Fatemi et al., 2012). Ceci est dû à son rôle central au niveau de la PSD de la synapse glutamatergique.

Le gène codant pour la protéine SH3 possède un modèle de transcription complexe, conduisant à de nombreuses isoformes de la protéine SH3 par épissage alternatif (figure 5A). L'expression des différentes formes de SH3 dépend du type cellulaire et est régulée au cours du développement. Il est fort probable que chaque isoforme de SH3 joue un rôle précis en influençant la composition ou l'organisation de la PSD selon le type cellulaire et l'environnement.On note que l'expression de SH3 est faible dans le cerveau des rats après la naissance, mais augmente rapidement durant les 2 semaines suivantes (Jiang & Ehlers, 2013). Les augmentations les plus importantes se font au niveau du striatum, de l'hippocampe et du cervelet (Böckers et al., 2001, 2004). Ces régions sont fréquemment affectées dans le cadre des TSA montrant ainsi l'importance du rôle de SH3 au cours du développement cérébral post-natal.

Étant donné l'épissage complexe de SH3, de nombreux modèles se basant sur la mutation decette protéine ont été créés, chacun utilisant une mutation différente.

2.1.2.2 Modèles animaux de mutations Shank3

On pourra citer parmi ces modèles, celui utilisant un KO complet de SH3. Ce modèle a été créé en utilisant la technique Cre/loxP pour cibler les exons 4 à 22 permettant ainsi d'inhiber l'expression de toutes les isoformes de SH3 (Wang et al., 2016). Les souris de ce modèle présentent un toilettage (grooming) excessif pouvant aboutir à de lésions (Wang et al., 2016). Ces animaux ne montrent pas de diminution du nombre d'interactions sociales, mais les individus SH3 KO effectuent des comportements non-sociaux durant les interactions comme le grooming.

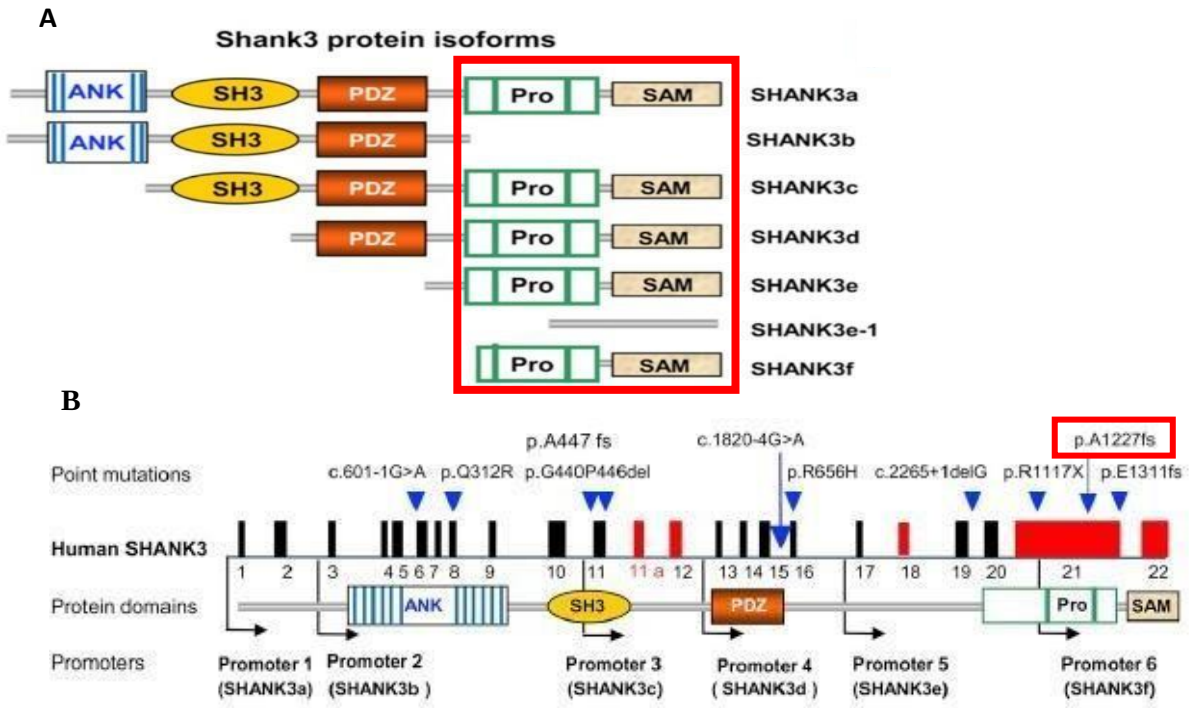


Figure 5 : (A) Les différentes isoformes de la protéines SH3 (Jiang and Ehlers, 2013) issus principalement par épissage alternatif. (B) Exemple de mutations de SH3 utilisées pour la création de modèle des TSA. *On notera que la délétion de la queue C-terminal dans le modèle Shank3 ΔC touchera principalement les domaines PRO et SAM de SH3*

Au niveau cellulaire, il a été observé des changements de la connectivité neuronale ainsi qu'une altération de la morphologie cérébrale chez ces animaux (Wang et al., 2016). Enfin, au niveau de la synapse, il a été montré que la PSD était significativement réduite chez les animaux SH3 KO. L'effet notable le plus important est la mauvaise organisation de mGluR5 et de la protéine se liant à ce récepteur Homer 1, une protéine d'échafaudage se liant au domaine Pro (promoteur 6) de Shank3 (Wang et al., 2016). Il faut aussi noter que l'utilisation d'un antagoniste de mGLUR5 permet de corriger les troubles comportementaux et neurologiques observés dans un modèle deKO complet de SH3 (Wang et al., 2016). Cependant, ce modèle s'il présente une bonne validité de construction et potentiellement une bonne validité prédictive, ne possède pas une forte validité d'apparence étant donné que les interactions sociales sont peu affectées.

Un autre modèle utilisé est celui avec le floxage de l'exon 9 de SH3. Cela aboutit à une délétion de cet exon et donc la perte de fonctionnalité du domaine ANK de la protéine. L'un des intérêts de ce modèle, est le fait que les isoformes de SH3 principalement affectées, sont celles retrouvées en grande quantité dans le striatum, l'hippocampe et le thalamus (Lee et al., 2015).

Ce modèle peut donc permettre de cibler spécifiquement certaines régions cérébrales. Dans ce modèle, les souris présentent un comportement globalement normal.

Toutefois, on note une légère augmentation du redressement des souris après introduction dansun nouvel environnement (probablement traduisant une anxiété) et une mémoire spatiale modérément impactée (Lee et al., 2015). Là où les souris de ce modèle diffèrent le plus, c'est auniveau du fonctionnement synaptique où elles présentent diverses altérations de la balance excitation-inhibition dans diverses régions cérébrales. Par exemple, la transmission excitatrice est réduite dans l'hippocampe avec une augmentation de la transmission inhibitrice. Alors que dans le cortex préfrontal, on observe une diminution de la transmission inhibitrice sans altération de la transmission excitatrice (Lee et al., 2015).

Ce modèle possède une bonne validité de construction due à la présence d'altération au niveau de régions retrouvées dans le cadre des TSA et à l'altération du fonctionnement synaptique. Maisla limite la plus importante de ce modèle, est le fait que les souris ne présentent pas de fortes altérations de leur comportement. De fait, ce modèle ne possède pas une forte validité d'apparence au niveau comportemental.

Pour mon étude, j'ai utilisé le modèle SH3 ΔC. Dans ce modèle, une base a été ajoutée au niveaude l'exon 21 du gène codant pour SH3, causant ainsi un décalage du cadre de lecture et créant un codon stop prématuré. Cette mutation engendre la délétion de la partie C terminale de la protéine SH3 et entraine, entre autres, la perte du site de liaison à Homer et du domaine SAM. Cette mutation peut donc avoir un impact notable sur l'interaction de SH3 avec les récepteurs mGluRs et sur l'interaction de SH3 avec le cytosquelette d'actine dû à la perte du domaine SAM.Cette mutation a été choisie pour plusieurs raisons. Premièrement, cette mutation est celle répliquant l'une des mutations décrites chez l'homme dans l'article de Durand et al (2007). Deuxièmement, cette mutation permet d'affecter le fonctionnement de la majorité des isoformesde la protéine SH3 (figure 5A). De plus, ce modèle présente une bonne validité d'apparence, car on retrouve des déficits sociaux et des comportements répétitifs similaires à ceux observés dansle cadre des TSA. On retrouve également une bonne validité de construction avec une altérationdu fonctionnement synaptique dans le cortex préfrontal. Enfin, ce modèle pourrait présenter potentiellement une bonne validité de prédiction à travers le ciblage pharmacologique des mGluR5s cibles de SH3 pouvant constituer une nouvelle voie thérapeutique à explorer pouvantaméliorer des comportements de type autistique (Ma et al., 2018).

2.2 Modèles environnementaux

En plus des causes génétiques, de multiples études ont montré un lien entre le risque de développer des TSA et les altérations de l'environnement durant la grossesse, avec des facteurs de risque à la fois pharmacologiques et immuns.

2.2.1 Modèle MIA

L'inflammation provoquée par la MIA a montré sa capacité à augmenter les risques d'apparitionde TSA, de SCZ et de microcéphalie (Rasmussen et al., 2016). Le lien entre MIA et TSA est établi suite à une infection virale durant le premier trimestre de la gestation (Atladóttir et al., 2010). Defait, la plupart des modèles inflammatoires de TSA se basent désormais sur une injection d'ARN double brin durant la gestation.

Basés sur ces informations, des modèles animaux ont été créés, tel que le modèle d'injection d'acide polyinosinique-polycytidylique (poly I:C), chez la souris gestante à 12, 5 jour de gestation(E 12,5). Poly I :C, est un ARN double brin synthétique avec une structure analogue à certains virus (U. Meyer, 2014). Quand celui-ci est injecté, il se fixe sur les récepteurs Toll-like 3 (TLR-3). Cette interaction causera une activation du TIR-domain-containing adapter -inducing interferon-B (TRIF) qui, une fois activé, activera les processus de réponses anti-viraux, tel que la production de cytokines pro-inflammatoire de protéines impliquées dans les processus apototiques (Reisinger et al., 2015; Yu & Levine, 2011). Ces cytokines auront la capacité d'altérer la migration des précurseurs neuronaux ainsi que la neurogénèse (Shi et al., 2009). Cela permettraainsi de reproduire le phénomène de MIA observé chez l'Homme.

Le comportement des rongeurs modèles MIA exposés in utero au poly I:C a été largement analyséafin de déterminer si le modèle présentait les symptômes caractéristiques des TSA. De nombreuses études, dont celles effectuées dans notre équipe, ont confirmé la présence dans cesmodèles de déficits des interactions sociales, ainsi que des mouvements répétitifs et stéréotypés (Haida et al., 2019; Patterson, 2009; Smith et al., 2007). En plus de ces symptômes comportementaux, ce modèle présente aussi des altérations au niveau du cervelet avec une diminution du nombre de PC (Haida et al., 2019). La combinaison de mécanismes et de symptômes similaires à ceux observés en clinique font de poly I:C un modèle pertinent des TSA.

2.2.2 Modèles VPA

2.2.2.1 Création des différents modèles VPA

Comme décrit précédemment, l'exposition au VPA durant la grossesse augmente les risques de développer des TSA chez la descendance. De ce fait, un modèle des TSA se basant sur l'administration du VPA chez la souris gestante a été créé en 1996 par Rodier et al., en effectuant une injection unique de VPA à 350mg/kg à E12,5 (Rodier et al., 1996). Depuis, de nombreuses procédures différentesont été utilisées (Tableau IV), avec souvent une administration de VPA autour de E12.5. En effet, c'est à ce moment, au cours du développement embryonnaire du rongeur, que s'effectuent la fermeture du tube neural, et la migration de cellules embryonnaires vers leur site final, étapes cruciales du développement cérébral (Favre et al., 2013).

Table 1
Autistic-like behavioral deficits induced by maternal challenge with valproic acid.

Strain/species	VPA dose/administration	Time of exposure	Outcome	Reference
Wistar rats	600 mg/kg; single i.p. injection	E12.5	↓ number of social explorations, ↓ prepulse inhibition, ↓ sensitivity to painful stimuli, ↓ exploratory activity, ↓ place aversion to naloxone, ↑ latency to social behavior, ↑ latency to reach home bedding, ↑ locomotor, repetitive/stereotyped activity, ↑ sensitivity to nonpainful stimuli, ↑ anxiety, delayed maturation and motor development	Schneider and Przewlocki, 2005; Schneider et al., 2006; Schneider et al., 2007; Schneider et al., 2008
Wistar rats	500 mg/kg; single i.p. injection	E12.5	↓ play behavior, ↓ social exploration, active avoidance of social interactions, ↓ sensitivity to thermal pain, ↓ prepulse inhibition, ↓ fear extinction, ↑ repetitive behavior, ↑ anxiety, ↑ amygdala-dependent fear memories, ↑ fear generalization	Markram et al., 2008
C57BL/6Hsd mice	600 mg/kg; single s.c. injection	E13	↓ pup distress calls, ↓ adult 70-kHz premating vocalizations, ↓ social preference, ↓ prepulse inhibition, ↑ repetitive self-grooming behavior, ↑ anxiety	Gandal et al., 2010
Hybrid of C57BL/6, CF-1, Swiss Webster and DBA-2 mouse strains	800 mg/kg; 1.5 g of peanut butter mixed with VPA	E11	lack of sociability, ↑ latency to find home bedding, ↓ general exploration	Roullet et al., 2010
Sprague-Dawley rats	400 mg/kg; single s.c. injection	E12	lack of sociability and preference for social novelty	Kim et al., 2011
C57BL/6Hsd mice	600 mg/kg; single s.c. injection	E13	↑ repetitive self-grooming and digging behaviors, ↑ anxiety	Mehta et al., 2011
ICR (CD-1) mice	500 mg/kg; single i.p. injection	E12.5	↓ social interactions, ↓ locomotor activity, ↓ general exploratory activity, ↑ anxiety	Kataoka et al., 2013
C57BL/6 J mice	600 mg/kg; single i.p. injection	E12.5	lack of sociability, ↓ social interactions, ↓ number of pup vocalizations, ↓ number of complex call types (Complex, Two-syllable, Downward, Composite, Harmonic, Frequency step), ↑ number of Flat call type, ↑ repetitive self-grooming and digging behaviors	Moldrich et al., 2013
Sprague-Dawley rats	400 mg/kg; single s.c. injection	E12	lack of sociability	Kim et al., 2014a
ICR (CD-1) mice	300 mg/kg; single s.c. injection	E10	lack of sociability, ↓ social interactions, ↓ nest building, ↑ repetitive digging behavior	Kim et al., 2014b

Tableau IV : Exemple de méthode de création de modèle des TSA par utilisation du VPA *(Nicolini & Fahnestock, 2018)****. On notera que la plupart des méthodes de création de ce modèle reposent sur une unique injection de VPA (entre 300mg/kg et 600 mg/kg) aux alentours de E12 (± 2 jours). On peut cependant observer que quelques études ont administré le VPA par mélange à la nourriture des animaux.***

2.2.2.2 Effets du VPA sur l'animal

L'administration de VPA a plusieurs effets notables sur le comportement de la progéniture (Schneider & Przewłocki, 2005). En effet, la progéniture montrera des déficits sociaux semblables à ceux observés chez les patients TSA. Il s'agit notamment d'une diminution du nombre d'interactions sociales, d'une augmentation du nombre de mouvements répétitifs et stéréotypés, ainsi que d'une diminution de la sensibilité à la douleur. En plus de ces changementscomportementaux, l'exposition au VPA a aussi de nombreux effets sur la neuroanatomie. En effet, on peut noter que l'exposition au VPA cause une réduction du nombre de neurones dans le cortex préfrontal et dans le cortex sensorimoteur (Kataoka et al., 2013). Ces régions sont impliquéesdans la communication sociale, le langage et la prise de décision. Il est aussi observé une diminution du nombre de PC dans le cervelet, indiquant ainsi que le VPA à un impact important sur le bon fonctionnement du cervelet qui pourrait être à l'origine des troubles moteurs observés(Al Sagheer et al., 2018). Enfin, au niveau cellulaire, on peut noter que le VPA entraîne une hyperplasticité dans le cortex sensorimoteur (Rinaldi, 2008). Cela est dû au fait que le VPA cause une augmentation de l'expression des sous-unités NR2A et NR2B des récepteurs NMDA ainsi que de CAMKII, augmentant la potentialisation synaptique à long terme. En plus de cet effet sur la transmission glutamatergique, l'exposition au VPA impacte aussi la voie PI3K-Akt-mTOR (Nicolini et al., 2015). Cette voie a été associée à des difficultés de reconnaissance des visages et des troubles sociaux chez les individus TSA (Nicolini et al., 2015; Pierce, 2004). Enfin, il a été montré que le VPA cause une diminution de l'expression de l'ARNm codant pour la neuroligine 3, protéine dont la mutation a été associée aussi aux TSA (Kolozsi et al., 2009).

Ainsi, le modèle VPA des TSA présente une forte validité d'apparence, de construction et potentiellement de prédiction, justifiant ainsi sa large utilisation en recherche expérimentale (Niolini and Fahnestock, 2018). Néanmoins, ce modèle présente aussi plusieurs limites. Tout d'abord, l'une des difficultés associées à l'utilisation de ce modèle, est le fait que l'administrationde VPA cause une baisse de la fertilité et une augmentation des pertes de 15 % chez la progéniture. Donc, il peut être plus difficile d'obtenir un grand nombre d'individus exposés VPA à des fins expérimentales. De plus, il est nécessaire, dans la majorité des méthodes de création de ce modèle, d'effectuer une injection chez la souris gestante, rajoutant ainsi une étape et doncune opportunité d'erreur supplémentaire lors de la création de ce modèle. Malgré ces limitations, le modèle VPA des TSA reste un modèle simple et rapide à créer, permettant une bonne modélisation des TSA tant au niveau comportemental que cellulaire et moléculaire.

Chapitre 3 : Analyse d'interaction social chez l'animal : intérêts et limites

3.1 Méthodes d'études actuelles

3.1.1 Le test à 3 chambres ou 3-CT

Le « Three-Chamber Test » (3-CT) est une méthode d'analyse de la motivation sociale des souris développée en 2004 (Nadler et al., 2004). Le 3-CT est composé d'une cage en plexiglas avec trois chambres séparées par des portes amovibles (figure 6). Le test repose sur le fait qu'une souris avec une sociabilité normale préférera passer plus de temps dans une chambre contenant un autre individu que dans une chambre vide ou contenant un objet non social. La chambre centralesera la position de départ de la souris testée. Dans les chambres latérales, on retrouvera les gobelets où seront placés les individus sociaux au cours du test. Ce test se déroule en 3 phases de 10 minutes (min) d'enregistrement précédées par une phase d'habituation de 5 minutes, la durée totale du test pour 1 individu est de 50 min en comptant les nettoyages entre les phases.

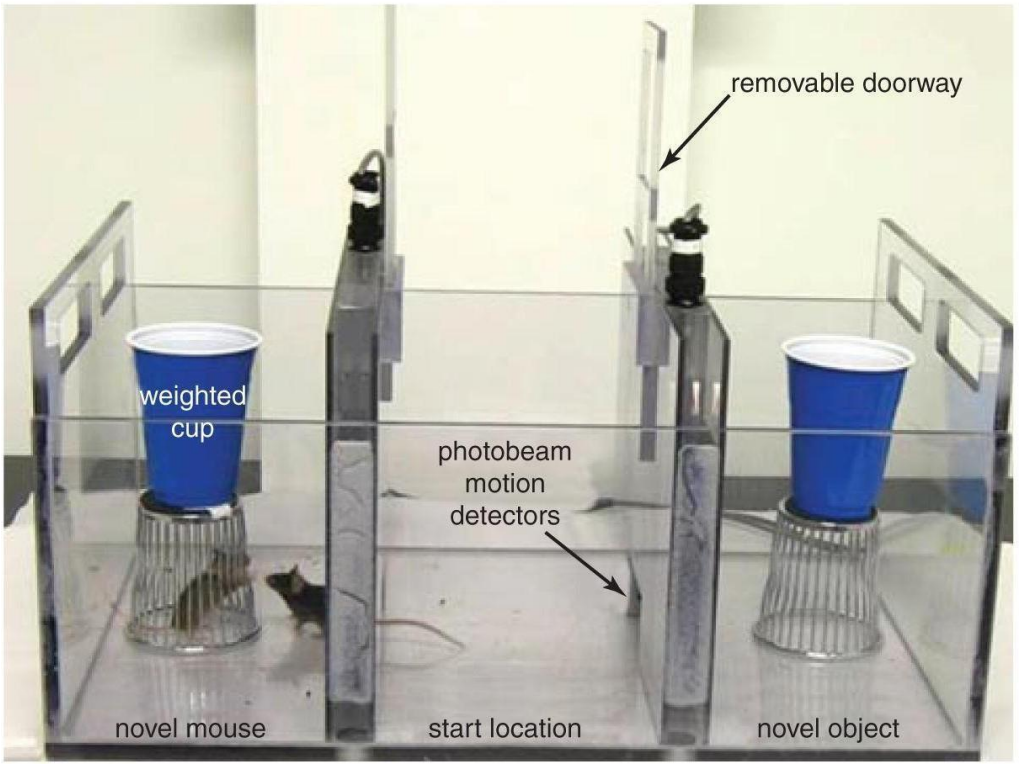


Figure 6 : **Photo d'un appareil de 3-CT au cours de la phase 2 (Yang et al., 2011).** La position de départ (start location) est la position de laquelle la souris test commence chaque phase. Ici on quantifie le temps passé par la souris à tester, dans la chambre contenant un individu social (novel mouse) ainsi que le temps passé dans la chambre contenant un objet non social (novel object), sinon vide. Les détecteurs photométriques (photobeam motion detectors) permettent de compter le nombre d'entrée de la souris test dans chaque chambre. Les coupes lestées (weighted cup) permettent d'éviter que les cages soient renversées.

Durant la phase d'habituation, la souris testée est placée dans la chambre centrale du 3-CT pendant 5 min. A la fin de cette phase d'habituation, la phase 1 commence. Les deux portes sontouvertes permettant à la souris l'accès aux autres chambres de 3-CT. L'enregistrement des déplacements de la souris se fait pendant 10 min pour chaque phase. Durant la première phase,il n'y a aucune souris dans le 3-CT à part la souris testée. Le but de cette phase étant de vérifier l'absence de préférence de place spontanée pour la souris test pour l'une des chambres, ce qui pourrait fausser la mesure de préférence de chambre. A la fin de la phase 1, la souris test est replacée dans la chambre centrale du 3-CT et les deux portes sont refermées. Ensuite, un individu social est placé sous le gobelet d'une des deux chambres latérales. Puis les deux portes sont ouvertes et la phase 2 commence. La phase 2 est la phase qui permettra d'évaluer la préférence sociale de la souris. Si la souris présente une sociabilité normale, elle passera significativement plus de temps dans la chambre avec l'autre individu. Si la souris présente des déficits de sociabilité alors elle n'aura pas de préférence pour l'une ou l'autre des chambres.

A la fin de la phase 2, la souris testée est là encore replacée dans la chambre centrale et les deuxportes sont refermées. Puis un second individu est placé dans la chambre qui contenait l'objet non-social. Une fois le nouvel individu social introduit, les deux portes sont ouvertes à nouveau et l'enregistrement est relancé. L'objectif de cette phase 3 est d'étudier la réaction de la souris testée à un nouvel individu, et de fait, sa préférence pour la nouveauté sociale. Une souris avec un comportement social normal aura tendance à passer plus de temps dans la chambre contenant le nouvel individu par rapport à la chambre contenant l'individu déjà connu. Cette méthode permet d'obtenir plusieurs informations sur la sociabilité des souris. La phase 2 permetd'évaluer la sociabilité de la souris test alors que la phase 3 permet d'étudier l'intérêt de l'individutesté pour la nouveauté sociale ainsi que sa capacité à reconnaître un individu déjà rencontré.

Le 3-CT présente plusieurs avantages, c'est une méthode simple à mettre en place, largement automatisée et rapide. En effet, elle permet de déterminer rapidement si l'individu testé présente des déficits sociaux et/ou des difficultés avec la nouveauté sociale. Néanmoins, cette technique présente aussi des nombreuses limites. En effet, l'analyse de la sociabilité est peu détaillée étant donné que l'on ne fait que comparer le temps que passe la souris testée dans différentes chambres. Ainsi, si le 3-CT permet une analyse qualitative (sociable ou pas sociable) il ne permet pas d'obtenir plus de précisions quant à la nature et l'amplitude de la sociabilité. De plus, le 3-CT ne permet pas non plus l'analyse du comportement d'un groupe de souris, ne pouvant analyser que le comportement d'une seule souris à la fois. Enfin, le 3-CT étant une méthode rapide, elle ne permet qu'une mesure de très courte durée, une photographie d'un instant donné (10 min.), ce qui n'est pas forcément en phase avec un comportement social qui par nature, s'étend sur de longues périodes.,

Enfin, il est aussi possible que les actions de l'expérimentateur entre chaque phase interfèrent avec le comportement de la souris testée.

En conclusion, le 3-CT est une méthode présentant de nombreux avantages : elle est rapide, simple et facile à mettre en place. Néanmoins, cette méthode ne permet pas l'analyse de comportements complexes, en groupe et sur une longue durée.

3.1.2 Visible Burrow system

Le Visible Burrow System (VBS) a été développé à la fin des années 80 et référencé en tant que tel pour la première fois en 1989 (Blanchard & Blanchard, 1989). Le VBS a été utilisé pour mesurer un grand nombre de comportements différents chez les rongeurs et notamment les comportements de défense (Blanchard & Blanchard, 1989), la formation de la hiérarchie sociale et le stress résultant de celle-ci (Herman & Tamashiro, 2017). Depuis, le VBS a été adapté afin d'étudier les performances sociales chez les souris modèles des TSA (Bove et al., 2018).

Le VBS est composé de deux parties (figure 7A) : un espace ouvert où les souris auront accès à l'eau et la nourriture sur un cycle jour/nuit de 12h, et une zone recouverte et sombre incluant des tunnels ainsi que des nids pouvant accueillir les souris. Le couvercle de cette zone est transparent aux infrarouges autorisant l'observation du comportement des individus avec une caméra infrarouge.

Dans le cadre de l'étude des interactions sociales chez les souris modèles des TSA, le VBS est utilisé pour comparer les différences de comportement entre des colonies modèles des TSA et des colonies de souris contrôles. Les animaux sont placés dans le VBS en colonie de 8 (6 mâles, 2 femelles). La présence des femelles vise à de réduire le niveau de stress dans les colonies de mâles. Les mâles sont marqués afin d'être facilement identifiables par les expérimentateurs, lesanimaux resteront dans le VBS pendant 8 jours. Le comportement sera observé sur des périodesde 10 min à différents points du cycle jour/nuit (figure 7B) (Bove et al., 2018). Les différents comportements sont comptés manuellement sur 5 jours par un expérimentateur en aveugle. Lescomportements sont analysés soit au jour le jour soit en effectuant la moyenne sur 5 jours.

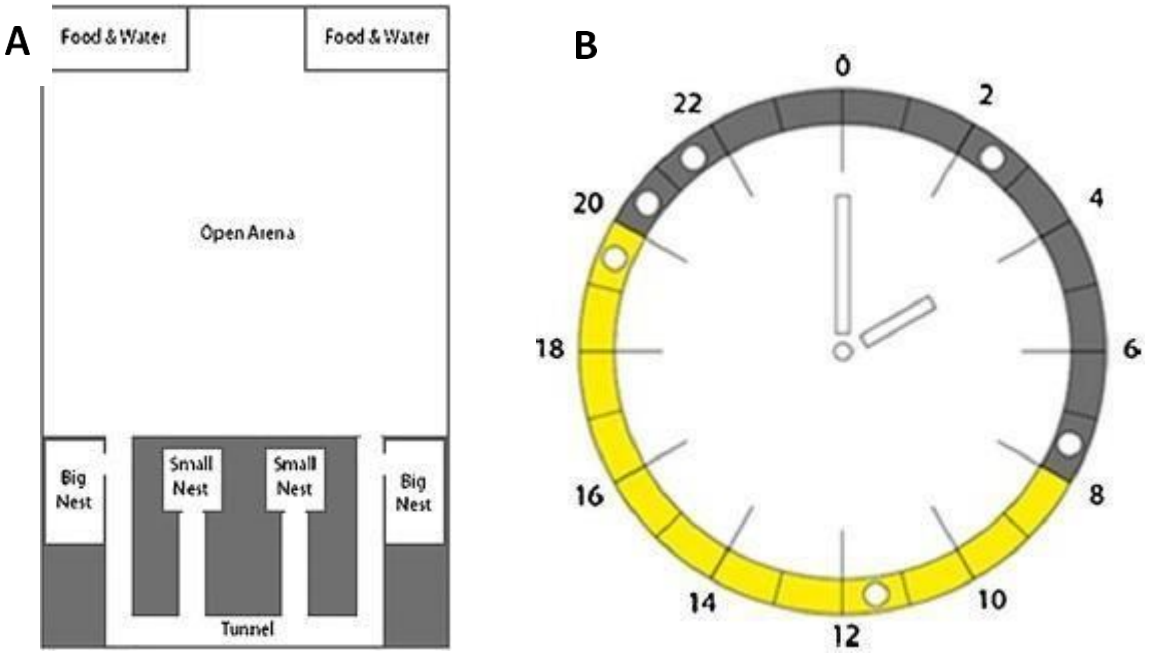


Figure 7 : Le Visible Burrow System. *En (A), Organisation de cette cage avec une zone ouverte en cycle jour/nuit (openArena) et une zone recouverte mais visible avec caméra infra-rouge, avec les tunnels et les nids (big et small nest). En (B), planning de mesure lors d'un cycle jour/nuit sur 2x12h avec changement de luminosité à 8h et 20h, les mesures se font sur 10 min à 8h, 12h, 19h, 20, 21h et 3h (Bove et al., 2018).*

Le VBS est une technique permettant d'évaluer différents comportements sociaux chez les colonies de souris sur du long terme, notamment la tendance des animaux à aller chercher un contact social, éviter les contacts sociaux et les temps passés seul ou en groupe (Bove et al., 2018).

Cette méthode présente de nombreux avantages : Elle est simple, facile à mettre en place et permet d'étudier de nombreux comportements sociaux sur plusieurs jours. Néanmoins, cette méthode présente de nombreuses limites : les mesures sont réalisées manuellement pouvant ainsi créer un risque d'erreur par l'expérimentateur. De fait, et même si le comportement est enregistré sur plusieurs jours, les données ne seront analysées que sur des tranches de 10 min sélectionnées sur l'intégralité de l'enregistrement. De plus les comportements sociaux identifiés,s'ils sont plus complexes que ceux observables avec le 3-CT, restent toujours relativement simples. En effet, il est possible d'observer si une souris essaie d'éviter les autres mais non, par exemple, si une souris suit une autre, ce qui constitue un comportement social plus complexe. Enfin, le VBS permet l'analyse de comportement social des souris à l'échelle de la colonie, mais pas à l'échelle de l'individu et donc ne permet pas d'identifier le comportement spécifique d'unesouris dans un groupe et n'autorisera pas l'analyse du comportement entre des groupes de sourismodèles TSA et des groupes de souris WT.

3.2 Le LMT : Nouvelle méthode d'analyse des comportements sociaux

3.2.1 Généralités sur le « Life Mouse tracker »

Le Live Mouse Tracker (LMT) est une nouvelle méthode d'analyse de comportements sociaux des souris créée par De chaumont en 2019 (de Chaumont et al., 2019). Le LMT permet le suivi et l'analyse en temps réel de comportements sociaux complexes d'un groupe de souris sur une longue durée dans un environnement standard. Le LMT est constitué d'une cage en plexiglas de50cmx50cm surmontée par une caméra d'acquisition infrarouge/RGBD reliée à un ordinateur (figure 8a). Cela permet au programme, par l'intermédiaire de la caméra, de détecter lesdifférents animaux et les événements sociaux qui leur seront associés. En-dessous de la cage, on notera la présence de 16 antennes reliées elles aussi à l'ordinateur (figure 8b). Ces antennes permettront au programme de vérifier de manière régulière et automatique l'identité des différents individus de la cage grâce aux puces d'identification par radio fréquences implémentée(RFID) dans chaque souris. Le LMT permet le suivit d'un groupe de 4 à 6 souris sur plusieurs heures voire plusieurs jours. Le nombre de souris analysable simultanément dépend de la puissance de calcul de l'ordinateur et de la taille de la cage.

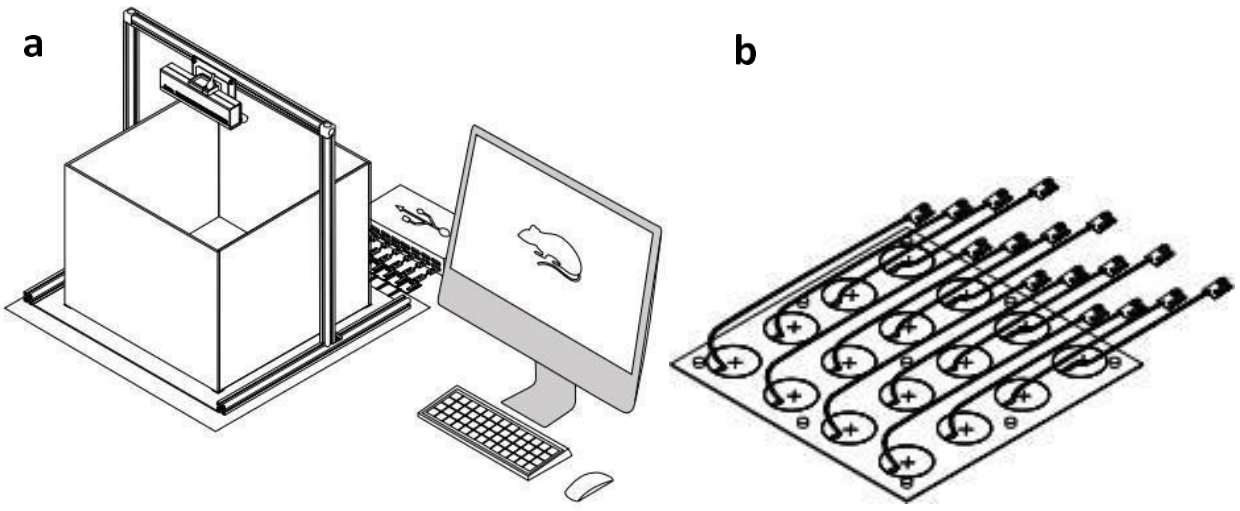


Figure 8 : schéma du LMT *avec (A) la cage de test et la caméra reliée à l'ordinatuer et (B) schéma de la plaque des antennes placées sous la cage pour permettre la détection des puces RFID par le LMT (manuel d'assemblage, https://livemousetracker.org/).*

3.2.2 Fonctionnement du LMT

Le LMT fonctionne en combinant l'utilisation de puces RFID avec un logiciel de « tracking » utilisant la caméra. Le principe du LMT est que la caméra fournit en continu des informations surla profondeur des différents éléments dans la cage ainsi que sur leur volume. Ces informations permettront au logiciel de déterminer la forme de l'animal ainsi que sa posture en identifiant entre autres la tête et la queue des souris. Le programme associé au LMT est ensuite capable, grâce à la vidéo obtenue par la caméra, d'identifier divers événements sociaux (figure 9), ainsi que les souris ayant participé à ces événements. Les puces RFID permettent de confirmer l'identité des différents individus. Quand les souris forment un groupe compact, le système a desdifficultés à identifier les différents individus. Cela peut causer au moment de la rupture d'un groupe un échange momentané d'identité entre deux individus. Ceci sera corrigé par la suite grâce aux antennes placées sous la cage et qui permettent d'effectuer des vérifications fréquentes, de détecter et signaler les erreurs d'identité puis de réassigner la bonne identité à chaque individu ainsi que les événements sociaux leur ayant été associés.

3.2.3 Données fournies par le LMT

Le LMT pouvant suivre en direct les déplacements des différents individus, cela permet d'obtenirun grand nombre d'informations sur les différents comportements sociaux des souris (figure 9). En effet, le LMT est capable de détecter les différentes postures adoptées par la souris au cours de l'enregistrement, permettant d'analyser les comportements individuels de chaque membre du groupe (figure 9a). Le système peut aussi enregistrer les différents événements sociaux ayantlieu entre 2 individus qu'ils soient statiques (figure 9c) ou dynamiques (figure 9d). Le LMT est aussi capable d'analyser les formations et ruptures de groupes (figure 9b et 9e) pouvant ainsi donner des informations au niveau individuel comme par exemple, identifier l'individu à plus forte tendance à former ou à casser les groupes. L'addition de l'ensemble des événements sociaux obtenus par le LMT permet ainsi de créer un profil social détaillé pour chaque membre d'un groupe donné, sur une longue période.



Figure 9 : Liste des événements mesurées par le LMT (De chaumont et al., 2019). *Le LMT est capable d'enregistrer les mouvement et postures d'une souris seule (A), le système détecte aussi les comportements de groupe (B), ainsi que les formations et ruptures de ces groupes (E). Enfin le système est capable d'analyser un grand nombre d'interactions entre 2 souris (C et D).*

3.2.4 Avantages et limites du LMT et comparaison par rapport au techniquesprécédentes

Le LMT est une méthode nouvelle présentant de nombreux avantages. En effet, elle permet d'analyser un grand nombre de comportements sociaux complexes sur des groupes de souris. Cela permet ainsi d'obtenir un profil social détaillé de chaque souris analysée et également de déterminer si les différents modèles animaux des TSA présentent des modifications de leurs comportements sociaux, en fonction par exemple de l'étiologie de la maladie (environnementalevs génétique par exemple).

De plus, le LMT autorise l'analyse simultanée de groupes d'animaux de phénotype/génotype etde sexe différent. A titre d'exemple, ceci peut être intéressant pour étudier si une souris contrôleprésente un comportement différent selon qu'elle soit avec d'autres souris contrôles ou avec dessouris modèles des TSA. Un autre avantage du LMT est que l'analyse est en temps réel et en continu, permettant ainsi d'obtenir une grande quantité de données sur le comportement des individus durant toute la période de test. Enfin, l'un des derniers avantages du LMT est que la méthode est automatisée, évitant ainsi la possibilité d'erreur lors d'enregistrements manuels, ainsi que la possibilité d'interférences dans le comportement des animaux testés dû aux actionsde l'expérimentateur. Enfin, Le LMT est également capable d'autocorriger des erreurs d'attribution de souris.

Néanmoins, le LMT présente aussi de nombreuses limites. L'une d'entre elles étant le fait que le système ne peut pas identifier les comportements sociaux agressifs ou sexuels. Il est à noter aussique la plus importante limite est la complexité du système. En effet contrairement aux précédentes méthodes, comme le 3-CT qui ne demandait que la mise au point d'une caméra et de vérifier que la luminosité est la même partout dans la salle de test, le LMT demande une préparation importante avant de pouvoir être utilisé. Le montage du LMT est relativement complexe et requiert un certain niveau de technicité électronique et informatique. Il ne s'agit pas d'un équipement « clé en main » mais d'un équipement qu'il faut construire pièce par pièceen acquérant soi-même les différents composants du système. Au-delà de la fabrication de l'équipement, il est aussi nécessaire de calibrer chaque antenne afin de s'assurer de la bonne détection des puces RFID. Une mauvaise calibration pouvant conduire à des difficultés sur la portée de détection des antennes et donc des problèmes pour l'identification des animaux par le logiciel. Il est aussi nécessaire de calibrer de manière précise la caméra et de vérifier fréquemment cette calibration, afin d'éviter des erreurs de détection et de séparation des individus. Enfin, le LMT requiert que chaque souris utilisée soit implantée avec une puce RFID. Bien que l'opération soit relativement simple et rapide, cette étape, et le délai de récupération des souris après l'opération, est un élément qui n'encombrait pas les méthodes précédentes.

Au final, le LMT est une méthode nouvelle et prometteuse. Elle permet l'analyse d'un grand nombre de comportements complexes en groupe et sur une longue durée, mais elle reste compliquée à mettre en oeuvre.

Objectifs

Comme décrit précédemment, les TSA sont des troubles neurodéveloppementaux caractérisés par des stéréotypies comportementales et des déficits dans les interactions sociales avec souvent des troubles moteurs et des troubles de la marche. Le diagnostic de cette pathologie se fait sur la base d'une évaluation des aptitudes sociales et cognitives des individus. Malgré leur caractère standardisé, ces tests sont difficilement réalisables chez les très jeunes enfants (moins de 3 ans). Une analyse des troubles moteurs, présents dans cette pathologie, mais ne faisant pas encore partie des critères diagnostiques, peut constituer un complément d'analyse de nature objective, quantitative et surtout précoce. D'après les résultats obtenus dans le laboratoire de thèse, ces comportements moteurs semblent être directement corrélés aux déficits d'interaction sociale (Al Sagheer et al., 2018; Haida et al., 2019; Matas et al., 2021)

Une autre des difficultés actuelles se trouve au niveau des modèles animaux des TSA. En effet, plusieurs modèles de TSA existent, notamment chez la souris, animal avec une sociabilité importante et vivant en groupe. Néanmoins, les méthodes actuelles d'analyse de la sociabilité des souris présentent de nombreuses limites et ne permettent pas l'analyse en groupe de comportements complexes et sur le long terme. Cela restreint significativement les possibilités pour déterminer des comportements potentiellement spécifiques aux différents modèles des TSA.

Basé sur des résultats obtenus dans le laboratoire chez deux modèles environnementaux des TSA, notre **première hypothèse** de travail est qu'il serait possible d'utiliser les troubles moteurs, retrouvés chez les patients atteints de TSA, comme un complément de diagnostic quantitatif, objectif et réalisable précocement.

Notre premier objectif est de fait d'investiguer ce qu'il en est dans un modèle génétique de TSA dû à une mutation de la protéine Shank 3. Plus précisément, nous avons visé à :

(i) Déterminer la présence ou l'absence de troubles moteurs et de la marche dans le modèle SH3 des TSA.

(ii) Déterminer si ces troubles pouvaient être corrélés avec des altérations au niveau cellulaire et notamment du nombre de PC dans le cervelet,

(iii) Et au niveau moléculaire sur l'expression de molécules impliquées dans la transmission synaptique dans les régions modulant la motricité comme le cervelet, le cortex moteur et le striatum.

Le second objectif de ma thèse est de déterminer s'il est possible de révéler des troubles fins d'interaction sociale avec une analyse groupée et prolongée dans le temps examinant de nombreux paramètres. Ainsi, **pour cette seconde partie,** j'ai œuvré à :

(i) Mettre en place une technique nouvelle permettant l'analyse détaillée de la sociabilité des souris en groupe et au long terme, le LMT

(i) Déterminer un profil de comportement social détaillé de souris modèles environnementaux des TSA précédemment utilisées au laboratoire, les souris modèles VPA.

Pour cela, j'ai œuvré dans un premier temps à la construction et la mise en place de l'appareil, puis je l'ai testé sur des souris contrôle.

(ii) Enfin, j'ai comparé la sociabilité des souris VPA dans un 3-CT à celle obtenue avec le LMT afin de déterminer la pertinence relative d'une procédure par rapport à l'autre.

Les TSA touchant d'avantage les hommes que les femmes, il était ainsi important de déterminersi le LMT est en mesure de révéler des troubles de la sociabilité chez les femelles non observablesavec le 3-CT. Cela pourrait potentiellement renforcer la théorie que les femmes seraient moins diagnostiquées que les hommes de par leur capacité d'adaptation et de compensation sociale qui seraient plus développées que chez les hommes.

Résultats

Chapitre1 : Major motor and gait deficits with sexual dimorphism in a Shank3 mutant mouse model

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Emmanuel Matas, Alexandre Maisterrena, Mathieu Thabault, Eric Balado, Maurren Francheteua, Anais Balbous, Laurie Galvan and Mohamed Jaber

1. Résumé

Deux études précédentes réalisées par notre équipe ont montré la présence de déficits sociaux, moteurs et de troubles de la marche dans deux modèles environnementaux des TSA. Ici, nous avons souhaité étudier ces mêmes troubles dans un modèle génétique des TSA, celui de SH3. L'objectif principal est d'explorer le caractère transversal et commun de ces troubles, ainsi que les variations à travers des modèles d'étiologie bien différentes pouvant reproduire le spectre de la pathologie en fonction des différentes étiologies des TSA. En effet, les TSA sont par définition un spectre de pathologies avec une myriade de troubles comportementaux dont la sévérité et l'étendue peuvent se manifester de manière bien différente d'un individu à l'autre.

Pour cela, nous avons utilisé une mutation de shank3 générée par l'addition d'une base au niveau de l'exon 21 du gène. Cette addition cause un décalage du cadre de lecture et produit l'apparition d'un codon stop prématuré, aboutissant à une délétion de l'extrémité C-terminal dela protéine. Il en résulte une perte du domaine SAM de cette protéine et du domaine permettantl'accrochage de HOMER1 à SH3. Ceci pouvant un avoir un impact majeur sur la capacité de SH3 à se lier au cytosquelette d'actine suite à la perte de SAM et sur sa capacité à adresser les récepteurs mGluRs à la membrane. Cette mutation spécifique a été sélectionnée, car elle possède une forte « validité d'apparence et de construction » (face and construct validity). En effet, elle reproduit une mutation retrouvée chez l'homme et de précédentes études ont montréla présence de troubles sociaux ainsi que de comportements répétitifs dans ce modèle.

Ainsi, les objectifs de cette étude étaient, dans un premier temps, de déterminer les capacités sociales, motrices et le comportement de la marche des souris dans ce modèle à la fois chez les souris +/- et -/- et en séparant les groupes de souris mâles et femelles. En effet, comme détaillé dans l'introduction, la mutation chez l'Homme est le plus souvent à caractère hétérozygote et les phénotypes observés présentent un dimorphisme sexuel. Dans un second temps, nous avons exploré les conséquences de cette mutation sur le plan histologique et moléculaire au niveau du cervelet, région impliquée dans de nombreux troubles associés aux TSA.

Pour cela, nous avons utilisé différentes techniques :

- Pour l'analyse des comportements sociaux, nous avons utilisé le 3-CT, qui est une technique permettant d'évaluer la sociabilité d'une souris en comparant le temps passé dans une chambre avec une autre souris vs le temps passé dans une chambre sans souris, comme précédemment décrit.
- Pour l'analyse des comportements répétitifs, nous avons observé le comportement de nettoyage ou « grooming » spontané des souris en les plaçant dans des cylindres transparents et en les filmant durant 10 min. Cette situation engendre un léger stress et permet ainsi d'exacerber l'apparition de ce comportement stéréotypé. Une fois l'expérience terminée, les vidéos sont analysées en aveugle, la durée et le nombre de grooming effectués par chaque animal sont relevés.
- Pour l'analyse des capacités motrices, nous avons utilisé la technique du Challenging Beam. Avec cette technique les souris traversent une poutre surmontée d'un grillage d'un mètre de long avec 4 sections de 25cm de largeur décroissante. Si lors de la traversée une de patte traverse le grillage et touche la poutre, une erreur est comptabilisée traduisant un déficit de la coordination motrice. La souris est filmée sur la durée de la traversée suite à quoi le temps de traversée, le nombre de pas, le nombre d'erreurs total ainsi que le nombre d'erreurs pour chaque section sont quantifiés. Une différence dans l'un de ces paramètres permettant d'identifier une altération des capacités motrice fine.
- Pour l'analyse de la marche des souris nous avons utilisé l'équipement du Gait Lab (ViewPoint, France). Au cours de ce protocole, les souris traversent une poutre en verre avec une lumière laser verte horizontale tout en étant filmées par une caméra de haute définition connectée à un programme d'analyse. Ce programme permet l'analyse d'un grand nombre de paramètres de la marche, avec des paramètres spatiaux (longueur d'un pas, écartement entre patte avant et arrière, écartement entre les deux pattes avant, force d'appui…), et des paramètres temporels (durée de pose de la patte, durée de balancement de la patte, fréquence des pas, ainsi que le ratio entre le temps de pose de la patte et le temps de balancement de la patte…).
- Pour les analyses post-mortem, nous avons réalisé un comptage stéréologique des PC dans crus I et crus II suite à une coloration crésyl violet. Les conséquences moléculaires de la mutation ont été étudiées par western blot et RT-qPCR sur des extraits de cervelet.

1.1 Résultats

1.1.1 Sur le plan comportemental

Nous avons montré que les souris -/- mâles et femelles présentent une augmentation significative du nombre de grooming (toilettage) effectué, confirmant la présence de comportements stéréotypés chez ces souris.

En termes de comportements sociaux, nous avons montré qu'aucun des groupes de souris ne montre de différences significatives de leur préférence sociale (phase 2 du test). Néanmoins, tous les groupes de souris mutées montrent des déficits significatifs de préférence à la nouveautésociale (phase 3 du test).

Avec le challenging beam nous avons pu montrer la présence de déficits de la coordination motrice seulement chez les souris -/- femelles sur la 4ème section du challenging beam, qui est la plus étroite et de fait, la plus difficile.

En ce qui concerne la qualité de la marche, nous avons pu montrer plusieurs troubles de la marche chez les mâles et les femelles transgéniques, avec des altérations de la fréquence des pas, de la durée du balancement de la patte et de la durée de pose de la patte. Il est à noter que plusieurs paramètres sont altérés uniquement chez les mâles.

Après avoir montré la présence de troubles sociaux, de comportements répétitifs ainsi que des troubles de la marche et de la motricité nous avons décidé d'étudier quels étaient les corolaires cellulaires et moléculaires de ces altérations comportementales au niveau du cervelet.

1.1.2. Sur le plan cellulaire et moléculaire

Nous avons effectué un comptage des PC dans les sous lobule crus I et crus II du cervelet, deux sous-régions importantes dans la gestion de la motricité et les interactions sociales. Nous avons pu observer que les souris -/- mâles présentaient une réduction significative du nombre de PC dans crus I et dans crus II. Cette réduction n'est observée que dans crus I chez les femelles +/-.

Au niveau moléculaire, nous avons identifié une diminution significative et spécifique de l'expression du récepteur mGluR5 dans le cervelet des souris mâles +/- et -/-.

1.2 Conclusion

En résumé, nous avons pu montrer dans cette étude la présence de troubles sociaux et des comportements répétitifs chez les souris mâles et femelles suite à la délétion fonctionnelle de plusieurs isoformes majeurs de Shank 3. Les troubles moteurs ne sont observés que chez les souris femelles, mais montrent une tendance vers la significativité chez les souris mâles. Les troubles de la marche eux aussi ont été observés chez les mâles et les femelles, mais ceux-ci sont plus prononcés chez les mâles. Enfin, au niveau cellulaire et moléculaire nous avons là aussi pu observer la présence d'altérations plus prononcées chez les mâles que les femelles.

En lien avec nos observations de déficits dans la préférence de la nouveauté sociale, il est intéressant de noter que les patients TSA présentent des déficits sociaux similaires avec une tendance à éviter les contacts sociaux avec des individus inconnus par rapport à des individus connus. On peut aussi noter que les souris ne présentent des troubles de la coordination motrice que sur la dernière section du challenging beam, qui est la plus difficile, et seulement chez les femelles. Il est notable que les troubles de la marche sont eux présents dans ce modèle et concernent essentiellement des paramètres temporels de la marche indiquant que les troubles de la marche ne sont pas dus à des altérations anatomiques, mais a des altérations de la coordination motrice de type cérébelleux. Il est à souligner que les souris mâles présentent des troubles de la marche plus importants que les souris femelles.

Au niveau cellulaire et moléculaire, il est à noter que les souris mâles présentent des altérations plus sévères et dans plus de régions cérébelleuses que les souris femelles. De plus, la diminution de mGluR5 n'est retrouvée que chez les mâles. Ceci pointe le rôle majeur de SH3 dans le bon adressage à la membrane de ce récepteur, et suggère une implication de ce récepteur dans les troubles comportementaux observés.

Cet article a permis de montrer que les troubles moteurs et de la marche sont une caractéristique commune aux TSA dans différents modèles animaux quelle que soit l'étiologie, environnementale ou génétique. Il a aussi permis de montrer que, d'une manière générale, ces troubles affectaient plus les mâles que les femelles, reproduisant ainsi les observations cliniques. Enfin, avec les travaux antérieurs de l'équipe, ce travail a démontré qu'il serait pertinent d'envisager de considérer les troubles moteurs et de la marche comme éléments diagnostiques de la pathologie, qui seraient des critères objectifs, précoces, facilement quantifiables et pouvant être testés de manière reproductible.

Néanmoins, il est aussi à noter que cet article pointe plusieurs limites des modèles SH3 des TSA. La première est le fait qu'un grand nombre de modèles SH3 sont réalisés avec des mutations différentes qui ne reproduisent pas toujours les mutations retrouvées chez l'homme. De fait, plusieurs de ces modèles ne reproduisent pas ou peu les déficits sociaux ou les comportements répétitifs. La seconde limite majeure est le fait que dans cette étude la majorité des déficits sont observés chez les souris -/- alors que la mutation est +/- chez l'homme remettant en question la « face validity » de ce modèle. Il est possible que chez l'Homme, une combinaison de facteurs de prédisposition génétique avec des facteurs environnementaux soit nécessaire pour une expression forte des TSA.

Molecular Autism

2. Article

RESEARCH Open Access

Major motor and gait deficits with sexual dimorphism in a *Shank3* mutant mouse model

Emmanuel Matas[1†], Alexandre Maisterrena[1†], Mathieu Thabault[1], Eric Balado[1], Maureen Francheteau[1], Anais Balbous[1,2], Laurie Galvan[1] and Mohamed Jaber[1,2*]

Abstract

Background: Contrasting findings were reported in several animal models with a *Shank3* mutation used to induce various autism spectrum disorder (ASD) symptoms. Here, we aimed at investigating behavioral, cellular, and molecular consequences of a C-terminal (frameshift in exon 21) deletion in *Shank3* protein in mice, a mutation that is also found in clinical conditions and which results in loss of major isoforms of Shank3. A special focus was made on cerebellar related parameters.

Methods: All three genotypes were analyzed [wild type (WT), heterozygote (Shank3 +/ΔC) and homozygote (Shank3 ΔC/ΔC)] and males and females were separated into two distinct groups. Motor and social behavior, gait, Purkinje cells (PC) and glutamatergic protein levels were determined. Behavioral and cellular procedures used here were previously validated using two environmental animal models of ASD. ANOVA and post-hoc analysis were used for statistical analysis.

Results: Shank3 ΔC/ΔC mice showed significant impairments in social novelty preference, stereotyped behavior and gait. These were accompanied by a decreased number of PC in restricted cerebellar sub-regions and decreased cerebellar expression of mGluR5. Females Shank3 ΔC/ΔC were less affected by the mutation than males. Shank3 +/ΔC mice showed impairments only in social novelty preference, grooming, and decreased mGluR5 expression and that were to a much lesser extent than in Shank3 ΔC/ΔC mice.

Limitations: As *Shank3* mutation is a haploinsufficiency, it is of interest to emphasize that Shank3 +/ΔC mice showed only mild to no deficiencies compared to Shank3 ΔC/ΔC.

Conclusions: Our findings indicate that several behavioral, cellular, and molecular parameters are affected in this animal model. The reported deficits are more pronounced in males than in females. Additionally, male Shank3 ΔC/ΔC mice show more pronounced alterations than Shank3 +/ΔC. Together with our previous findings in two environmental animal models of ASD, our studies indicate that gait dysfunction constitutes a robust set of motor ASD symptoms that may be considered for implementation in clinical settings as an early and quantitative diagnosis criteria.

Keywords: Gait, Sociability, Motor coordination, Cerebellum, Crus I, Crus II, Purkinje cells, mGluR5

Background

Autism spectrum disorder (ASD) is a set of heterogeneous neurodevelopmental alterations characterized by persistent difficulties in verbal and nonverbal communication with restricted and repetitive patterns of behavior [1]. ASD can be diagnosed during childhood and affects 3 times more males than females [2]. The basis for the

*Correspondence: mohamed.jaber@univ-poitiers.fr
†Emmanuel Matas and Alexandre Maisterrena have contributed equally to this work
[1] Université de Poitiers, INSERM, Laboratoire de Neurosciences Expérimentales et Cliniques, 86073 Poitiers, France
Full list of author information is available at the end of the article

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male bias in ASD is unknown with theories including the "extreme male brain", hormonal differences, and genetic influences [3]. For instance, increased levels of testosterone have been correlated with autistic traits in toddlers and young children [4]. Male bias in ASD and intellectual disability have also been associated with rare mutations at chromosome Xp22.11 but that affect less than 1% of patients [5]. In the absence of biological markers, ASD is currently diagnosed based on clinical scales focused on behavioral symptoms. There is presently no cure for ASD but only symptomatic relief to some of its comorbidities such as anxiety, sleep disorders, or seizures [6].

ASD etiology remains elusive, and both genetic and environmental components have been identified [7]. Genetic factors have long been suspected to be involved in ASD, as this is a highly heritable psychiatric disorder [8]. Hundreds of genetic mutations have been associated with ASD in genes such as neurexin (NRXN), neuroligin (NLGN), SH3 and multiple ankyrin repeat domains proteins (SHANK), tuberous sclerosis (TSC)1/2 and fragile X mental retardation (FMR)1, each one contributing to only a small percentage of the disease [9]. Although these mutations only account for approximately 20–30% of ASD cases, many of them seem to converge towards synaptic dysfunction as they are implicated in the formation, the function, and maintenance of synapses, mainly glutamatergic [10, 11]. Based on these findings, the current working hypothesis is that synaptic dysfunction would lead to functional and cognitive impairments observed in ASD [12].

Several genetic mutations linked to these genes have been modeled in mice and were shown to induce behavioral symptoms reminiscent of ASD: deficits in social interaction, impairments in communication, and dysfunctional behaviors [12]. Among these mutations, *Shank3* has received a great deal of attention this last decade [13–15]. Shank (also known as ProSAP) proteins consist of three major isoforms (Shank1, Shank2, and Shank3), and are master scaffolding proteins at excitatory synapses [13, 14]. They interact with more than 30 synaptic proteins through multiple domains and are essential for synaptic formation, glutamate receptor trafficking, and neuronal signaling.

The *Shank3* gene has 22 exons that give rise to numerous mRNA and protein isoforms deriving from multiple intragenic promoters and alternative splicing [11, 13]. Mouse models bearing mutations or deletions in these isoforms have been generated and have been extensively studied this last decade [16]. Although many of these animal models exhibit autism-associated behaviors, suchas social deficits and repetitive behaviors, they do so to different extents and with a great deal of variability in terms of neuropathology and behavior [17–27]. These myriad outcomes can be linked to (1) the existence of several *Shank3* animal models in relation with the tar- geted isoform, (2) lack of systematic use of heterozygote mice, whereas several *Shank3* mutations are in fact a haploinsufficiency in clinical settings, (3) lack of systematic use of female mice in some of these studies and even mixing females with males within the same group of mice, (4) and lack of standardized and robust behavioral procedures.

Here, we aimed at investigating the consequences of a *Shank3* mutation on behavioral parameters that we previously investigated in two different environmental animal models of ASD [28, 29]. These models were obtained following prenatal injection of either valproic acid (Depakine), an anti-epileptic drug targeting the GABA neurotransmission system, or a double stranded RNA (poly IC) mimicking a viral infection and inducing a maternal immune activation. We previously reported that these models recapitulate several ASD symptoms, although at different degrees and magnitude, with a clear sexual dimorphism. Using a full-scale behavioral analysis, we have shown in the VPA model that males and females were differently affected by the treatment with males showing motor, gait and social deficiencies while females showing only motor and gait deficiencies with normal social behavior. At the cellular level, decreased number of cerebellar Purkinje cells (PC) did not affect the same cerebellar sub-regions in males and females. Similar results were obtained with the poly IC injection, although at lower magnitude, where males were also more affected by the treatment than females. These findings recapitu-late the spectrum distribution of ASD that affect differ- ently patients in magnitude and in symptomatology witha clear sexual dimorphism.

In line, we investigate here behavioral, cellular, and molecular consequences of lack of *Shank3* expression [17]. In this animal model Shank3 is lacking the entire C-terminal region, including the Homer-binding site in the sterile alpha motif (SAM) domain. This results in a partial or total loss of the major naturally occur- ring isoforms of Shank3 proteins in heterozygotes and homozygotes, respectively. This mutation was chosen as it has strong construct validity given that it mimics a human mutation, which is not the case of several other *Shank3* mutations in mice. Major behavioral deficits were reported in these mice, including social preference deficits and repetitive behaviors, but not always using both males and females or Shank3+/ΔC and Shank3 ΔC/ΔC mice [22–25]. Here we used wild type, Shank3+/ΔC, and Shank3 ΔC/ΔC mice, and we analyzed males and females separately. A special focus was made on cerebellar-related parameters as we have previously shown that motor dysfunction and gait deficits are strongly correlated with the

degree of severity of ASD in terms of social symptomsand loss of PC. Standardized behavioral procedures were used and were previously validated in two environmental models of ASD using a prenatal injection of either valproic acid (VPA) or the double-stranded RNA Poly: IC, inducing a maternal immune activation (MIA) [28, 29].

We report here that loss of major Shank3 isoforms induces several social, motor, and gait behavioral phenotypes reminiscent of ASD symptoms in humans. These are accompanied by a restricted decrease in both cerebellar PC cell number and mGluR5 levels. While social interaction deficits were observed in Shank3 ΔC/ΔC and Shank3+/ΔC, in both males and females, other parameters investigated affected mostly male Shank3 ΔC/ΔC mice.

Methods

Design, and setting

Shank3+/ΔC mice with a C-terminal deletion of *Shank3* at exon 21 (Shank3tm1.1Pfw/J) were obtained from Jack- son laboratories and are on a C57BL/6J genetic back-ground (Jackson Laboratories, USA); they were originallydonated by Paul Worley from Johns Hopkins Univer- sity School of Medicine [17]. Shank3+/ΔC mice were mated to generate all mice of all three genotypes: WT, Shank3+/ΔC, and Shank3 ΔC/ΔC. Sex and age-matched pups were separated from the dams on postnatal day 28 (P28) and were then genotyped and raised by groups of4 of the same genotype in a randomized fashion to avoid littereffects. All litters of the three genotypes and two sexes were used for this study. Outliers and mice that did not perform a given test appropriately (not finishing the walking beam, jumping out of the apparatus, stalling or reversing during gait…) were taken out from the study. Comprehensive behavioral screening was performed between postnatal days 50 and 60 (P50 and P60) as we previously described in two environmental ASD mouse models [28, 29]. Mice were tested during their light cycle and the investigators were blind to genotype. At the end ofthe behavioral experiments, mice were sacrificed and brains were harvested for histological analysis, RT-PCRq, or western blotting experiments.

Animals

Animal housing and experimental procedures were performed under the European Union directive (2010/63/ EU) and validated by the regional ethical committee (Approval # 2015020415093780). Mice were housed in ventilated cages with access to food and water ad libitum. Room temperature was maintained at 23 °C on a 12 h light/dark cycle. Offspring were segregated into 6 differ-ent groups: wild type males (n = 47), Shank3+/ΔC males (n = 71), Shank3 ΔC/ΔC males (n = 27), wild type females (n = 39), Shank3+/ΔC females (n = 58) and Shank3 ΔC/ΔC females (n = 26). A set of these mice was used for every procedure, as detailed below.

Behavioral analysis

All behavioral tests were performed as we previously described in detail in two different environmental ASD mice models [28, 29].

Social interaction was assessed using the three- chambers test (3-CT) in an apparatus that consists of a Plexiglass box (60 × 45 × 22 cm) partitioned into three chambers with retractable doorways. Mice were moni- tored using an unbiased automated video tracking sys- tem (VideoTrack, Viewpoint-France) which calculates several parameters for each observed animal such as position, speed, trajectory, and various other behaviors set up by the experimenter. Data were retrieved using the VideoTrack 3.2 software (Viewpoint-France). Micewere habituated to the 3-CT for 20 min 2 days before phase 1 that consists of two identical non-social stimuli (inverted wire-cups) placed in the opposite chambers. The second phase comprises a non-social stimulus and a social stimulus (a naïve mouse with no previous contact with the tested mouse). The naïve mouse is of the samesex and age and is usually used for two tests per day at the most, with a 2–3 h rest between tests; no mouse is used more than 2 consecutive days. In the third phase, an additional and novel mouse was placed in the cup pre-sent in the opposite chamber. After a habituation phase of 10 min in the central chamber, mice underwent the three phases of the experiment, with each phase being of 10 min each during which time spent in each chamber and around each cup was recorded. Mice used for this procedure were Wild type males (n = 13), Shank3+/ΔC males (n = 20), Shank3 ΔC/ΔC males (n = 18), wild type females (n = 14), Shank3+/ΔC females (n = 12), Shank3 ΔC/ΔC females (n = 16).

Spontaneous grooming behavior was scored in mice placed individually in a clean and transparent cylinder with house bedding. Each mouse was videotaped and rated for 10 min. Cumulative time spent grooming and grooming frequency were evaluated. Wild type males (n = 20), Shank3+/ΔC males (n = 12), Shank3 ΔC/ΔC males (n = 7), wild type females (n = 12), Shank3+/ΔC females (n = 12), Shank3 ΔC/ΔC females (n = 13).

Motor coordination was evaluated using the challeng- ing beam test and which consists of four Plexiglass sec- tions (25 cm each) starting with a width of 3.5 cm and gradually narrowed to 0.5 by 1 cm decrements. Mice werefirst trained for 2 days to traverse the beam, starting at the widest section and ending at the narrowest section that led into the home cage. On the test day, a mesh grid(1 cm squares) was placed over the beam surface. Mice

were videotaped while traversing the grid-surfaced beam for five trials. Mice that stalled during the beam walkor tried to reverse course were not included in the final analysis. Time to traverse, errors, number of steps, and errors per step made by each mouse were measured and averaged. Wild type males (n = 10), Shank3+/ΔC males (n = 20), Shank3 ΔC/ΔC males (n = 14), wild type females (n = 14), Shank3+/ΔC females (n = 17), Shank3 ΔC/ΔC females (n = 12).

Gait was analyzed during spontaneous walk using an automated gait analysis system (Gaitlab, Viewpoint, France). The apparatus is made of a 1.5 m long glass corridor with dim green light beamed into the glass walkway. Each mouse was assessed individually for 3 consecutive runs. Mice that stalled during the beam walk or tried to reverse course were not included in the final analysis. All gait parameters were analyzed with a special focuson (1) stride length: distance between two consecutive placements of the same paw, (2) limb base of support: distance between two pair prints at contact during each step cycle, and (3) pair gap: gap between the placement of the two trailing feet, which measures spatial coordi- nation between the two pairs. Wild type males (n = 11), Shank3+/ΔC males (n = 27), Shank3 ΔC/ΔC males (n = 15), wild type females (n = 11), Shank3+/ΔC females (n = 21), Shank3 ΔC/ΔC females (n = 12).

Tissue processing and immunohistochemistry

Males and females from each genotype were randomly selected for either histological, mRNA, or protein analysis. For histological analysis, mice (wild type males (n = 8), Shank3+/ΔC males (n = 11), Shank3 ΔC/ΔC males (n = 8), wild type females (n = 8), Shank3+/ΔC females (n = 9), Shank3 ΔC/ΔC females (n = 6)) were deeply anesthetized with ketamine-xylazine (120–20 mg/kg) and transcardially perfused with 0.9% saline at 37 °C followed by 4% paraformaldehyde (PFA) at 4 °C. Brains were post-fixed in 4% PFA at 4 °C for 24 h before cryoprotection in 30% sucrose for 48 h. Serial 50 µm (cerebellum) free-floating sections were collected and stored at — 20 °C until use in an anti-freeze solution. Every fourth cerebellar section was mounted on gelatin-coated slides for PC quantification. PC were identified based on their morphology on cresyl violet staining. The PC phenotype was confirmed using calbindin immunohistochemis- try (1:2500; Swant, Cb-38a) [30]. Stereological estimates were performed using the optical fractionator method and systematic random sampling to obtain the total num- ber of cerebellar PC. Each region of interest was out- lined based on the Franklin and Paxinos's mouse brain atlas [31] at 2.5 × objective. Neurons were counted at 40 × objective using the Mercator image analysis system (Explora Nova, France). Upper and lower guard zones of 1 µm were set at the top and bottom of the section to exclude lost profiles and each neuron or visible nucleus was counted as previously described [28, 29].

Quantitative immunoblot analysis

Mice from each group [wild type males (n = 4), Shank3+/ ΔC males (n = 8), Shank3 ΔC/ΔC males (n = 8), wild type females (n = 5), Shank3+/ΔC females (n = 8), Shank3 ΔC/ΔC females (n = 9)] were sacrificed, brains were retrieved, and cerebellum region was dissected out and frozen in – 80 C until use. For control purposes, we also used cerebella obtained from offspring of pregnant mice that received VPA injections at E12.5, as previ- ously described [28]. Proteins were extracted using a 1% sodium dodecyl sulfate (SDS) solution in Tris Hcl 0.1 M with EDTA 0.01 M and PMSF, protease inhibitor, and phosphatase inhibitor at 1%. Equal amounts of proteins from cerebellum lysates were separated by SDS-PAGE and migrated proteins were transferred to nitrocellulose membranes (Bio-Rad). After blocking the membranes at room temperature for 1 h in Tris Buffer Solution with Tween-20 0,1 M (TBST) and (pH 7.4, TBS) 5% non-fat milk, the blots were incubated with corresponding pri-mary antibodies at room temperature for 3 h. The blots were then washed three times in TBST and incubated with HRP-conjugated secondary antibodies overnight at4 °C. Following 3 washes in TBST, the blots were incu- bated with ECL reagent (GE Healthcare Life Sciences,NJ, USA). For quantification, the films were scanned bya PXi image system and gray signals were analyzed by GeneTools software (Syngene, Cambridge, UK), and nor- malized to that of corresponding internal controls (actinor β-tubulin III). The primary antibodies mTOR 7C10, P- mTOR ser 2448, ERK1/2, and P-ERK1/2 (Cell Signal- ing Technology, Leiden, The Netherlands) were used atthe dilution 1/1000 to 1/1500. NR2A, NR2B, GluR1, and GAPDH antibodies (Millipore, Paris, France) were used at the dilution of 1/500 to 1/1500. The following dilu- tions were used for antibodies against CAMKII (1/1000), α-tubulin (1/10,000), and β-actin (1/10,000) (Sigma- Aldrich, Lyon, France). Shank3 and mGluR5 antibodies (Abcam, Paris, France) were used at the dilution of 1/500 and 1/5000, respectively. NR1 antibodies (Antibodies Incorporated, California, USA) were used at the dilution of 1/500. GluR2 antibodies (Neuromab, California, USA) were used at the dilution of 1/1000.

RT-PCRq analysis of mGluR5 mRNA levels

Mice from each group (wild type males (n = 7), Shank3+/ ΔC males (n = 8), Shank3 ΔC/ΔC males (n = 8), wild type females (n = 7), Shank3+/ΔC females (n = 8), Shank3 ΔC/ΔC females (n = 8)) were sacrificed, brains were retrieved, and cerebellum region was dissected out

and frozen in -80C until use. Total RNA was isolated using TRIzol Reagent/chloroform, then purified using a NucleoSpin RNA kit (Macherey-Nagel) and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was performed on 1 μg of total RNA for each sample using Verso cDNA Synthesis kit (Thermo Fischer Scientific). qPCR was performed on LightCycler 480 system (Roche Diagnostics, France). Ct values were averaged from triplicates. Results were subtracted to the mean Ct of the housekeeping genes Gapdh, Beta actin and cyclophilin A (ΔCt) and log transformed ($2^{-\Delta Ct}$). mGluR5 primers sequence was designed using Primer-Blast (NCBI); primers were synthesized by IDT: 5′-aggacagataaaggtgatcc-3′ and5′-agatactggactgggatcaa-3′.

Statistical analyses

Data are expressed as mean ± Standard Error of the Mean (SEM) and analyzed using GraphPad Prism-7 software (La Jolla, California, USA). Data followed normal distribution as evidenced by the Shapiro–Wilks normality test with the hypothesis for normality rejected at a p value less or equal to 0.05. Data were analyzed using Student's t test, one-way or two-way analysis of variance (ANOVA) when relevant and as stated for each experiment. Tukey's or Fisher's LSD post-hoc multiple comparisons were applied. Outliers were identified using the Grubbs statistical test with alpha at 0.05. For all analyses, a p value < 0.05 was considered significant.

Results

Shank3+/ΔC and Shank3 ΔC /ΔC mice show impairments in social novelty preference

Social preference and motivation in mice have strong face validity to simple social approach behaviors in humans, which are frequently impaired in ASD [32]. We have used herea standardized 3-CT procedure, as previously described [28, 29], and evaluated social preference and novelty. Surprisingly, we observed social preferences with all genotypes (Males: One-way ANOVA, [F(5,96) = 23.69, $p < 0.0001$]; Females: One-way ANOVA, [F(5,76) = 8.852, $p < 0.0001$]) and in both males (Tukey's post-hoc test: WT, $p < 0.0001$; Shank3+/ΔC, $p < 0.0001$; Shank3 ΔC/ΔC, $p < 0.0001$) and females (Tukey's post-hoc test: WT, $p = 0.026$; Shank3+/ΔC, $p = 0.0039$; Shank3 ΔC/ΔC, $p = 0.023$) (Fig. 1b, d). Indeed, mice from all genotypes and sex spent more time around the cage that contained another mouse than around an empty cage (phase 2). Preference for social novelty is defined as a significant propensity to spend more time with a new mouse than with a familiar mouse. When a novel mouse was then introduced in the opposite chamber (phase 3), only WT mice of both sexes showed social preference to the novel mouse (Males: One-way ANOVA, [F(5,94) = 7.729, $p < 0.0001$]; Females: One-way ANOVA, [F(5,78) = 10.21, $p < 0.0001$]). Tukey's post-hoc test revealed a signifi- cant increase (males: + 94.65%; females: + 146.70%) in the time spent within the cage area of the novel mouse only in the WT group of males ($p = 0.0016$) and females ($p < 0.0001$) but not in either the Shank3+/ΔC or the ΔC/ ΔC groups of males or females (Fig. 1f, h).

These results indicate that Shank3+/ΔC and Shank3 ΔC/ΔC males and females show social preference and motivation but have impaired preference for social novelty as they show no preference for a newly introduced mouse compared to a mouse with which they have previously interacted.

Shank3 ΔC/ΔC mice show increased self-grooming frequency

Self-grooming is an innate behavior in rodents and its alteration is used as an indication of compulsive and repetitive behavior [33]. Motor stereotypies, repetitive and uncontrollable motor behaviors are among the core symptoms of ASD. Most *Shank3* mouse models were shown to display increased self-grooming although with conflicting results in relation to the genotype and the sex of mice [17, 18, 21–24]. Here we have assessed grooming in a novel environment (a transparent cylinder) with home cage bedding (Fig. 2). We report that time spent grooming is not significantly different in relation with genotype in males (One-way ANOVA, [F(2,36) = 2.653, $p = 0.0842$]) and females (One-way ANOVA, [F(2,34) = 0.232, $p = 0.79$]) (Fig. 2a, b). However, there was a significant effect in the number of grooming episodes in relation with genotype (Males: One-way ANOVA, [F(2,36) = 14.71, $p < 0.0001$]; Females: One-way ANOVA, [F(2,34) = 7.951, $p = 0.001$]). Indeed, Tukey's post-hoc analysis showed that the number of grooming episodes increased in both the Shank3 ΔC/ΔC groups of males and female mice ($p < 0.0001$ and $p = 0.03$, respectively) (Fig. 2c, d). These results are in line with previous reports in several mouse models bearing a *Shank3* mutation indicating increased grooming in these mouse models as a key behavioral feature.

Shank3 ΔC/ΔC mice show motor coordination deficits

Motor coordination deficits were found in ASD patients but are not used in the diagnosis criteria [34]. Motor coordination can be explored using the challenging beam test, usually performed in animal models of pathologies affecting motor behavior such as Parkinson's disease [35]. We have recently implemented and validated this procedure in two environmental mouse models of ASD [28, 29]. Here, we report that Shank3 ΔC/ΔC mice display

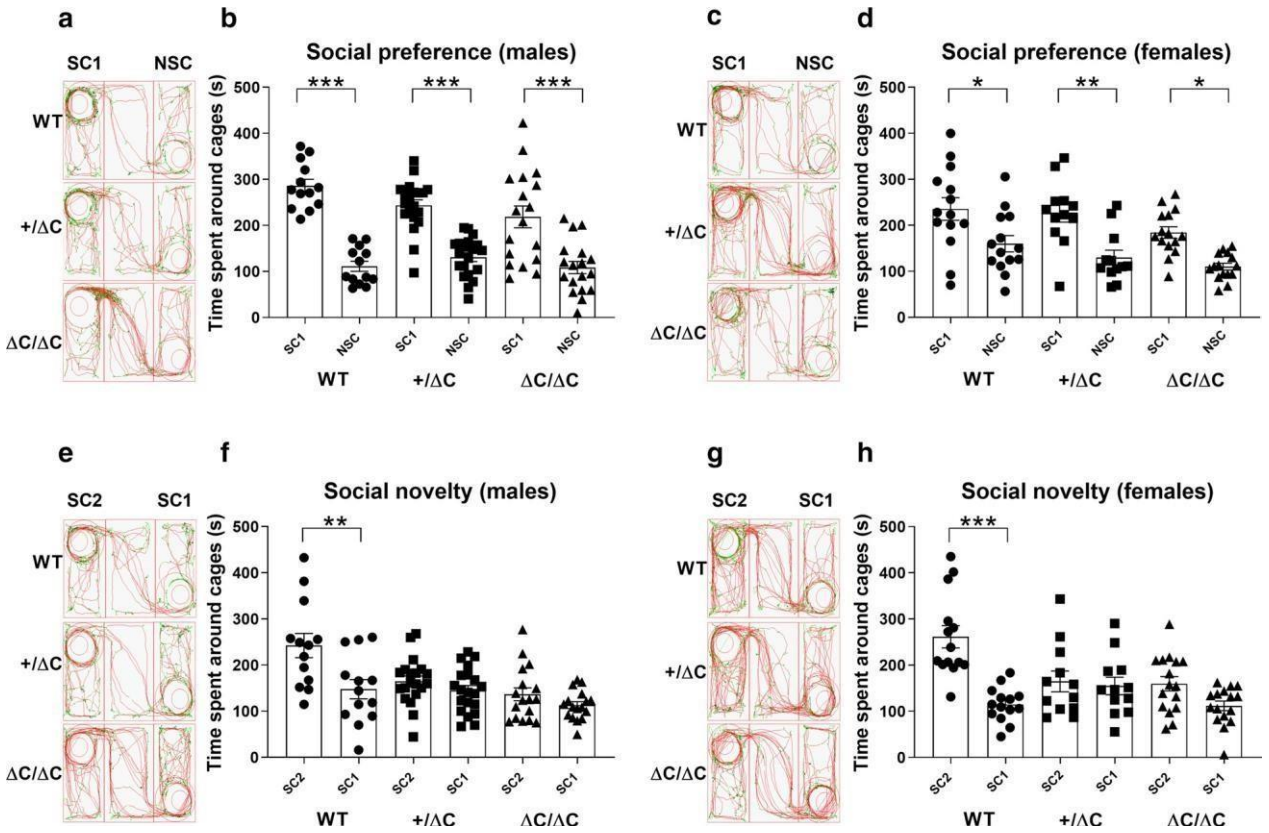


Fig. 1 Social preference in *Shank3* mice assessed through the three-chambers test (3-CT). **a**–**c**–**e**–**g** Representative tracks of mice in the 3-CT. **b**–**d**–**f**–**h** Social behavior analysis. **b**–**d**. Following habituation, mice were put in the 3-CT with one chamber containing an encaged mouse (social chamber 1: SC1) and one chamber with an empty cage (Non-social chamber: NSC) to evaluate social preference. Note that all mice, whatever the sex and the genotype, spent more time in SC1 than in NSC. **f**–**h** Then, a novel mouse was introduced in the chamber 2 (social chamber 2: SC2) in addition to the encaged mouse in SC1 to evaluate social novelty recognition. Note that Shank3 ΔC/ΔC and Shank3+/ΔC mice of both sexes spend equal time interacting with the novel mouse in SC2 than with the mouse in SC1. In comparison, wild type mice spent more time interacting with SC2 than with SC1. All data are expressed as means ± SEM; One-way ANOVA followed by Tukey's multiple analysis was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). WT males $n = 13$; Shank3+/ΔC males $n = 20$; Shank3 ΔC/ΔC males, $n = 18$; WT females $n = 14$; Shank3+/ΔC females n = 12; Shank3 ΔC/ΔC females, $n = 16$

mild motor coordination deficits (Fig. 3). Indeed, there was a significant effect of genotype (One-way ANOVA, [$F(2,40) = 10.07$, $p = 0.001$]) in the number of mistakes while traversing the last, and thus the narrowest, section of the beam in the Shank3 ΔC/ΔC female group (Tukey's post-hoc analysis, $p = 0.0008$) (Fig. 3b). In male Shank3 ΔC/ΔC mice, there was clearly a tendency towards motor deficits but that did not reach significance (One-way ANOVA, [$F(2,41) = 2.758$, $p = 0.075$) (Fig. 3a). Shank3+/ΔC mice did not show any deficits, whatever the sex and the section of the beam.

Shank3 ΔC/ΔC mice show gait abnormalities

Although gait disturbances and motor development delays have been constantly reported in ASD patients [36], they are still not included in the diagnosis criteria. We have previously shown in the VPA and Poly IC ASD mouse models significant alterations in several gait parameters explored here (Figs. 4, 5).

We found that Shank3 ΔC/ΔC mice display several gait abnormalities in greater number in males than in females. For example, in the male group these parameters include the following: (1) swing time (fore left paw: One-way ANOVA, [$F(2,49) = 12.48$, $p < 0.0001$] and fore right paw: One-way ANOVA, [$F(2,49) = 6.869$, $p = 0.002$]). Tukey's multiple analysis showed that fore left paw was significantly decreased by 12.84% in Shank3 ΔC/ΔC mice compared to wild type ($p = 0.011$) and the fore right paw was significantly decreased by 11.23% ($p = 0.036$) (Fig. 4c); (2) stance time was significantly affected in Shank3 ΔC/ΔC mice for the fore right, hind left and hind right paws whereas only a trend towards a decrease was observedfor the fore left paw as revealed by post-hoc analysis.

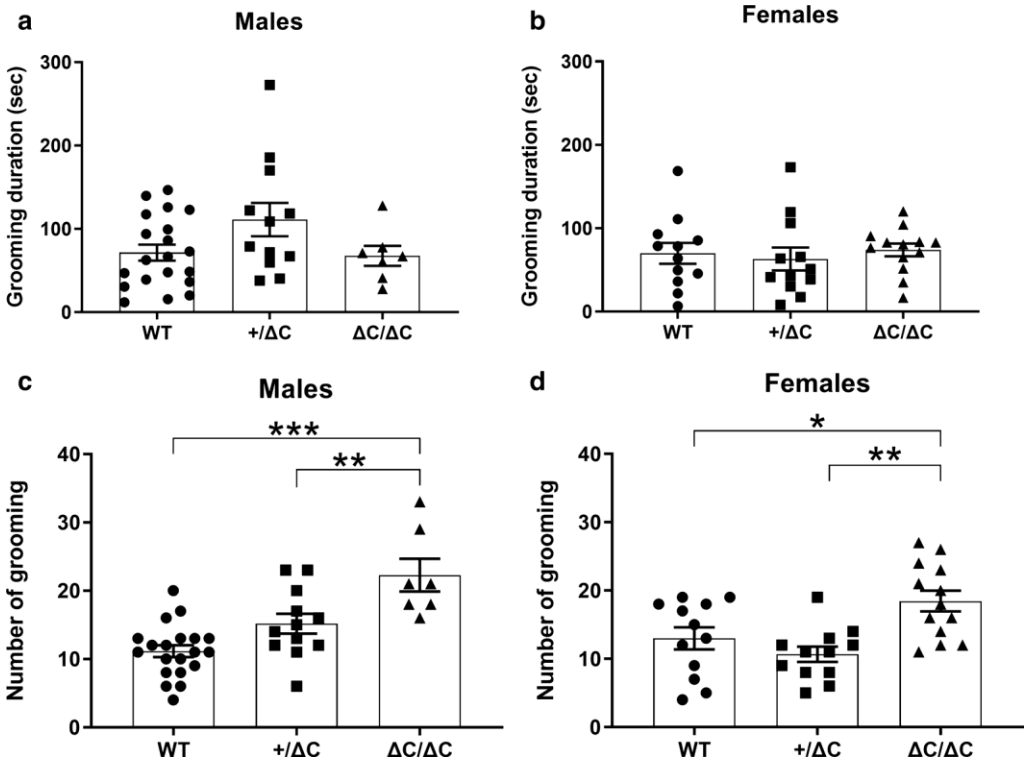


Fig. 2 Stereotyped behavior in *Shank3* mice. **a**–**d** Grooming time and frequency in *Shank3* mice. Mice were placed in a clean cylinder and videotaped. **a**–**c** There was no difference in the time spent grooming within all groups of mice. **b**–**d** However, compared to WT, grooming frequency was increased in Shank3 ΔC/ΔC and Shank3+/ΔC males. Only Shank3 ΔC/ΔC females showed increased grooming compared to WT and Shank3+/ΔC (B). All data are expressed as means ± SEM. One-way ANOVA followed by Tukey's multiple analysis was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) WT males $n = 20$; Shank3+/ΔC males $n = 12$; Shank3 ΔC/ΔC males, $n = 7$; WT females $n = 12$; Shank3+/ΔC females $n = 12$; Shank3 ΔC/ΔC females, $n = 13$

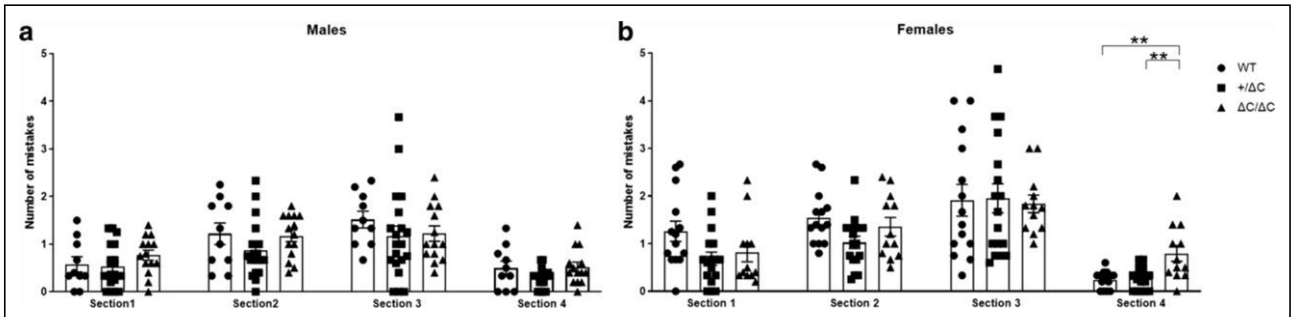


Fig. 3 Motor coordination deficits in *Shank3* mice. Time to traverse the beam was only slightly affected in the last section of the beam, and which is the narrower, in Shank3 ΔC/ΔC females. This is indicative of motor coordination deficits. All data are expressed as means ± SEM. One-way ANOVA followed by Tukey's multiple analysis was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) WT males $n = 10$; Shank3+/ΔC males $n = 20$; Shank3 ΔC/ΔC males, $n = 14$; WT females $n = 14$; Shank3+/ΔC females $n = 17$; Shank3 ΔC/ΔC females, $n = 12$

Tukey's multiple analysis showed a significant decrease of the stance time by 26.18% for Shank3 ΔC/ΔC mice hind left paw [$F(2,49) = 9.003$, $p > 0.01$] (Fig. 4b); (3) stride fre- quency was also affected (One-way ANOVA followed by Tukey's multiple analysis (WT vs Shank3 ΔC/ΔC);

Fore left paw: [$F(2,49) = 9.431$, $p = 0.02$], fore right paw: [$F(2,49) = 9.253$, $p = 0.02$], hind left paw: [$F(2,49) = 8.265$, $p = 0.03$] and hind right paw: [$F(2,49) = 8.675$, $p = 0.03$]) (Fig. 4a); (4) left pair gap (One-way ANOVA followed by Tukey's multiple analysis (WT vs Shank3 ΔC/ΔC):

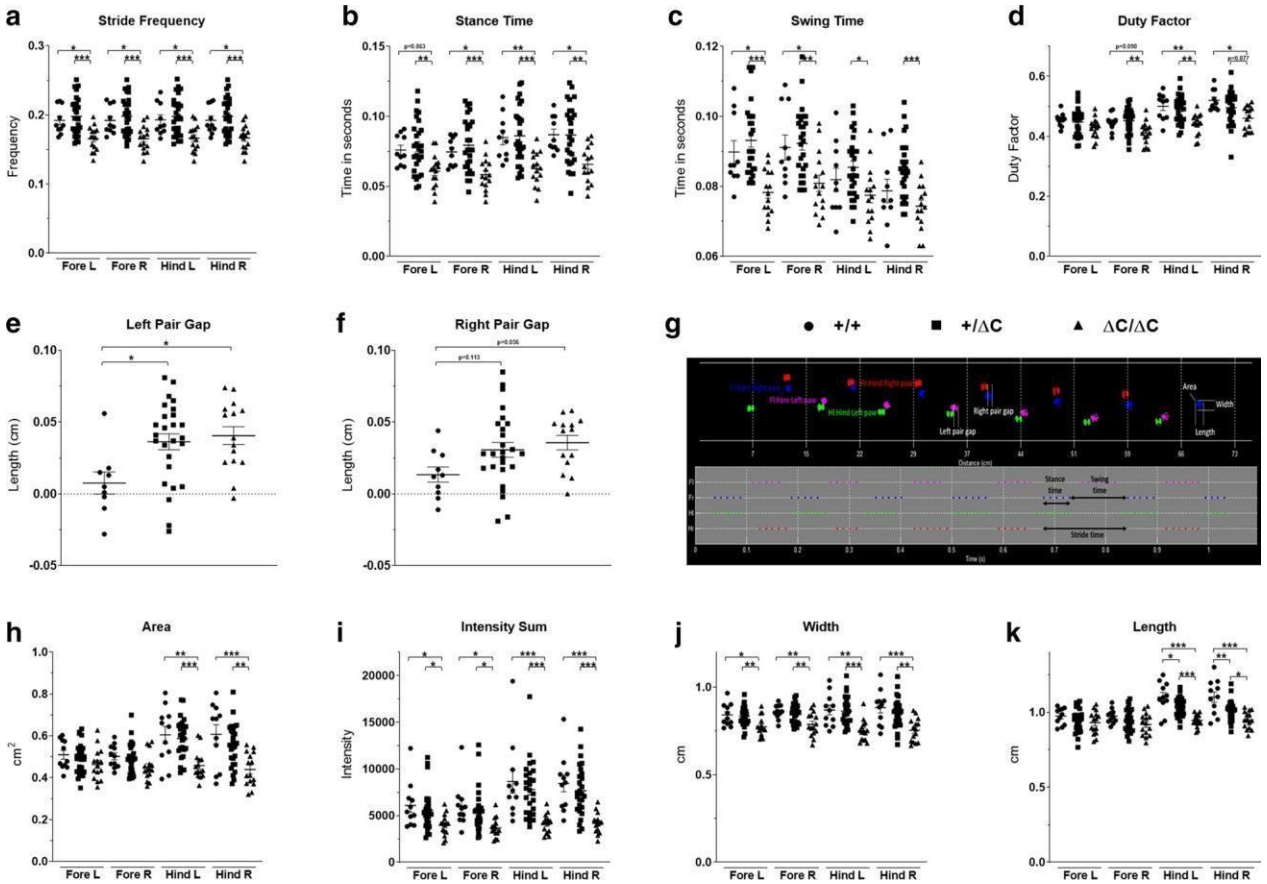


Fig. 4 Gait analysis in *Shank3* males. Gait was analyzed during spontaneous walk using an automated gait analysis system (Viewpoint, France). Ten parameters were analyzed: **a** stride frequency, **b** stance time, **c** swing time, **d** duty factor, **e**, **f** pair gap, **h** area, **i** intensity sum, **j** width, **k** length and **g** representative spatio-temporal gait analysis. All data are expressed as means ± SEM. One way ANOVA followed by Tukey's multiple analysis was performed. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) WT males $n = 11$; Shank3+/ΔC males $n = 27$; Shank3 ΔC/ΔC males, $n = 15$

([F(2,47) = 5.026, $p = 0.012$]) (Fig. 4e, f) and also area (Fig. 4h), width (Fig. 4j) and length (Fig. 4k) of the footsteps. Duty factor, which is the ratio of the limb stance time and limb stride time, was decreased only in Shank3 ΔC/ΔC males (One-way ANOVA followed by Tuk- ey's multiple analysis when applicable: Fore left paw:[F(2,49) = 1.62, $p = 0.21$]. Fore right paw: [F(2,49) = 5.372, $p = 0.1$], hind left paw: [F(2,49) = 8.365, $p = 0.01$] and hind right paw: [F(2,49) = 4.894, $p = 0.011$]. Intensitysum, defined as the sum of the work exerted through body movement and the resting, was decreased in both male and female Shank3 ΔC/ΔC compared to wild type (One-way ANOVA followed by Tukey's multiple analysis: ([F(2,50) = 4.423, $p = 0.027$] and [F(2,41) = 6.165, $p = 0.003$], respectively) (Figs. 4i, 5h).

Similar alterations were also observed in the Shank3 ΔC/ΔC female group but implicating less gait param- eters than in males. For instance, Tukey's multiple analysis showed a significant decrease of the swing time by 19.71% for Shank3 ΔC/ΔC mice fore left paw [F(2,41) = 15.25, $p < 0.0001$] and by for 20.79% the fore left paw [F(2,41) = 15.01, $p < 0.0001$]. In addi- tion, one-way ANOVA followed by Tukey's multiple analysis showed that swing time fore left paw [F(2,41) = 15.25, $p = 0.004$] and swing time fore right paw [F(2,41) = 15.01, $p = 0.016$] were significantly reduced in female Shank3+/ΔC mice compared to WT (Fig. 5c). Stance time was also significantly affected in Shank3 ΔC/ΔC and Shank3+/ΔC females. For exam- ple, One-way ANOVA followed by Tukey's multiple analysis showed the following differences between WT and Shank3 ΔC/ΔC in fore leftpaw [F(2,41) = 6.908,$p = 0.002$], fore right paw [F(2,41) =6.797, $p = 0.002$], fore left paw [F(2,41) = 6.908, $p = 0.05$] and fore right paw [F(2,41) = 6.8, $p < 0.05$]) (Fig. 5b). For stride frequency, a significant decrease was observed for the fore and hind paws of Shank3 ΔC/ΔC females com- pared to WT: fore left paw [F(2,41) = 10.29, $p = 0.0001$],

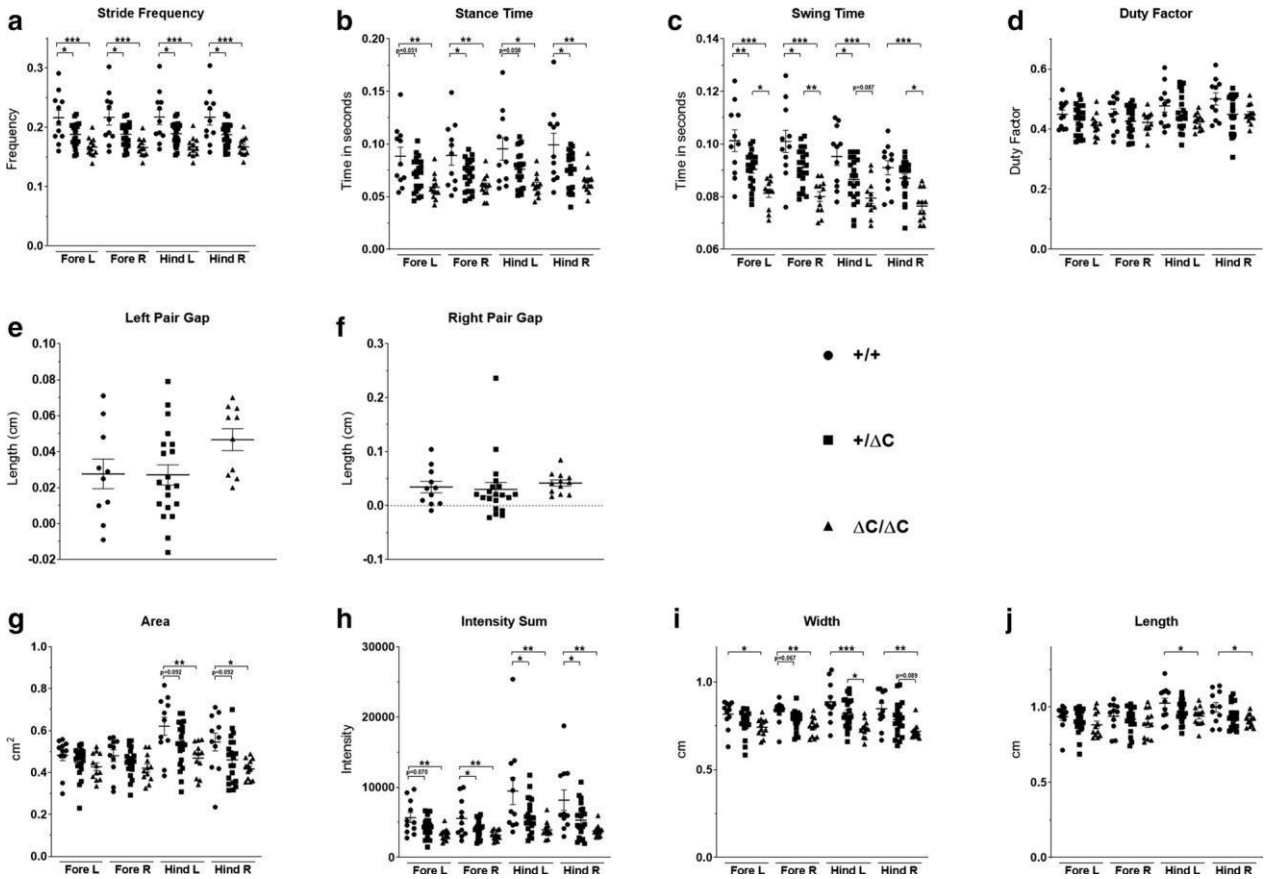


Fig. 5 Gait analysis in *Shank3* females. Gait was analyzed during spontaneous walk as with males. The following parameters were analyzed: **a** stride frequency, **b** stance time, **c** swing time, **d** duty factor, **e**, **f** pair gap, **g** area, **h** intensity sum, **i** width, **j** length. All data are expressed as means ± SEM. One way ANOVA followed by Tukey's multiple analysis was performed. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) WT females $n = 11$; Shank3+/ΔC females $n = 21$; Shank3 ΔC/ΔC females, $n = 12$

fore right paw [F(2,41) = 10.35, $p = 0.0001$], hind left paw [F(2,41) = 10.55, $p = 0.0001$] and hind right paw [F(2,41) = 10.09, $p = 0.001$]). There were also differ- ences in this parameter in Shank3+/ΔC females com- pared to WT: Fore left paw [F(2,41) = 10.29, $p = 0.017$], fore right paw [F(2,41) = 10.35, $p = 0.018$], hind left paw [F(2,41) = 10.55, $p = 0.018$] and hind right paw [F(2,41) = 10.09, $p = 0.015$]) (Fig. 5a). Duty fac- tor was not affected in Shank3 ΔC/ΔC females com- paredto WT: Fore left paw [F(2,41) = 1.861, $p = 0.17$],fore rightpaw [F(2,41) = 1.277, $p = 0.29$], hind left paw [F(2,41) = 2.524, $p = 0.09$] and hind right paw [F(2,41) = 3.206, $p = 0.051$]) (Fig. 5d).

Taken together, gait analysis indicates major abnor- malities mainly in Shank3 ΔC/ΔC mice, with more parameters encountered in males than in females.These abnormalities are often of spatio-temporal naturerather than anatomical [37].

Male Shank3 ΔC/ΔC mice show decreased Purkinje cell number

We aimed at determining the effect of *Shank3* mutation on cell numbers in the Crus I and Crus II cerebellar sub- regions that are known to play a major role in movement regulation, exploratory behavior, stereotyped and repeti- tive behaviors [38] but also in social interactions [39]. Several clinical studies in ASD [40, 41] and explorations in animal models, including our own [28, 29, 42], have consistently shown a decrease in the number of these neurons in the cerebellum and mainly within the Crus I and/or Crus II sub-regions.

Here, we report that the *Shank3* mutation resultedina decreased number of PC in Crus I in both malesand females (Fig. 6). Indeed, two-way ANOVA anal- ysis showed a clear genotype (Two-way ANOVA, [F(2,44) = 5.92, $p = 0.0053$]) and sex effect (Two-way ANOVA genotype x sex interaction, [F(2,44) = 3.741,

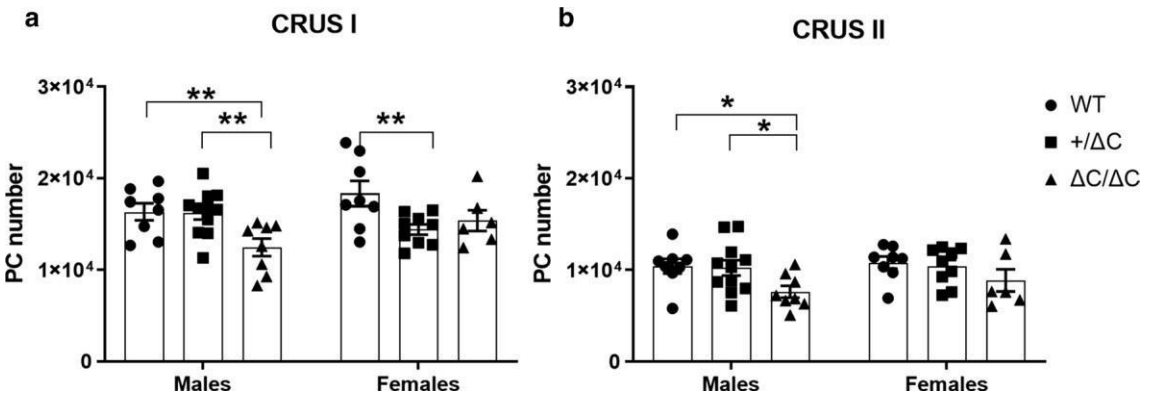


Fig. 6 Purkinje cell (PC) number in *Shank3* mice. Stereological PC counts on coronal sections of the cerebellum in the Crus I (**a**) and the Crus II (**b**) sub- regions. Only male Shank3 ΔC/ΔC mice showed a decrease in the number of PC in the crus I and Crus II cerebellar sub-regions. Female + / ΔC mice showed a slight decrease in PC number in the Crus I subregion. All data are expressed as means ± SEM. Two-way ANOVA was performed (*p < 0.05, **p < 0.01, ***p < 0.001) WT males n = 8; Shank3+/ΔC males n = 11; Shank3 ΔC/ΔC males, n = 8; WT females n = 8; Shank3+/ΔC females n = 9; Shank3 ΔC/ΔC females, n = 6

p = 0.032]). Fisher's LSD post-hoc analysis further indicated that the Shank3 ΔC/ΔC male group had a lower number of PC than wild type in both the Crus I and CrusII cerebellar area (p = 0.0066 and p = 0.0206, respec- tively) (Fig. 6a, b). Females Shank3+/ΔC mice showed a mild but significant decrease in PC number in the CrusI subregion (Two-way ANOVA followed by Fisher's LSD post-hoc analysis, [F(2,44) = 5.92, p = 0.005]) (Fig. 6a). In conclusion, the lower number of PC affects mainly Shank3 ΔC/ΔC male mice as also reported in the VPA and poly IC mouse models.

Male Shank3 mice show decreased cerebellar mGluR5 levels

The *Shank3* C-terminal region (exon 21–22), which is mutated in these mice, contains binding sites for mGluR5 and plays a crucial role in the synaptic targeting and postsynaptic assembly of Shank3 scaffolding complex [43–45]. We thus explored the cerebellar levels of this receptor and associated scaffolding proteins, along with a set of other glutamate receptors and postsynaptic proteins (Fig. 7). Surprisingly, only mGluR5 levels were dramatically decreased and this was observed only in Shank3+/ΔC and Shank3 ΔC/ ΔC males. Indeed, ANOVA analysis showed a genotype effect (One-way ANOVA, [F (2,16) = 6.368, p0.0092]) and Tukey's multiple analysis revealed decreased lev- els of mGluR5 in males Shank3+/ΔC and Shank3 ΔC/ΔC mice compared to WT (p = 0.022 and p = 0.009, respectively). This decrease was dramatic in mag- nitude (down to − 71.32%) in male Shank3 ΔC/ΔC mice (Fig.7a). In order to determine whether this decrease was specific to the *Shank3* mutation, we also evaluated levels of mGluR5, and NR1, NR2A and NR2B glutamate receptors' subunits in a mouse model of prenatal VPA injection as described [28], and found no modifications in these levels (t(8) = 0.8569, p = 0.4164 for mGluR5, t(8) = 0.8427, p = 0.4239 for NR1, t(8) = 1.574, p = 0.1540 for NR2A, t(8) = 1.31, p = 0.2266 for NR2B). We further evaluated mGluR5 mRNA levels by RT-PCRq in order to determine whether the decrease in mGluR5 protein levels wasdue to transcriptional or translational regulations and found no alterations in the corresponding mRNA levels. Males: One-way ANOVA, [F(2,20) = 1.748, p = 0.1996]) and females: One-way ANOVA, [F(2,19) = 1.985, p = 0.1648]).

Discussion

This study aimed at investigating several behavio- ral, cellular, and molecular outcomes related to ASD in Shank3+/ΔC and Shank3 ΔC/ΔC male and female mice with a mutation that is also observed in human conditions. The *Shank3* gene encodes a master scaffolding protein in the postsynaptic density where it interacts with mul- tiple key synaptic components, mainly implicating the glutamate receptor clusters, along with the cytoskeleton and signal transduction cascades [10, 26, 43, 44, 46]. The gene is located on chromosome 22q13.3 in humans and was first implicated in ASD in the 22q13.3 microdeletion syndrome, also known as Phelan-McDermid Syndrome [13]. Haploinsufficiency of the *Shank3* gene accounts for 0.5–2.0% of ASD and intellectual disability cases [16, 47]. Human genetic studies of ASD have found that the

(See figure on next page.)

Fig. 7 Brain levels in PSD proteins and mRNA in ASD mouse models. **a**–**c** Expression profile of Shank3 and associated postsynaptic scaffolding proteins in males and females *Shank3* mice within the cerebellum by immunoblot analysis. **b**–**d** Representative figures of the western blot. Note that only mGluR5 was significantly decreased in Shank3+/ΔC and ΔC/ΔC males compared to wild type. No decreases were present in Shank3 ΔC/ΔC and Shank3+/ΔC females. Experiments for western blots were repeated at least three times and statiscal analysis were performed with a one-way ANOVA followed by Tukey'smultiple analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) WT males $n = 4$; Shank3+/ΔC males $n = 8$; Shank3 ΔC/ΔC males, $n = 8$; WT females $n = 5$; Shank3+/ΔC females $n = 8$; Shank3 ΔC/ΔC females, $n = 9$. **e** No difference in the protein levels of several glutamate receptors was found in the male cerebellum of VPA ASD mouse model whatever the prenatal treatment (Saline, $n = 5$; VPA, $n = 5$, Student's t test, $p > 0.05$). **f** No difference in the mGluR5 mRNA levels was found in the cerebellum whatever the genotype or sex (wild type males ($n = 7$), Shank3+/ΔC males ($n = 8$), Shank3 ΔC/ΔC males ($n = 8$), wild type females ($n = 7$), Shank3+/ΔC females ($n = 8$), Shank3 ΔC/ΔC females ($n = 8$), One-way ANOVA, $p > 0.05$). All data are expressed as means ± SEM

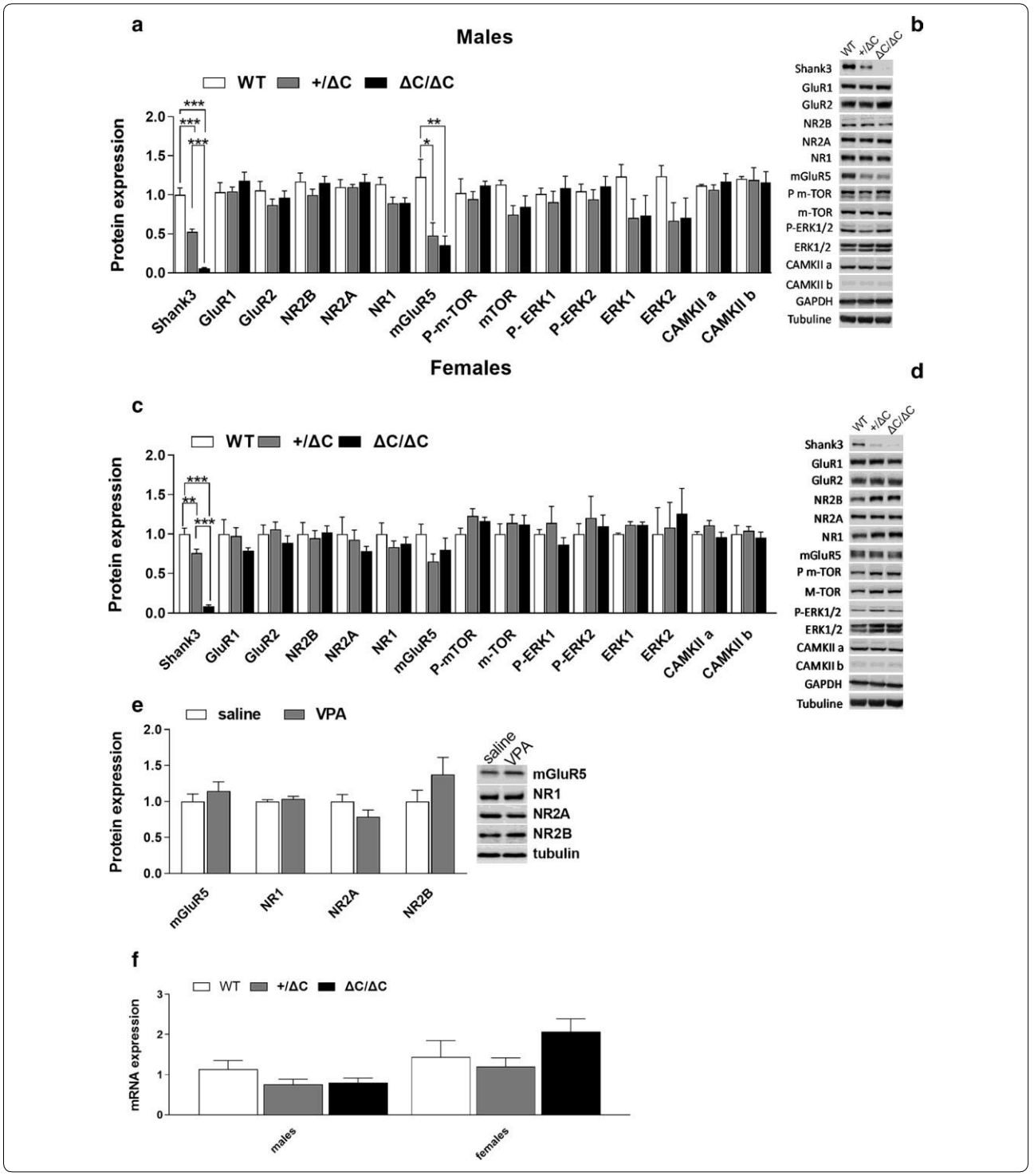
C-terminal region (exon 21) of *Shank3* harbors several mutations [16]. This region encodes binding sites for actin/Cortactin and mGluR5/Homer and plays a crucial role in the synaptic targeting and postsynaptic assembly of Shank3 scaffolding proteins [48]. A dozen different genetically alerted mice with various *Shank3* point mutations or deletions of a given exon have been constructed [17–19, 24, 26, 42, 49]. These mice show variable outcomes at the behavioral, cellular, and molecular levels.

We have set up our behavioral, cellular, and molecular analysis in a *Shank3* mouse model [17] based on our previous and recently reported findings in two different environmental mouse models of ASD [28, 29]. These models were obtained following prenatal injection of either VPA or poly IC and recapitulate several ASD symptoms, although at different degrees and magnitude, with a clear sexual dimorphism.

Here, we show that a *Shank3* mutation affects mostly male homozygote mice in several social and motor behavioral parameters. This is accompanied by a significant reduction in cerebellar PC and specific decreased levels of mGluR5 proteins. We first investigated social preference parameters, as they constitute one of the hallmarks of ASD [50]. We report that Shank3+/ΔC and Shank3 ΔC/ΔC males and females both have normal social preference and motivation but show impairments in social novelty preference. Of interest is the fact that ASD patients are reported to avoid unfamiliar social partners and display diminished interest in novelty [50]. Indeed, ASD patients tend to avoid social contacts with a new individual compared to someone familiar [51].

Self-grooming is a core behavior for mice that spend about 40% of their waking time performing it. It is aimed to maintain physiological stasis and comfort and thus tends to increase under stressful situations and in path- ological conditions implicating motor brain centers as reported in clinical and animal models of ASD [33]. Here, we replicated findings in these animal models showing increased grooming, reflective of stereotyped repeated behavior. We further show that this increased grooming affects both Shank3 ΔC/ΔC males and females.

In the initial paper using this line of mice [17], authors have investigated a wide range of behavioral parameters including motor-coordination deficits, grooming and novelty avoidance, but only in homozygote mice. Of interest is the observation that homozygote mice showed social preference in the phase 2 of the 3-CT but no preference for social novelty (phase 3), a finding that we replicated here both in males and females. In the report by Duffney et al., (2015), Shank3+/ΔC did show social preference in phase 2 trial but that was of a lesser magnitude than in wild type animals [18]. Qin et al. [19] used the 3-CT with only two phases and showed no social preference in Shank3+/ΔC mice, mixing measurements of 'investigating behavior" and time spent in the area surrounding the cup with the social cue. Using a similar mutation leading to a premature stop codon in exon 21 of *Shank3*, Speed et al. [24] found no social interaction deficits in both heterozygote or homozygote mouse mutants. Also in this paper, homozygote mice, but not heterozygotes, showed deficits in hippocampus-dependent spatial learning, impaired motor coordination, altered response to novelty, and sensory processing deficits. Jaramillo et al. [22] used a different approach to generate Shank3 mutant mice with a loss of the two higher molecular weight isoforms by disrupting the PDZ domain with a transcrip- tional stop cassette prior to exon 13. Homozygote mice with this mutation, but not heterozygotes, displayed a preference for the social target over the inanimate object (phase 2), a finding that we replicated here. In the phase3 of the 3-CT, neither heterozygote nor homozygote mice displayed preference to social novelty as also we show here for homozygote mice. In addition, interaction time and approach were altered only in homozygote animals, in line with our findings of altered social behavior only in homozygotes. In the grooming behavior, both heterozygotes and homozygotes displayed increased time spent grooming compared to wild type, but only homozy- gotes showed increased grooming bouts, a finding thatwe also replicate in the *Shank3* mouse models that we used. Although apparently conflicting, collectively these results show alterations in grooming and social behavior in *Shank3* mutant mice, which are often present only in



homozygotes or at least with a much higher degree than in heterozygote mice.

Several reports in ASD described impairments in visio-motor and manual dexterity tasks, limb coordination during tasks requiring balance, agility, and speed, as well as in gait and ataxia [52]. Furthermore, motor impair-ments may be amongst the earliest signs of some formsof ASD and their assessment might help the early and quantitative diagnosis of the pathology and the identi- fication of dysfunctional brain regions and circuits in

ASD [53]. Here, we found only mild motor coordination impairments in this mouse model. These impairments were revealed only when the task required significant challenge (a very narrow beam with a grid mech above it). This is in line with our findings with a Poly IC environmental model where no deficiencies were observed in this task [29].

One of the most robust and consistent findings in our previous studies and here are anomalies in gait. Early studies in ASD patients have revealed irregular gait [54]. Quantitative measures indicated increased variabilityand irregularity in gait suggestive of cerebellar, rather than basal ganglia, involvement. Later studies reported increased missteps, increased step width, and higher ataxia ratios similar to what is found in patients with cerebellar lesions [55]. In fact, several spatiotemporal gait parameters of children with ASD were similar to those of patients with cerebellar ataxia [56]. Of interest is the fact that abnormal gait patterns seem to be correlated with the severity of social impairments in both humans [57] and ASD animal models [28]. Here, we have used an automated rodent quantitative gait analysis system with optical touch sensors, which records and tracks rodents' footprints as they move freely in a walkway. This allowed qualitative and quantitative readout of footprint pattern and contacts, highlighting gait abnormalities. As nearly all gait parameters are correlated, a collection of these parameters is needed to determine the potential altera- tions' nature. Gait is made up of strides that comprise a stance phase with the foot in contact with the ground anda swing phase with the foot off the ground. Fundamental descriptors of gait can be divided into two main sections:
(1) spatial patterns that include stride length, step length, and step width and (2) temporal parameters including duty factor (limb stance time divided by stride time) and limb phase (the time between forelimb and hindlimb foot- strike on the same side divided by the stride time). We report here that several temporal parameters were altered and that these alterations were mainly found in Shank3 ΔC/ΔC mice and to a wider extend in males.Interestingly, spatial patterns did not seem to be alteredby the mutation (stride length did not differ between groups), indicating no major anatomical alterations in these mice. However, all temporal parameters measured were reduced in Shank3 ΔC/ΔC mice (stance time, swing time, stride frequency, and duty factor). Along with these temporal deficiencies, there were also alterations of the way paws are applied to the surface in terms of surface contact and strength, and that were also reduced mainlyin both males and females Shank3 ΔC/ΔC.Also of inter- est is the fact that more gait parameters wereaffected in males than in females. The temporal and surface altera- tions observed are probably not due to change in velocity as only females, but not males, Shank3 ΔC/ΔC mice showed a mild increase (less than 20%) in velocity; in addition, there was no change in the regularity of gait nor the stride length. Altogether, these gait elements argue against an anatomical alteration and a primary motor disorder implicating the basal ganglia. In contrast, they seem to point to a cerebellar dysfunction as they mainly affect movement coordination, a cerebellar function, which is in line with clinical findings (see for instance reference [56]).

Gait disturbances, and more generally behavioral abnormalities in neurodevelopmental disorders including ASD, are a consequence of underlying alterations in circuit maturation and function. Post-mortem and brain imaging studies have consistently identified the cerebellum as one of the most abnormal brain regions associated with ASD with a specific reduction of cerebellar PC [40, 41]. Here we show a decreased number of PC within the hemispheric part of lobule VII, within the in Crus I and Crus II subregions and only in Shank3 ΔC/ΔC males. Females showed only a mild decrease in PC number in Shank3+/ΔC. This is in line with VPA and Poly IC models showing also decreases of PC in these brain areas and that were more pronounced in males than in females. Cerebellar defects seem to be widespread in ASD mouse models. Using five different ASD mouse models, including the one used in this study, Kloth et al. [49] have shown major cerebellar associative sensory learning deficits with the eyeblink conditioning task, a task that relies on cerebellar plasticity. Further on, mice with a Tsc1 mutation specifically in cerebellar cells PC showed ASD-related altered behavior that was rescued with rapamycin treatment targeting mTOR signaling further pointing to these cells as key players in ASD [42].

In this line, we investigated the levels of dozens of cerebellar proteins implicated in the cerebellar glutamatergic transmission and signal transduction. In accordance with previous findings in cortical or hippocampal areas, we did not detect any difference in the cerebellar expression level of several glutamatergic related proteins [18, 19, 24]. We further extend these findings and show here a dramatic decrease in the levels of cerebellar mGluR5 protein, and only in male Shank3 ΔC/ΔC, pointing to that receptor as the major target of Shank3 and perhaps even the major component underlying the observed behavioral deficits linked to this mutation. These decreased protein levels were probably due to post-translational regulation as we did not detect any alterations in the corresponding mRNA levels. Of interest is also the fact that such a decrease was not observed in the VPA environmental ASD mouse model. Shank3 is essential to mediating mGluR5 signaling by recruiting Homer1b/c to the postsynaptic density [17, 18, 44]. Pharmacological

enhancement of mGluR5 activation in *Shank3* knock-out mice, through the administering 3-Cyano-*N*-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide, ameliorated functional and behavioral defects [58], suggesting that pharmaceutical treatments that increase mGluR5 activity may represent an interesting approach for treating ASD patients with a *Shank3* mutations [18, 59, 60]. In this line, restoration of Shank3 expression in adult using a *Shank3* conditional knock-in procedure in mice, rescued several ASD phenotypes through improvements in synaptic protein composition, spine density, and neural function [27]. This is of interest as it further emphasis the major roleof Shank3 in postsynaptic function and indicates that this neurodevelopmental pathology may in fact be manageable at adult age in patients with *Shank3* mutations.

Shank3 mutations in ASD patients are either point mutations in one copy of the *Shank3* gene or haploinsufficiencies where a single functional *Shank3* allele is insufficient to maintain a normal behavior [14, 61]. Thus, one would have expected that Shank3+/ΔC mice would show clear behavioral alterations relevant to ASD. In our hands however, Shank3+/ΔC mice showed deficienciesin the social novelty recognition task and did not show other behavioral or cellular dysfunction, despite over a 50% decrease in mGluR5 expression. This is in line with previous publications with this or similar mouse model [17, 24] but at odds with other previous reports exploring behavioral and cellular consequences only in males [18, 19].

Several studies utilizing *Shank3* mutations or deletions do not report measurable changes in animal behavior. For instance, exons 4–9 of *Shank3* (JAX 017890) show only mild ASD-related phenotype and have normal sociabil- ity and social novelty in the 3-chamber test [61]. Muta- tions targeting exons 4–7 only yielded mild behavioral phenotypes, while targeting exons 13–16 (JAX 017688) showed profound phenotypes such as impaired sociabil- ity and preference for social novelty, as well as reduced pair interaction, and profound self-grooming leading to skin lesions [62]. Targeting exon 11 induced prominent self-grooming and skin lesions [63], while targeting exon 21 of *Shank3* (JAX 018398) results in normal sociability but impaired social novelty in the 3-chamber test [17], as observed in our study here.

Limitations

The construct validity of several, but not all, of the *Shank3* mouse lines is strong as they tend to reproduce human mutations. However, several of these mouse models may have limited face validity, given that they sometimes fail to reproduce behavioral and cellular symptoms reported in ASD patients. Another element to take into consideration is the fact that each of these mutants targeted only a subset of *Shank3*'s coding exons. Consequently mice only lacked a subset of *Shank3* protein isoform, which may have differential function across brain regions, developmental time periods, or cell types [64]. Another limitation is the possibility that some *Shank3* mutations lead only to subtle or limited forms and symptoms in the autism spectrum and that are difficult to detect in mice (such as intellectual disabilities).

Conclusion

Several major conclusions can be drawn from our study and several previous related findings: (1) the need to include females and heterozygote mice in separate groups in ASD animal models. (2) Even though *Shank3* mutations have strong construct validity when producedin a way to mimic the genetic deletions and mutations observed in humans, there is a need to better evaluate Shank3 mouse models' face validity. For instance, in our hands and in previous reports [17, 24], Shank3+/ΔC did not show major ASD-related behavioral deficits, contrary to the haploinsufficiency *Shank3* deletions in humans. (3) Our findings, along with our previous reports in environmental mouse models of ASD, clearly point to gait disturbances as being key behavioral features in ASD, whatever the disease's etiology, with symptoms that are in line with clinical findings. This is of interest as gait investigations can be implemented early during the diagnosis proce- dures when ASD is suspected; they are also quantitativein an objective and reproducible manner. As such, gait needs to be considered as a credible new diagnostic cri- terion in ASD.

Abbreviations

SHANK: SH3 and multiple ankyrin repeat domains; ASD: Autism Spectrum Disorders; PC: Purkinje cells; ANOVA: Analysis of variance; mGluR5: Metabotropic glutamate receptor 5; NRXN: Neurexin; NLGN: Neuroligin; TSC1/2: Tuberous sclerosis 1/2; FMR1: Fragile Mental Retardation 1; VPA: Valproic acid; MIA: Maternal immune activation; 3-CT: Three-chambers test; PFA: Paraformaldehyde; SDS: Sodium dodecyl sulfate; TBST: Tris Buffer Solution Tween; P-mTOR: Phosphorylated mammalian target of rapamycin; mTOR: Mammalian target of rapamycin; ERK1/2: Extracellular signal regulated kinase; NR2A: *N*-Methyl-d-aspartate receptor subunit 2A; NR2B: *N*-Methyl-d-aspartate receptor subunit 2B; GluR1: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit 1; GAPDH: **D**-Glycéraldéhyde-3-phosphate déshydrogénase; NR1: *N*-Methyl-d-aspartate receptor subunit 1; GluR2: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit 2.

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Authors' contributions

EM and AM performed all behavior experiments, western blotting and histological studies; they performed all statistical analysis, constructed the figures and wrote the paper. MT performed some of the stereotyped behavior

and genotyped mice. EB helped setting up, performing and supervising several behavior experiments. AB helped with the western blot studies and was in charge of interactions with the animal facility and ethical committee. MF helped with the histological studies and basic laboratory procedures. LG supervised the Shank3 colony, helped with the immunohistochemistry and discussed findings. MJ designed experiments, supervised the project, discussed findings, sought funding and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Most of the data generated and analyzed during this study are included in this published article. All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Animal housing and experimental procedures were performed under the European Union directive (2010/63/EU) and validated by the regional ethical committee (Approval# 2015020415093780).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Université de Poitiers, INSERM, Laboratoire de Neurosciences Expérimentales et Cliniques, 86073 Poitiers, France. [2] CHU de Poitiers, 86000 Poitiers, France.

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Chapitre 2 :
The state of dopaminergic and glutamatergic systems in the valproic acid mouse model of autism spectrum disorder

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Alexandre Maisterrena, Emmanuel Matas, Helene Mirfendereski, Anaïs Balbous, Sandrine Marchand, MohamedJaber.

Cerebellar and striatal implication in autism spectrum disorders : fromclinical observations to animal models

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Mathieu Thabault, Valentine Turpin, Alexandre Maisterrena, Mohamed Jaber, Matthieu Egloff and Laurie Galvan

1. Résumé article, The state of dopaminergic and glutamatergic systems in the valproic acid mouse model of autism spectrum disorder :

Les TSA sont un ensemble de troubles neurodéveloppementaux caractérisés par des déficits d'interactions sociales et des comportements stéréotypés et répétitifs. Dans ce papier, je me suis intéressé à l'état des systèmes dopaminergiques et glutamatergiques dans le modèle VPA des TSA, un modèle dans lequel nous avions précédemment décrit la présence d'altérations au niveau comportemental et au niveau cellulaire dans le cervelet.

Ici, nous n'avons pas observé d'altérations du nombre de neurones dopaminergiques au niveau du mésencéphale ni d'altérations des niveaux de tyrosine hydroxylase dans le striatum et le Nac. Nous avons ensuite mesuré les niveaux de dopamine et de ses métabolites dans le striatum, le Nac, le mésencéphale et le striatum dorsal par HPLC et par spectrométrie de masse. Nous avons observé une diminution des niveaux de DA dans le CPU dorsal des souris VPA femelles, accompagnées d'une augmentation du ratio DA/DOPAC indiquant une augmentation de la demi-vie de la dopamine. Nous avons aussi observé une diminution des niveaux de DOPAC dans le mésencéphale des souris VPA mâles et femelles ainsi qu'une diminution des niveaux de DA chez les souris mâles VPA. Ces résultats semblent indiquer que le système dopaminergique n'est que peu altéré par l'exposition prénatale au VPA.

Cette hypothèse a pu être renforcée avec nos analyses au niveau moléculaire dans lesquelles, à part une augmentation des niveaux d'ARNm des récepteurs D1 et D2 dans le Nac, nous n'avons pas observé d'altérations au niveau des récepteurs glutamatergiques ni au niveau de plusieurs protéines impliquées dans la transduction des signaux glutamatergiques et dopaminergiques.

L'absence d'altérations majeures au niveau des systèmes glutamatergiques et dopaminergiques, cibles fréquentes d'essais cliniques, peut être expliquée de deux manières : (i) il est possible que les techniques utilisées pour les analyses n'étaient pas assez précises et il aurait été

intéressant d'utiliser d'autres techniques de mesures de la quantité de protéines comme le fractionnement synaptique pour obtenir une vue plus détaillée de l'état de ces systèmes. Cette possibilité ne s'applique pas aux mesures des niveaux de dopamine et ses métabolites. En effet, nous avons utilisé initialement la technique d'HPLC suivie de la technique de spectrophotométrie de masse, de plus haute précision, pour conforter nos résultats. (ii) Ainsi, la seconde possibilité que l'on pourrait avancer serait en lien avec la grande variété de facteurs de risque des TSA. En effet, il est possible qu'il n'existe pas un unique système déréglé de manière importante, mais plusieurs systèmes déréglés de manière modérée conduisant ainsi à un déséquilibre de l'homéostasie de la transmission synaptique et à l'apparition des symptômes des TSA. Cette hypothèse semble plus probable, car comme observé avec les modèles SH3, il apparait qu'une hypo ou une hyperactivation du système glutamatergique soit capable d'induire l'apparition de troubles comportementaux.

Dans le cadre de ce travail, j'ai participé à la rédaction d'une revue s'intéressant aux altérations des différents neurotransmetteurs au niveau du striatum et du cervelet. Dans cette revue, je décris que, selon le modèle animal utilisé, les altérations observées sont différentes. Ceci renforce l'hypothèse de l'altération modérée de plusieurs systèmes conduisant à un déséquilibre qui serait à la base de l'apparition des TSA.

2. Résumé revue, sur implication cérébelleuse et striatale dans les troubles du spectre autistiques : des observations cliniques aux modèlesanimaux.

Ma contribution, dans cette revue collective, s'est essentiellement concentrée sur les altérations moléculaires au niveau des systèmes de neurotransmission au niveau du cervelet et du striatum. Comme l'indique le titre de la revue, ses principaux objectifs sont (i) de proposer un état des lieux des altérations du cervelet et du striatum dans le cadre des TSA, et ce du point de vue clinique et expérimental ; (ii) de présenter le lien entre les altérations décrites au niveau cellulaire et moléculaire et les déficits comportementaux observés ; et enfin (iii) de souligner le rôle important des troubles moteurs et des déficits de la marche dans le cadre des TSA et d'émettre des propositions pratiques en terme diagnostiques en prenant en compte ces différents paramètres. Pour cela, nous nous sommes principalement intéressés aux altérations moléculairesau niveau du cervelet et du striatum, deux régions impliquées à la fois dans les troubles moteurs,mais aussi d'interaction sociale dans les TSA.

2.1 Altérations des systèmes GABA et glutamate au niveau du cervelet

Au niveau du cervelet, les deux principaux neurotransmetteurs impliqués dans son activité sont le glutamate et le GABA créant une balance excitation/inhibition à ce niveau. Dans le cadre des TSA, cette balance est déséquilibrée avec des altérations à la fois du système glutamatergique et du système gabaergique.

Concernant le système glutamatergique, il a été observé une diminution de la densité des récepteurs AMPA chez les patients. Partant de cette observation, et du fait du rôle des protéines Grip1/2 dans l'adressage des récepteur AMPA, la signalisation AMPA au niveau des cellules des PC PC a été étudiée chez des souris KO pour Grip1/2. Cette étude a mis en évidence la présence de toilettage excessif ou « grooming » chez ces animaux et d'une dépression à long terme (LTD) altérée des récepteurs mGluRs. De plus, il a été décrit que l'activité de mGluR5 était augmentée et ceci potentiellement afin de compenser le manque de recyclage des AMPA, en l'absence de Grip1/2. Une augmentation des niveaux et de l'expression des transporteurs d'acides aminés excitateurs EAAT1 et EAAT2 a aussi été notée dans le cervelet des patients TSA. Étant donné que les niveaux de ces transporteurs sont en lien avec ceux de glutamate, ceci pourrait indiquer que les niveaux de glutamate sont augmentés chez ces patients. Cette augmentation pourrait se traduire par une activation des PC par le système glutamatergique conduisant ainsi à une inhibition de la sortie cérébelleuse.

Différentes altérations du système glutamatergique ont aussi été observées dans plusieurs modèles animaux de TSA. C'est le cas chez les souris Fmr1 KO, modèle du syndrome d'X fragile lié aux TSA, où une augmentation des niveaux de mGluR5 a été observée. Dans nos mains, nous avons aussi observé que les niveaux de mGluR5 étaient diminués dans le cervelet de souris mâles $SH3^{+/\Delta c}$ et $SH3^{\Delta c/\Delta c}$ et que ceci est accompagné par des déficits à la préférence sociale. Ces résultats soulignent le rôle important de l'extrémité C terminale de Shank 3 pour le bon adressage à la membrane de mGluR5 et l'implication potentielle de ce récepteur dans les interactions sociales. En lien avec nos études et conclusions, une étude indépendante a également montré que l'administration d'agonistes de mGluR5 dans un modèle d'inactivation de Shank3 était capable de réduire les déficits comportementaux associés au TSA.

Au niveau du système gabaergique, il a été observé dans le cervelet des patients une diminution des niveaux des récepteurs GABA-A (avec une activité inhibitrice rapide) et GABA-B (régulant la balance excitation/inhibition), ainsi que des protéines GAD65 et GAD67, les principales protéines responsables de la production de GABA. Cependant, il a été observé une

augmentation des concentrations de GABA plasmatique chez les patients dont l'origine et les conséquences restent à déterminer.
De plus, il a aussi été observé une diminution des niveaux de la sous-unité GABAρ3 du récepteur GABA-A, dans le cervelet des souris exposées en prénatal au VPA. Cette sous-unité joue un rôle important dans la régulation de la transmission gabaergique lors du développement du cervelet. La diminution des niveaux de cette sous-unité, observée au niveau du lobule X du cervelet, semble être impliquée dans les déficits observés dans la coordination du regard, une fonction atteinte chez les patients TSA. Il est intéressant de noter que dans ces cas-là l'administration d'un agoniste de GABA-A ou de GABA-B permet une correction des déficits sociaux observés et des altérations au niveau moléculaire.

En conclusion, nous pouvons observer dans les TSA, un déséquilibre de la balance excitation/inhibition et un déséquilibre dans la balance GABA/Glutamate. Ces déséquilibres seraient dus à la fois à une réduction de la quantité de GABA suite à une réduction des niveaux des enzymes GAD65 et GAD67, mais aussi à une réduction de l'activité de récepteur GABA pouvant conduire à son tour à une augmentation de l'activité du récepteur mGluR5. Cependant, il faut aussi noter qu'une diminution de l'activité de mGluR5 apparaît aussi être impliquée dans des déficits sociaux. De fait, une action pharmacologique ciblant ces systèmes doit auparavant tenir compte de l'état précis de ce déséquilibre : dans le cas d'une diminution de l'activité du système gabaergique, il convient de cibler un traitement pharmacologique activant le système gabaergique ou inhibant mGluR5 alors que dans le cas d'une baisse de l'activité des mGluR5 un traitement l'activant pourrait être efficace pour la gestion de certains symptômes des TSA et permettre un rétablissement de la balance excitation/inhibition.

2.2 Altérations des systèmes GABA, glutamate et dopamine au niveau du striatum

Au niveau du striatum, plusieurs études ont montré des altérations des systèmes glutamatergiques, gabaergiques, mais aussi dopaminergiques.
Les altérations du système glutamatergique au niveau du striatum ont été retrouvées dans plusieurs modèles de TSA, dont certains décrits plus haut : Nlgn1 KO, Ngln3 KO, Shank3 et Shank2 KO. Notamment, chez les souris Nlgn1 KO, le ratio NMDA/AMPA est réduit au niveau du striatum dorsal avec une augmentation des comportements de « grooming ». Il est aussi noté dans ce modèle une diminution similaire de ce ratio dans les neurones striataux épineux de taille moyenne (MSN) de la voie directe induite par une diminution du courant NMDA à travers la

sous-unité GluN2A. Il est intéressant de noter que, dans ce modèle, l'utilisation d'un co-activateur de NMDA permet de restaurer le ratio NMDA/AMPA ainsi que de réduire les comportements de « grooming ». On peut aussi noter que dans le modèle Shank3B$^{-/-}$, une diminution de la réponse post-synaptique AMPA a été observée ainsi qu'une diminution du nombre de synapses fonctionnelles.
Concernant le récepteur mGluR5 impliqué dans la potentiation des réponses NMDA, il a été observé une diminution dans les MSN des souris SH3$^{e4\text{-}22\text{-}/\text{-}}$ et ceci est accompagné d'une diminution de l'excitabilité et de la plasticité de ces neurones suite à une perte de la LTD.
Pris ensemble, ces études suggèrent que les altérations du système glutamatergique au niveau du striatum seraient dues à une diminution de l'activité des récepteurs NMDA. Une étude a aussi renforcé cette hypothèse en montrant que l'utilisation d'un agoniste de mGluR5 permettait un retour à la normale de l'activité NMDA dans le modèle SH3Δ11$^{-/-}$.

Pour le système gabaergique striatal, il a été décrit chez les souris Chd8+/- au niveau du striatum ventral une diminution de l'amplitude des courants miniatures inhibiteurs post-synaptiques (mIPSCs) avec une augmentation du nombre de courants excitateurs post-synaptiques spontanés. Ceci indique qu'une augmentation de l'activité excitatrice striatale serait due en partie à une réduction de l'activité inhibitrice locale. Ces résultats suggèrent que les altérations gabaergiques observées au niveau des MSN de la voie directe (exprimant le RD1) au niveau du striatum ventral seraient responsables du déséquilibre observé de la balance inhibition/excitation. Au niveau du striatum dorsal, peu d'études se sont intéressées au système gabaergique. Dans un modèle Fmr1$^{-/-}$ de TSA, il a été montré une augmentation de la fréquence des mIPSCs et des courants inhibiteurs spontanés post-synaptiques (sIPSC) sans changement de l'amplitude de ces courants. Ensemble, ces résultats suggèrent que les altérations du système gabaergique sont probablement situées en pré-synaptique mais avec une action et des conséquences post-synaptiques.

Pour le système dopaminergique, une analyse post-mortem du striatum de patients TSA a montré une augmentation de l'ARNm des RD2, suggérant un déséquilibre de la balance dopaminergique en faveur de la voie indirecte. Il est intéressant de noter que l'utilisation d'antagonistes D2 a conduit à une diminution des déficits sociaux et des comportements stéréotypés chez les patients. Cependant, il apparaît que la voie directe est dysfonctionnelle seulement quand elle est activée durant une tâche.

Une étude chez de jeunes adultes atteints deTSA a montré une diminution de la libération de dopamine phasique dans le putamen et le noyau caudé qui pourrait sous-tendre des déficits de l'apprentissage et du renforcement des comportements orientés vers un but (goal-directed).
Dans les modèles animaux, il a été montré qu'une stimulation optogénétique de la voie nigrostriatale induisait l'apparition de déficits sociaux et de redressement (rearing), mais sans changement au niveau des comportements de toilettage (grooming). Au niveau moléculaire, une augmentation de p-ERK1/2 et de p-CAMKIIα a été observée au niveau du striatum dorsal. Ces études suggèrent que la voie indirecte serait moins altérée que la voie directe dans ces modèles de TSA.
D'autres altérations ont été aussi décrites par exemple chez les souris BTBR et les souris FMR1 KO avec une diminution du transporteur de la dopamine (DAT) qui permet de réguler les niveaux de dopamine extra-cellulaire. Dans ces deux modèles, il a été documenté une augmentation du couplage entre les neurones TH+ et les neurones Vglut1+. Un lien fort existe entre Vglut1 et mGluR5 et une activation du mGluR5 astroglial régule l'activité de Vglut1. Il est intéressant de noter que l'inactivation de l'adénylate cyclase 5 (AC5), chez les souris, qui est un effecteur de D2 et de mGluR3 dans le striatum, induira une augmentation du grooming et une réduction de la préférence sociale dans le test à trois chambres, deux phénotypes associés aux TSA. Il faut aussi noter que mGluR3 à un effet antagoniste sur mGluR5 similaire à celui entre D1 et D2. Dans le cas de souris sauvages, l'activation de mGluR5 (avec DHPG, un agoniste) ou l'inactivation de mGluR3 (avec LY341495, un antagoniste) ainsi que l'inactivation de GluA1 ou GluN2B sont suffisantes pour induire l'apparition de déficits sociaux.

Cette revue a permis de montrer qu'une meilleure compréhension des rôles du cervelet et du striatum dans les TSA a été développée ces dernières années. Cependant, on peut aussi noter deux limites à ces études et qui auront besoin d'être adressées dans l'avenir. Tout d'abord, il sera nécessaire d'effectuer une meilleure discrimination des groupes en séparant systématiquement les mâles des femelles. En effet, plusieurs de ces études ne distinguent pas homme et femme chez les patients et mâles et femelles dans les modèles animaux, et ce alors que les TSA montrent un dysmorphisme sexuel important. Dans la continuité de ce point, il serait aussi intéressant d'étudier les TSA chez les patients adultes ou âgés et pour les modèles animaux chez des souris plus âgées. En effet, de nombreuses études se sont intéressées au TSA chez l'enfant et le jeune adulte, mais peu chez les patients âgés où l'évolution de la pathologie pourrait être différente. Ces approches prenant en compte le sexe et l'âge des patients pourraient aider à développer des études visant à explorer de nouvelles voies de traitements permettant de réduire les symptômes comportementaux observés dans les TSA.

3. Article

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Original article, special issue dedicated to the memory of Marc G Caron

The state of the dopaminergic and glutamatergic systems in the valproic acid mouse model of autism spectrum disorder

Alexandre Maisterrena[1#], Emmanuel Matas[1#], Helene Mirfendereski[2,3], Anaïs Balbous[1,3], Sandrine Marchand[2,3], Mohamed Jaber[1,3*]

[1] Université de Poitiers, Inserm, Laboratoire de Neurosciences Expérimentales et Cliniques U1084, Poitiers, France
[2] Université de Poitiers, Inserm, Pharmacologie des Agents Anti-infectieux et Antibiorésistance U1070, Poitiers, France
[3] CHU de Poitiers, Poitiers, France
[#] These two authors contributed equally to this work
[*] Correspondence: Mohamed Jaber, mohamed.jaber@inserm.fr

Abstract: Autism Spectrum Disorder (ASD) is a progressive neurodevelopmental disorder characterized mainly by deficits in social communication and stereotyped behaviours and interests. Here, we aimed at investigating the state of several key players in the dopamine and glutamate neurotransmission systems in the valproic acid (VPA) animal model that we had previously characterised at the behavioral and cellular levels. We report no alterations in the number of mesencephalic dopamine neurons nor in protein levels of tyrosine hydroxylase in either the striatum or the nucleus accumbens. Levels of dopamine were slightly decreased in the dorsal striatum while levels of D1 and D2 dopamine receptors mRNAs were increased in the nucleus accumbens suggesting an up-regulation of the corresponding receptors. We found no alteration in levels of several striatal, cortical, or cerebellar transduction and apoptotic proteins such as mTOR, ERK, Akt and LC3. mRNA and protein levels of glutamate receptor subunits NR1, NR2A, NR2B were not altered by VPA, nor were protein levels of BDNF and TrkB. To few exceptions, these results indicate a rather stable signalling status of the dopamine and glutamate transmission. This is of interest as clinical trials are being considered aiming at these systems.

Keywords: dopamine; glutamate; autism spectrum disorder; valproic acid; mouse models; striatum; cerebellum; accumbens.

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1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting an increasing proportion of the population and that is characterized mainly by the presence of social deficits and restricted and repetitive behavior and interests [1]. Management of the disorder targets mainly associated comorbidities while effective pharmacological therapies for the core symptoms are yet unavailable [2,3]. This is in great part due to the ASD's etiology and physiopathology that are still at an early stage of scientific discovery. Over a 1000 genes have been identified as being ASD-related and that mainly target the neurotransmission systems at the levels of synapses [4,5]. Exposition of pregnant mothers to several environmental factors have also been described as increasing the risk factor to develop ASD in the offspring [6–9]. These environmental factors include numerous factors among which the most documented ones are of pharmacological nature (Depakine, through its active ingredient the valproic acid, VPA) [10] or infectious (inducing a maternal immune activation, MIA) [11].

Despite the high heterogeneity between ASD cases and its various etiology, the occurrence of shared symptoms suggests common deficits in common neurodevelopmental pathways [12–15]. Given that neurotransmitter systems take a major part in shaping the nervous system during development, a dysfunction at this level may contribute to the pathophysiology of ASD. Indeed, normal brain development and functioning depends in large part on a physiological equilibrium of the neurotransmission systems. An imbalance in the glutamate and dopamine neurotransmission systems is thought to be a main cellular and molecular component of ASD pathophysiology [14,15]. Disturbances in these neurotransmission systems during development can originate from a myriad of heterogeneous origins, whether genetic or environmental, and that may converge to the common behavioral symptoms that are the hallmark of the ASD. Determining the biological basis of ASD is of relevance at this may lead to better diagnosis and potential pharmacological therapy for a psychiatric condition that is in dear need.

Many genes within the glutamatergic system have been associated with ASD. These include, but are not limited to, *NRX1-3* (neurexin 1-3), *NLGN1, 3, 4* (neuroligin1, 3, 4), *CNTNAP2* (contactin associated protein 2) and *SHANK 1-3* (SH3 and multiple ankyrin repeat domains proteins) [14,16,17]. However, whether ASD patients and animal models present a hyper or a hypoglutamatergic state is still debatable as two opposite theories are proposed by Carlsson et al., (1998) [18] and Fatemi et al (2008) [19] respectively. On one hand, high levels of glutamate were reported in ASD patients' blood [20] and were linked to ASD severity [21]. In the VPA animal model, the NMDA-R (N-methyl-D-aspartate receptor) subunits GluN2A and GluN2B levels were upregulated and AMPA-R (a-amino-3- hydroxyl-5-methyl-4-isoxazole-propionate receptor) subunits GluN2A and GluN2B levels were also upregulated while AMPA receptor antagonists ameliorated social deficits [23]. On the other hand, glutamatergic receptor agonists were shown to improve some of ASD symptoms in patients and in animal models [24]. These variabilities may stem from the wide spectrum of the disorder in terms of its environmental and genetic etiology but also in terms of phenotypic expression. The main hypothesis on the pathophysiology of ASD stipulates that there is an excitation/inhibition (E/I) imbalance underlying the disorder and implicating the glutamatergic and the GABAergic systems [25]. This, along with the fact that several genes implicated in ASD are major actors of the glutamatergic transmission [14], have spurred a number of clinical trials and experimental assays aiming at targeting the glutamatergic neuronal transmission.

Dopamine plays a critical role in motivated behavior, action selection and reward processing [26]. In addition, dopamine seems also to be a major actor in social function as dopamine is released in responses to social cues and this release is driven by social seeking behavior [27]. Positron emission imaging studies in ASD patients have shown a significant reduction in prefrontal cortical levels of dopamine [28] and more recent MRI studies have shown several alterations in dopaminergic connectivity within the caudate nucleus in relation with ASD [29]. Of interest is the fact that the extend of these alterations were correlated with the severity of ASD symptoms [30].

Beyond imaging studies, post-mortem studies are hindered by the limited availability of brain samples and tissues from ASD patients. Conveniently, ASD animal models, whether genetic or environmental, have regularly been proven to present a strong construct and face validity, with some showing promises for their predictive validity [31–34]. We have recently reported findings in this line, with genetic (Shank3) or environmental (VPA and MIA) ASD models [10,11,35]. To various degrees, these models reproduced the spectrum of the disease as they all showed major behavioral alterations that were reminiscent of ASD core symptoms, with clear-cut social deficits and stereotyped behavior. These alterations were also accompanied by gait and motor deficits and were correlated with loss of Purkinje cells (PC) within restricted cerebellar subregions. Additionally, we also reported alterations in the synaptic structures of striatal medium spiny neurons as well as in their excitability [36].

Here, we focused at determining the state of several key players of the dopamine system within the ventral mesencephalon, the striatum the accumbens and the frontal and motor cortex in the VPA animal model of ASD. We also examined the state of several glutamate receptors in these brain regions as well as of Brain-Derived Neurotrophic Factor (BDNF) and neurotrophic tyrosine receptor kinase beta (TrkB). This was performed by stereological counting of dopamine cells, analysis of dopamine levels by High performance Liquid Chromatography and mass spectrometry, quantitative polymerase chain reaction (qPCR) and membrane blotting.

2. Materials and Methods

Experimental design

C57BL/6J Mice (Charles Rivers, France) were housed at the local Prebios animal facility, in ventilated cages with access to food and water *ad libitum*. Room temperature was maintained at 23°C on a 12h light/dark cycle.

All animals used here previously underwent full scale behavioral analysis before being sacrificed [10]. For this, 20 females and 10 males were used for mating. 3 females were placed with a single male and left for 3 days, and males were retrieved from the cage following mating as evidenced by the presence of a vaginal plug. Pregnant mice received a single i.p. injection of either VPA (450 mg/kg) or NaCl 0.9 % at gestational day 12,5 (E12.5) when the neuronal tube is closing in rodents, followed by neurogenesis and neuronal migration [32][10]. Following mating, pregnant mice were left undisturbed until they gave birth. At weaning on postnatal day 21 (P21), sex and age matched pups were separated and raised by groups of 4 in a randomized fashion to avoid littermate effects. All mice were identified by ear tags. Mice were then allocated to four experimental groups depending on prenatal treatment and sex: VPA males (n=34), saline males (n=27), VPA females (n=25), and saline females (n=30). The experiment timeline was previously provided in our initial paper [10]. Following behavioral analysis, animals were assigned to different experimental groups and were sacrificed according to the corresponding required procedure then brains were harvested for further analysis as described below. The experimenter was blind to the treatment until all studies and analyses were completed.

Tyrosine hydroxylase (TH) immunohistochemistry at the ventral mesencephalon level

Mice (saline (n=11) and VPA (n=7)) were deeply anesthetized with ketamine-xylazine (120-20 mg/kg) and transcardially perfused with 0.9% saline at 37°C followed by 4% paraformaldehyde (PFA) at 4°C. Brains were post-fixed in 4% PFA at 4°C for 24h before cryoprotection in 30% sucrose for 48 h. Serial 50 µm free-floating sections were collected and stored at -20°C until use in an anti-freeze solution. Every fourth section was mounted on gelatin-coated slides for quantification of TH-positive neurons that were further identified based on their morphology and localization. Stereological estimates were performed using the optical fractionator method and systematic random sampling to obtain the total number of TH-positive neurons. Neurons were counted at 40x objective using the Mercator image analysis system (Explora Nova, France). Upper and lower guard zones of 1 µm were set at the top and bottom of the section to exclude lost profiles and each neuron or visible nucleus was counted as previously described [11].

Quantitative immunoblot analysis

Mice (saline (n=14) and VPA (n=14)) were sacrificed, brains were retrieved, regions of interest were either dissected out (cerebellum) from the frozen brain or through punches (striatum, nucleus accumbens, motor cortices) and were frozen in -80C until use. Proteins were extracted using a 1% sodium dodecyl sulfate (SDS) solution in Tris Hcl 0.1M with ethylenediaminetetraacetyl (EDTA) 0.01M and phenylmethylsulfonyl fluoride (PMSF), protease inhibitor, and phosphatase inhibitor at 1%. Equal amounts of proteins from lysates were separated by SDS-PAGE and migrated proteins were transferred to

nitrocellulose membranes (Bio-Rad, France). After blocking the membranes at room temperature for 1 hour in Tris Buffer Solution with Tween-20 0,1M (TBST) at pH 7.4 and 5% non-fat milk, blots were incubated with corresponding primary antibodies at room temperature for 3 hours. The blots were then washed three times in TBST and incubated with HRP-conjugated secondary antibodies overnight at 4°C. Following 3 washes in TBST, the blots were incubated with ECL reagent (GE Healthcare Life Sciences, USA). For quantification, the films were scanned by a PXi image system and gray signals were analyzed by GeneTools software (Syngene, UK), and normalized to that of corresponding internal controls (actin or β-tubulin III). The primary antibodies mTOR 7C10, P-mTOR ser 2448, ERK1/2, and P-ERK1/2 (Cell Signaling Technology, Netherlands) were used at the dilution 1/1000 to 1/1500. NR2A, NR2B, GluR1, and GAPDH antibodies (Millipore, France) were used at the dilution of 1/500 to 1/1500. The following dilutions were used for antibodies against CAMKII (1/1000), α-tubulin (1/10000), and β-actin (1/10000) (Sigma-Aldrich, France), Shank3 (1/500), mGluR5 (1/5000) (Abcam, Paris, France), NR1 (1/500) (Antibodies Incorporated, USA) and GluR2 (1/1000) (Neuromab, USA).

qPCR analysis

Mice (saline (n=9) and VPA (n=8)) were sacrificed, brains were retrieved, and striatal, cerebellar and cortical regions were dissected out and frozen in -80°C until use. Total RNA was isolated using TRIzol Reagent/chloroform, then purified using a NucleoSpin RNA kit (Macherey-Nagel, France) and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was evaluated by an Agilent 2100 Bioanalyzer System. All RNAs had an RNA integrity number above 9. Reverse transcription was performed on 1µg of total RNA for each sample using Verso cDNA Synthesis kit (Thermo Fischer Scientific, USA). qPCR was performed on LightCycler 480 system (Roche Diagnostics, France). Ct values were averaged from triplicates. Results were subtracted to the mean Ct of the housekeeping genes *Gapdh*, *Beta actin* and *Hrpt1* (ΔCt) and log transformed ($2^{-\Delta Ct}$). Primer sequences are provided in supplementary Table I.

Chromatography and spectrometry analysis

Mice were deeply anesthetized with ketamine-xylazine (120-20 mg/kg) and transcardially perfused with 1X PBS at 37°C. Brains were retrieved, regions of interest were either dissected out (cerebellum) from the frozen brain or through punches (striatum, nucleus accumbens, motor cortices) and were frozen in -80C until use.

High-performance liquid chromatography (HPLC) (5200a Coulochem III, ESA, Chelms- ford, USA) was used to quantify dopamine levels within the striatum and accumbens of mice as described [37] ((Saline (n=12) and VPA (n=10). The mobile phase contained 100mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM n-octyl sulfate, and 18% (v/v) methanol (pH adjusted to 5.5 with Na_2HPO4). Assay sensitivity for dopamine was 2 fmoles per sample.

Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry detection was used to quantify dopamine and 3 of its metabolites, dihydroxyphenylacetic acid (Dopac), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) in striatal regions and accumbens nucleus following protein precipitation by CAN ((females saline (n=18), females VPA (n=18), males saline (n=18), males VPA (n=20)). The equipment was composed of a quadrupole tandem mass spectrometer (QT3500, Sciex, USA) and HPLC (Nexerra XR -LC system, Schimadzu, Japan) with a data acquisition station (Analyste version 1.6.3). The analytical column was a Luna® omega Polar C18 column (100Å, 5µm, 2.1 x 150 mm) (Phenomenex, USA). The mobile phase consisted of a mixture of water and acetonitrile (ACN) using a gradient mode at 30°C with a flow as 0.2 ml/min. For the MS/MS detection, electrospray ionization (ESI) was used in positive mode for dopamine and 3 MT, and in negative mode for Dopac and HVA. Mass spectra were acquired by multiple reaction monitoring. The specific transition used for quantification was 153.98 to 91.1 (Dopamine), 166.97 to 123.0 (Dopac), 180.99 to 121.9 (HVA), 168.0 to 119.0 (3-MT). For the internal

standards (IS), the transition was 153.1 to 95.0 (Dopamine IS), 171.0 to 128 (Dopac IS), 187.02 to 142.7 (HVA IS) and 172.1 to 94.9 (3-MT IS). For all compounds, the range concentration was between 10 to 5000 ng/ml. IS of dopamine, Dopac, HVA and 3-MT were [2H4]-Dopamine, [2H5]-acide dihydroxyphenylacetique, [13C6]-acide homovanillique, [2H4]-3- methoxytyramine, respectively. The within run accuracy and precision were performed by assaying the quality control (QC) samples with three concentration levels (low, medium and high) three times in a single analytical run while the between run accuracy and precision were performed by assaying the QC samples with tree concentration levels four times in at least four separate days. The accuracy error was expressed as a bias and the precision was expressed as the coefficient of variation (CV). The acceptance criteria for QCs were met for all compounds during the analytical analysis (Bias = +/- 15% and CV ≤ 15%). Experiments were performed under ISO 9001 procedures.

Data analysis

Data are expressed as mean ± Standard Error of the Mean (SEM) and analyzed using GraphPad Prism-7 software (La Jolla, California, USA) unless otherwise indicated in the legends of figures. Data that followed a normal distribution was analyzed using Student's t-test or two-way analysis of variance (ANOVAs) whenever appropriate. Upon significant main effects, Tukey's or Fisher's LSD multiple comparisons were performed for behavioral or histological measures, respectively. When data did not follow a normal distribution (grooming and rearing behavior), we conducted Mann-Whitney non-parametric tests followed by Dunn's multiple comparison tests. Pearson correlations between behavioral and neuroanatomical readouts were performed using SPSS Statistics version 21 (IBM, France). For all analyses, a p value of <0,05 was considered significant.

3. Results

3.1. Dopamine neurons and tyrosine hydroxylase levels are not altered by VPA treatment

We have previously shown that mice pre-exposed to VPA (450 mg/kg) at E 12.5 showed major social deficits [10], in line with clinical observations [38]. Given the described role of accumbal dopamine in modulating social interactions, we aimed here at investigating the number of dopamine cells within the ventral mesencephalon and the corresponding dopamine levels within target areas. We found that the number of dopamine neurons, as evidenced by TH+ labelling, was not altered by VPA (Figure 1A). Accordingly, protein levels of TH were not altered in either the striatum or the nucleus accumbens (figure 1B). These results suggest that dopamine levels are not altered within the mesolimbic and the nigrostriatal pathway.

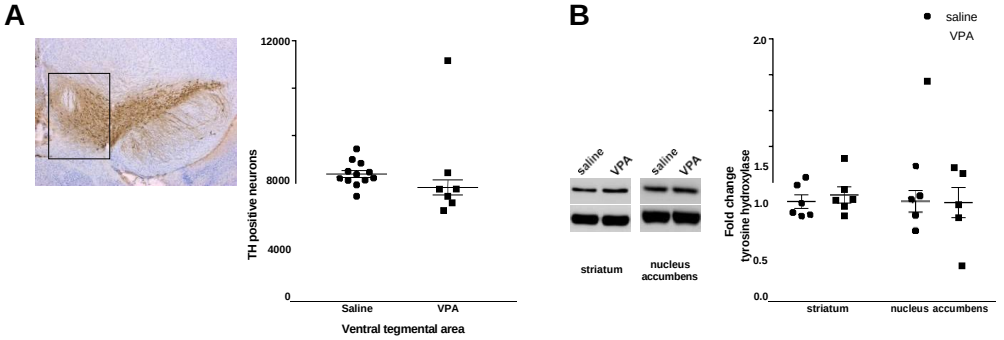


Figure 1. Dopamine neurons number and tyrosine hydroxylase levels are not altered in relation with VPA prenatal exposure. (A) No significant difference is observed in TH+ cell number in relation with treatment within the ventral mesencephalon following stereological quantification. (B) No alterations were found either in the striatal and accumbal levels of TH. All data are expressed as means ± SEM.

3.2. Dopamine and metabolites levels are not altered by VPA treatment, except in the females' dorsal striatum

We first measured dopamine levels by HPLC in saline and VPA mice in groups of mixed sexes and found no alterations within the striatum or the nucleus accumbens (Figure 2A). In order to further detail these findings, we explored levels of dopamine and metabolites using the more sensitive spectrometry procedure in groups of separate males and females and within several brain regions that are the ventral mesencephalon (Mes), the nucleus accumbens (Nac), the whole striatum (CPU) and the dorsal striatum (CPU dorsal) (Figure 2B). We found that female VPA mice showed a slight decrease in the levels of dopamine within the dorsal CPU ($p<0.05$) associated with an increase of the DOPAC/DA ratio ($p<0.05$) that generally indicates the turnover rate of dopamine metabolism. A decrease of dopamine in the Mes of VPA male and in DOPAC in the Mes of VPA males and females was also present (<0.05). No other alteration in the levels of dopamine, DOPAC, HVA and 3-MT in any of the brain regions explored was found.

These results indicate a slight but significant decrease in dopamine levels in several brain areas but that are of low magnitude suggesting that the dopamine nigro-striatal and mesolimbic pathways may not be the main pathways that underly social deficits and motor disorders in VPA mice.

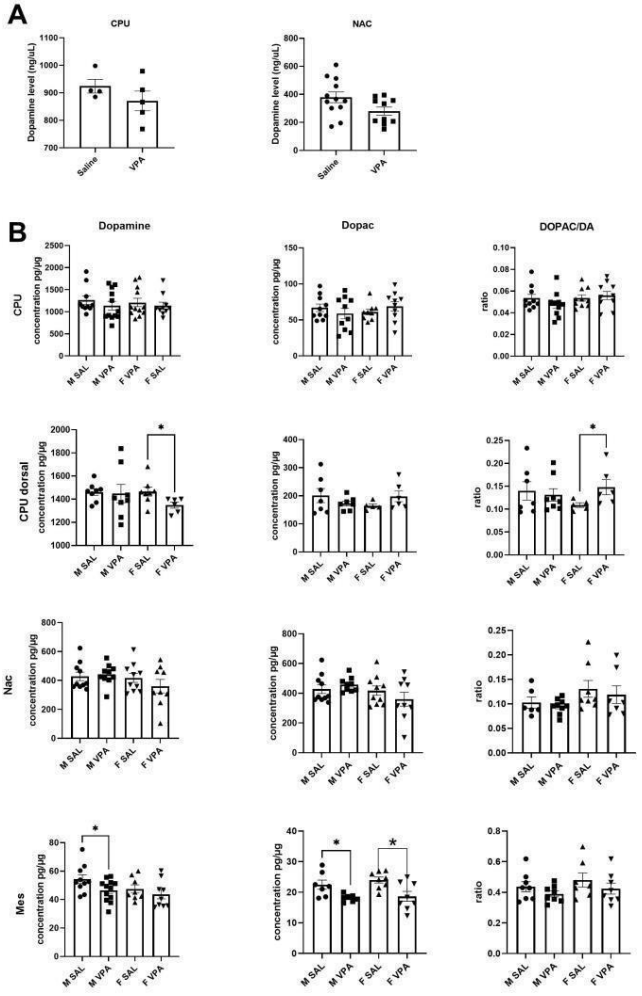


Figure 2. Dopamine and metabolite levels in various brain regions as measured by HPLC (A) and mass spectrometry (B). No alterations were found in the levels of dopamine within the striatum or nucleus accumbens as measured by HPLC. A slight decrease in the levels of dopamine was found in the group of VPA females within the dorsal striatum and in the mesencephalon of VPA males. Abbreviations: ventral mesencephalon (Mes), Nucleus accumbens (Nac), striatum (CPU). All data are expressed as means ± SEM; Mann-Whitney or Student t-test test were performed (*$p < 0.05$). WT males (n=10); VPA males (n=12); WT females (n=11); VPA females (n=12).

3.3. Dopamine and glutamate receptors' levels in relation to VPA treatment

We measured mRNA and protein levels of major dopamine and glutamate receptors within target brain areas that are suspected to be implicated in the major ASD social and motor symptomatology: the nucleus accumbens (Figure 3A), the striatum (Figure 3B), the motor cortex (Figure 3C) and the cerebellum (Figure 3D). The only noticeable alterations that we report here are in the mRNA levels of D1 and D2 dopamine receptors (DRD1 and DRD2 respectively) that were increased by almost two folds within the accumbens (Figure 3). This would normally indicate an upregulation of the corresponding receptors as a compensation of decreased dopamine levels as reported here.

Glutamate is a major excitatory neurotransmitter that regulates cognitive functions such as attention, learning and memory, which are affected in ASD. Indeed, of the several genes that are implicated in ASD, many relate to glutamatergic transmission and synapses. We thus investigated levels of key glutamatergic receptors and receptor subunits in relation with VPA prenatal exposure. We report here no alteration in the mRNA levels of *NR1, NR2A, NR2B, mGluR1, mGluR4 and mGluR5* in any of the brain regions examined (nucleus accumbens, striatum, M1/M2 motor cortices and cerebellum) (Figure 3). Additionally, there were also no alterations in protein levels of NR1, NR2A, NR2B in these brain regions (Figure 4).

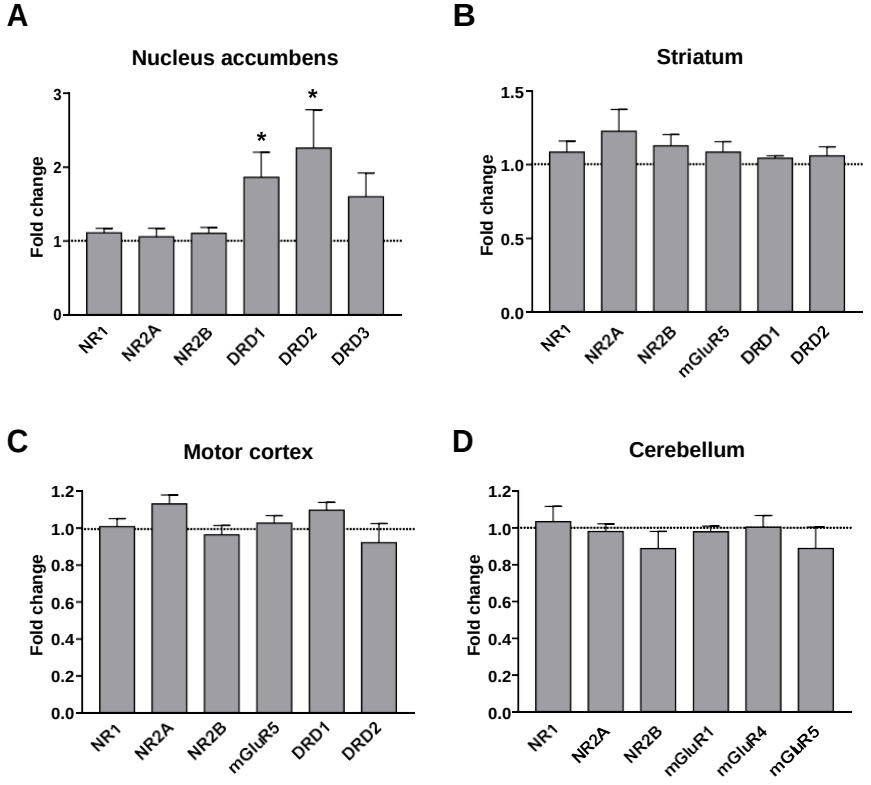


Figure 3. mRNA levels of glutamate and dopamine receptors in relation with VPA prenatal exposure. Note that D1R and D2R mRNAs are significantly increased in VPA mice compared to saline, in the nucleus accumbens. No other alteration is observed in mRNA levels of NR1, NR2A, NR2B, mGluR1, mGluR4 mGluR5. All data are expressed as means ± SEM; One-way ANOVA followed by Tukey's multiple analysis was performed (*$p < 0.05$).

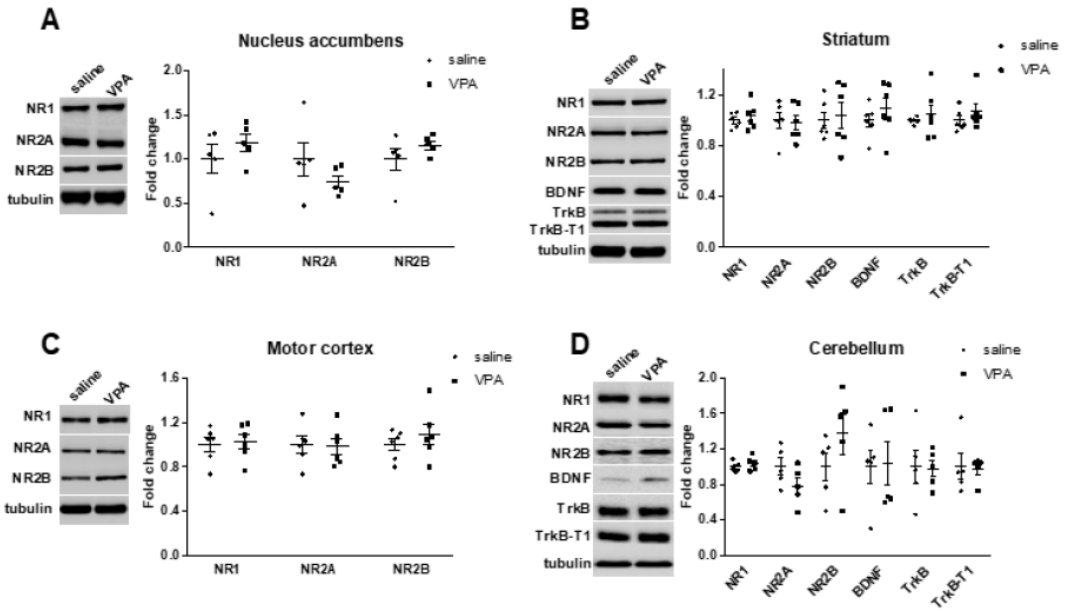


Figure 4. Protein levels of several signaling proteins are not altered in relation with VPA prenatal exposure. This was the case for the phosphorylated and the non-phosphorylated forms of TrkB, ERK1 and ERK2 mTOR, Akt 308, Akt 473 as well as in levels of LC3-I and LC3-II in all brain regions examined. All data are expressed as means ± SEM.

3.4. Signal transduction pathways within the dopaminergic and glutamatergic system

We underwent a further analysis of major molecules implicated in the dopamine signaling in main dopaminergic brain regions immunoblotting (Figure 4). We analyzed levels of dopamine- and camp-regulated phosphoprotein 32kDa (DARPP-32), considered as a major integrator of dopamine transmission [39].

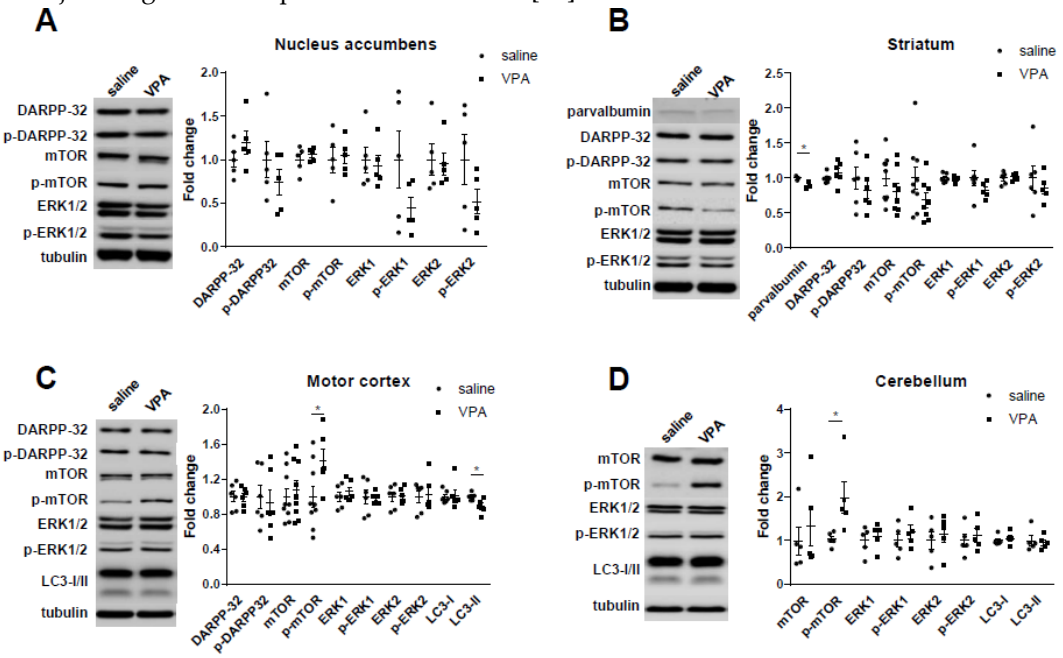


Figure 5. **Protein levels of NR1, NR2A and NR2B are not altered in relation with VPA prenatal exposure.** All data are expressed as means ± SEM.

We report here no alterations in DARPP 32 and p-DARPP 32 in either the nucleus accumbens, striatum or motor cortices (Figure 5). We also analyzed levels of phosphorylated and non-phosphorylated TrkB, Erk1 and 2, mTOR or Akt as these signal transduction effectors are highly regulated in relation with neuronal plasticity and thus are recognized indicators of alterations in neuronal signaling [40]. We found no modifications in the levels of any of these proteins whether in their phosphorylated or non-phosphorylated forms within the striatum, the nucleus accumbens, the M1/M2 motor cortices or the cerebellum (Figure 5). We also investigated levels of striatal parvalbumine (PV) as PV inter - neurons provide the strongest inhibitory inputs to striatal medium spin neurons MSNs and are in charge of the feed forward inhibition. In addition, PV as shown to be decreased in ASD patients [41] and in ASD animal models [42]. We confirm here these findings and report a slight decrease in PV striatal protein levels (data not shown).

4. Discussion

Given the major implications of the dopaminergic and glutamatergic neurotransmission systems in the pathophysiology of ASD, we aimed at determining the state of various players in these systems in the VPA animal model. As a prerequisite, we previously characterized this model at the behavioral and cellular levels and reported major deficits in social activities, gait and motor coordination. These deficits were directly correlated to the severity of PC loss within the cerebellum [10].

Among the few modifications that we found are (i) slight decreases in the levels of dopamine accompanied by (ii) increases in mRNAs coding for the D1 and D2 dopamine receptors within the nucleus accumbens. This suggests that the dopamine transmission is down regulated following VPA prenatal exposure with corresponding upregulation of the two major dopamine receptors. No alteration was detected in any transduction system related to dopamine in any of the brain regions investigated. In addition, mRNA and protein levels of several glutamate receptors and subunits were not altered either.

Several evidence point to a possible dysfunction within the dopamine system that may underlie ASD symptomatology [15,43]. Dopamine is the major catecholamine in the central nervous system and is involved in the regulation of a variety of functions, including locomotor activity, emotion and affect, motivated behavior and reward processing, all affected in ASD. Indeed, alterations in the dopamine system is directly linked to several neurological and psychiatric disorders such as Parkinson's disease and schizophrenia [44,45]. The link between the dopamine system and ASD stems from several observations and can be traced back to the seminal work of Antonino Damasio's team in the late 70's describing a neurological model of childhood autism [46]. In this paper, and based on analogies and signs in adult neurology, authors propose that ASD results from dysfunction in several brain areas including the striatum raising the possibility that dysfunction of the dopamine system is related to ASD core symptoms. Later on, imaging studies have provided consistent findings indicating alterations in the dopamine system in ASD patients. These include reduction of fluorodopa accumulation [28], abnormalities in dopaminergic structures and connectivity as revealed by MRI [47–49], enlargement of caudate volume [29,50] and reduction in grey matter within the fronto-striatal network [51]. These alterations were sometimes correlated with the severity of ASD symptoms [43]. The reported clinical evidences were also mirrored in various ASD animal models. For instance, several genetic mutations linked to ASD were performed in the Drosophila as was the case with the FMR1 mutation mimicking the Fragile X Syndrome, the most frequent monogenic mutation linked to ASD [52]. Authors report decreased synthesis of brain dopamine and elevated levels of vesicular monoamine transporter mRNA, implicated in the vesicular release of dopamine. The dopamine transporter (DAT) is a major element regulating dopamine transmission [45]. Inactivation of DAT in the Drosophila induces behavioral abnormalities related to ASD [53]. In the Zebrafish, social interaction was shown to

be directly related to the maturation of the dopamine system [54] while D1 antagonists reduced social behavior [55]. In mice, several reports have also linked dopamine to ASD. For instance, Mecp2-null mice, a mouse model of Rett syndrome with several ASD related behaviors, show abnormalities in dopamine synapses and a decrease in the number of TH+ neurons within the SNc [56]. Shank 3b-null mice have an enlargement of the striatum and morphological alterations of the striatal medium spiny neurons [57].

Although consistent, these set of findings cannot determine whether the alterations reported in the dopamine system constitute the molecular and cellular basis of ASD or whether they are merely a consequence of alterations in other systems, that may or may not be related to neuronal transmission. In our hands, and in the VPA mouse model where VPA was injected at E12.5 as a single dose of 450 mg/kg, we found slight but significant alterations within the dopamine system indicating that the dopamine transmission is down regulated. However, the low magnitude and extend of modifications suggest that dopamine may not be the major component underlying the reported behavioral symptoms in this animal model [10]. We cannot rule out however, that other alterations in the dopamine system, that we have not yet investigated, may be at play.

As with dopamine, early studies of ASD patients indicated alterations in the glutamatergic system [18,19]. Blood levels of glutamate in ASD patients were found to be increased while glutamine levels were decreased [20,22] and these alterations were correlated with the severity of ASD symptoms [21]. Glutamate/glutamine ratio was also found to be increased in several brain regions of ASD patients as measured by magnetic resonance spectroscopy [58,59] although other studies reported a decrease of these levels [60]. But perhaps the most compelling evidence of implication of glutamate in ASD arise from genetic studies. Indeed, amongst the hundreds of candidate genes related to ASD, several seem to be directly associated with the glutamate transmission [17]. These include genes encoding for glutamate receptor subunits, receptor regulatory subunits, glial glutamate transporters, and several signal transduction and scaffolding proteins among which members of the shank family coding for proteins associated with glutamate receptors [61]. In addition, several studies on ASD animal models report an E/I imbalance that may reflect changes in the GABA and/or glutamate transmission [36]. This E/I imbalance may be due to alterations in excitatory or inhibitory currents and is thought to be directly related to ASD-related behavior. In our hands, and in the VPA ASD animal model, we did not detect any alteration in the mRNA or protein levels of several metabotropic or ionotropic glutamate receptors or receptor subunits. This raises the possibility that the reported E/I imbalance may not be directly due to levels of glutamate receptors but rather to that of GABA receptors or to the function of associated inotropic receptors regulating membrane excitability and currents and that are yet to be identified.

5. Conclusions

The quest to identify molecular targets to ASD is of major importance as no pharmacological management of this neurodevelopmental disorder is yet available other than symptomatic. In this line, our set of results seem to indicate that the dopamine and the glutamatergic systems may be only indirectly implicated in the pathophysiology and the subsequent symptomatology of ASD. Given the very wide range of possible etiologies of ASD, whether environmental or genetic, it is possible that a myriad of systems are potentially affected. These systems may converge towards a cluster of players implicating the dopamine and glutamate systems leading to the spectrum of the disease. As such, this does not necessarily translate into alterations of major components of these neurotransmission systems but rather to a global disequilibrium away from the physiological homeostasis. If true, the quest for a single regional, cellular or molecular target to manage all ASD symptoms in all patients might not be fruitful without prior clear identification of the nature of alterations that might be different from one patient to another, in relation with the ASD etiology.

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Review

Cerebellar and Striatal Implications in Autism Spectrum Disorders: From Clinical Observations to Animal Models

Mathieu Thabault [1,†], Valentine Turpin [1,†], Alexandre Maisterrena [1], Mohamed Jaber [1,2], Matthieu Egloff [1,2,†] and Laurie Galvan [1,*,†]

[1] Laboratoire de Neurosciences Expérimentales et Cliniques, Institut National de la Santé et de la Recherche Médicale, Université de Poitiers, 86073 Poitiers, France; mathieu.thabault@univ-poitiers.fr (M.T.); valentine.turpin@univ-poitiers.fr (V.T.); alexandre.maisterrena@univ-poitiers.fr (A.M.); mohamed.jaber@univ-poitiers.fr (M.J.); matthieu.egloff@univ-poitiers.fr (M.E.)
[2] Centre Hospitalier Universitaire de Poitiers, 86021 Poitiers, France
* Correspondence: laurie.galvan@univ-poitiers.fr
† These authors contributed equally to this work.

Abstract: Autism spectrum disorders (ASD) are complex conditions that stem from a combination of genetic, epigenetic and environmental influences during early pre- and postnatal childhood. The review focuses on the cerebellum and the striatum, two structures involved in motor, sensory, cognitive and social functions altered in ASD. We summarize clinical and fundamental studies highlighting the importance of these two structures in ASD. We further discuss the relation between cellular and molecular alterations with the observed behavior at the social, cognitive, motor and gait levels. Functional correlates regarding neuronal activity are also detailed wherever possible, and sexual dimorphism is explored pointing to the need to apprehend ASD in both sexes, as findings can be dramatically different at both quantitative and qualitative levels. The review focuses also on a set of three recent papers from our laboratory where we explored motor and gait function in various genetic and environmental ASD animal models. We report that motor and gait behaviors can constitute an early and quantitative window to the disease, as they often correlate with the severity of social impairments and loss of cerebellar Purkinje cells. The review ends with suggestions as to the main obstacles that need to be surpassed before an appropriate management of the disease can be proposed.

Keywords: ASD; cerebellum; striatum; epigenetics; motricity; autism

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1. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition manifested by early onset of (i) persistent deficits in communication and social interactions and (ii) restricted patterns of behaviors, activities or interests (DSM5). The severity range of each symptom, and the fact that other abnormalities can accompany ASD, led to the "spectrum" umbrella term. Comorbidities vary among patients, but the most common ones are: sleep disorders (up to 80%), intellectual deficits (45%), epilepsy (up to 30%) and, interestingly, motor abnormalities (79%) [1]. The WHO considers that ASD affects 1 in 160 children worldwide following a ratio of 3 boys for 1 girl [2], suggesting male susceptibility. To this day, the diagnosis age ranges from infant to adult, depending on the severity of the symptoms and the social environment.

The etiology of ASD is still uncertain, but evidence has strongly linked genetic, epigenetic and environmental factors to it. The gathered knowledge in this domain is interdependent with technological advances. The possibility to collect and analyze broad genetic information for the past ten years has led to breakthroughs. The first pieces of evidence of genetic involvement in ASD were the case studies of monozygotic twins. These twins displayed a 60% probability to share the same ASD diagnosis, whereas dizygotic

twins' probability was at 10% [3–5]. More importantly, the incidence of having an ASD child is proportional to the child's shared genomic percentage with an ASD parent or sibling [6–8]. The advancement of sequencing technics led to the possibility of analyzing the patient's whole genome, which helped identify more than 1200 susceptibility genes (SFARI gene, https://www.sfari.org/resource/sfari-gene/, accessed on 14 September 2021). These genes can be broadly categorized into two families, involving either chromatin modeling or synapse formation [9].

Perinatal factors have been identified as increasing ASD incidence, including maternal infection and maternal toxin exposure. A 2016 meta-analysis of more than 40,000 ASD cases highlighted an increased risk of ASD in children whose mother had a viral infection during pregnancy, leading to hospitalization [10]. One hypothesis underlying this observation is that maternal immune regulation leads to increased production of inflammatory cytokines. There is not a consensus about how the cytokines could reach the fetus brain. They could reach it by disrupting the brain–blood barrier (BBB) [11] or by the actions of proinflamma- tory cytokines able to cross the BBB (for review, see [12]). Several pharmacological agents are also responsible for increasing ASD incidence in children exposed during prenatal development. For instance, valproic acid (VPA) is widely used as an antiepileptic drug and a mood stabilizer. It has various effects, from inhibiting the histone deacetylase [13] to the GABA signaling potentiation [14]. Pregnant women under VPA medication are four to five times more likely to have an ASD child even when VPA was taken at the lowest doses [15] (for review, see [16] and Table 1). We will focus on the VPA as an environmental model of ASD in this review event, though; others have been described as the phthalic acid ester exposition (for review, see [17]).

Some of the mutations and perinatal factors increasing ASD incidence have led to the generation of corresponding animal models, with some showing good face validity compared to clinical conditions. However, not all ASD symptoms can be reproduced in rodents. For example, while social interaction and motor stereotypy can be robustly observed and scored in rodents using various and complementary behavioral tests, cognitive stereotypy and intellectual disability cannot be adequately assessed. Speech deficits are also a symptom in patients, which cannot be directly translated in the mouse models. The closest feature that can be measured is ultrasonic vocalizations (USVs). USVs are used by pups separated from their mother or littermates, by juveniles when playing and by adults during social interactions or mating. Although their exact significance is unclear, many different USVs parameters were modified in ASD mice compared to wild type. Shank1$^{-/-}$ pups emitted less isolation-induced USV with a shift in frequencies than control mice [18], but not Shank3$^{-/-}$ pups, where other parameters were modified [19]. Additionally, no difference in the number of USVs emitted by pups was found between the Frm1$^{-/-}$ mice and wild type but rather a change in specific frequency [20]. Thus, it seems challenging to observe consistent and robust USV changes through different ASD mouse models.

Since 1943 and the first description of autistic features by Leo Kanner [21], followed almost concomitantly in 1944 by Hans Asperger [22], motor and cognitive impairments have been within the core of ASD symptoms. Currently, studies focus more on cognitive aspects of ASD and the corresponding physiopathology and brain regions [23,24]. The notion linking ASD to a focal cerebral dysfunction has drastically evolved toward a consensus of cerebral multi-regional reorganization during development. We will focus this review on two complex brain areas involving sensory and motor functions: the cerebellum and the striatum.

Table 1. Various outcomes in various animal models of valproic acid administration during development in relation with species, dose and age. This table recapitulates the different VPA models and their phenotypes. IP: Intraperitoneal injection, USV: Ultrasonic vocalizations, D1R and D2R: D1 dopamine receptor and D2 dopamine receptor, PC: Purkinje cells, MOR: Mu opioid receptor, PV: Parvalbumin interneurons, mPFC: median Prefrontal cortex, GABAP3: GABA receptor subunit p3.

Species	Treated Animal	Periodicity	Dose	Age of Treatment	Phenotype
Xenopus [13]	Embryo	24 h exposition of the eggs	• 1 mM • 2.5 mM • 5 mM	Stage 8 embryo	• 5 mM: loss of anterior structures and shortening of anterior-posterior axis in 88% of embryos
C57BL/6J mice [25]	Pregnant female	One IP injection	450 mg/kg	E12.5	• ↗ Eye-opening delay • ↗ Time to climb the wire and the grid • ↗ Immobility in males • ↘ Crossing in males • ↗ Grooming duration • ↘ Rearing in males • ↘ Sociability index in males • ↗ Errors during challenging beam test • ↗ Gait abnormality • ↘ PC number in Crus 1/2 and M1/M2
C57BL/6J mice [16]	Pregnant female	One IP injection	600 mg/kg	E12	• ↗ PC density in young mice at P13 • ↘ PC number in males at P40
C57BL/6J mice [26]	Pregnant female	One oral administration	600 mg/kg	E12	• ↘ PV expression level but not PV neurons • ↗ mPFC volume • ↘ KCNC1 mRNA level in forebrain • ↘ Kv3.1b mRNA level in forebrain • ↗ HCN1 mRNA level in forebrain
CD-1 and GFAP-eGFP mice [27]	Pregnant female	One IP injection	500 mg/kg	E12.5	• ↘ CB+ cells CB intensity and GABAP3 intensity in lobule X at P8 • ↘ Expression of GABP3 in ependymal glial cells • ↗ Disrupted GABAP3 expression through development

Table 1. *Cont.*

Species	Treated Animal	Periodicity	Dose	Age of Treatment	Phenotype
FVB/NJ mice [28]	Pregnant female	One IP injection	400 mg/kg	E11.5 or E12.75	E11.5 • ↘ MOR1 expression in caudal striatum at P14 • ↗ Abnormal cell aggregation into striosomal patch E12.75 • ↘ MOR1 expression in caudal striatum at P14 • ↗ CB number rich matrix in rostral in the ventro-medial striatum at P14 • ↗ Abnormal cell aggregation into striosomal patch • ↗ FOXP2+ cell in layer 5 (S1 cortex) and layer 6 (S2 cortex) • ↘ Corticostriatal synapse in rostral striatum • ↘ Number, duration, peak frequency and peak amplitude in USV
Long Evans rats [29]	Pregnant female	One IP injection	600 mg/kg	E12.5	• ↘ PC number in vermis, anterior, posterior lobes • ↘ Granule layer volume in vermis, anterior, posterior lobes
Long Evans rats [30]	Pregnant female	One oral administration	800 mg/kg	E12	• ↘ Brain weight • ↗ Errors in T-maze • ↗ Deficits in females in skilled reaching but improvement in male • ↗ Time in open arms in elevated plus maze • ↘ Dendritic branching in male • ↘ Dendritic length; decrease spine density, decrease cortical thickness
Sprague-Dawley rats [31]	Pregnant female	Two oral administrations	800 mg/kg	E10 and E12	• ↘ Body weight between P21 and P28, ↘ brain weight • ↘ Body length • ↘ Purkinje cell size in all ten lobules • ↘ Number of calbindin positive PC in all ten vermal lobules • ↘ Primary dendrite thickness. • ↗ Time to righting • ↘ Length in limb stride • ↗ Time to complete static beam and number of errors and of failed attempts

Table 1. *Cont.*

Species	Treated Animal	Periodicity	Dose	Age of Treatment	Phenotype
Winstar rats [32]	Young rat	Daily intragastric administration	200 mg/kg	1 month 3 months 6 months 9 months 12 months	• ↗ Swelling of perikarya and dendritic process • ↗ Abnormalities of mitochondria • 9 and 12 months: severe degenerative changes • ↗ Swollen Bergmann astrocytes
Winstar rats [33]	Pregnant female	One IP injection	500 mg/kg	E12.5	• ↘ Social play behavior and sociability in 3-CT • ↘ Social discrimination ability • ↗ D2R expression in Nucleus accumbens • ↗ D1R expression in Nucleus accumbens • ↘ Resting potential in MSNs • ↗ Altered action potential discharge pattern in MSN

2. Cerebellar Involvement in ASD

2.1. Structure and Function of the Cerebellum

All vertebrates have a cerebellum, although not all have a cortex [34]. The cerebellum might have first emerged in fish, providing computational analysis of sensory feedback from their lateral lines and hair cells on their skin that help them detect their environ- ment [35]. The primary role of the cerebellum has long been linked to coordination and control of movement. Its implication in higher function has first been proposed by Leiner, following the observation in higher primates of an enlargement of the cerebellum that paralleled that of the frontal cortex during phylogenic evolution [36]. A few years later, Middelton and Strike provided anatomical evidence of cerebellar and basal ganglia involve- ment in higher cognitive function [37]. Since then, many studies highlighted the eminent role of the cerebellum in cognitive and emotional function, and that was attributed to its connections with cortical and subcortical centers [38,39]. In addition, extensive connections between the cerebellum and frontal associative cortical areas suggest a critical role in treat- ing sensory motor information from visual, auditive and sensory input, as well as a role in memory, language processing and planification [40].

Although relatively simple in its general structure, as well as afferent and efferent projections and cellular organization, the anatomy of the cerebellum can be difficult to describe. This is mainly due to three factors: (i) the structure/function of the cerebellum is quite different depending on whether one is considering the anteroposterior or parasagittal plans; (ii) each part of the cerebellum can have several names depending on the user and the current trend; (iii), not all authors agree on what each part encompasses. When viewed from the anteroposterior axis, the cerebellum is formed from three lobes: the vestibulocerebellum, the posterior cerebellum and the anterior cerebellum, which are separated by deep fissures. Each lobe is constituted by lobules numbered I to X, giving the cerebellum its folded shape. The vestibulocerebellum (also called lobule X) comprises a median nodule and flocculus lateral ones. It is directly connected to the vestibular nuclei within the brainstem and receives projections from the auditory nerve. Most of the efferent projections from the cerebellum originate from four deep cerebellar nuclei within the vestibulocerebellum that receive projections from collateral fibers, parallel fibers and Purkinje cells (PC). This is the oldest part of the cerebellum phylogenetically, and it is shared among all vertebrates. It handles perception of self-motion and spatial orientation through the auditory system ofthe head's position and motion. The anterior lobe is composed of lobules I–V and includes the medial vermis part. It receives proprioceptive information from the body and limbs, as well as from the visual and auditive systems. It sends projections toward the cortex and the spinal cord, modulating the descending motor system. The anterior lobe is responsible for balance, posture and consequent movement adjustments in time and space. The posterior lobe is composed of lobules VI–IX. This lobe constitutes the cerebellar hemispheres and is the most recent and evolved part of the cerebellum. It is only present in higher mammals and is particularly enlarged in the human species. The posterior lobe receives projections exclusively from the cerebellar cortex and sends projections to the ventrolateral part of the thalamus. It is thought to be implicated in movement planification, motor learning, timing, language processing and emotional, cognitive and social functions.

From a parasagittal perspective, the central part of the cerebellum is constituted by the vermis, followed by the paravermis and ending laterally by the cerebral hemispheres, which constitute the most significant part in the primate's cerebellum. Each lobule can have a different function and set of projections, depending on its parasagittal plan. Of interest to us here are crus I and crus II that constitute the lateral parts of lobules VI and VII, respectively, within the posterior lobe and that are affected in ASD. Crus I and crus II are homologous in human and non-human primates. However, rodent crus I corresponds to crus I and crus II in primates [41], while rodent crus II may be homologous to HVIIB.

At the cellular level, the cerebellar cortex is organized into three layers that are, from the surface to the white matter: (i) the molecular layer, a cell-poor layer containing stellate and basket cells as well as PC dendrites connected to parallel fiber axons from granule

cells and to climbing fibers. The climbing fiber axons are also found in the granular layer, (ii) the PC layer, a thin single cell layer and (iii) the granule cell layer containingwhat is thought to constitute more than 50% of whole nerve cells within the brain (up to 10^{11} neurons) [42]. Granule cells receive projections from mossy fibers originating from the vestibular nuclei. In turn, they project excitatory parallel fibers to PC dendrites within the molecular layer, each parallel fiber contacting 100 PC. Each PC receives an approximate number of 150,000 synapses from parallel fibers and projections emanating from a single climbing fiber [43] originating from the inferior olive. The PC are large neurons with flatand rich dendritic arborization. Their inhibitory GABAergic axon projects through the granular layer to the vestibular nuclei and constitutes the sole efferent projection from the cerebellar cortex (Figure 1).

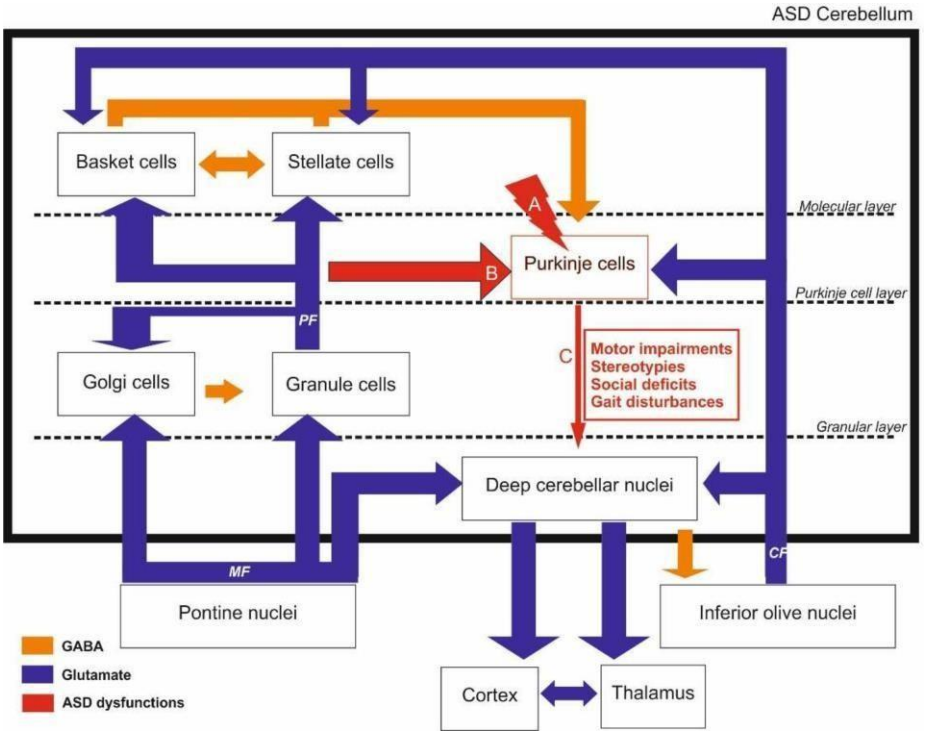


Figure 1. The cerebellum is involved in both motor and social impairments reported in ASD. Dysfunctional Purkinje cells (PC) seem to be at the center of these impairments as they represent the sole output of the cerebellum and receive inputs from both inhibitory and excitatory cells. PC dysfunctions are reported in ASD clinical settings and in animal models (A, B, C). PC intrinsic changes (A) such as reduced PC density was shown in Fmr1 KO mice [44], TSC2f/- mice [45], Mecp2 deficient mice [46], VPA mice [47], polyI:C mice [48] and Shank3ΔC/ΔC mice [49], with regional lobular differences between males and females. Low PC numbers associated with a decrease in soma size and an increase in ectopic PC number were also reported in VPA rats [50], especially in lobule VII hemispheres (crus I and II) that are involved in sensorimotricity. Impaired inputs from granule cells through parallel fibers (B) were also reported. For instance, the mGluR-long term depression (LTD) was altered at the PF-PC synapse in both PC-specific Grip1/2 KO mice and Fmr1 KO mice [51,52]). Furthermore, PC abnormal dendritic branching and reduced density of dendritic spines were found to impair synapse formation in VPA rats [53,54] and in Fmr1 KO mice [44,51]. Outputs from PC onto deep cerebellar nuclei (C) are also impacted, as PC firing pattern is impaired in mice lacking Calbindin, with decreased complex spike duration and pause, as well as decreased simple high spike firing rate [55]. In the Shank2 KO mice, an increased irregularity in simple spike PC firing accompanied by increased inhibition was only found in the posterior cerebellum [56]. Altogether, these PC-focusedalterations would lead to dysfunctional cerebellar loop up to the thalamus and cortex leading to both motor and cognitive impairments. MF: Mossy fibers, CF: climbing fibers, PF: parallel fibers.

The various functions of the cerebellum that have been described to date, whether motor or cognitive, seem to converge toward a peculiar capacity of the cerebellum to estimate and keep track of time. James Albus proposed that the anatomy and physiology of the cerebellum both point toward a pattern-recognition data processing that allows the handling and storage of information based on trial (intent) and error (action) and that this is achieved by weakening the synaptic weights rather than by strengthening them [36,57]. Clumsiness and deficits in motor coordination and manual dexterity, abnormal balance gait and posture are all dependent on the cerebellar function and are affected in ASD [58–60]. These deficits can be detected even in the first months of life, with affected babies exhibiting difficulties positioning their body when carried, hypotonia and uncoordinated movements. Based on the existing knowledge of the sensory motor role of the cerebellum and suspecting its implication in psychiatric disorders, Dow and Moruzzi developed test batteries early in 1958 on patients with various psychiatric disorders. They reported severe cerebellar-type impairments in what was referred to as patients suffering from autistic and Asperger syndromes [61]. Since then, a growing number of papers have reported substantial implications of the cerebellum in motor and non-motor deficits in ASD. From this aspect, the severity of cerebellar injury in premature infants is predictive of the severity of ASD symptoms in adult age [62]. Indeed, there are three cerebellar-related deficits reported in ASD patients based on imaging and post-mortem histological observations: (i) a decrease in the number of PC, (ii) reduced cerebellar volume and (iii) disrupted circuitry between the cerebellum and connecting brain areas, such as the thalamus, the pons and the cortex [63,64]. As PC are inhibitory, this, in turn, leads to hypersensitivity of cerebellar target areas [65,66].

2.2. *Anatomical Evidence of Cerebellar Involvement in ASD*

The involvement of cerebellum in the ASD context is also supported by genetic evi- dence. Indeed, 38 genes, whose expression is enriched in the cerebellum, were identified as ASD-linked susceptibility genes [25]. Several studies showed that ASD patients displayed hypoplasia (−12% [67,68], for review, see [63]). Using magnetic resonance imaging (MRI) in both adult ASD and healthy patients, Murakami et al. reported a reduced size of both vermis and hemispheres in ASD patients without and with mild mental retardation [67]. This alteration is not always found in ASD mouse models and could be due to the spectrum of the disease or the heterogeneous way of assessing it at the levels of (i) methodology (stereology vs. MRI), (ii) diversity of genetic background of ASD animals, (iii) animal age and (iv) gender (for review, see [69]). A decrease in cerebellar volume has also been reported in multiple genetic pathologies related to ASD, including fragile X syndrome (FXS) [70,71], Rett syndrome [70–72], the Phelan–McDermid syndrome, including *Shank3* deletion [73,74] (SH3 and multiple ankyrin repeat domains 2), and *NLGN4* (Neurologin-4) associated non-syndromic X-linked ASD [46]. Decreases in cerebellar volume can also be observed in corresponding animal models, such as the Fmr1 Knock-out (KO) [44] and Nlgn4 KO [75] mice models (Table 2).

Table 2. Cerebellar dysfunctions in ASD. This table recapitulates cerebellar dysfunctions found in patients and animal models.

		Children		Teenagers	Adults	Animal Models
		<5 years old	5 to 14 years old	14 to 21 years old	>21 years old	
Anatomical impairment	Global cerebellar volume	• N/A	• N/A	• N/A	• Hypoplasia [55,56]	• No consistent changes [57] ↘ Fmr1KO [64] and Nlgn4KO^{-} [65], = Shank3$^{\Delta c/\Delta c}$, VPA and Poly I:C models [25,66,67]
	White matter (WM) changes	• ↗ WM [68,69]	• No change [69] • Local thickening in boys [70,71]	• No change [69]	• N/A	• N/A • ↗ in at least 30 mouse lines for Crus I/II projections [78]
	Connectivity	• N/A	• ↗ in the right Crus I and left inferior parietal lobule [76] • ↗ Right during motor task [72] ↗ in the left supplementary motor areas [72] Global right overconnectivity [73]		• ↗ in right Crus I and left mPFC [77]	• ↗ PC firing in left mPFC when right crus I is inhibited [76]
Cellular correlates	Purkinje cell (PC)	• ↘ axon numbers [79]	• ↘ axon numbers [79]	• ↘ PC density [80]	• ↘ PC density [51,80–82] • ↘ soma size & ectopic PC in molecular layer [81,82]	• ↘ PC density [25,66,67,83] ↗ PC density [63] ↘ PC arborization in LPS rats [84] ↘ Dendritic spine density in VPA rats [30]
	Bergmann cells	• N/A	• N/A	• N/A	• Activated/reactivated in PC loss area [51,85]	• ↗ Increased ectopic PC in VPA rats [29,30] • Activated /reactive in PC loss area in Fmr1$^{-/-}$ [64] and VPA rat [32]
	Microglia	• N/A	• High global microglial activation [85]			• No changes in poly I:C mice [67]
Neurotransmission	Glutamate	• N/A	• ↘ AMPA-R density [86] ↘ glutamate & glutamate metabolite levels [87]	• ↘ AMPA-R density [86] • ↘ glutamate & its metabolite levels [87]	• ↘ AMPA-R density [86] • ↗ levels of EAAT1/2 [88]	• Impaired mGluR LTD [89–91] • ↗ mGluR5 expression in Grip1/2-PC, Fmr1$^{-/-}$ and Shank2$^{-/-}$ [90,92–94] ↘ mGluR5 expression in Shank3$^{\Delta c/\Delta c}$ [66]
	GABA	• ↘ GABA-A β3 level [95]	• ↘ GABA-A β3 level [95]	• ↘ GABA-A β3 level [95]	• ↘ GABA-A α, GABA-B R1 density, GAD65/67 levels [96,97] • ↘ GABA-A β3 level [95]	• Abnormal PC firing pattern in Calbindin deficient mice [98] ↘ GABA-A ρ3 levels in VPA model [27] ↘ GABA-A β1 & β2 levels in Fmr1 KO [99]

N/A: not applicable.

PC loss has been consistently described in ASD patient brains, and cerebellar hypoplasia was found in most cases [49,62,63,75]. In our previous articles, three ASD mice models (Shank3$^{\Delta C/\Delta C}$ [49], VPA [47] and polyinosinic:polycytidylic acid (poly I:C) [48]) displayed no global changes in cerebellum size, although they did show PC loss predominantly in crus I and II. Beyond the cell count, cerebellar connectivity is also affected in ASD, and white and gray matter abnormalities have been observed by voxel-based morphometry. Analysis of cerebellar white matter in low- and high-functioning ASD young male children shows a significant increase in white matter volume compared to a typical children group [77]. That characteristic has been robustly used as a prediction for ASD diagnosis in this same study. A specific change in cerebellar white matter is typical in 2–3-year-old ASD children, which is not observed in older ASD children and adolescents [78]. A few studies have investigated fiber tracts and myelin differences in ASD patients using diffusion tensor imaging (DTI), revealing changes in white matter structure and projections. The evaluation of white matter in male ASD children (6–12 years old) shows a thickening of the left cerebellar peduncle and of both middle cerebellar peduncles compared to typical children [76]. Interestingly, motor-related structures were also affected, such as the left putamen and the corticocortical pathway [76]. Considering motor deficits in ASD, another study assessed, contingently, motor function and DTI in ASD children (5–14 years old, twelve males and one female) compared to a non-ASD control age group [100]. In this study, ASD children displayed the poorest motor function scores (manual dexterity, ball skills and balance) compared to neurotypical children. Additionally, these motor deficits were correlated with a decrease in fractional anisotropy (white matter microstructural integrity index) bilaterally in the superior cerebellar peduncle [100], the efferent cerebellar tract to the midbrain being mainly composed of the cerebellothalamic tract. Taken together, these white matter analyses reveal an alteration of cerebellar motor pathways in ASD.

Lateralization of functional connectivity patterns is modified in ASD, revealing changes in functional topography. Noonan et al. assessed the functional connectivity of ASD brains by asking adult patients to perform a memory task while undergoing an MRI. They selected high functioning ASD adult males who showed the lowest score at general word recognition and source recognition performance compared to a control group. Although ASD and control groups showed no differences in functional connectivity in the left hemisphere, ASD patients showed greater connectivity in the right hemisphere, including the right supplementary motor areas and the cerebellum [101]. This right overconnected lateralization of the ASD cerebellum was also observed in ASD children and adolescents [81]. Interestingly, the performance of a simple motor task (self-paced button press with the dominant thumb) by young ASD adults and control patients while being imaged revealed that the magnitude of activation of the ipsilateral anterior cerebellum is strongly increased in ASD patients [68]. Moreover, the authors found that contralateral and posterior cerebellar regions that are not generally associated with simple motor tasks were abnormally active in ASD patients [68].

Consequently, based on the over-recruitment of crus I and II in ASD patients, as well as in cerebral regions involved in both cognitive and motor tasks, these regions might be essential for understanding the behavior impairments in ASD patients. From this aspect, MRI analysis in young children showed that ASD children with repetitive movements had a negative correlation with cerebellar vermis area of lobule VI and VII (crus I andII) [82]. Evidence from 10-year-old ASD children revealed higher functional connectivity between the right crus I and the left inferior parietal lobule than neurotypical children [102]. This connectivity is not the only one impaired in ASD. Similar observations were reported regarding projections originating from the right crus I to the left mPFC in ASD patients [79] and in 30 out of 94 ASD mice models [83]. Consequently, chemogenetics-specific inhibition of PC in the right crus I in the tuberous sclerosis complex 1 (Tsc1) ASD mouse model led to increased firing in the left mPFC associated with improved sociability behavior, inflexibility and motor stereotypies [80]. This pathway is polysynaptic, as it anatomically relays on the ventral medial thalamus. In the Tsc1 ASD mouse models, the optogenetic inhibition

of these specific thalamic neurons was sufficient to prevent both social impairments and repetitive behavior [83].

The posterior cerebellar circuits allow the processing and integration of multisensory information, including visual, proprioceptive and somatosensory, which may be reduced in ASD patients. This raises the hypothesis of compensating mechanisms in ASD patients regarding their sensory forward control deficits in prehension, gait and postural control. When high-functioning ASD children have been asked to perform a simple motor task, such as a "grip", they displayed an increase in the grip to load force, suggesting temporal dyscoordination. Since Vilensky's study in the 1980s, ASD patients' walking features have been highly investigated, and a dozen differences were found, such as increased stance duration, reduced stride length, toe striking and increased angular motions [103]. Interestingly, these features are also modified in some ASD mice models. ASD patients seem to slow down their movements in relation to task difficulty and need more practice than typical individuals. These deficits in complex social motor skills could be associated with social and imitation impairments and over-reliance on proprioceptive feedback in motor control and learning (for review, see [104]).

2.3. Cellular Correlates

The PC number decreases from 35 to 95% in ASD patients [49,63,75,84], and their soma size and, consequently, their density [29,84], are affected too. Differences in the extent of the reported decreases seem to result from a high variability both in subjects and methods. One of the pioneer post-mortem studies found PC loss and ectopic PCs within the molecular layer only in adults, whereas PC inclusions were described in a sole child case [30]. In parallel, fewer axons were found in PCs from ASD children (male and female, 3.6–13.3 years old) using diffusion MRI tractography [31]. These findings highlight age as a crucial parameter in ASD cellular consequences. The spectrum of symptoms in this disorder might account for the difficulty in obtaining homogenous results in both patients and animal models. This is even the case with genetic mouse models of ASD, where different cellular outcomes have been reported. For instance, PC density was reduced in both Fmr1 KO mice (C57BLJ6J, P30) [44] and mice with a specific deletion of the tuberous sclerosis complex 2 (TSC2) [45], whereas an increase was observed in Mecp2-deficient mice [46]. In our study, Shank3$^{\Delta c/\Delta c}$ mice displayed an extensive loss of PC in both crus I and II, but only in males [49]. While gait was disrupted only in males, deficits in social novelty were observed in both sexes. In environmental ASD models, our previous studies showed PC loss in both crus II and 7cb in males and PM in females, with poly I:C prenatal insult [48]. In the VPA mouse model (E12.5; 450 mg/kg) (Table 1), PC number was reduced in crus I in males and crus II in females [47]. This sex-specific reduction was correlated with behavioral tests assessing sociability and motor impairments. Sexual dimorphism was also found in another study that reported PC decrease only in VPA males in the cerebellar lobules VI, VIII, IX and the paramedian one [16]. Clinical studies also established correlations between sex and regional-specific PC loss. For instance, Skefos et al. found that ASD male patients (7–56 years old) exhibited a 21% decrease in regional volume-weighted mean compared to females (4–21 years old). Only males presented a reduction in PC in the lobule X of the flocculonodular lobe. The posterolateral region seems to be the most affected, specifically the lobule VII hemispheres, which are crucial sensorimotor areas with reciprocal interaction with the PFC and the posterior parietal cortex [105] (Table 2).

Differences between males and females in ASD seem to find their origin in the brain masculinization during brain development, a critical period that puts males at risk regarding neurodevelopmental disorders. During the second postnatal week, arachidonic acid and estradiol production peak. It has been shown that inflammation or nonsteroidal anti-inflammatory drugs (NSAIDs) result in impaired play behavior in males [106]. Even though it is still assumed that the cerebellum is not sexually dimorphic, as opposed to the preoptic area, for example, specific subregions such as lobules VI and VII have been demonstrated to represent a particular sexual orientation dimorphism related to emotion and

sensation [106]. In the cerebellum, prostaglandins stimulate aromatase and local estradiol production. PCs respond to prostaglandin E2 (PGE2) and 17-estradiol (E2) levels. Indeed, E2 induced BDNF expression at physiological levels and promoted PC dendritic growth, spinogenesis and synaptogenesis during neonatal life. High E2 levels stop dendritic growth and reduce excitatory synapses number [89,106]. Given that E2 is the primary hormonein females, this raises the hypothesis that E2 could protect females from environmental insults, whether toxic, pharmacologic or immune. Indeed, only male rats exposed to LPS or PGE2 during the second postnatal week displayed reduced PC arborization and impaired juvenile social play behavior [107] (Table 1).

Unfortunately, not all studies separate male and female groups; many use only males, and some studies even pool the data from both sexes, undermining sex differences. For instance, rats (male and female pooled) prenatally exposed to a single VPA dose exhibited a reduced number of neurons in the cerebellum, abnormal dendritic branching and reduced density dendritic spines affecting axonal projections of PC [53,54]. Double-dose VPA-exposed rats (male and female pooled, E10 and E12 800 mg/kg) exhibited an increased number of ectopic PCs correlated with a 21% reduction in soma size compared to control ones in all ten vermal lobules. While lobules IV and VIc were both affected in decreased PC soma size (30 and 39%, respectively) and PC number (55 and 36%, respectively), lobule VII seemed to be less affected, with only a 9% decrease. In the same study, VPA gestational exposure induced a reduction in Calbindin across all ten vermal lobules. Only 65% of vermal PC were Calbindin positive compared to control animals that were 90% PC Calbindin positive [50]. In this model, Calbindin-positive PC dendrites were shorter and showed reduced branching complexity, in accordance with a previously reported slight increase in spine length and volume in Calbindin KO mice [85], suggesting that a lack of Calbindin could lead to impaired spine morphology, hence reducing synapse formation. Interestingly, no significant changes were observed in the Parvalbumin KO mice. Nonetheless, double transgenic KO mice for the two EF-hand type Ca^{2+} binding proteins (Calbindin and Parvalbumin (PV)) showed significant differences in PC spine morphology compared to wild type [85]. Synapse establishment is a crucial process for PC to engage in cognitive and motor tasks. It has been known that mutations of genes coding for SHANK are impaired in autistic patients [32]. These mutations lead to fewer mature dendritic spines due to impaired spine induction and morphology. In detail, this leads to reduced mature glutamatergic synapses, which would affect cognitive functions. Studies on the male Fmr1 KO mouse model also showed dendritic impairments in the cerebellum, with PC exhibiting longer and immature dendritic spines [44,88]. The eye-blink conditioning, a behavior managed by the cerebellum and mainly the interpositus nucleus, was impaired in both Fmr1 KO and PC-specific Fmr1 KO mice [88] (Table 1). Although eye-blink conditioning was impaired in FXS patients [88,108], repeated training led to improvements in adult patients [108].

Bergmann cells are cerebellar astrocytes that are crucial for PC dendritic formation and maintenance. Post-mortem analysis of six male ASD patients revealed that these cells were activated and reactive in cerebellar areas where PC were reduced [64,90]. This was also observed in VPA rats [86] and Fmr1 KO mice [44]. The microglia oversee the proper neuronal development and have been demonstrated to be involved in synaptic density, spatial localization, morphology, process retraction and thickening, resulting in synaptic pruning. The co-activation of microglia and astroglia seems to correlate with degenerating PC, granule cells and axons. Indeed, post-mortem analysis of ASD patients' cerebellum (male and female, 5–44 years old) showed a pattern of high microglial activation compared to control tissues [90]. However, in our study, no change in microglia was found in the poly I:C animals (P45) [48]. Since microglia are known to be activated during a specific time win- dow, a more detailed investigation of the timing of these processes is needed. In addition, microglia and astroglia activation in post-mortem samples could be an independent event that may not be linked to ASD but rather to traumatic cause of death (listed in [90]). PCs seem to be at the center of the gliosis found in ASD patients and animal models through

auto-toxic mechanisms, independent of adaptative immunity. Indeed, markers found in the perineural compartment of PCs raise the hypothesis of complement system involvement in immunopathogenic means, similarly to what is reported in neurodegenerative disorders. These findings indicate that cerebellar abnormalities found in ASD patients could be due not only to prenatal developmental insults but also to glia-mediated chronic neuroinflammatory processes persisting throughout life.

Other cerebellar cell types, such as basket and stellate cells, seem to be preserved in ASD patients [109]. These observations reinforce the hypothesis that PC density loss could be due to an insult during late prenatal periods, when PCs and inferior olivary neurons establish their connections [105]. Only one study found a decreased granule cell number in ASD patients [90], but findings in this cellular population rarely constitute a perfect match through animal models of various cerebellar pathologies.

2.4. Neurotransmission Systems Implicated

Glutamate and GABA have consistently been reported to be the most affected neurotransmission systems in ASD. The nature of motor and cognitive impairments in ASD points toward an excitatory/inhibitory imbalance within the cerebellum (for review, see [87]). The GABA–glutamate imbalance was thought to be responsible only for seizures occurring in some schizophrenic and ASD patients, but recent evidence has proven a wider array of pathologies [51]. For instance, glutamate has been extensively related to neurogenesis, synaptogenesis and neuronal maintenance in relation with emotional behavior acquisition. The cerebellum contains various AMPA, NMDA and kainate glutamate receptors. Using microarrays, autoradiography and western blot on post-mortem patient samples, Purcell et al. compared markers of glutamatergic neurotransmission in ASD subjects (5–54 years old) and neurotypical ones (2.4–53 years old) and reported a decreased AMPA receptors (AMPAR) density in patients' cerebellum [110]. Since glutamatergic interacting proteins 1/2 (Grip1/2) regulate AMPAR trafficking and synaptic strength, PC–AMPAR signaling in ASD was recently examined in a PC-specific knockout mouse model. The specific loss of expression of Grip1/2 in PC resulted in an increased repetitive self-grooming in 3-month-old male mice and impaired mGluR long-term depression (LTD) at the parallel fiber (PF)-PC synapses [51]. In addition, mGluR5 and Arc were increased in a possible attempt to compensate for AMPAR inefficient recycling in the absence of Grip 1/2 [51]. However, no PC loss was found in this animal model [109]. Both excitatory amino acid transporter 1 (EAAT1) and 2 (EAAT2) mRNA and proteins were increased in ASD patients [87]. The EAAT1 and 2 are mainly expressed by Bergmann astroglia in the cerebellum, which is highly activated in ASD patients. The glutamatergic extracellular concentrations are thus suspected to be abnormally elevated in ASD subjects, resulting in an imbalance in excitation/inhibition. This may implicate a glutamate-mediated strong activation of PCs, which in turn would lead to cerebellar inhibition. Interestingly, the precursor of the glutamate synthesis, glutamine, is also upregulated in the left cerebellum of ASD patients [52]. However, a few studies did not report any increase in the ASD glutamatergic system. For instance, DeVito et al. used proton magnetic resonance spectroscopic imaging (1H MRSI) to detect various low-molecular-weight metabolites in vivo in young male ASD patients (6–17 years old) and control subjects (6–16 years old) [111]. Among the studied metabolites, both N-acetyl aspartate and glutamine were reduced in the cerebellum of ASD patients compared to controls, suggesting not only neuronal loss or dysfunction but also reduced levels of glutamate. These results suggest widespread reductions in gray matter neuronal integrity and a dysfunction of cerebellar glutamatergic neurons in ASD patients. However, it is to be noted that no females were used in this study, contrary to the work from Hassan et al. [52]. This is of relevance, as the menstrual cycle influences women's neurotransmitter levels across cortical regions [112]. Further studies with a larger cohort, including sex, age and menstrual cycle parameters, are required to determine parameters influencing the glutamate and glutamine levels in the ASD cerebellum [113]. Glutamatergic transmission is also modified in ASD animal models. Shank2-deficient mice

(Shank2$^{-/-}$) displayed abnormal and repetitive behaviors, as well as autism-like social deficit behaviors [114]. The cerebellar synaptosomes from these mice had fewer AMPA receptor subunits (GluA1 and GluA2) than control without affecting dendritic arborization and postsynaptic density. Electrophysiological recordings in these animals revealed deficits in long-term potentiation (LTP) in PF–PC [114]. In line with these findings, mice with a Shank2 deletion restricted to PC (*Pcp2-Cre;Shank2fl/fl* mice) displayed an interesting pheno- type that only partially related to ASD symptomatology [98]. Indeed, social behavior and repetitive behaviors were not observed in this mouse line, as these transgenic mice showed mainly motor coordination impairments and increased anxiety. The PC lacking Shank2 protein (*Pcp2-Cre;Shank2fl/fl* mice) displayed fewer miniature excitatory postsynaptic cur- rents (mEPSC) and fewer GluA1,GluA2,GluN2C, VGluT1 and GluD2 protein levels than control [98]. Specific loss of the TSC1 in the PC (*L7Cre;Tsc1flox/1* and *L7Cre;Tsc1flox/flox*) results in ASD behaviors [96]. Indeed, mice carrying this mutation displayed an increase in stereotypic movements, abnormal behavior and changes in PC electrophysical properties. PC lacking Tsc1 had a decrease in action potential frequency and EPSCs, but not IPSCs. This modification led to a decrease in the Excitation:Inhibition (E:I) ratio in mutant mice compared to control [96].

GABA has been studied to a lesser extent in the cerebellum compared to glutamate. Indeed, even though PC generate a GABAergic output, their cerebellar inputs are mainly glutamatergic, except for interneurons regulating PCs' firing pattern. Interestingly, Calbindin reduction in mice resulted in abnormal firing patterns in PC, such as decreased complex spike duration and pause and simple high spike firing rate [55]. These findings suggest that GABAergic inputs on the PC could be partially dependent on Calbindin levels. Lower densities of the two GABA receptor subtypes—GABA-A and GABA-B—have been found in ASD individuals. The GABA-A receptor is known for its fast inhibitory action, whereas GABA-B receptor activation results in excitatory/inhibitory regulation. The subunits GABA-A α protein levels and GABA-B R1 receptor density and levels of glutamic acid decarboxylase (GAD) 65 and 67 proteins, in charge of glutamate to GABA conversion, were all found to be decreased in the cerebella of ASD patients [97,115]. Furthermore, ASD children (5–15 years) showed increased GABA concentration in their plasma [116]. GABAρ3 is a subunit of the GABA-A receptor, with a high affinity for GABA, providing the receptor with low desensitization upon activation. The GABAρ3-composed GABA-A receptor plays an important role in regulating GABAergic transmission during the postnatal development of the cerebellum [27]. In the VPA model, GABAρ3 is decreased by 43% in the lobule X [99], in charge of the gaze coordination. Importantly, GABAρ3 level linearly increases during typical development, but not in the VPA model, as it chronically decreases at each studied time point (P4:—54%, P30:−83%) [99]. A significant reduction of GABA-Aβ1 and GABA-Aβ2 levels were also observed in the cerebellum of Fmr1 KO mice. Interestingly, the decrease in GABA- Aβ1 mRNA is only observed in the cerebellum, whereas GABA-Aβ2 mRNA levels also drop in the cortex, hippocampus and diencephalon [117]. In this mouse model, the administration of a GABA-A (Diazepam) or GABA-B (STX209) agonists results in the improvement of several behavioral deficits and a partial rescue at the molecular level [95,118] (Table 2).

Excitatory/inhibitory imbalance is one of the major hypotheses explaining ASD symptoms. Of interest is the finding that both ASD patients and ASD mouse models (FMRP) displayed surprising GABA dysregulations and glutamate receptor subunit changes, even in *post-mortem* cerebella of ASD subjects, without FXS or FMRP being downregulated, which was associated with increased levels of mGluR5 and decreased levels in GABA-A β3 subunits [92]. Fmr1 KO mouse models confirmed these findings, as PSD-95 (Postsynaptic Density protein 95) and mGluR5 were found to be increased in the cerebellum [93,94,119]. In our study, mGluR5 levels were reduced in the cerebellum of Shank3$^{\Delta C/\Delta C}$ males [49]. These findings suggest that mGluR5 plays a crucial role in the synaptic targeting and postsynaptic assembly of the Shank3 scaffolding complex. Drug therapies targeting this protein could be of interest, as the administration of 3-Cyano-N-(1,3diphenyl-1H-pyrazole-

5-yl) benzamide, which increases mGluR5 activity, was shown to alleviate functional and behavioral ASD defects [91]. In another study, the Fmr1 KO mouse model showed abnormal presynaptic vesicle dynamics and increased LTD induction at the PF-PC synapse [88]. Other models, such as Shank2 KO mice and specific patDP/+ mutant mice, also showed impaired LTD associated with dysfunctional intrinsic plasticity [56]. The latter is based on findings in ASD patients with mutations in 15q11-q13, a genetic region involved in GABA A β3 subunits and two other subunit candidate genes for ASD [120]. The patDp/+ mice mutants within the cerebellum exhibited impairments in motor coordination, learning, eye-blink conditioning, along with abnormal climbing fiber elimination of the PCs [56]. In the Shank2 KO mice, an increased irregularity in simple spike PC firing, accompanied by increased inhibition, was only found in the posterior cerebellum, possibly underlying cognitive impairments in this model. mGluR1 receptors are expressed by PC mediating LTD plasticity with parallel fiber [121]. In the postnatal developing cerebellum, the mGluR1 activating pathway is involved in axon pruning [122]. The inactivation of this receptor in mice ($mGluR1^{-/-}$) leads to a lack of motor coordination [123]. Even though the role of these receptors is crucial in glutamatergic transmission in the cerebellum, there is yet no direct evidence of mGlur1 dysfunction in ASD. In conclusion, there is an imbalance of the excitation/inhibition inputs in the cerebellum of ASD animal models and patients, with a significant reduction in GABA amount due to a reduction of its synthesizing enzymes GAD65 and GAD67. This is accompanied by a decrease in GABA receptors' activity, causing an increase in the activity of mGluR5. The mGluR signaling has been shown to be involved in GABA-A receptor stabilization at the synaptic membrane [124] in a healthy context. This may underly the fact that mGluR dysfunctions in ASD are often linked to GABA-A dysregulations. However, it appears that a reduction of the activity of mGluR5 can also lead to social deficits. Pharmacological treatments in animal models indicate that either activating the GABAergic system or inactivating the mGluR5 receptor may be of interest in managing some of the ASD symptoms where a reduced activity of the GABAergic system is reported. In the case of a decrease in mGluR5 levels or activity, the activation of mGluR5 reduces social deficits by restoring the excitation/inhibition balance.

2.5. Evidence from Our Previous Work

We have recently set up a series of studies on environmental and genetic animal models of ASD [47–49]. Our choice of the models was based mainly on their reported strong construct and face validity, as they were known to replicate both the etiology of the disease and at least some of its cardinal behavioral symptoms. The predictive value of animal models is hard to achieve, especially when no known treatment for the corresponding illness is available, which is the case for ASD. Our main aims when starting these studies were: (i) to determine whether different animal models of the same pathology would yield a spectrum of behavioral and cellular outcomes, mirroring the large and variable range of ASD symptoms in clinical settings, i.e., whether the nature (motor, social, gait) and the severity of the symptoms are variables depending on the etiology of the disease; (ii) to determine whether we can replicate the sexual dimorphism reported in clinical settings, as ASD affects three times more males than females [2], and again, whether sexual dimorphism is observed whatever the animal model and with the same proportion; (iii) to determine whether motor and gait deficits are observed in all animal models and whether they are correlated with the severity of social deficits. All experiments were performed within a relatively short timeframe (4 years in total), in the same laboratory and animal facility, using the same behavioral and bench equipment and software. For this, we have chosen two environmental animal models: the VPA and the poly I:C, a maternal immune activation (MIA) animal model and a genetic animal model bearing a Shank3 deletion. All three animal models showed major motor and gait deficits that were more pronounced in males than in females, but to a variable extent.

VPA animal models were obtained by injecting the drug i.p. at 400 mg/kg to pregnant females at E12.5 [47,125] (Table 1). In this model, we have found that the male offspring, but not females, expressed severe social deficits. However, both sexes showed motor coordination and gait deficits that were more pronounced in males than in females in their severity and variety. Cellular consequences accompanied these differential social and behavioral phenotypes. We reported a decrease in the number of PC found in the crus I cerebellar subregion in males and in crus II in females. In addition, only males showed a reduction in the number of neurons within the motor cortex. No neuronal decrease was found in the striatal region. Of interest is the finding that the severity of motor and gait disturbances was directly and strongly correlated to deficits in social behavior, as mice that had the most motor coordination deficits were the ones that had major social deficits and the highest decrease in PC within the cerebellar cortices.

In order to induce MIA, pregnant mice received a single i.p. injection of poly I:C (20 mg/kg, a double-stranded RNA analog polyinosinic:polycytidylic acid, which presents strong construct and face validity toward ASD and is the preferred MIA paradigm com- pared to direct injection of viruses [48,126]. Mice that received a Poly I:C injection at theprenatal age of 12.5, inducing an MIA phenotype, showed less dramatic social and motorbehavior alterations than those following VPA injection [48]. Only males showed deficits in social behavior and motor coordination. Of interest is that neither gait nor walking skillswere affected in either males or females. A reduced number of PC in the cerebellum was found to be more widespread and within distinct lobules in males than in females.

The Shank3$^{\Delta c/\Delta c}$ mice that we have used for our studies are those with C-terminal 508 deletion in the Shank3 gene following a frameshift in exon 21, which includes the homer-binding site in the sterile alpha motif domain. Consequently, there is a partial or total loss of the major naturally occurring isoforms of Shank3 proteins in heterozygotes and homozygotes, respectively [127]. This mutation has a strong construct validity, as it mimics a human mutation, which is not the case for several other Shank3 mutations in mice [127]. In homozygote animals, we reported significant impairments in social novelty preference, stereotyped behavior and gait. These were accompanied by a decreased number of PC in restricted cerebellar sub-regions and decreased cerebellar expression of mGluR5. Heterozygote mice showed impairments only in social novelty preference, grooming and decreased mGluR5 expression, but to a much lesser extent than in homozygote mice. All reported deficits were more pronounced in males than in females (Table 2).

Several elements of conclusion can be drawn for our studies: (i) The severity of ASD phenotypes, whether behavioral, cellular or molecular, varies from one animal model to another. This recapitulates in some way the spectrum of the disease, where variability may be due to its etiology, i.e., to what initially caused it. In our study, the VPA model yielded the most robust and severe phenotypes at all explored behavioral and cellular levels. (ii) Females are globally less affected than males in all the paradigms explored, whatever the treatment or the mutation. This is in line with clinical settings, where ASD is reported to be present three times more in males than in females. Notably, females showed no social deficits but still exhibited motor and gait alterations. In addition, the decrease in the number of PC was found in different sub lobules in females than in males. This suggests that ASD may be expressed differently in relation to sexual dimorphism. Thus, the proportion of affected ASD females may be higher than previously suspected if one also investigates motor and gait behavior. Such behaviors may be of relevance to implement in clinical exploration to help diagnose the disease. (iii) When the phenotypes explored are robust, as with the VPA animal model, the magnitude of social deficits can be correlated to both motor and gait deficits and PC cell number. In line with the previous conclusion, this further suggests that exploring motor and gait deficits may constitute an objective, early and quantitative diagnosis tool in ASD.

3. Striatal Involvement in ASD

3.1. Anatomical Evidence of Striatal Involvement in ASD

The basal ganglia are a group of subcortical nuclei involved primarily in motor skills. The term "Basal Ganglia" refers to the striatum and the globus pallidus, while the substantia nigra (mesencephalon), the subthalamic nuclei (diencephalon) and the pons are related nuclei [128]. The basal ganglia and their related nuclei can be split into three groups: input nuclei receiving incoming information from different cerebral areas, output nuclei sending basal ganglia information to the thalamus and intrinsic nuclei located in between, playing the role of a relay. The striatum is the largest subcortical structure and the only input nucleus of the basal ganglia (Figure 2). Although it has been remarkably conserved through the evolution of the vertebrate lineage for 530 million years, the striatum enhanced its role from primarily motor relay in amniotes to a complex circuitry capable of motor control (action selection), dealing with emotions and motivational state in mammals [129,130]. In primates and humans, the striatum is formed by the caudate and the putamen, separated by the internal capsule in the dorsal part and the nucleus accumbens in the ventral part. For the rest of the mammalians, the striatum is divided into two parts: the dorsal and ventral striatum. The dorsal striatum receives inputs from the sensory and motor cortices, the insular cortex and the orbital cortex on its lateral part (i.e., on the putamen in primates and humans) and the visual cortex, the anterior cingulate cortex, the ventral hippocampus and the amygdala on its medial part (i.e., on the caudate). The nucleus accumbens receives inputs from prelimbic and infralimbic cortices, the amygdala, the hypothalamus and the ventral hippocampus. This input organization leads to each territory's specializations from the motor to associative and cognitive mechanisms, from dorsolateral to dorsomedial and ventral striatum [129,130].

In the late 20th century, several studies took an interest in symptoms and brain structures other than the cardinal ones by studying the link between ASD and the cerebellum, as we discussed before. First evidence of the involvement of the striatum in the physiopathology of ASD was obtained from imaging studies [131–133]. Studies over the last decades highlighted anatomical differences between ASD and non-ASD subjects regarding the striatum and reported a wide range of modifications and inconsistencies. On the one hand, only the putamen and nucleus accumbens volumes appear to increase by, respectively, 22% and 34% in ASD patient brain compared to age-matched controls. The caudate volume was not significantly different between groups [84]. On the other hand, all the striatum, i.e., the caudate, was found to be more prominent in ASD patient brains than age-matched controls in magnetic resonance imaging studies [134–136]. Thus, the larger right caudate volume seems to be positively correlated with repetitive and stereotyped behavior [134–136] and negatively correlated with insistence on sameness [135]. Interestingly, neuroanatomical changes of the striatum are also found in animal models of ASD but can be different from one model to another: Neuroligin3 Knock-In (B6/129F2 strain, 15 weeks of age), Shank3$^{-/-}$ (B6/SV129 strain, 22–23 weeks of age) and Cntnap2$^{-/-}$ (C57BL/6J strain, 8–9 weeks ofage) transgenic mice, for example, have bigger striatum than control littermates [69], while 16p11$^{+/-}$ mice (B6/129F2 strain 32–35 weeks of age) have a bigger nucleus accumbens [137]. Idiopathic models of ASD, such as the BTBR mice (C57BL/6J strain, 11 weeks of age) show a reduction of their striatal volume [138]. Although no modification of the global striatal volume has been reported in FVB/NJ mice exposed in utero to valproic acid (VPA) (Na- tional Laboratory Animal Center, Taipei, Taiwan), the balance between striosomes and the matrix is reported to be impaired at postnatal day 8 (P8) depending on the time of the VPA administration. These two compartments receive different cortical inputs: the matrix compartment receives inputs from the cortical sensorimotor areas, while the striosomal compartment receives inputs from prelimbic and insular cortices. The authors reported that both striosomal and matrix areas were affected and could be linked with both social and motor impairments in ASD, highlighting the heterogeneous nature of the disease [28] (Table 2). These differences align with the inconsistencies reported in humans and reflect the large scale of striatal neuroanatomical changes. More generally, all three parts of the

striatum are involved in functions that are altered in one way or another in ASD. The dorsolateral striatum has been linked to motor stereotypies in a large range of species, from rodents [139] to monkeys [140], and to insensitivity to reward devaluation [141]. The dorsomedial striatum has been linked to executive dysfunctions and abnormal reactivity [142], and the nucleus accumbens has been linked to the inability to process and respond to social cues [33].

Taken together, these sets of results indicate that all three striatal parts are affected in ASD patient brains and animal models of ASD, reinforcing the link between the striatum and ASD symptoms.

3.2. Cellular Consequences Correlates

Despite increasing their volume, the numerical density of neurons is reported to decrease by 15% in the nucleus accumbens and 13% in the putamen in patients diagnosed with ASD and comorbidities [84]. These findings need to be more thoroughly replicated, as 7 out of 14 patients included in the study (50%) were diagnosed with seizure, and death was seizure related for 5 of them. Similar variations in striatal cellular alterations were also found in ASD animal models. In VPA mice and rats, no drastic striatal neuronal loss has been described. A recent paper strongly suggested that the lack of PV staining observed in the striatum of VPA mouse model should not be attributed to cell loss but rather to a decrease in PV protein contents within the interneurons, as the cell can still be observed using another marker (Vicia Villosa lectin, VVA) [26,143]. Nevertheless, the general cellular organization within the striatum appears to be disturbed, with an impaired aggregationof striosomal cells into cell clusters [28] (see Table 1). This could cause (or enhance) an imbalance between sensorimotor and cognitive inputs on the striatum, leading to social and motor impairments.

Representing more than 95% of the striatal neuronal population, medium spiny neurons (MSNs) are the striatum's main neuronal population and are the striatum's only output. They are segregated into at least two subtypes according to their axonal projection patterns: striatonigral MSNs, or direct pathway MSNs, expressing dopamine receptor D1 (RD1) and striatopallidal MSNs, or indirect pathway MSNs, expressing dopamine receptor D2 (RD2) [129,144]. All MSNs share a similar morphology and exhibit similar properties, except for excitability, which appears to be increased in DRD2-expressing MSN [145]. Male and female 5-week-old Shank3$^{B-/-}$ MSNs exhibit increased neuronal complexity, increased total dendritic length and surface area, but a lower spine density compared to Shank3$^{B+/+}$ littermates [146]. At the age of 2–4 months, a reduced MSNs spine density is also found in Shank3$^{B-/-}$mice, in both males and females, only in DRD2-expressing MSNs [147]. This is particularly relevant to human findings, where regional quantification revealed that RD2, but not RD1, was significantly more expressed in both caudate and putamen of 4– 20-year-old ASD male patients, with multiple ethnicities and non-comorbidities-related death [148] (Table 2).

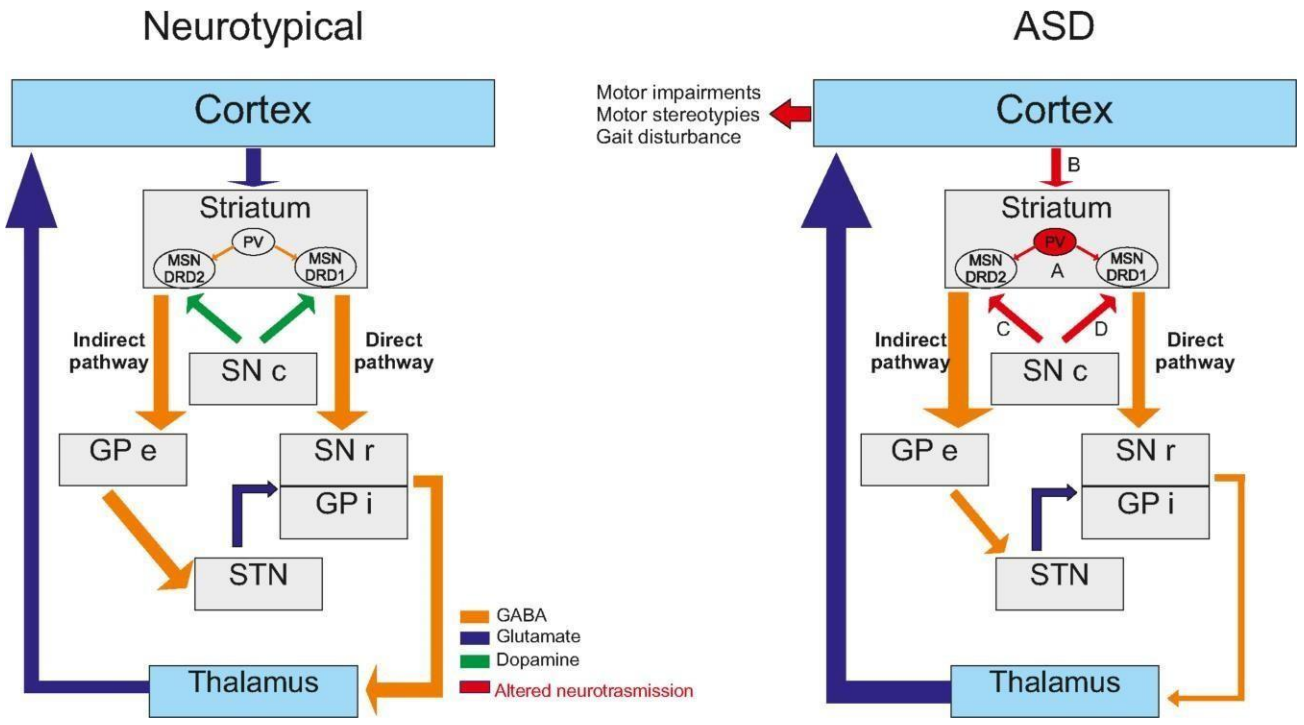


Figure 2. Basal ganglia modifications in ASD. Basal ganglia network is shown to be deeply affected in ASD models. The main alterations are found at 4 points of interest (A,B,C,D). Striatal local inhibition mediated by PV interneurons. (A) is altered in some ASD models like Shank1$^{-/-}$ and Shank3B$^{-/-}$ mice [149], VPA mice [26] or Cntnap2$^{-/-}$ mice [143]. Responsible of feed-forward inhibition, alteration of these interneurons may lead to altered MSNs' functionality. Interestingly, corticostriatal pathway (B) is widely described as altered in ASD models. For instance, in Ngln$^{-/-}$ mice, Ngln3$^{-/-}$ mice, Shank3$^{-/-}$ mice [150] or Shank2$^{-/-}$ rats [151], alterations of the glutamatergic transmission onto MSNs are reported. Thus, alterations of plasticity were also highlighted in Shank3e4-22$^{-/-}$ mice [91]. However, the dichotomy between direct and indirect pathway allows to highlight some pathway-specific alteration. On the one hand, some alterations were found to be specific of the indirect pathway (C), like an increase of DRD2 expression in the striatum, leading to a dopaminergic imbalance in favor of the indirect pathway [148,152]. On the other hand, some alterations were found to be specific to the direct pathway (D) as alteration of synaptic transmission has been highlighted in DRD1 MSNs but not DRD2 in NL3-cKO mice [153]. Thus, in Nlgn1$^{-/-}$ mice, the AMPA/NMDA ratio was found to be reduced specifically in direct pathway MSNs [154], leading to an altered response.

The striatal interneurons are responsible for local inputs and regulation, which play a major role in the striatum's functionality. A conjoint depletion of the PV fast-spiking and cholinergic interneurons in the dorsal striatum leads to autism-like behavior in male mice [155], but not in females, suggesting that interneurons, including cholinergic interneu- rons, could be involved in ASD physiopathology and could take part in the reported sexual dimorphism in ASD patients and animal models [47–49]. The central role of cholinergic interneurons is further highlighted, as they are also involved in other brain pathologies. For instance, they have been directly linked to Tourette's syndrome or attention deficit hyperactivity disorder [156] and motor stereotypies, as a lesion of these cells in the dor- sal striatum significantly prolonged their duration [157,158]. A significant increase in the stereotyped behavior was also found in mice (C57BL/6, 6–21 weeks) with a loss of MeCP2 in somatostatin-expressing interneurons, including in the striatum [159]. Nevertheless, there is no available information regarding the involvement of striatal somatostatin-expressing interneurons in ASD physiopathology.

The striatal PV fast-spiking interneurons, responsible for gamma oscillations and feed-forward inhibition onto MSNs, are important in the excitatory/inhibitory balance [129,160]. The inactivation of the PV gene in mice leads to reduced social interactions, reduced ultrasonic vocalizations and repetitive and stereotyped patterns of behavior [161]. This ASD-like phenotype of $PV^{-/-}$ mice is also directly correlated with brain morphological abnormalities similar to those reported in patients. This is accompanied by functional disturbances, as $PV^{-/-}$ fast-spiking interneurons exhibit a lower excitatory post-synaptic current facilitation, i.e., a lower excitatory synaptic plasticity [161]. Interestingly, these striatal PV fast-spiking interneurons are not only related to ASD-like mechanisms when targeted but are commonly affected in ASD models. PV expression levels are reduced in the striatum of $Shank1^{-/-}$ and $Shank3^{B-/-}$ mice, the two mice models of ASD, without a loss of PV interneurons [149]. Similar findings are reported in VPA mice [26] or $Cntnap2^{-/-}$ mice [143]. Given that $PV^{-/-}$ mice ASD-like phenotype is strongly relevant to human ASD core symptoms and related abnormalities and that PV fast-spiking interneurons are affected in a wide range of ASD models, a PV hypothesis of ASD has been formulated recently [162] (Table 3).

Glial cells, including astrocytes and microglia, are now well known to influence synapse formation and function, as astrocytes, for example, can make contact with multiple neurons and express receptors and ion channels that are also expressed in neurons [163]. The role of astrocytes in ASD physiopathology has been demonstrated by re-expressing Mecp2 in whole-brain astrocytes in $Mecp2^{-/-}$ mice (C57BL/6 strain, 4–8 weeks of age), leading to the recovery of motor symptoms [164]. However, no striatum-specific astroglial changes in $Shank3^{+/\Delta C}$ and $Cntnap2^{-/-}$ male mice (C57BL/6J strain, 5–6 months of age) were reported [165]. The overexpression of the eukaryotic translation initiation factor 4E (eIF4E) in macrophages in mice, i.e., in microglia in the central nervous system, leads to elevated protein synthesis and abnormal morphology of microglia, as well as an increase in their size and number within the striatum of both males and females (C57BL/6 strain, 2–6 weeks of age). This overexpression of eIF4E in microglia has no phenotypic conse- quences in females, but it causes ASD-like phenotype in male mice [166], suggesting that microglial impairment could cause ASD with a sexual polarity. Thus, a significant increase in the size and number of microglial cells in the striatum has been reported in $Pten^{+/-}$ and $Fmr1^{-/-}$ mice [166]. These two models exhibit core ASD features, such as social deficits [167,168] and repetitive behavior [169,170], suggesting that microglia could also be impaired due to ASD.

3.3. Neurotransmission Systems Implicated

In the early 2000s, John Rubenstein and Michael Merzenich formulated the excitation/inhibition (E/I) imbalance hypothesis of ASD, suggesting that the physiopathology of ASD and their related comorbidities may reflect a disturbance in such a balance [87]. Even though their work focused only on the cortical networks, it may be easily extended to striatal networks, as the striatum receives major excitatory inputs from cortices areas and major inhibitory inputs from the local interneurons network [142]. Added to the dopaminergic and serotoninergic neuromodulations, this would lead to a balance within the striatum, which is essential for its functioning. Another argument suggests an E/I imbalance in the striatum is found in the numerous genes involved in ASD physiopathology. According to the SFARI database, nearly one-third of genes (68 out of 213) used to model ASD in mice directly concern synaptic establishment, strength and/or transmission or are associated with receptors in pre- and/or post-synaptic compartments. Most of these genes are expressed in the striatum, although not exclusively.

Table 3. Striatal dysfunctions in ASD. This table sums up the striatal dysfunctions identified in patients and animal models of ASD.

		Children		Teenagers	Adults	Animal Models
		<5 yo	5 to 14 yo	14 to 21 yo	>21 yo	
Anatomical impairment	*Volume*	• N/A	• ↗ Volume of putamen and nucleus accumbens but not of the caudate [82]	• ↗ Volume of putamen, accumbens & caudate [133–136]		• ↗ Striatal volume in cntnap2 ko mice [57] • ↘ Striatal volume in btbr mice [137]
	Matrice/striosome		• N/A			
	organization	• N/A		• N/A	• N/A	• Impaired balance in vpa exposed mice [28]
Cellular correlates	*Medium spiny neurons*	• N/A	• Decreased neuronal density in nucleus accumbens and putamen [82]			• ↗ Neuronal complexity, ↗ dendritic length and surface area, ↘ Spine density in Shank3B KO mice [146] • ↗ Spine density in DRD2-expressing msns in Shank3B $^{-/-}$ mice [147] • ↘ ePSCs [155]
	PV interneurons	• N/A				• ↘ PV expression [26,143] • No cell loss [143–156]
	Astrocyte	• N/A	• N/a			• No striatum-specific changes in shank3$^{+/\Delta c}$ or cntnap2 ko mice [160]
	Microglia	• N/A	• N/a			• ↗ Size and number of microglial cells [161]
Neurotransmission	*Glutamate*	• N/A	• N/A	• N/A	• N/A	• ↗ sEPSCs amplitude and frequency [171] • ↘ NMDA/AMPA ratio [170] & ↘ NMDA-R function [172] • ↘ AMPA mediated mEPSCs amplitude & frequency, no defect in paired-pulse ratio [146] • ↘ NMDA/AMPA ratio but no changes in mEPSCs amplitude or frequency [173] • Abnormal distribution and ↗ expression of mGluR5 in MSNs in [119] • ↘ mIPSCs amplitude [171]
	GABA	• N/A	• N/A	• N/A	• N/A	↘ mIPSCs frequency in ventral DRD1-expressing MSNs only [174] ↗ sIPSCs & mIPSCs frequency in Fmr1 KO mice [175]
	Dopamine	• N/A	• N/A	• ↗ DRD2 mRNA in MSN [148]	• N/A	• ↘ DAT level in BTBR and Fmr1 KO mice [176]

N/A: not applicable.

Both ionotropic and metabotropic receptors mediate the glutamatergic responses in the striatum and are differentially expressed throughout the striatal territories at the pre-or post-synaptic compartments. While metabotropic receptors are involved in long-term synaptic plasticity, the ionotropic receptors, i.e., AMPA-R and NMDA-R, are responsible for the neurotransmission itself in the striatum [172,177]. In ASD, alterations of the gluta- matergic transmission are reported in several models, such as Nlgn1$^{-/-}$ mice, Ngln3$^{-/-}$ mice, Shank3$^{-/-}$ mice [150] or Shank2$^{-/-}$ rats [151]. In Ngln1$^{-/-}$ mice (C57BL/6 strain, P15- P38), the NMDA/AMPA ratio is reported to be decreased in the dorsal striatum and can be restored to WT levels with the application of D-Cycloserine (DCS), a co-activator of NMDA-R. This rescue of NMDA-R function also lowers the increased grooming behavior observed in Nlgn1$^{-/-}$ mice [178]. Interestingly, in the same mice that are 2–3 weeks old, the NMDA/AMPA ratio is reduced in the direct pathway (DRD1-expressing MSNs) but not in the indirect pathway (DRD2-expressing MSNs) and is directly driven by a decreasein GluN2A-containing NMDA-R currents. However, the strength of the synapse, measured by input/output curves, as well as the short-term plasticity, are not reported to be impaired in KO mice compared to WT littermates [154]. Such a dichotomy between direct and indirect pathway has also been described regarding endocannabinoid (CB)-mediated LTDin a model of selective loss of TSC1 in either DRD1-expressing MSNs or DRD2-expressing MSNs (C57BL/6 strain, P40 to P50) [179]. Here, the authors highlighted impairments of CB- mediated LTD in TSC1$^{-/-}$ DRD1-expressing MNSs but not in DRD2-expressing MSNs. In Fmr1$^{-/-}$ mice (C57BL/6J, males only, adult), a loss of LTD has been described inventral MSNs, without any CB1-R functional alteration [173]. These findings suggest that impairments of glutamatergic neurotransmission and plasticity are directly linked to the neuromodulation system specificity in ASD.

In another neuroligin model of ASD (R451C-NL3 male mice, B6/J strain, 2 months), a high-frequency stimulation (HFS) protocol failed to induce LTD in R451C-NL3 mice, while it did do so in WT littermates. Furthermore, the application of quinpirole to activate D2 receptors, known to be involved in the establishment of LTD during HFS protocol, did not prevent the induction of LTD in the dorsal striatum of mutated mice [180]. Male and female Shank3B$^{-/-}$ mice (C57BL/6 strain, 5 weeks) exhibited a lower MSNs popula- tion spike amplitude than control and reduced miniature AMPA-mediated post-synaptic currents (mEPSCs) amplitude, suggesting a reduction of the post-synaptic response on available synapses [146]. Shank3B$^{-/-}$ MSNs also exhibited a reduced mEPSCs frequency,but there were no defects in the paired-pulse ratio, suggesting that the number of func- tional synapses is decreased. These findings indicate that cortico-striatal impairments are occurring at the post-synaptic, i.e., striatal disturbances. In another Shank3 mouse model (Shank3^{e4-9} mice, C57BL/6 strain, 3–4 weeks), the NMDA/AMPA ratio signifi- cantly decreased at glutamatergic synapses on MSNs from both Shank3$^{e4-9+/-}$ and KO mice compared to WT. Nevertheless, no difference in mEPSCs amplitude of frequency has been highlighted [181]. However, in another mouse model of ASD (eIF4E overexpressingmice, males only, 2–6 months), increased mEPSCs amplitude but not frequency has been highlighted [182]. The authors suggested that the increased expression of this translation initiation factor may lead to exaggerated cap-dependent protein synthesis, such as mGluR5 pathway, as it has been described in the hippocampus of Fmr1$^{-/y}$ mice (C57BL/6J, males only, 3–6 weeks). Considering that no AMPA-mediated disturbances have been reported in several models and that mGluR5 are involved in the potentiation of NMDA response,these findings suggest that the disturbances at glutamatergic synapses are due to decreased NMDA-R function. The mGluR5 receptors are mainly involved in the potentiation of NMDA responses in striatal MSNs and are reported to be abnormally distributed and accumulated in striatal MSNs in Shank3$^{e4-22-/-}$ mice (C57BL/6 strain, 2–5 months). These same mice's MSNs also exhibit enhanced excitability and a loss of plasticity through impair- ments of high-frequency stimulation (HFS)-induced LTD. All this information suggests that mGluR5 receptors could be dysfunctional and involved in electrophysiological changes observed at excitatory synapses on striatal MSNs in mice models of ASD. To further test

such a hypothesis, the authors proposed a pharmacological enhancement of mGluR5 with 3-cyano-N-(1,3-diphenyl-1H-pyrazole-5-yl)-benzamide (CDPPB) and showed that it restores Shank3$^{e4-22-/-}$ MSNs normal excitability and plasticity, as well as behavioral ASD-like behavior [91]. In Shank3Δ11$^{-/-}$ mice (C57BL/6 strain, 3 months), pharmacological enhancement of mGluR5 with CDPPB leads to a total recovery of NMDA receptor functions, compared to WT littermates [91].

Although GABAergic transmission is crucial for proper striatal function, this transmission is far less studied in an ASD context. The first apparent involvement of the GABAergic transmission in ASD physiopathology is following the knock-out of either GABA-A receptor subunit α5 or β3 in mice that leads to ASD-like phenotype [171,174]. In Chd8$^{+/-}$ mice (C57BL/6 strain, males only, 6–8 weeks), the MSNs of the ventral striatum exhibited decreased miniature inhibitory postsynaptic currents (mIPSC) amplitude, compared to WT littermates, while no changes were observed in mIPSC frequency. The authors also report increased spontaneous excitatory postsynaptic currents (sEPSC) amplitude and frequency but no changes in mEPSCs [175]. This suggests that the observed enhancement of the excitatory transmission is partly allowed by a local decrease in inhibitory transmission. In the NL3-cKO mice (C57BL/6 strain, males only, 4–6 weeks), a reduction in the mIPSC frequency, but not amplitude, is reported in ventral striatum D1-MSNs only. The authors also report no changes in the paired-pulse ratio of evoked IPSCs or short-term plasticity and no changes in excitatory transmission. Added to a significant decrease in inhibition/excitation ratio, measured by the ratio between GABA receptor-mediated inhibition and AMPA receptor-mediated excitation, this suggests that the GABAergic alterations observed in inhibition of D1-MSNs of the ventral striatum could be responsible for a shift of balance between synaptic excitation and inhibition [153]. Little is known regarding the implication of dorsal striatum GABAergic transmission in ASD. To date, the most comprehensive study on that topic concerns the Fmr1$^{-/-}$ mouse model, in which extensive GABAergic disturbances have been reported [183]. In this study, the authors found that dorsal striatal MSNs of Fmr1$^{-/-}$ mice (C57BL/6 strain, 2–3 months) have a higher sIPSCs and mIPSCs frequency than WT littermates, but no changes were found in sIPSCs and mIPSCs amplitude and kinetic properties. In addition, the paired-pulse ratio of evoked IPSCs is lower in Frm1$^{-/-}$ mice than WT littermates at 50 ms interstimulus interval, but not at 80 ms interstimulus interval. Taken together, these findings suggest that alterations
may occur in pre-synaptic neurons, but in an action-potential-independent manner.

Post-mortem analysis of striata of ASD young patients revealed an increase in RD2 mRNA within MSNs in both the caudate and putamen using radioisotopic in situ hybridization histochemistry [148]. This suggested a dopaminergic imbalance in favor of the indirect pathways in ASD patients [152]. This may support dopaminergic targeted therapeutics that are currently used to manage ASD-related symptoms, such as irritability or aggression. Indeed, treatments with D2 antagonists result in improved sociality (under risperidone) [184] and a decrease in stereotypy scores (under aripiprazole) [185]. Interestingly, it seems that the direct pathway is only dysfunctional when activated during a task. A study in young ASD adults using PET ([^{11}C]raclopride, a D2-antagonist) simultaneously with magnetic resonance scanning during an incentive task revealed a decrease in phasic dopaminergic release in the putamen and caudate nucleus, suggesting an impairment in the learning and goal-directed reinforcement [186]. The dopaminergic content is not extensively studied in ASD due to the result disparateness among animal models, especially regarding the striatal dopaminergic content, tyrosine hydroxylase staining and dopaminergic transporter (DAT) levels (for review, see [176]). Dysfunction in the striatal direct pathway in ASD animal models leads to deficits in social interactions and an increase in grooming [153,187,188]. Optogenetic stimulation of the nigrostriatal pathway (ii) was shown to reduce the preference for the social target in the 3-chamberts test (3-CT) with an increase in rearing but
no change in grooming or digging behaviors. The stimulation also caused an increase in p-ERK1/2 and p-CAMKIIα levels in the dorsal caudate nucleus-putamen (CPu) [187]. The indirect pathway seems to be not as affected as the direct pathway in ASD mouse

models, but studies on early life stress in $D2R^{+/-}$ heterozygous mice showed that these mice display ASD-like phenotype [188] (Table 3).

Alterations in the dopaminergic and glutamatergic systems have been found in several animal models of ASD. For example, a diminution of DAT level is observed in the BTBR mice and Fmr1 KO mice models [188]. It is also interesting to note that in both models, there is an increase in tyrosine hydroxylase-positive neurons (TH+) coupling with VGlut1+ neurons [188] due to the existence of a strong link between VGlut1 and mGluR5 [189]. The inactivation of adenylyl cyclase 5 (AC5), an effector of the D2R and mGluR3 receptor inthe striatum, induces ASD-like behaviors in mice [190]. Interestingly, mGluR3 has an antagonistic effect on mGluR5, mirroring the interrelation between D1R and D2R. In the case of WT mice, either the activation of mGluR5 (with DHPG) or the inactivation of mGluR3 (with LY341495) is sufficient to induce ASD-like behaviors [190], as is the inactivation of either GluA1 or GluN2B.

4. Epigenetic Alterations in the Brain of ASD Animal Models

Interactions between specific genes and environmental factors play an essential role in the development of ASD, acting directly or indirectly on the transmission and transduction systems detailed above [191]. Environmental factors can affect gene expression without causing changes in the DNA sequence, but by acting via epigenetic mechanisms: DNA methylation, histone modifications or post-transcriptional regulation by non-coding RNAs. Thus, the exposure to environmental factors can cause changes in the expression of critical genes in a crucial period of embryonic and fetal development, resulting in an increased risk of inducing ASD [192]. This is a significant focus of current research, specifically in deciphering the link between environmental insults that occur very early in life anda pathological phenotype that is only observed later in childhood or even adulthood. Epigenetic modifications may be essential molecular mechanisms that can translate early aggressions into lasting brain pathologies that could even be transmitted to the descendants.The best- characterized epigenetic modification is DNA cytosine methylation [193]. This mechanism is already known to be involved in neurodevelopmental disorders, such as Prader Willy/Angelman or Fragile X syndromes, which are major causes of ASD with intellectual disability that have been known for a long time in humans [194]. Methylated cytosines are mainly localized at CpG islands, corresponding to regions of the genome enriched in GC and essentially present in the promoter regions of genes. The primary known function of cytosine methylation at these islands is to repress transcription of the downstream gene(s) [195]. However, there are many exceptions, and the mechanism by which DNA methylation regulates transcription may be specific to different contexts, such as gene content, locus and time of development. Furthermore, DNA methylation is thought to be more abundant in genebodies, where it probably plays a primary role in fine alternative splicing and expression regulation [196].

In the mouse prefrontal cortex, significant reconfiguration of the methylome occurs throughout synaptogenesis from fetus to young adult [197]. This is thought to be a critical process in defining the molecular identity of neurons [197]. In humans, DNA methylation alterations in the cortex and anterior cingulate gyrus have been observed in patients with ASD [198]. The differentially methylated genes were primarily related to microglialcell specification and synaptic pruning during brain development [198]. More recently, Nardone et al. refined these observations more selectively on neurons after FACS cell sorting of the prefrontal cortex. In doing so, they identified differences in DNA-specific methylation profiles in neuronal nuclei affecting genes related to synaptic, GABAergic and immune processes [199]. This is the first characterization of neuron-specific DNA methylation changes in ASD and is consistent with observations by Lister et al. in mice that showed a specific distribution of methylation in neurons that appeared very different from other tissues, with as many methylated cytosines outside of GC-rich regions as in CpG islands [197]. Epigenetic changes in specific brain structures other than the cortex have only rarely been reported. Notably, the differences in methylation and acetylation

of lysine 27 of histone 3 (H3K27) in the cerebellum have been detected in autistic patients compared to controls [200]. Furthermore, in the developing human prefrontal cortex, the 3D organization of chromatin forms loops around synapse-related genes, allowing the regulation of their expression [201]. The formation of these loops is probably regulated by DNA methylation [196]. Other yet unresolved functions of DNA methylation likely play major roles in brain development and neurodevelopmental disorders such as ASD.

Regarding transmission systems, the GABA and Glutamate pathways have already been linked to epigenetic changes. Indeed, an increase in glutamate concentration was identified in the blood of ASD patients compared to healthy subjects [201]. This increase in glutamate metabolism, which could be associated with ASD in children, could be genetic in origin and under epigenetic control. Similarly, the alterations in synaptic physiology in PV interneurons previously described may be related to a deficiency in genes encoding GABA-related enzymes, which alters neuronal GABA content [202].

Concerning the link between epigenetics and environmental insults known to increase the risk of ASD, Richetto et al. [203] showed that maternal infections mimicked by poly I:C injection result in persistent changes in DNA methylation. These changes are visiblein many different genomic regions and are related to the timing of prenatal infection.Indeed, late prenatal infection during pregnancy induced methylation changes in significant genes of GABAergic cell development, while early prenatal infection seemed to affect mainly the WNT signaling pathway, involved in the nervous system development [203]. These epigenetic mechanisms have been suggested to be involved in maintaining the long- term effects of prenatal insults, as well as maintaining the cellular dysfunctions they generate [203,204].

In addition, a growing body of evidence proposes the gut microbiota as a likely key player modulating the effect of environmental factors on brain function. This could occur through changes in the immune system and epigenetic machinery. Changes in microbiota composition have been found in patients with behavioral disorders and autism spec- trum disorders [205], adding further complexity to the already multifactorial pathogenic mechanisms associated with neurodevelopmental and psychiatric disorders. Epigenetic changes in ASD could notably be induced by metabolites produced by an abnormal gut microbiota [206].

5. Conclusions and Future Direction

Although we better understand the implication of the cerebellum and the striatum in ASD, further studies are needed on at least two aspects. First, more knowledge should be gathered on a widespread population of ASD patients without gender or age bias. This means systematically including women and middle-aged and senior ASD patients that are often left out of clinical studies. The state of the brain and its maturation are both sex- and age-dependent. Second, there is an urgent need to focus on developing effective treatments to alleviate ASD symptoms, and knowledge of cellular and molecular correlates of the disease is key to help in this research avenue. Motor social and cognitive functions are common features between the cerebellum and the striatum, which are only two synapses away from each other. A better and more thorough assessment of the corresponding interconnections may lead to breakthroughs in this field.

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Chapitre 3 : Complex social behaviour during an extended period of time in a valproic acid animal model of autism spectrum disorder

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Alexandre Maisterrena, Fabrice de Chaumont, Jean-Emmanuel Longuevile, Eric Balado, Elodie Ey, Mohamed Jaber

1. Résumé

Les déficits de l'interaction sociale figurent parmi les caractéristiques principales des TSA. Plusieurs méthodes chez l'animal sont actuellement utilisées pour étudier ces interactions et ainsi valider les modèles utilisés. La procédure la plus utilisée est celle du 3-CT. Cette méthode présente plusieurs limitations : (i) il n'est pas possible d'analyser le comportement d'un groupe de souris (ii) il n'est pas possible d'analyser les comportements sociaux complexes, les souris étant le plus souvent séparées par une cage limitant les interactions. Enfin, (iii) la durée d'étude est courte (10 minutes) ne permettant pas l'analyse de l'évolution de ces comportements sociaux. Ainsi, nous avons décidé de mettre en place une technique récemment développée au sein de l'Institut Pasteur, le LMT, afin d'analyser des comportements sociaux complexes d'un groupe de souris sur une longue durée, et ainsi nous permettre d'établir un profil social détaillé de notre modèle.

Pour cette étude, nous avons décidé d'utiliser un modèle environnemental de TSA basé sur l'exposition in utero au VPA (E 12.5, 450 mg/kg), le principe actif de la Dépakine®, l'un des modèles les plus utilisés et l'un des plus fiables.

Comme indiqué précédemment, le LMT est une méthode nouvelle d'analyse des comportements sociaux complexes d'un groupe de souris sur une longue durée. Dans notre cas, chacun des groupes est composé de 4 animaux et les groupes sont observés sur 3 jours. Le LMT est constitué d'une cage d'acquisition de 50 cm X 50 cm surmontée d'une caméra RGB-D infrarouge ; sous la cage d'acquisition est placée une plaque couverte d'antenne RFID. Ces antennes permettront de détecter les puces RFID implantées en sous-cutané chez chaque souris, ce qui permettra d'identifier les différents individus au cours de l'expérience. La caméra permettra de suivre les individus au cours de l'expérience et de détecter les différents événements sociaux.

1.1 Rappels méthodologiques

Le VPA est injecté à 450mg/kg en i.p. à E.12,5 chez la femelle gestante. Au moment du sevrage, les souris vont subir plusieurs procédures : (i) elles seront séparées par sexe et par traitement en évitant que deux individus de la même portée soient hébergés dans la même cage (ii) elles subiront une chirurgie de 10 min afin d'implanter une puce RFID en sous-cutanée permettant d'identifier les individus durant les expériences de LMT et (iii) elles seront baguées à l'oreille pour un suivi plus facile.

Dans le cadre du LMT toutes les expériences s'effectueront avec des groupes de 4 souris. Dans un premier temps, nous utiliserons des groupes constitués de 4 souris SAL ou de 4 souris VPA (désigné SAL HOMO (n=16) et VPA HOMO (n=20)) ce qui nous permettra de définir les déficits sociaux observables dans un groupe homogène de souris, ayant a priori les mêmes phénotype et symptomatologie. Dans un second temps, nous utiliserons des groupes composés de 3 souris SAL et 1 souris VPA ce qui nous permettra dans ce cas-là d'observer les effets de la présence d'individus sociaux de phénotypes HÉTÉROGÈNE (SAL HÉTÉROGÈNE n=27 ; VPA HÉTÉROGÈNE n=9) sur les comportements sociaux.

La mise en place du LMT s'est faite en 3 étapes principales. (i) La cage d'acquisition d'une dimension de 50cm*50cm pour 30cm de haut a été construite localement à l'aide de plaques de plastique transparent. En parallèle, un support métallique a été construit pour la caméra d'acquisition placée 62cm au-dessus du sol de la cage d'acquisition. (ii) Les antennes RFID ont été connectées aux puces électroniques correspondantes et les antennes ont toutes été réglées pour émettre à une fréquence de 134,2 kHz ± 1kHz. Une fois toutes les antennes réglées, celles-ci sont collées à une plaque placée sous la cage d'acquisition. (iii) Enfin, l'ordinateur dans lequel a été installé le logiciel ICY avec les modules pour le LMT est connecté à l'ensemble comprenant la caméra d'acquisition ainsi que les antennes RFID. Le bon fonctionnement du suivi ou 'tracking' est tout d'abord vérifié grâce à l'utilisation de peluches en forme de souris et déplacées à l'aide d'un fil sur le sol de la cage d'acquisition. Une fois complété, le LMT est placé dans l'animalerie et une seconde série de tests est réalisée avec des souris contrôles. Après cela, une carte électronique de type « arduino » est connectée à l'ordinateur afin de permettre le suivi des conditions d'enregistrement (son, lumière, température, humidité) au cours de l'expérience.

Les données générées par le LMT sont en grand nombre, et seront analysées à l'aide de scripts en python. Les données devront être modifiées notamment pour inclure une durée minimale pour qu'un événement social soit considéré valide et une durée maximale pour laquelle si deux événements sociaux identiques ont lieu entre les mêmes souris, ces deux événements seront fusionnés pour constituer un seul événement.

En plus de ces modifications, nous avons dû aussi créer de nouveaux scripts afin de permettre des analyses complémentaires. Notamment, nous avons créé un script permettant d'obtenir le nombre de comportements sociaux pour chaque souris, pour chaque heure sur la durée de l'expérience afin de créer une courbe d'évolution des contacts sociaux au cours du temps. Une version modifiée de ce script nous a permis ensuite d'étudier de manière spécifique les interactions entre chaque souris pour chaque heure sur la durée de l'expérience afin de créer une matrice d'interaction entre les souris et d'étudier comment les souris interagissaient dans les différents groupes.

1.2 Résultats

1.2.1 3-CT

En amont des analyses par LMT, nous avons procédé à une mesure des comportements sociaux des souris saline et VPA grâce au 3-CT précédemment décrit. Chez les femelles VPA, nous n'avons pas observé de déficits à la préférence sociale en phase 2 (avec une souris dans la cage au sein d'une chambre), mais un déficit de préférence à la nouveauté sociale a pu être observé en phase 3 (avec une nouvelle souris dans la cage de la chambre opposée).

1.2.2 LMT à 3 jours

Nous avons commencé l'expérience en analysant l'intégralité des 3 jours d'expérience et en comparant les comportements sociaux entre les groupes dits homogènes composé de 2 souris saline ou 4 souris VPA et les groupes dits hétérogènes composé de 3 souris salines et une souris VPA. Dans ces conditions, nous n'avons pas observé de différences de comportements entre les groupes salines homogènes et VPA homogènes ; le même résultat est observé en comparant les souris salines hétérogènes aux souris VPA hétérogènes.

1.2.3 LMT à différents points dans le temps

À la suite de ces résultats, nous avons décidé de comparer les comportements sociaux à différents points dans le temps. Ainsi, nous avons examiné les comportements sociaux pour la 1ère heure d'expérience, les 3 premières heures et les 24 premières heures. Pour la comparaison des groupes homogènes, nous avons pu observer une diminution significative du nombre de contacts sociaux à 3 heures et à 1 jour d'expérimentation chez les souris VPA. Tandis qu'à une heure d'expérimentation chez les groupes homogènes, nous n'observions plus de différences sur le nombre de contacts sociaux, mais on notait une diminution du nombre d'approches sociales chez les souris VPA homogènes. Chez les groupes hétérogènes, nous continuions à n'observer aucune différence entre les souris saline hétérogène et les souris VPA hétérogènes et ce à aucun point dans le temps.

1.2.4 Courbe d'évolution des interactions au cours du temps

Enfin, nous avons décidé de créer une courbe permettant de visualiser l'évolution du nombre de contacts cumulés au cours du temps. Nous avons pu observer 3 éléments principaux. (i) les souris des groupes saline homogène sont celles effectuant le plus de contacts sociaux de tous les groupes. (ii) Les souris des groupes VPA homogène sont celles effectuant le moins de contacts sociaux de tous les groupes. (iii) Les courbes des souris salines hétérogènes et VPA hétérogènes sont quasi-identiques et se trouvent à un nombre de contacts sociaux les plaçant à un point intermédiaire entre les groupes salines homogènes et VPA homogènes.

1.3 Conclusion

En résumé, nous avons pu montrer dans cette étude que le LMT avait la capacité de détecter des déficits sociaux qui n'avaient pas pu être observés avec le 3-CT. Avec le LMT nous avonspu montrer que les souris VPA homogène présentaient dans un premier temps des déficits des approches sociales, puis dans un second temps des déficits des interactions sociales. On notera cependant qu'après une certaine durée, les souris VPA homogène et les souris saline homogène ne sont plus différentes dans l'expression des comportements sociaux analysés. Ceci suggère que dans le cadre du modèle VPA, les déficits sociaux semblent être sur la mise en place puis le renforcement de ces interactions sociales, et non pas sur les capacités et les qualités d'interaction en elles-mêmes une fois les interactions établies.

Dans un second temps, nous avons pu montrer que la présence de souris sociales avec une souris VPA permettait d'améliorer significativement les capacités sociales de la souris VPA. A l'inverse, la présence de cette même souris VPA diminuait de manière significative les capacités sociales des souris SAL.

Cette étude a permis de montrer la capacité du LMT à établir un profil social détaillé des souris ainsi que la capacité d'étudier l'établissement des interactions sociales au cours du temps. Ces travaux montrent tout l'intérêt de cette technique dans les études nécessitant une analyse approfondie des comportements sociaux.

Cette étude nous a aussi permis de montrer l'impact important de l'environnement social sur les capacités sociales des souris, et ce de manière tant bénéfique que délétère. Le fait que la souris VPA soit capable de dégrader les capacités sociales des souris SAL indique qu'une attention particulière doit être portée pour l'hébergement des individus afin d'éviter que la présence des souris « autistes » ne dégrade les capacités sociales des souris contrôles.

Cependant, le LMT présente plusieurs limitations majeures à prendre en considération. Cette technique est beaucoup plus complexe à mettre en place que le 3-CT et nécessite plusieurs compétences spécifiques, au niveau technique, électronique et informatique. De plus, due à sa grande précision, la méthode de formation des groupes placés dans la cage d'acquisition doit être bien définie à l'avance afin d'éviter les biais liés à la manipulation ou à l'hébergement. Il convient également de prendre en compte le temps d'expérimentation rendant difficile la manipulation d'un grand nombre d'animaux à un âge donné précis, l'expérimentation pouvant prendre pour chaque phase, plusieurs jours. Enfin, le LMT produit une très grande quantité de résultats analysés par des scripts nécessitant des compétences en langage informatique.

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Original article-New Results

Complex social behaviour during an extended period of time in a valproic acid animal model of autism spectrum disorder

Alexandre Maisterrena[1], Fabrice de Chaumont[2], Jean-Emmanuel Longueville[1], Eric Balado[1], Elodie Ey[3], Mohamed Jaber[1,4 #]

1 Université de Poitiers, Inserm, Laboratoire de Neurosciences Expérimentales et Cliniques U1084, Poitiers, France
2 Institut Pasteur, CNRS, Gènes synapses et cognition, Paris, France
3 CNRS, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France
4 CHU de Poitiers, Poitiers, France

#: Corresponding author. Université de Poitiers, Inserm, Laboratoire de Neurosciences Expérimentales et Cliniques, Bâtiment B36, 1 rue Georges Bonnet, BP 633, TSA 51106, 86073 POITIERS cedex9 – FRANCE.
Email: mohamed.jaber@univ-poitiers.fr
Phone: 33-(0) 549 453 985
ORCID: 0000-0003-2536-1913

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Abstract

Autism Spectrum Disorder (ASD) is a progressive neurodevelopmental disorder characterized mainly by deficits in social communication and stereotyped and restricted interests.

Deficits in social interactions in ASD animal models are generally analysed using the three chamber's test paradigm that is simple to implement and use but fails to detect subtle social deficits or complex social behavior on an extended period of time within a group of mice. Here, we set up a novel procedure entitled the Live Mouse Tracker (LMT) that detects a great number of complex social behaviours that we recorded continuously for up to three days in groups of 4 mice. This was performed in the valproic acid (VPA) mouse model where VPA (450 mg/kg) was injected to pregnant females at E12.5. Studies were performed with a special focus on females given that ASD is 3-4 times more diagnosed in males than in females and that several ASD models failed to detect major social deficits in females, contrary to males. Comparisons were made within groups of 4 female animals with same treatment or within groups of different treatments (saline versus VPA).

We report that VPA females show several types of social deficits and that are different in nature and magnitude in relation with time (from 1 hour to 3 days). These deficits were also different when VPA mice were tested together compared to when they were mixed with saline treated mice. Indeed, while social behavior was improved in VPA mice with the presence of saline mice that of saline mice was negatively affected by the presence of VPA mice.

This study indicates that female VPA mice show several social deficits, contrary to the common knowledge. It further implies that ASD related behavior alters normal behavior in a mixed group of mice.

Keywords: autism spectrum disorder, machine learning, Live Mouse Tracker, social behavior, female, valproic acid, mouse models.

Statements and Declarations: Authors declare no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

Introduction

Autism spectrum disorder (ASD) is a pervasive developmental disorder that is currently diagnosed based on two main clusters of behaviors as per the current version of the Manual of the American Psychiatric Association: (i) deficits in social communication and interactions and (ii) repetitive and restrictive behavior and thoughts. Symptoms need to be present since childhood and affect negatively the person's life[1]. Symptoms and comorbidities vary in their expression and severity leading to a spectrum of disorders. ASD is one the leading developmental disorders with a significant burden on the affected person, its family and the society in general. Its prevalence is in constant increase probably due to a combination of factors[2]. These include a heightened awareness of the disease by family and practitioners leading to a better diagnosis and recognition but also to possibly increased environmental insults from our modern way of living[3,4]. ASD has a prevalence of 3:1 in males to females although this proportion is still a matter of debate[5]. Several hypotheses are associated with this sexual dimorphism, among which is the possibility that females are underdiagnosed as ASD symptoms may be expressed differently in relation to gender[5].

The specific causes of ASD are still not clearly identified but there is a general consensus that there is no single etiology, rather a combination of both genetic and environmental factors[6]. There are over 1000 genes that have been implicated in ASD, each being responsible for only a minor proportion[7]. A common ground for most of these genes is that they seem to be implicated in neuronal transmission with a great part being at the interface of glutamatergic and GABAergic synapses[8–10]. Gene mutations all together are thought to account for only 25% of ASD cases so that environmental factors may account for the rest. Effects of these environmental factors are most inflicting if occurring during prenatal development and include toxins, pharmacological agents and viral and bacterial infections[11].

Knowledge of genetic and environmental determinants of ASD has led to the development of various genetic and environmental mouse models mimicking the main ASD symptomatology and thus showing good construct and face validity for most of them, or at least striving towards that

direction[12,13]. Among these animal models, the valproic acid (VPA) administration as a single dose (250-500 mg/kg) to pregnant females at a critical age of embryonic development (E 12.5) has been constantly shown to be a reliable ASD model recapitulating several behavioral, cellular and molecular phenotypes of the disorder[13–18].

As stated above, social deficits are amongst the hallmark symptoms of ASD and have been reported in these ASD models using various procedures, the most common one being the 3-Chamber test (3- CT) apparatus that was first set in place by Crawley in 1999[19]. This is a simple test measuring a mouse social behavior in a small surface subdivided into three chambers and counting the time, inframes of 10 minutes, that a mouse spends in a chamber containing another mouse compared to anempty chamber. If a new animal is thereafter introduced, one can also measure the preference to social novelty of the analyzed mouse, a behavior that is also affected in ASD patients. The 3-CT and similar procedures, including measuring social behavior in an open field, are widely used amongst researchers dealing with social determinants of ASD; they have the benefits of being easy to set in place and yield straightforward scores to analyze statistically[20]. As such, they are very useful for initial screenings but do not permit the analyses of complex social behavior, during an extended period of time, within a group of mice.

Here, we have implemented the recently described Life Mouse Tracker (LMT) procedure that enables automatic live tracking and subsequent analyses of complex social behavior in a group of mice with virtually no time limit but the one set by the experimenter[21]. This is achieved through the combination of computer vision through a depth-sensing infrared camera combined with machine learning and radio frequency identifying chips for animal identification. The social profile of each animal can thus be obtained, and its individual and group behavior analyzed in depth. Currently, this procedure must be set up starting from its basic hardware components by the experimenter. The corresponding software is in open access and can be downloaded directly but must be adapted to each's needs.

Using this procedure, we analyzed a wide range of collective and individual social behaviors continuously for 3 days in groups of female mice that were prenatally exposed to either a saline solution or VPA (450 mg/kg) at E12.5. Analysis was performed on groups of 4 mice that were either homogeneous or mixed regarding their treatment. Our study provides a wide scale behavioral analysis of several parameters related to social behavior at different periods of time ranging from 1hr to 3 days and reveals a wide spectrum of deficits in ASD mouse models that can also affect control mice when housed together.

Materials and Methods

Hardware- and software-related information

The LMT equipment needs to be built from scratch by the user according to the previously described procedure as no commercial version is yet available[21]. This system enables individual identification of groups of mice over several days, their automatic live tracking using a random-forest machine learning process for animal and posture identification, radio-frequency identification (RFID) chips detected by floor antennas for mice identification, and an infrared-depth RGB-D camera. For this, a Kinect camera is set 63 cm above a 50 x 50 cm2 cage, perpendicular to the floor. RFID antennas (134-125 kHz) are placed under the cage and induce an electric current in the RFID chips carried by a mouse thus allowing their identification. The depth map is represented as an image of 512 x 424 pixels and the segmentation map represents all the animals and objects in the field with a depth sensitivity equal to 1,4 mm.

Behavioural labelling is performed in real time using the continuous stream of depth and volume information to characterize the shape and posture of each individual, as well as the relative position between individuals. The segmentation process detects all moving objects (a mouse size corresponds to 60 pixels approx.) and filters small ones (less than 30 pixels). To obtain all individual segmentations, a connected component extraction is performed. An additional filter is set

to determine that the moving objects are indeed mice, and this is achieved through a machine learning algorithm that is trained during the first seconds of the observations. A detection splitter recovers animals fused into a single segmentation, based on an index map that corresponds to the identity index of each pixel of the mask. Thus, the system determines the outline mask and orientation of each mouse and builds a comprehensive repertoire of individual and social behavioural events from these data.

At the end of the detection procedure, different processes are used to associate a detection with a track or identity. The feature vector for machine learning is computed for each detection and allows machine learning algorithm to classify objects; it is composed of 33 values, the first being the ID of the animal, 16 values are the infrared histogram, and the 16 others are the depth histogram. The feature vector will therefore act as the object signature. The machine learning algorithm is involved in preparing the data, searching for the identity in concurrence with RFIDs and post-processing detections for the orientation of the animal.

Information on the detection, tracking and RFID readings of mice is stored in a database and can be freely accessed live or later as needed. Video and background maps are simultaneously recorded as videos and image series, respectively.

All software can be downloaded directly from the following site: http://www.bioimageanalysis.org/lmt/. Full source code is available at http://icy.bioimageanalysis.org/plugins/livemousetracker. This includes Java code and CAD hardware resource files. Python analysis scripts are available at https://github.com/fdechaumont/lmt- analysis.

Animals

Animal housing and experimental procedures were performed in accordance with the European Union directive (2010/63/EU) and validated by the regional ethical committee (Approval #

2020022610505052, 2021011214447548 and 2022021015414724). C57BL/6Jrj Mice (Janviers labs, France) were housed at the local Prebios animal facility, in ventilated cages with access to food and water *ad libitum*. Room temperature was maintained at 23°C on a 12h light/dark cycle.

Experimental Design

27 females and 22 males were used for mating. For this, 2 females were placed with a single male and left overnight, and males were retrieved from the cage following mating. Pregnant mice received a single i.p. injection of either VPA (450 mg/kg) or NaCl 0.9 % at gestational day 12,5 (E12.5) when the neuronal tube is closing in rodents, followed by neurogenesis and neuronal migration[17,22]. Following injection, pregnant mice were left undisturbed until they gave birth. At weaning on postnatal day 28 (P28), sex and age matched pups were separated and raised by groups of 4 in a randomized fashion to avoid littermate effects. Thus, LMT measurements were performed on groups of 4 mice that were not previously housed together. All mice were identified both by ear tags and with a RFID tag inserted subcutaneously under isoflurane anaesthetic with local analgesia (lidocaine 2% in ointment form). Female mice were then allocated to two experimental groups depending on prenatal treatment: VPA females (n=37), and saline females (n=37). The experiment timeline is presented in Figure 1. Behavioral analysis was performed for 3 continuous days for LMT and during 30 min within the light cycle for the 3-CT procedures. At the end of the behavioral experiments, mice were sacrificed, and brains harvested for further analysis as described below.

Individual social behavior as measured with the three chambers test (P30-P60)

Social interaction was assessed using the 3-CT as previously described[16] on the VPA females (n=20), and saline females (n=15). The apparatus consists of a Plexiglas box (60×45×22 cm) partitioned into three chambers with retractable doorways. The first phase (PHASE-I) comprises two identical non-social stimuli (inverted wire-cups) placed in the opposite chambers. The second phase (PHASE-II) comprises a non-social stimulus and a social stimulus (a naïve age and sex-matched mouse with no previous contact with the tested mouse). Each phase was of 10 minutes during which time spent in each chamber and around the inverted cup was recorded using a video tracking system. Subsequently, a sociability index (SI) was calculated as follows: (time exploring

social chamber – time exploring non-social chamber) / (time exploring social chamber + time exploring non-social chamber).

Group behavior as measured with the Live Mouse Tracker (LMT) (P35-P105)

Data obtained through the LMT system served as the basis for computing several events that were inferred from shape geometry. Overall, 35 behavioural events were defined related to intrinsic and relative positions of the mice that were extracted automatically as previously described[21]. Behavioral features were split into five categories: (i) individual behavior, (ii) social dyadic events between two mice, (iii) dynamic events between two mice (approach, escape, following each other…), (iv) subgroup events with 2, 3 or 4 mice, (v) group making and group-braking events. Details of these events can be found in the initial report of LMT and are reported in the results section[21]. It is to be noted however, that all behavior from a given category does not yield values of equivalent scale. Thus, and for the sake of presentation, behaviors with similar scales were gathered within a single figure, independent of their behavior category.

Data analysis

Data are expressed as mean ± Standard Error of the Mean (SEM) and analyzed using GraphPad Prism-7 software (La Jolla, California, USA). Data that followed a normal distribution was analyzed using one-way or two-way analysis of variance (ANOVAs) whenever appropriate. Upon significant main effects, Tukey's or Fisher's LSD multiple comparisons were performed for behavioral or histological measures, respectively. When data did not follow a normal distribution, we conducted non-parametric tests (Kruskal-Wallis or Mann-Whitney as indicated) followed by Dunn's multiple comparison tests. For all analyses, a p value of $<0,05$ was considered significant. All detailed statistical analysis and raw data can be obtained upon request to the authors.

Results

Social interaction analysis using the three-chamber test

The 3-CT was used to assess individual social behavior of female mice prenatally exposed to VPA (450 mg/kg) or saline at E12.5 as previously described[16]. Using this procedure, we have previously shown that only male mice that were exposed prenatally to VPA showed a dramatic decrease (down 7-fold) in their social behavior where VPA females had normal sociability[16].

Here, none of the treatment groups, regardless the sex, showed spontaneous preference for any of the chambers during the 10 minutes habituation (PHASE I) (Figure 2). Both saline and VPA females spent more time in the social chamber than in the empty chamber (62% *versus* 38%, and 69% versus 30%, respectively) [$F(3, 64)=43$, $p<0,0001$], although VPA mice seem to show an even better preference for the social chamber than the saline group ($p<0.05$) (Figure 2). However, when a female mouse was introduced in the opposite chamber, VPA female mice failed to show social novelty preference contrary to saline female mice (figure 2) [$F(3, 64)=6,8$, $p<0.001$]. Of interest is also the finding that saline mice preferred the new social chamber (SC2) more than the VPA mice ($p<0.05$).

Social interaction analysis using the Live Mouse Tracker system for 3 continuous days

We analyzed several variables of social behavior as described in the materials and methods section, for 3 days, on homogenous (same treatment within the group) or mixed groups (one VPA and threesaline mice within the group) of 4 female mice that were prenatally (E 12.5) exposed to either salineor VPA (450 mg/kg). Mice behavior was segmented into 5 groups of behavior as described in the methods section and as initially proposed[21]. As reported in figure 3, few altered behaviors can be identified at the end of the three days period in VPA groups of mice compared to control, whetherin males or females. Indeed, only a few social behaviors were affected by the VPA treatment. This

is for instance the case for the number of following behavior and movement in contact with another mouse for which VPA females performed less than saline.

We then performed the same analysis on heterogenous groups of mice of the same sex. For this, we introduced in the LMT arena one VPA mouse and three saline mice of the same sex and monitored their behavior for three continuous days. As shown in figure 3, we found that VPA mice created significantly more nests of 3 mice than the saline mice in the group ($p<0,05$). No other differences were found between the VPA and saline mice when analyzed together within a heterogeneous group.

Altogether, analysis of social behavior for a total of three days did not show major social deficits in the various groups of mice. This may be due to a flattening effect of behavioral deficiencies in the VPA group on a 3 days scale. In order to test this hypothesis, we underwent a further analysis of the data by time segments as detailed below.

Social interaction analysis using the LMT on various time periods

Group social interactions can vary greatly in time. Analyzing results on the total time period of three days may mask major differences in the behavior of different groups and that may be due to reaction to novelty, whether environmental or social. In order to determine the behavior of homogenous and mixed groups of female mice, we further analyzed the data obtained from the LMT system and segmented it to periods of 1h, 3h and 1 day (Figure 4).

We report here that after one hour of experiment, there were few differences in the number of contacts between saline and VPA mice in both the homogeneous and the mixed groups (figure 4A and D). For example, VPA female mice of the homogenous groups performed significantly fewer social approaches than saline mice (-12% for SA versus saline, $p<0.01$) (figure 4B). No such differences were identified within the mixed groups (figure 4E). After 3h of experimentation, several deficits were identified in the number of social contacts within the VPA mice of the

homogenous groups (-27% for oral-oral contacts $p<0.0001$; -42% for oral-genital contacts $p<0.0001$) (figure 4G) and some deficits in social approaches were also noticeable (-10% approachto contact, $p<0.05$) (figure 4H). Again, no major difference was found between saline and VPA mice in the mixed groups (figure 4J-L). We then analyzed the behavior after 24hours of experimentation. Again, we observed several deficits in the number of social contacts in the VPA mice in the homogenous groups (-11% oral-oral contacts $p<0.0001$; -13% oral-genital contacts, $p<0.0001$) (figure 4M), along with a slight reduction in the number of social approaches (-5% approach to contact, $p<0.05$) (figure 4N). Once more, no major differences were found in the mixed groups (figure 4P-R).

Evolution in time of social behavior using the LMT

When analyzing social behavior in relation with time, we have identified major differences on individual and group behaviors. To further reveal these differences, we plotted social behavior of several chosen variables in relation with time (figure 5). We show here that across behaviors, the main differences between groups and treatment are observed during the first 24h to 48h of the experiment. For instance, we found significant alterations in the behavior of saline mice within the heterogenous groups during the first 24h (figure 5A). this was also the case in the VPA mice in the homogenous groups (figure 5A). Across time, differences between saline mice in homogenous groups (all saline) compared to the heterogenous groups (3 saline and one VPA mice) were observed but that were of a lesser magnitude than differences between saline and VPA mice in homogenous groups (figure5B-D).

Discussion

Social deficits are one the two hallmarks of ASD symptoms as defined by the American Manual of Psychiatry in its latest version[1]. As face validity is key in animal modeling of diseases and

disorders, it has become mandatory for ASD animal models, whether genetic or environmental, to express some forms of social deficits before being considered for further analysis and characterization[19,20,23–25].

Social deficits in ASD children are expressed in the form of social play behavior deficits and lack of non-verbal social gestures such as pointing, showing, and giving[26–29]. Mice are very social mammals and exhibit a wide range of social behavior such as direct cross interactions, sniffing, following, regrouping, collective nesting, maternal care, general play behavior and sexual approaches[30–32]. A significant number of reports have described social impairments in a large majority of ASD animal models, including the VPA models[13,17,26].

Since its initial implementation, the 3-CT has become rapidly the gold standard to measure social approach, social preference and, more generally, exploratory locomotion[31]. The procedure is now automated and is now widely used, in its original form or with slight variations, amongst teams working on various animal models of ASD[33]. This automated test was referred to by the team as being a "basic protocol for a standardized, high throughput social approach test for assaying mouse sociability" and that "allows to quantify direct social approach behaviors when a subject mouse is presented with the choice of spending time with either a novel mouse or a novel object"[33]. Despite the many advantages of this simple and efficient procedure, the 3-CT suffers from several drawbacks as it cannot evaluate complex social behaviors, in a group of mice, and for a long period of time.

Recently, we have published a series of studies on 3 different ASD animal models, genetic (Shank3) and environmental (VPA and poly I:C) all showing major deficits in social behavior thatwere almost exclusively present in male ASD mice but not in females [13,16,16,34]. These deficits werealso variable in their magnitude and expression in relation with ASD's etiology, and they include deficits in social preferences and/or social novelty. However, both males and females showed abnormal gait and motor coordination behaviors and correlative loss of cerebellar Purkinje cells. This raised the question of whether ASD females were somehow protected from contracting social

deficits, whatever the disorder's etiology, or whether the procedure that is used to evaluate social behavior is not sensitive enough to detect mild or specific social deficits. Of interest is the fact that loss of PC cells was restricted to different cerebellar subregions between males and females, and this may possibly underly differences in the behavioral expression of ASD in relation with mice gender[13].

Here, we aimed to explore whether we can identify complex social deficits in female VPA mice and determine the extent and the nature of these social deficits. For this, we have used in the widely used VPA ASD animal model and that we have previously characterized extensively in our experimental settings, where VPA is injected prenatally to the pregnant female at E12.5 at 450 mg/kg in a single i.p. dose. We implemented the recently developed LMT apparatus and performed the experiments on female mice. We measured social behavior on groups of 4 mice randomized from different litter of the same sex and of the same treatment (homogeneous groups) or with a mixed treatment of saline and VPA. Experiments were performed for three consecutive days and data was analyzed following 1hr, 3 hrs, 24 hrs and three days.

VPA, is a potent antiepileptic drug[35] and mood stabilizer[36] that modulates GABA neurotransmission[37] and regulates gene expression through inhibition of histone deacetylase (HDAC) activity[38]. It has been known since 1991 that VPA is teratogenic when administered during pregnancy[39] and later it was consistently reported that it also significantly increases the risk of developmental delays including ASD[40]. Prenatal exposure to a single dose of VPA around the date of E12.5 is now widely used to induce in offspring the two cardinal ASD symptoms, social deficits and stereotyped behavior. The VPA model is considered a reliable model bearing a very strong construct and face validity[17,18,26]. In our hands, we found that the VPA animal model was the most robust of all three models that we had explored in our experimental settings (Shank 3 ΔC or prenatal exposure to either Poly I:C or VPA). Indeed, we previously reported that VPA mice showed significant deficits in social interactions along with major gait and motor disorders that were even detected at early perinatal ages[16]. Of interest is also the fact that social deficits were

directly correlated in their severity with motor and gait deficits and also with loss of PCs within restricted cerebellar areas.

We report here that: (i) VPA females show several forms of social deficits as compared to saline females; (ii) social deficits were complex in their nature as they implicated several behavioral traits and variables such as approaches, following, direct interactions, breaking a group of mice and nesting ; iii) these social deficits appear early on during the experiments then tend to disappear within three days implicating possibly a reaction to novelty; (iv) one of the most intriguing findings that we report here is that VPA mice, when placed within a group of saline mice, show significantimprovement in several social variables ; (v) conversantly, saline mice within mixed groups showed degradations of several variables linked to social behavior in the presence of a VPA mouse.In other words, placing an ASD mouse within a group of normal mice improves the social behaviorof the VPA mouse but deteriorates that of normal mice. These findings need to be confirmed withfurtherstudies in ASD animal models and more importantly in humans, as they may have repercussions regarding the current policies of integrating ASD patients within groups of typical individual. A such, these results may also suggest that some form of follow up and companionshipis needed to increase the chances of success of including ASD patients in regular schools. Althoughresearch reports on this subject are very scarce, it was shown that when intervention therapies are implemented in schools, ASD children had significantly higher social network inclusion and received more friendship nominations than ASD children without such an intervention[41,42].

To our estimate, the 3-CT should always be the default procedure to use for screening social behavioral deficits in animal models of ASD or other psychiatric illnesses. The LMT is to be used when precise and detailed exploration of social deficits are needed as is the case with females who do not show such deficits in the 3-CT whatever the ASD etiology. The LMT is also useful to study long term social behavior in a homogeneous or heterogeneous groups of mice. Currently, the LMT does not allow measurements of other social behaviors such as mating or fighting. Surely, with the current developments, this may be overcome in the future.

Figures Legends

Figure 1: Experimental design and timeline. Pregnant female mice were i.p. injected at E12.5 with either saline (0.9% NaC) or a single dose of VPA (450 mg/kg). Offspring were weaned at 4 weeks pf age, implanted with a RFID tag and were allocated to 2 experimental groups in relation with prenatal exposure. Behavioral analysis was performed for 3 continuous days for the Live Mouse Tracker (LMT) and for 30 min. within the light cycle for the 3-Chamber test (3-CT) procedure.

Figure 2: Social behavior as analyzed in the 3-chamber test (3-CT) procedure. A) Following habituation (Phase I) mice were put in 3-CT with one chamber containing an encaged mouse (social chamber 1: SC1) and one chamber with an empty cage (non-social chamber: NSC) to evaluate social preference (Phase II). (B) a novel mouse was introduced in the chamber 2 (social chamber 2: SC2) in addition to the encaged mouse in SC1 to evaluate social novelty recognition (Phase III). During Phase III, VPA female mice spent equal time interacting with SC1 and SC2 while saline mice spent more time interacting with SC2. All data are expressed as means ± SEM; One way ANOVA followed by Tukey's multiple analysis was performed (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Saline females (n=14), VPA females (n=20).

Figure 3: Social behavior during three continuous days as measured using the Life Mouse Tracker (LMT) procedure. (A) Number of various social contacts within the homogenous groups (mice of the same treatment) during the three days of experiment. (B) Number of various social approaches within the homogenous groups. (C) Number of "following behavior" and of clusters of 2 and 3 mice within the homogenous groups (D). Number of "making and breaking of groups" of

clusters of 3 and 4 mice and number of “trains” of 2 mice within the homogenous groups. (E) Number of “moving behavior” and “rearing” within the homogenous groups. (F) Number of “wall jump”, “water zone interaction” and “nest of 3” within the homogenous groups. (G) Number of various social contacts for the mixed groups (3 saline and 1 VPA). (H) Number of various social approaches within the mixed groups. (I) Number of “following behavior” and of clusters of 2 and 3 mice within the mixed groups (J) Number of “making and breaking of groups” of clusters of 3 and 4mice and number of “trains” of 2 mice within the mixed groups. (K) Number of “moving behavior” and “rearing” within the mixed groups. (L) Number of “wall jump”, “water zone interaction” and “nest of 3” within the mixed groups. All data are expressed as means ± SEM; Mann-Whitney test was performed (*p<0.05, **p<0.01, ***p<0.001). Saline females homogenous group (n=16); VPA females homogenous group (n=20); saline females mixed group (n=27); VPA female mixed group (n= 9).

Figure 4: Social behavior at various time periods (1h, 3h and 12h) as measured using the Life Mouse Tracker (LMT) procedure. Number of various social contacts (A), social approaches (B) or of “following” and making groups of 2 and 3 (C) within the homogenous groups for 1 hour. Number of various social contacts (D), social approaches (E) or of “following” and making groupsof 2 and 3 (F) within the mixed groups for 1 hour. Number of various social contacts (G), social approaches (H) or of “following” and making groups of 2 and 3 (I) within the homogenous groups for 3 hours. Number of various social contacts (J), social approaches (K) or of “following” and making groups of 2 and 3 (L) within the mixed groups for 3 hours. Number of various social contacts (M), social approaches (N) or of “following” and making groups of 2 and 3 (O) within the homogenous groups for 24 hours. Number of various social contacts (P), social approaches (Q) or of “following” and making groups of 2 and 3 (R) within the mixed groups for 24 hours. All data are expressed as means ± SEM; Mann-Whitney test was performed (*p<0.05, **p<0.01, ***p<0.001).

Figure 5: Evolution in time of social behavior using the Life Mouse Tracker (LMT) procedure. Number of (A) oral-genital contacts, (B) oral-oral contacts, (C) social approaches, (D) social contacts over the three days of the experiment. All data expressed as means ± SEM; two-Way ANOVA with post-hoc Tukey was performed. Only selected differences were highlighted for the sake of clarity (*: differences between saline homogenous and VPA homogenous mice; +:differences between saline homogenous mice and saline mixed groups mice; -: differences between VPA homogenous and VPA mixed groups mice).

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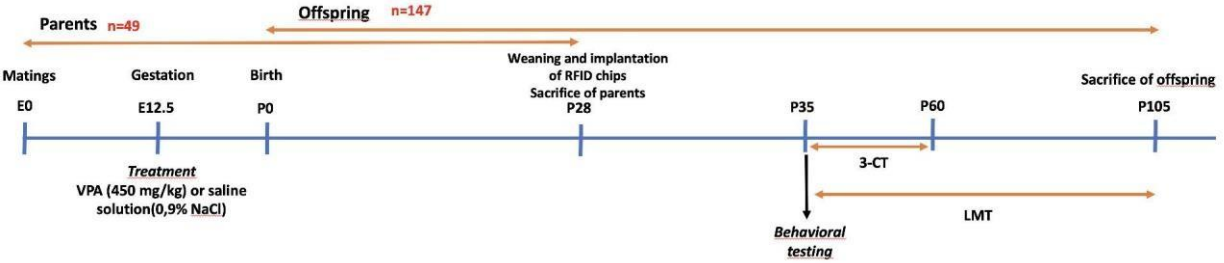
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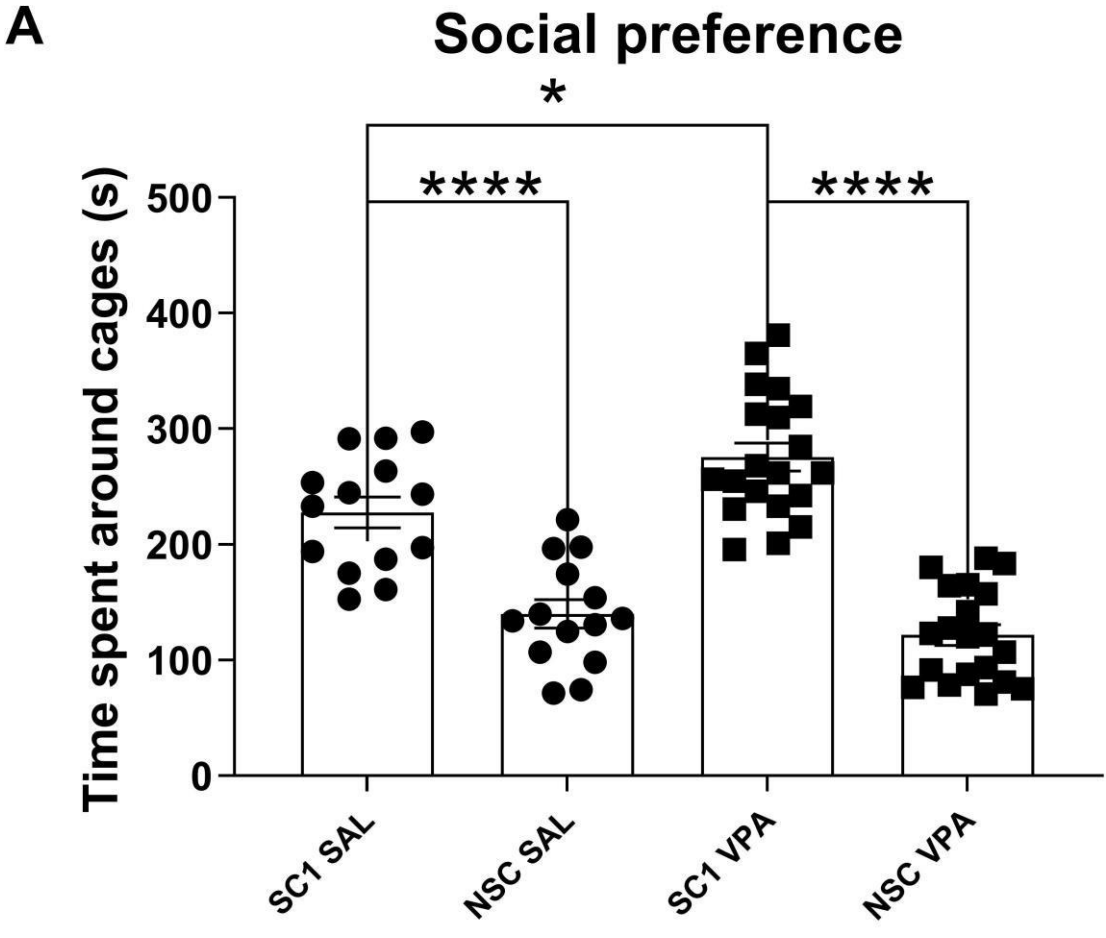
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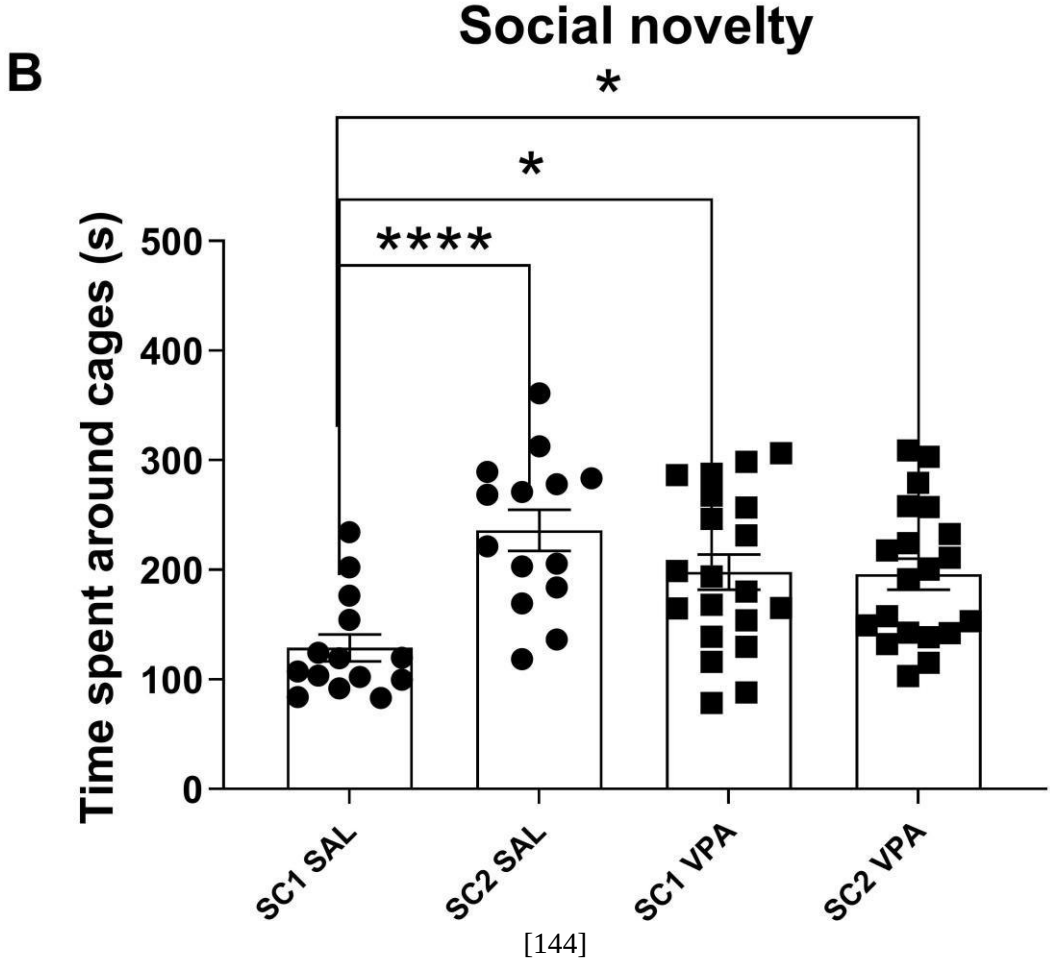
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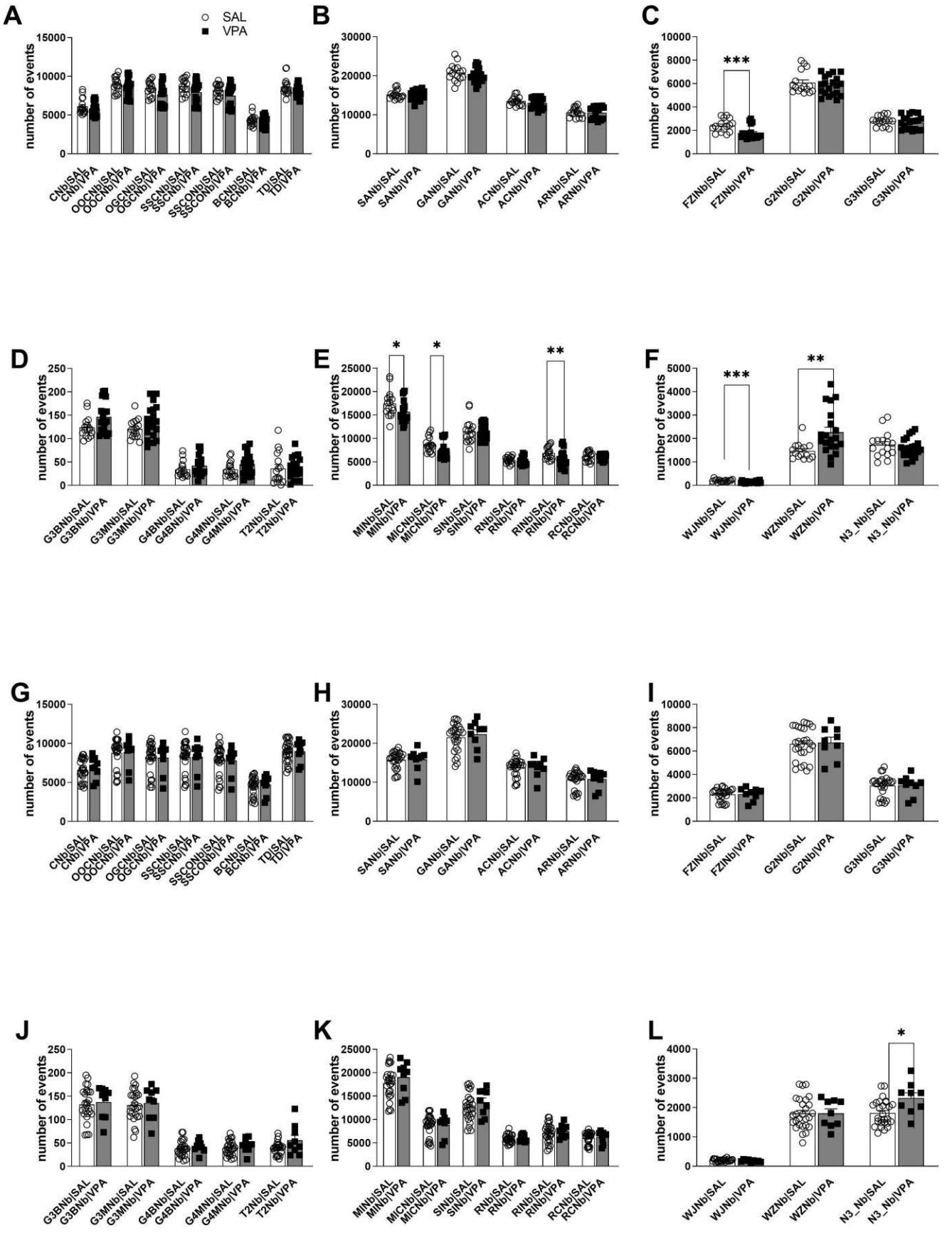
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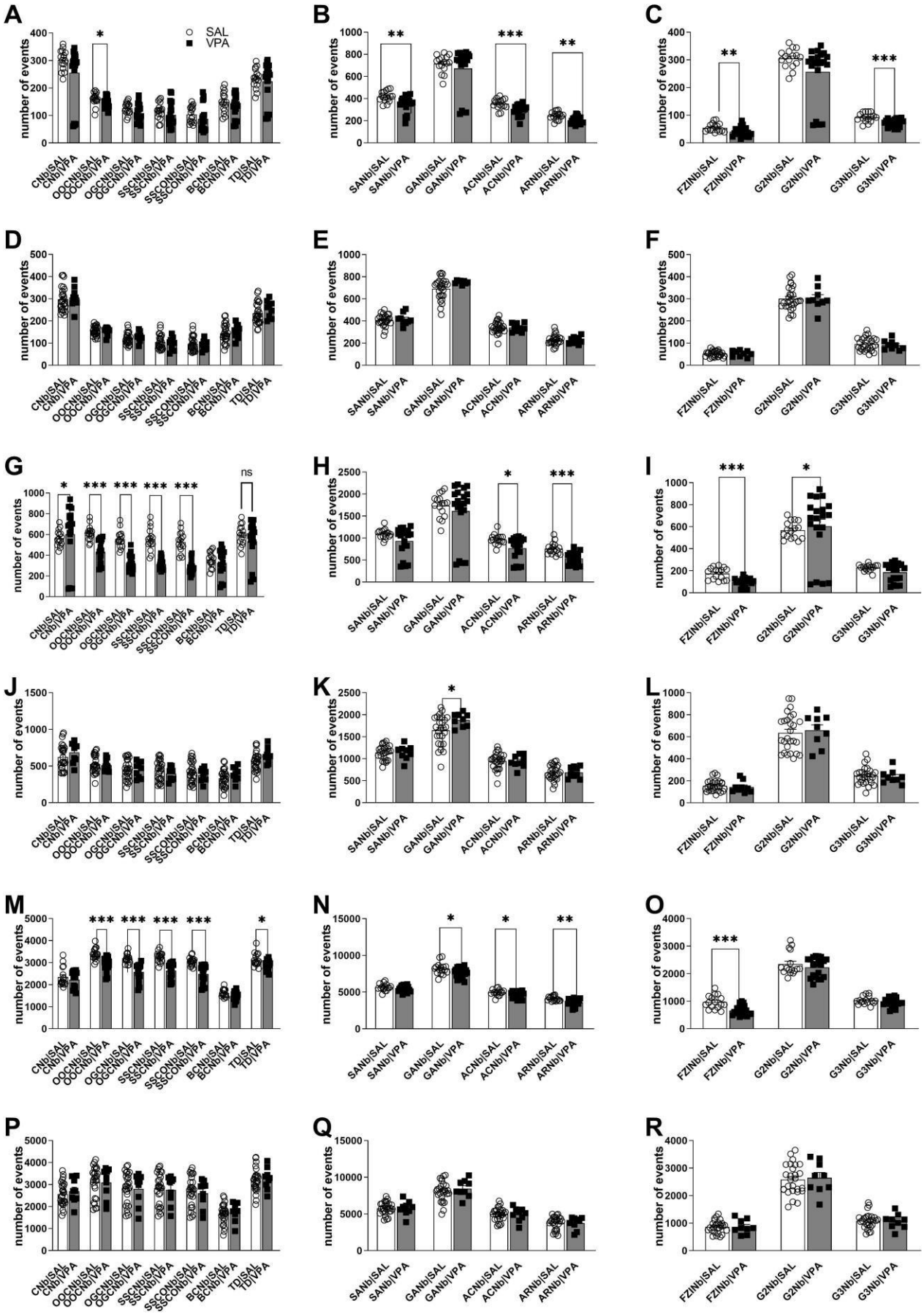
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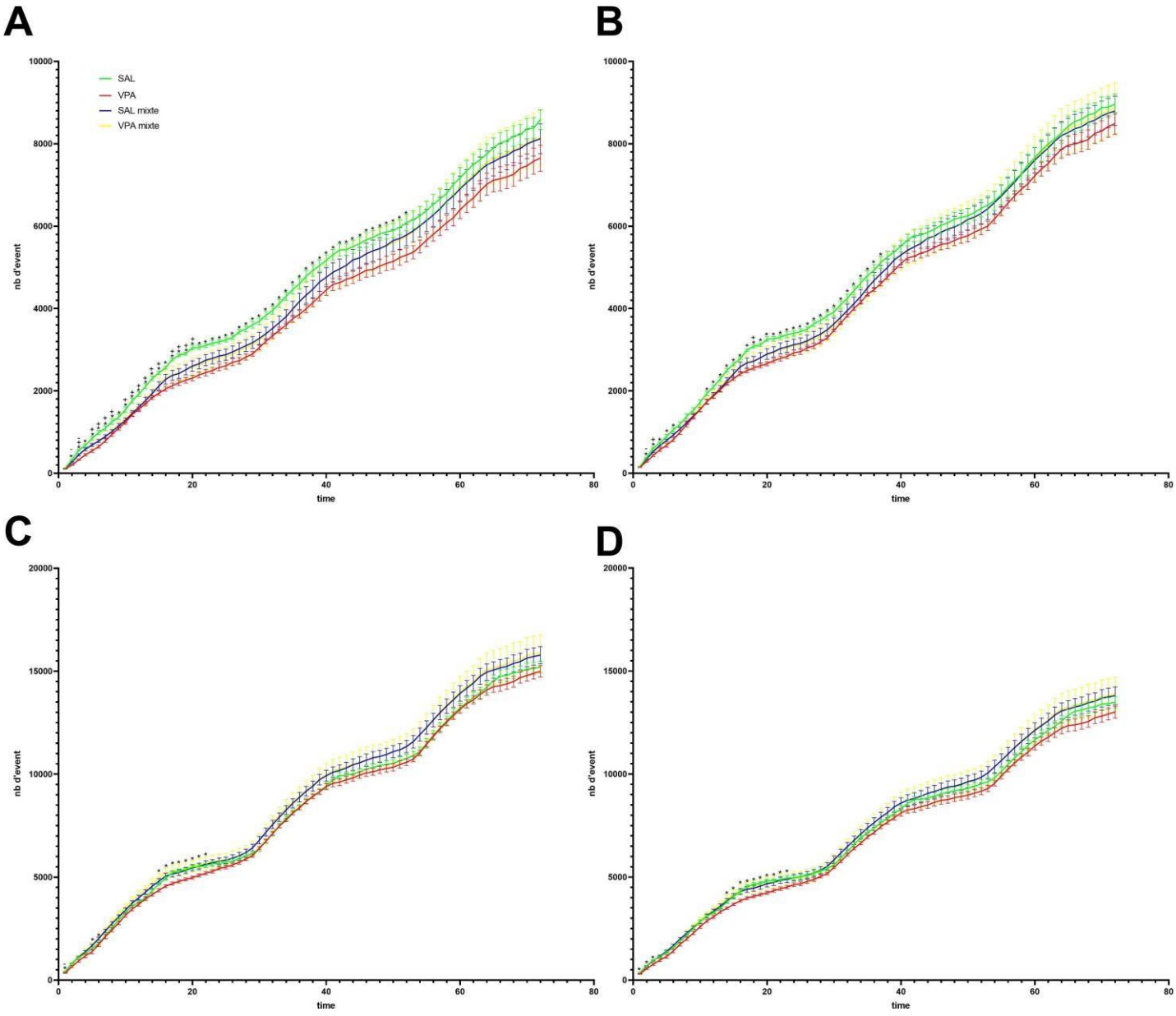

Parents n=49
Offspring n=147
Matings
E0
Gestation
E12.5
Birth
P0
Weaning and implantation of RFID chips
Sacrifice of parents
P28
P35
P60
Sacrifice of offspring
P105
Treatment
VPA (450 mg/kg) or saline solution(0,9% NaCl)
3-CT
LMT
Behavioral testing


A
Social preference
*
****
****
Time spent around cages (s)
500
400
300
200
100
0
SC1 SAL
NSC SAL
SC1 VPA
NSC VPA


B
Social novelty
*
*
****
Time spent around cages (s)
500
400
300
200
100
0
SC1 SAL
SC2 SAL
SC1 VPA
SC2 VPA


A
B
C
D
E
F
G
H
I
J
K
L
SAL
VPA
number of events
CNbISAL
CNbIVPA
OOCNbISAL
OOCNbIVPA
OGCNbISAL
OGCNbIVPA
SSCNbISAL
SSCNbIVPA
SSCONbISAL
SSCONbIVPA
BCNbISAL
BCNbIVPA
TDISAL
TDIVPA
SANbISAL
SANbIVPA
GANbISAL
GANbIVPA
ACNbISAL
ACNbIVPA
ARNbISAL
ARNbIVPA
FZINbISAL
FZINbIVPA
G2NbISAL
G2NbIVPA
G3NbISAL
G3NbIVPA
G3BNbISAL
G3BNbIVPA
G3MNbISAL
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RCNbISAL
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SAL
VPA
number of events
CNbjSAL
CNbjVPA
OOCNbjSAL
OOCNbjVPA
OGCNbjSAL
OGCNbjVPA
SSCNbjSAL
SSCNbjVPA
SSCONbjSAL
SSCONbjVPA
BCNbjSAL
BCNbjVPA
TDjSAL
TDjVPA
SANbjSAL
SANbjVPA
GANbjSAL
GANbjVPA
ACNbjSAL
ACNbjVPA
ARNbjSAL
ARNbjVPA
FZlNbjSAL
FZlNbjVPA
G2NbjSAL
G2NbjVPA
G3NbjSAL
G3NbjVPA
ns


A
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C
D
SAL
VPA
SAL mixte
VPA mixte
nb d'event
time
0
2000
4000
6000
8000
10000
5000
10000
15000
20000
20
40
60
80

[147]

Discussion Générale

1. Rappel de la problématique générale de la thèse

Cette thèse avait pour objectif d'étudier deux problèmes distincts liés aux TSA. Le premier est la difficulté à établir un diagnostic précoce, objectif et quantitatif des TSA avec les méthodologies actuelles. Cela est dû au fait que, comme précédemment indiqué, ces tests se basent principalement sur une évaluation des capacités sociales, du raisonnement logico-mathématique et des fonctions intellectuelles des patients. Ainsi, ces tests sont difficilement réalisables chez le très jeune enfant retardant la pose du diagnostic et la mise en place des thérapies comportementales, réduisant ainsi potentiellement l'efficacité de ces thérapies. Afin d'apporter des éléments de réponses à cette problématique, le premier objectif de cette thèse a été de déterminer si les déficits moteurs observés chez les enfants TSA peuvent être utilisés comme éléments de diagnostic objectif et quantitatif. Pour cela, nous avons caractérisé ces déficits dans un modèle génétique des TSA. Nous avons aussi cherché à établir si ces troubles et leur gravité pouvaient être accompagnés par des altérations au niveau moléculaire et cellulaire.

Mes travaux sur les troubles moteurs et de la marche chez des souris modèles de TSA s'inscrivent dans un projet plus large au niveau régional (Bordeaux, La Rochelle, Poitiers, Limoges) visant à déterminer en condition clinique la pertinence de ces mesures dans le diagnostic précoce des TSA. Ces projets cliniques sont issus directement de nos travaux chez la souris.

Le second problème sur lequel nous nous sommes penchés est la grande variabilité dans la présence et la gravité des troubles d'interaction sociale d'un modèle TSA à l'autre, mais aussi au sein du même modèle en fonction des laboratoires. C'est le cas par exemple des modèles génétiques basés sur la mutation de Shank3, dans lesquels les différentes mutations génèrent un profil comportemental différent avec certaines mutations présentant d'importants déficits sociaux et des stéréotypies motrices, d'autres en présentant peu ou aucun. L'absence de méthodes d'analyses fines des comportements sociaux chez les souris modèles de TSA limite la caractérisation précise de chacun des modèles. Pour répondre à cette problématique, j'ai mis en oeuvre une nouvelle méthode d'analyse des comportements sociaux, le LMT, permettant l'analyse de comportements sociaux complexes sur une longue durée dans un groupe de souris. L'objectif affiché était d'établir un profil social détaillé d'un modèle établi des TSA, le modèle VPA, en mettant à profit la puissance de mesure

et d'analyse du LMT. Nous avons ainsi déterminé quelles informations sur les capacités sociales il était possible d'obtenir avec cette technique comparée aux techniques utilisées habituellement, telle que le 3-CT. L'ensemble de mes résultats sur cette problématique devrait contribuer à proposer une meilleure standardisation des techniques de mesure d'interaction sociale pouvant ainsi révéler des troubles qui n'étaient pas détectables auparavant entre les différents modèles mais aussi chez les femelles.

2. Troubles moteurs et de la marche dans un modèle génétique d'autisme

2.1 Rappel des principaux résultats Shank 3

Pour notre étude utilisant le modèle SH3 ΔC/ΔC, nous avons commencé par caractériser la sociabilité de nos souris en utilisant le 3-CT. Nous avons pu observer que les souris SH3+/ΔC et les souris SH3 ΔC/ΔC mâles et femelles ne présentaient pas de déficits à la préférence sociale, montrant que la mutation de SH3 n'a pas affecté la valeur de l'interaction sociale chez ces souris. Cependant, les souris SH3+/ΔC et les souris SH3 ΔC/ΔC mâles et femelles montrent une absence de préférence à la nouveauté sociale, indiquant que la mutation de SH3 a affecté la valeur d'un nouvel individu par rapport à celle d'un individu déjà connu. Ces résultats nous permettent de conclure que les déficits sociaux chez les souris SH3 mutées sont peu prononcés et concernent uniquement la nouveauté sociale.

Dans un deuxième temps, nous avons décidé d'étudier les troubles moteurs chez nos souris SH3. Là encore, peu d'altérations de la motricité fine ont été observées. Seules les souris SH3 ΔC/ΔC femelles ont montré des déficits moteurs sur la partie la plus fine du challenging beam.

Enfin, nous nous sommes intéressés à la marche des souris SH3. Nous avons pu observer de nombreuses altérations de la marche des souris SH3+/ΔC et des souris SH3 ΔC/ΔC mâles et femelles au niveau de la fréquence des pas, la durée du balancement de la patte et de la durée de la pose de la patte. Il est à noter que certains paramètres étaient affectés uniquement chez les souris mâles. Nous avons pu noter deux éléments principaux, le premier était que les altérations touchaient principalement des paramètres temporels de la marche indiquant que les déficits observés n'avaient pas une origine anatomique. Le second était que plusieurs

paramètres n'étaient altérés que chez les mâles indiquant une relative protection des femelles contre les effets de la mutation, dont l'origine est à déterminer.

Ayant montré la présence de troubles comportementaux et moteurs, nous avons par la suite étudié l'histologie du cervelet où nous avons pu observer une diminution significative des PC dans crus I et crus II chez les mâles et dans crus I chez les femelles. Il est à noter donc que les altérations cellulaires sont plus marquées chez les mâles et chez les femelles, de manière parallèle aux observations comportementales.

Enfin, au niveau moléculaire, on notera une diminution significative de la protéine mGluR5, mais seulement dans le cervelet des souris mâles, en phase avec les observations précédentes. Ce résultat permet aussi de souligner l'importance de SH3 dans le bon adressage de mGluR5 à la membrane.

Un point important à noter est le fait que les souris mâles qui présentaient des troubles de la marche plus importants que les femelles, présentent aussi des altérations cellulaires et moléculaires plus importantes. Il reste à démontrer un lien direct entre la diminution des PC, la diminution de mGluR5 et les troubles de la marche et de la coordination.

2.2 Comparaison des résultats avec d'autres études et différents modèles Shank3

Les résultats concernant l'expression de mGluR5 dans les modèles SH3 sont discordants. En effet, selon le modèle utilisé, la région étudiée, mais aussi le laboratoire dans lequel les expérimentations sont réalisées, une augmentation ou une diminution des niveaux de mGluR5 ont été observées.

Plusieurs explications à cela sont possibles : (i) les variations sont fonction du modèle SH3 utilisé. En effet, selon le modèle, différentes isoformes de SH3 sont affectées avec différentes formes tronquées pouvant avoir différentes activités possibles. (ii) L'expression de SH3 varie grandement selon les régions (Monteiro & Feng, 2017) et il est possible que SH2 puisse en partie remplacer SH3 dans les régions ou SH3 est peu exprimé. (iii) Les procédures expérimentales utilisées sont aussi variables avec certaines mesurant la quantité de protéines par western blot, avec ou sans fractionnement synaptique ou cytosolique, et d'autres de

niveaux d'ARNm par qPCR. Enfin (iv), certains modèles de mutation SH3 ne représentent pas de phénotypes liés aux TSA, mais simplement des déficits intellectuels (Soorya et al., 2013).

De fait, et en conséquence de cette variabilité des résultats concernant mGluR5, des traitements par des agonistes et antagonistes de mGluR5 ont été décrits comme étant capables d'avoir un effet positif sur les comportements autistiques observés (Vicidomini et al., 2017). On pourra citer comme exemple Wang et al. (Wang et al., 2011) qui, après avoir observé une augmentation de mGluR5 dans le striatum de souris SH3 KO, ont utilisé un antagoniste de mGluR5 (MPEP) réduisant ainsi les comportements de grooming et améliorant les déficits sociaux. A l'inverse, l'utilisation d'un agoniste de mGluR5 (CDPPB) causait une augmentation des comportements de grooming (Wang et al., 2011). À l'opposé de ces résultats, on pourra citer les travaux de Vicidomini et al., qui, après avoir observé une diminution de mGluR5 dans le striatum et le cortex de souris SH3 Δ 11/Δ 11, ont utilisé CDPPB pour réduire les comportements de grooming et les déficits sociaux (Vicidomini et al., 2017).

Cette dualité rappelle celle en cours sur l'activité glutamatergique chez les patients atteints de TSA proposant, avec d'un côté l'hypothèse d'une hyperactivité du système glutamatergique défendue par Carlson. (Carlson, 2012), et d'un autre côté celle d'une hypoactivité glutamatergique défendue par Fatemi et al (Fatemi, 2008). Dans les deux cas, ces études ne sont pas sans rappeler la théorie suggérant que la cause des TSA serait liée à un déséquilibre dela balance excitation/inhibition (Uzunova et al., 2016). De fait, il serait indiqué de mieux déterminerle rôle du GABA dans les différents modèles de SH3 (Yoo et al., 2018).

2.3 Conclusion des travaux Shank3 : avantages et limites de ce modèle

En conclusion, notre étude a montré une diminution de l'activité glutamatergique au sein du cervelet de nos souris accompagnée par une diminution du nombre de PC ainsi que par des déficits marqués de la marche et de légers déficits moteurs et sociaux. Un consensus sur le niveau d'altération du système glutamatergique dans les différents modèles SH3, et en condition clinique, n'a pas pu être trouvé.

Un élément supplémentaire à noter rendant la situation encore plus complexe, est le fait que la plupart des modèles SH3 ne présentent que de légers déficits sociaux. Ce fait, combiné au

lien entre SH3 et déficit intellectuel, rend difficile la classification et la comparaison des modèles SH3 entre eux et entre les différents autres modèles de TSA.

En conséquence, il est devenu déterminant de mieux caractériser les interactions sociales des souris modèles de TSA. En effet, une étude plus détaillée de ces déficits pourrait potentiellement permettre d'écarter définitivement certains modèles SH3 comme étant peu/pasliés aux TSA. Dans cette optique, le second objectif de ma thèse a été la mise en place d'une nouvelle méthode d'étude de ces interactions : le LMT, dans un modèle éprouvé de TSA, celui de VPA.

3. Mise au point du Life mouse Tracker pour l'Étude d'interactions sociales complexes dans un modèle environnemental d'autisme

3.1 Rappel de la problématique des études d'interaction sociale dans des modèles animaux

Comme présenté avec le modèle SH3 ΔC, bien que les déficits d'interactions sociales soient l'un des symptômes principaux des TSA, les méthodes d'analyse actuelle restent limitées dans leurs capacités à détecter et créer un profil social détaillé des souris. En effet, les méthodes actuelles de manière générale quantifient la préférence des souris pour un stimulus social plus que les capacités sociales intrinsèques des souris (Crawley, 2004). De plus, l'étude de ces comportements nécessite le transfert de la souris test dans un environnement nouveau. Il est donc possible que le stress engendré par le changement d'environnement affecte de manière significative le comportement des souris modèles des TSA (Beery & Kaufer, 2015). Enfin,on pourra noter que les méthodes actuelles ne permettent pas l'étude des comportements d'un groupe de souris ou l'étude sur du long terme limitant là encore les possibilités pour l'analyse des comportements sociaux des souris modèles des TSA.

Les limites des méthodes actuelles d'analyse de la sociabilité des souris rendent plus complexe la caractérisation des nombreux modèles de TSA, et ne permettent pas d'éliminer certains modèles de TSA qui seraient peu ou pas liés à la pathologie. Pour commencer à apporter un élément de réponse à cette problématique, mon second objectif a été la mise en place d'une nouvelle méthode d'études des interactions sociales, le LMT. Mon objectif avec cette technique est de générer un profil social détaillé pour des souris VPA modèles des TSA, puis d'étudier l'impact de la présence de souris contrôles sur les capacités sociales des souris VPA.

3.2 Mise en place du LMT : difficultés et intérêts

Comme décrit précédemment, le LMT est une technique permettant l'analyse (i) de comportements sociaux complexes, (ii) sur un groupe de souris et (iii) à long terme. L'addition de ces trois éléments ouvre un grand nombre de possibilités pour l'analyse des interactions sociales.

Premièrement, grâce au grand nombre de comportements étudiés, il est possible de différencier les comportements d'approches sociales que l'on peut associer à la mise en place des interactions, à ceux des contacts sociaux que l'on peut potentiellement associer plus aux renforcements des interactions sociales. Plutôt que de simplement comparer la valeur d'un stimulus social versus un stimulus non-social, il devient possible d'étudier de manière détaillée la mise en place des interactions sociales ainsi que leur renforcement.

Deuxièmement, la possibilité de réaliser les études sur un groupe de souris ouvre là encore de nombreuses possibilités pour l'étude des interactions en fonction des traitements. En effet, en créant des groupes ayant un phénotype différent (TSA versus contrôle) il devient possible d'étudier le comportement des différents individus du groupe, confrontés à un individu de phénotype différent. En variant la composition de ces groupes, il est aussi possible d'étudier comment les différents phénotypes influencent la mise en place des interactions sociales. Ainsi, et à titre d'exemple, on pourrait étudier quelles sont les différences entre un groupe comportant 3 souris contrôles et 1 souris TSA comparées à un groupe contenant 2 souris contrôle et 2 souris TSA et un groupe contenant 1 souris contrôle et 3 souris TSA. Enfin, une dernière possibilité est la capacité d'étudier la réponse d'un groupe déjà formé à un nouvel individu qui serait introduit au cours de l'expérimentation.

Troisièmement, la capacité de réaliser les expérimentations à long terme permet, comme indiqué précédemment, d'étudier à la fois les interactions sociales dans un groupe déjà formé et les approches sociales au début de l'expérience. De plus, il sera aussi possible d'étudier comment les différents groupes réagissent et s'habituent à un environnement nouveau, les mesures par LMT pouvant être réalisé sur plusieurs mois. Ainsi, une étude possible serait de comparer les effets au long terme d'un environnement enrichi sur des groupes de souris contrôles ou de souris TSA (Chen et al., 2022).

Cependant le LMT présente de nombreuses limites compliquant sa mise en place à grande échelle. On pourra ainsi citer (i) la complexité de mise en place du système (ii) l'encombrement du système (iii) la fragilité du système, et enfin (iv) la complexité de l'analyse des résultats.

(i) Le LMT est complexe à installer, car celui-ci requiert plusieurs compétences spécifiques techniques, électroniques et informatiques. De plus, une infrastructure sera nécessaire pour la création et la construction des différentes pièces (imprimante 3D, matérielle de découpe de métal, etc.) ainsi que leur calibration (hauteur spécifique pour la caméra et réglage de la fréquence des antennes) demandant ainsi un investissement en temps beaucoup plus important que les méthodes utilisées précédemment, afin de rendre le système fonctionnel.

(ii) Le LMT est un système demandant beaucoup de place de par sa taille, chaque système pouvant requérir sa propre salle. De plus, le LMT est un système contraignant par le temps requis pour réaliser les expériences. En effet, chaque expérience pouvant durer plusieurs jours, bien que l'expérimentateur ne soit pas requis pour suivre constamment les animaux dans le LMT, cela limite l'accès aux salles utilisées pour le LMT, limitant ainsi le nombre d'expériences pouvant être réalisées en parallèle.

(iii) Le LMT est un système fragile, notamment au niveau des antennes utilisées pour la détection des puces RFID. La connexion de ces antennes à l'ordinateur d'analyse est fragile et il est facile d'endommager ces antennes en déplaçant le LMT, limitant ainsi la capacité d'identification des individus.

(iv) Le LMT génère une grande quantité de données, celles-ci sont analysées avec des scripts en python permettant un traitement rapide des données. Néanmoins, ces scripts sont complexes et la moindre modification de ceux-ci pourra changer de manière radicale les résultats obtenus. Ainsi, une bonne connaissance du langage informatique python est nécessaire pour permettre le traitement des données.

3.3 Discussion des résultats obtenus

Souhaitant obtenir des conditions d'activité similaires à celle de la cage d'élevage et sur les conseils des constructeurs qui avaient initialement mis au point la technique (de Chaumont et al., 2019), nous avons décidé de réaliser nos expériences sur 3 jours.

Pour notre étude, nous avons utilisé des groupes de souris dit homogènes, composés soit de 4 souris saline soit de 4 souris VPA ainsi que des groupes hétérogènes, composés de 3 souris salines et 1 souris VPA. Nos objectifs étaient (i) d'étudier les différences comportementales entre les souris salines et VPA et (ii) d'étudier l'impact de la mise en contact d'une souris VPA avec un groupe de souris salines. Nous avons commencé par analyser les comportements des groupes homogènes et hétérogènes séparément.

A la fin des 3 jours d'expérimentation, aucun des groupes ne présentaient de différences majeures quant à leurs comportements sociaux. Les différences les plus notables étaient une diminution des comportements de mouvement au contact et de « following » chez les souris VPA des groupes homogènes, indiquant la présence de légers déficits sociaux. Ce résultat n'est pas sans rappeler celui de De chaumont et al (2019) avec les souris SH3 KO qui présentaient peu d'altérations comportementales après une journée d'expérience (de Chaumont et al., 2019).

L'absence de différences significatives dans nos groupes après 3 jours d'expérience et après 24H d'expérimentation avec le modèle de De chaumont nous a donc conduit à étudier les comportements des groupes homogènes et hétérogènes à différents points dans le temps sur les premières 24h. Nous avons choisi d'étudier les comportements après une heure, trois heures et 24 heures d'expériences. L'objectif est de représenter respectivement, le début de l'exploration de la cage et d'approche des individus sociaux inconnus, le moment où les interactions sociales se mettent en place de manière plus prononcée et le moment où les interactions sociales ont fini de se mettre en place.

Après une heure d'expérimentation, nous avons observé une diminution du redressement ainsi qu'une diminution significative du nombre de comportements d'approches sociales effectuées par les souris VPA des groupes homogènes. Ceci indique une diminution de l'exploration du nouvel environnement social et de la cage par les souris VPA. Ces différences peuvent être expliquées par le fait que les souris VPA ont un niveau d'anxiété plus élevés (Gao et al., 2019). Cette anxiété a pu avoir un impact négatif sur l'exploration de l'environnement

des souris. Un second élément soutenant cette hypothèse est le fait que le stress est une comorbidité fréquente dans les TSA qui aura un impact négatif sur les capacités sociales des patients (Bishop-Fitzpatrick et al., 2015; Fuld, 2018). Si on se réfère au papier de Rusu (2022) qui ont étudié les déficits sociaux avec le LMT dans un modèle de TSA basé sur la délétion de la région 16p11.2, une seconde explication est possible (Rusu et al., 2022). Dans leur étude Rusu et al ont montré que quand on leur présente le choix d'interagir soit avec une souris WT ou une souris mutée, les souris mutées, ainsi que les souris WT, préfèrent interagir avec les souris WT. Ainsi, il est possible que les déficits observés chez les souris VPA soient dûs à un manque d'intérêts pour l'interaction avec les autres souris VPA (Rusu et al., 2022). Un point de différence cette étude et la nôtre, est que dans l'étude de Rusu et al les souris mutées ne présentent pas de déficits sociaux à court terme (15min). Cela peut probablement être expliqué par deux facteurs : (i) les souris utilisées par Rusu et al provenaient des mêmes cages d'hébergement évitant ainsi l'apparition d'un stress sociale ; cette absence a pu engendrer une diminution des déficits sociaux des souris mutées. (ii) L'analyse à court terme s'est faite après 15min seulement et il est donc possible que les souris étant familières avec les autres se sont concentrées sur l'exploration de l'environnement plus que sur les approches sociales. Chez les groupes hétérogènes, aucune différence n'a été observée à ce point chez les souris VPA.

Après 3 heures d'expérimentation, on observe chez les groupes homogènes trois éléments notables : (i) La diminution de redressement observé précédemment chez les souris VPA n'était plus présente, montrant que les femelles explorent normalement la cage, (ii) les déficits d'approche sociale persistent chez les souris VPA et (iii) la diminution significative du nombre de contacts sociaux effectués par les souris VPA. Ces résultats suggèrent que la mise en place des interactions sociales est retardée chez les souris VPA. Il est également possible que le retard d'exploration de l'environnement à une heure a induit un retard de la mise en place des interactions sociales. Chez les groupes hétérogènes, la seule différence notable est une augmentation des comportements d'évitement chez la souris VPA, indiquant la présence de léger déficits sociaux.

Après 24 heures d'expérimentation, nous avons continué à observer les mêmes déficits d'approche et de contacts sociaux qu'à 1 heure et 3 heures chez les femelles VPA dans les groupes homogènes. L'absence d'altération des comportements sociaux à ce point invalide

notre hypothèse d'un retard de mise en place des interactions sociales. Après 24h d'expérimentation on s'attendrait à ce que les souris VPA aient au moins compensé leurs déficits d'approche sociale. Ainsi, il semble peu probable qu'un retard d'exploration au début de l'expérience soit la cause des déficits observés ce qui conforte les hypothèses d'un stress causé par le nouvel environnement social ou du manque d'intérêt à établir des interactions. On note aussi que les souris VPA ont parcouru une distance moins importante que les souris salines indiquant une possible hypoactivité. Les souris VPA ayant montré précédemment une hyperactivité (Choi et al., 2014; Sakade et al., 2019) cette hypoactivité peut être expliquée par la diminution du nombre d'approches et de contacts sociaux. Pour les groupes hétérogènes, aucune différence significative n'est observée entre les souris salines et VPA.

Notre étude des interactions sociales à différents points dans le temps durant les 24 premières heures d'expérimentation a montré que les souris VPA présentaient des déficits sociaux importants. L'absence de déficits après 3 jours d'expérimentation indique que ces déficits concernent la mise en place et le renforcement des interactions sociales. Ces déficits peuvent être expliqués soit par un stress plus important des souris face au nouvel environnement, soit par un manque d'intérêt à interagir avec d'autres souris VPA.

Ayant observé l'absence de différences comportementales entre les souris salines et VPA au niveau des groupes hétérogènes, ainsi que l'évolution des comportements sociaux au cours du temps, nous avons alors décidé d'étudier l'évolution de différents comportements sociaux et de comparer les souris des groupes homogènes avec les souris des groupes hétérogènes.

Les résultats obtenus pour les groupes homogènes confirment la présence de déficits dans la mise en place et le renforcement des interactions sociales. La majorité des différences étant observées durant les 24 premières heures.

Lors de la comparaison entre les groupes homogènes et groupes hétérogènes, nous avons pu noter plusieurs points importants :

(i) Les souris salines des groupes hétérogènes effectuent significativement moins de contacts oraux-génitaux que les souris salines des groupes homogènes.

(ii) Les souris VPA des groupes hétérogènes effectuent significativement plus de contact oraux-génitaux que les souris VPA des groupes homogènes.

(iii) Tout au long de la durée de l'expérimentation, les souris VPA des groupes hétérogènes ne présentent pas de différences significatives par rapport aux souris salines des groupes homogènes.

Ceci nous amène à constater que dans un groupe hétérogène, la présence d'une souris VPA a un impact négatif sur les capacités sociales des souris salines et que les souris salines ont un impact positif sur les capacités sociales des souris VPA.

Nous sommes actuellement en train d'approfondir nos analyses afin de déterminer la cause de cette différence. Nos théories étant que la diminution de la capacité sociale des souris salines hétérogènes serait due : (i) soit à un comportement d'évitement de la souris VPA causant les déficits observés chez les souris des groupes hétérogène, (ii) soit à la présence de la souris VPA qui cause une diminution globale du nombre d'interactions sociales dans la cage. Les résultats obtenus par Rusu (2022) semblent favoriser la première hypothèse (Rusu et al., 2022).

En conclusion, nos résultats nous ont permis de souligner l'importance de la mise en place de méthodes d'analyse plus détaillées des comportements sociaux en permettant une caractérisation plus fine des déficits observés dans le modèle VPA. Cela pourrait permettre une meilleure sélection des modèles TSA, notamment SH3. Nos travaux montrent l'importance de séparer les souris selon les catégories de traitement ou de génotype en cas d'études de modèle de TSA, étant donné l'impact significatif des mélanges de souris à phénotypes différents sur les comportements sociaux.

Conclusions

Pour commencer, nos travaux avec le modèle Shank3 ont renforcé notre hypothèse que les troubles de la marche pourraient constituer un complément objectif et quantitatif de diagnostic des TSA. En effet, bien que nous n'ayons pas observé de déficits sociaux chez les souris femelles ou chez les souris hétérozygotes dans les deux sexes, nous avons pu observer des troubles de la marche chez les mâles et les femelles. Nous avons aussi pu noter que les souris mâles présentaient des troubles de la marche plus importants que les souris femelles. Cette observation est peut-être liée avec le fait que seules les souris mâles homozygotes présentaient des déficits sociaux. Ensuite, au niveau cellulaire et moléculaire, une diminution des PC a été observée dans le cervelet des souris mâles et femelles, mais on notera là encore que les altérations sont plus prononcées chez les souris mâles que chez les souris femelles. Enfin, au niveau moléculaire nous n'avons retrouvé des altérations que chez les souris mâles. De fait, la gravité des troubles de la marche serait en lien avec celle des déficits sociaux et des conséquences moléculaires. Ceci renforce notre hypothèse que les troubles de la marche pourraient être un élément de diagnostic quantitatif, objectif et potentiellement précoce.

Au cours de cette étude nous avons pu noter qu'étant donné le grand nombre de modèles SH3 existants, il était difficile de tirer des conclusions concernant ces modèles quant aux altérations collectives et transversales au niveau comportemental, cellulaire et moléculaire. Cela est due en partie au fait que la plupart des modèles SH3 ne présentent que de faibles altérations comportementales sans lien évident avec la symptomatologie des TSA.

Pour cette raison, nous avons décidé de tester une nouvelle méthode d'analyse des comportements sociaux complexe afin de voir si celle-ci était capable d'offrir un profil social plus détaillé du comportement des souris. Nous avons réalisé nos travaux chez les souris VPA constituant le modèle animal le plus reconnu pour ses valeurs de construit et de face. En effet, le LMT nous a permis de mettre en évidence la présence de plusieurs altérations comportementales précédemment non observées chez les souris VPA. Au début de l'expérimentation, nous avons noté des déficits d'exploration et d'approches sociales suggérantque l'introduction à un nouvel environnement pouvait induire un stress important chez les sourisVPA. Les déficits d'approches persistant au cours de l'expérimentation et auxquels s'ajoutent des déficits de contacts sociaux suggèrent deux causes potentielles : (i) le stress causé par le nouvel environnement social qui impacterait à long terme les interactions

sociales des souris, (ii) le peu d'intérêt des souris VPA à interagir avec d'autres souris VPA. Cette seconde possibilité semble plus probable, car l'introduction d'une souris VPA dans un groupe de souris salines permet de compenser l'ensemble des déficits sociaux observés. En parallèle, on notera aussi que les souris salines exposées à une souris VPA effectuent significativement moins d'interactions sociales que les souris salines d'un groupe homogène. Des analyses complémentaires sont en cours afin de déterminer si cette différence est due à un comportement d'évitement de la souris VPA ou à une diminution globale du nombre d'interactions sociales.

En conclusion, nos travaux soutiennent la possibilité que les troubles de la marche constituent un moyen de diagnostic complémentaire efficace des TSA. Le LMT quant à lui est une méthode prometteuse pour l'étude des comportements sociaux complexes chez la souris pouvant aboutir à une meilleure caractérisation des différents modèles de TSA utilisés actuellement.

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Résumé

Les troubles du spectre autistique (TSA) sont un ensemble de troubles neurodéveloppementaux caractérisés par deux symptômes principaux : des déficits d'interaction sociale et des comportements stéréotypés et répétitifs. Les TSA ont un impact sociétal important avec une prévalence d'un enfant sur 100. Il n'existe aujourd'hui aucun traitement curatif des TSA, leur prise en charge passant principalement par des thérapies comportementales. Ces thérapies sont plus efficaces si implémentées tôt chez l'enfant. Or le diagnostic des TSA est difficile et long, reposant principalement sur une évaluation des capacités sociales conduisant à une grande variabilité de la prévalence selon les méthodes diagnostics utilisées. Ainsi, le premier objectif de ma thèse a été d'étudier la présence de troubles moteurs et de la marche dans un modèle génétique avec une mutation du gène shank3 (SH3) des TSA et d'explorer les mécanismes cellulaires et moléculaires sous-jacents. Ceci est d'intérêt afin de proposer d'implémenter en clinique ces mesures pour contribuer à un diagnostic objectif et précoce.

La relative simplicité des techniques utilisées actuellement pour caractériser les interactions sociales chez les modèles animaux de TSA ne permet pas une étude prolongée dans le temps de ces déficits ni de déterminer leur nature dans un groupe d'animaux. Ainsi, le second objectif de ma thèse a été la mise en place d'une nouvelle méthode d'étude des interactions sociales, le Live Mouse Tracker (LMT), pour déterminer le comportement social dans un groupe de 4 animaux sur une durée pouvant aller jusqu'à 3 jours. Ceci a été réalisé dans modèle des TSA éprouvé, celui de préexposition prénatale à l'acide valproïque (Dépakine) (VPA). Avec cette procédure, j'ai pu montrer la présence de déficits sociaux chez les femelles VPA qui n'étaient pas détectables auparavant avec la technique du test à 3 chambres (3-CT). J'ai aussi pu caractériser de manière détaillée ces troubles en montrant que les souris VPA présentent principalement des difficultés à établir et renforcer les contacts sociaux mais une fois que ceux-ci ont été mis en place, les souris VPA présentent des interactions sociales similaires aux souris contrôles. De plus, j'ai pu montrer que la présence de souris contrôles avaient un impact positif sur les capacités sociales des souris VPA, mais que la présence de souris VPA avait-elle un impact négatif sur les capacités sociales des souris contrôles.

Enfin, j'ai contribué à un travail visant à déterminer l'état de la transmission glutamatergique et dopaminergique dans ce modèle VPA de TSA. Se faisant, j'ai pu montrer que ces deux systèmes majeurs de transmission étaient faiblement impactés par la pathologie.

En conclusion, au cours de ma thèse j'ai pu dans un premier temps montrer la présence de troubles significatifs moteurs et de la marche dans un modèle génétique des TSA. Puis dans un second temps j'ai caractérisé le profil social détaillée d'un modèle environnemental des TSA,

ainsi que mis en évidence l’importance de l’environnement social sur les capacités socialesdes souris.

Summary

Autism spectrum Disorder (ASD) is a group of neurodevelopmental disorders defined by two main symptoms: deficits in social interaction and stereotyped and repetitive behaviour. ASD has an important impact on society due to its prevalence of around 1 for every 100 children. To date there are no curative treatment for ASD. Patient care for ASD revolves around behavioural therapy for the main symptoms. These approaches are most effective when implemented early in childhood. ASD is currently diagnosed based mainly on the evaluation of the social capacities of the child leading to a great variability of prevalence depending on the methods used. Thus, the first aim of my thesis was to study the presence of motor and gait disorders in a genetic model of ASD with a mutation of the shank3 (SH3) gene and to explore potential underlying mechanisms. The goal was to determine whether motor and gait deficits are of relevance to be implemented in clinical settings as early and quantitative diagnostic criteria.

The relative simplicity of the methods currently used to study social interactions in animal modelsof ASD does not allow long term investigations or study of complexe interactions in a group of animals. Thus, the second aim of my thesis was the implementation of a new method to study social interactions, the Live Mouse Tracker (LMT). The LMT allows the study of complex socialinteractions in a group of 4 mice for a duration up to 3 days. This study was performed using a robust model of ASD, the valproic acid (VPA) model based on the prenatal exposition to the drug.With this procedure, I was able to detect social deficits in females VPA mice which were not detectable previously with the 3-CT technique. I was also able to characterise in a detailed mannerthose deficits, showing that VPA mice mainly have difficulty to establish and reinforce social interactions but that once those were in place, social interactions became similar to control mice. Finally, by using groups of mice with mixed treatment within the LMT, I was able to prove that on one hand the presence of control mice had a positive impact on the social capacity of the VPAmice, but on another hand the presence of VPA mice had a negative impact on the social capacityof the control mice.

Last, I also contributed to a work aimed at determining the state of glutamatergic and dopaminergictransmission in this VPA model of ASD. In doing so, I was able to show that these two major transmission systems were weakly impacted by the pathology.

In conclusion, during my thesis, I was first able to show the presence of significative motor and gait deficits in a genetic model of ASD. Then I was able to create a detailed social profile of an environmental model of ASD, which revealed the importance of the social environment on the social capacity of the mice.