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Identifying the Critical Therapeutic Window for Molecular and Behavioural Correction of Angelman Syndrome

Sara Silva Santos

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Identifying the Critical Therapeutic Window for Molecular and Behavioural Correction of Angelman Syndrome

Tese de Candidatura ao grau de Doutor em Neurociências através do Programa Doutoral em Áreas da Biologia Básica e Aplicada (GABBA) submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Photograph of Pedro de Mello Costa Duarte, the inspiring "Angel"
who significantly influenced the progress of this research.

"*By understanding the critical developmental window for UBE3A expression, we have the potential to design rational, targeted therapies that may be more effective for treating Angelman syndrome and other neurodevelopmental disorders.*"

- **Dr. Benjamin Philpot**, University of North Carolina at Chapel Hill

(Quote from an interview published on the website of the UNC school of Medicine)

To my parents, who never stopped believing in me.
To my husband, Ricardo, for the never-ending love and support.

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LIST OF ABREVIATIONS AND ACRONYMS

Abbreviations/Acronyms	Meaning
2'-MOE	2'-O-methoxyethyl
AAV	Adeno-associated virus
AAV-9	AAV serotype 9
AIS	Axon initial segment
AS	Angelman syndrome
AS-IC	Angelman syndrome imprinting centre
AS-PWS	Angelman syndrome - Prader-Willi syndrome
ASD	Autism spectrum disorder
ASOs	Antisense oligonucleotides
ATFs	Artificial transcription factors
ATP	Adenosine triphosphate
Atp10a	ATPase Phospholipid Transporting 10A
ATS	Anti-sense transcript
AZUL	Amino-terminal Zn-finger of Ube3a Ligase
BMAL1	Brain and muscle Arnt-like protein-1
BP	Breakpoints
Cas9	CRISPR-associated protein 9
CB1	Cannabinoid receptor type 1
CG1	Cingulate cortex area 1
CG2	Cingulate cortex area 2
ChABC	Chondroitinase ABC enzyme
CNS	Central nervous system
CPs	Critical periods
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS-GAG	Chondroitin sulfate glycosaminoglycans
CSF	Cerebrospinal fluid
Cys	Cysteine
DMD	Duchenne's muscular dystrophy
DNA	Deoxyribonucleic acid
E	Embryonic days
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-conjugate enzymes
E3	Ubiquitin-protein ligases
E6-AP	Ubiquitin-protein ligase E6-associated protein
ECM	Extracellular matrix
EEG	Electroencephalogram

ENS	Enteric nervous system
ERT	Enzyme replacement treatment
Fmr1	Fragile X messenger ribonucleoprotein 1
Fos	Fos proto-oncogene, AP-1 transcription factor subunit
FST	Forced swim test
GABAA	Gamma-aminobutyric acid type A
GABRA5	Gamma-aminobutyric acid type A receptor subunit Alpha5
GABRB3	Gamma-aminobutyric acid type A receptor subunit Beta3
GABRG3	Gamma-aminobutyric acid type A receptor subunit Gamma3
GAG	Glycosaminoglycans
GR	Glucocorticoid receptor
hATTR	Hereditary transthyretin amyloidosis
HECT	Homologous to E6-associated protein carboxy-terminal domain
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2
hESC	Human embryonic stem cell
HPV	Human papillomaviruses
HSC-GT	Hematopoietic stem cell gene therapy
HSPCs	Hematopoietic stem and progenitor cells
hUBE3A	Human ubiquitin protein ligase E3A
hUBE3Aopt	Human ubiquitin protein ligase E3A codon-optimized
IBR	In-between-RING
IC	Inferior colliculus
IC	Imprinting centre
ICRs	Imprinting control regions
ICV	Intracerebroventricular
ID	Imprinting defects
IL	Infralimbic
IL2rg	Interleukin-2 receptor subunit gamma
IPW	Imprinted in Prader-Willi Syndrome gene
K	Lysine
kb	Kilobase
kDa	Kilodaltons
KO	Knockout
KRAB	Kruppel-associated box
LncRNA	Long noncoding RNA
LoxP	Locus of X-over P1
LTD	Long-term depression
LTP	Long-term potentiation
M1	Methionine at position 1

M1	Primary motor cortex
M2	Supplementary motor cortex
MAPK1	Mitogen-activated protein kinase 1
MB	Marble Burying
MNTB	Medial nucleus of the trapezoid body
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mUBE3A	Murine ubiquitin protein ligase E3A
NB	Nest Building
NBS	Neonatal bloodspot screening
NCBI	National Centre for biotechnology information
NDDs	Neurodevelopmental disorders
Ndn	Necdin
Nestin	Neuroepithelial stem cell protein
OF	Open Field
OMIM	Online Mendelian inheritance in man
P	Post-natal day
PCD	Post-conception day
PFC	Prefrontal cortex
PMd	Premotor cortex
PMd	Dorsal premotor cortex
PNN	Perineuronal net
pre-SM	Presupplementary motor area
PSMD4	Proteasome 26S Subunit Ubiquitin Receptor, Non-ATPase 4
PV	Parvalbumin
PWS	Prader-Willi syndrome
PWS-IC	Prader-Willi syndrome imprinting centre
qPCR	Quantitative polymerase chain reaction
RBR	RING-BetweenRING
RCR	RING-Cys-Relay
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNase	Ribonuclease
RPN10	Regulatory particle non-ATPase 10
RR	Rotarod
SCN	Suprachiasmatic nucleus
SMA	Spinal muscular atrophy

SMA	Supplementary motor area
SNHG14	SNRPN sense/UBE3A antisense transcript
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SNURF	SNRPN upstream open reading frame
SSRI	Selective serotonin reuptake inhibitors
TAT	Transactivator of transcription
Ub	Ubiquitin
UBE3A	Ubiquitin protein ligase E3A
Ube3a-ATS	Ubiquitin protein ligase E3A antisense
UBE3A-Iso	Ubiquitin protein ligase E3A isoform
UPD	Uniparental disomy
UPD15	Uniparental disomy of chromosome 15
UPP	Ubiquitin-proteasome pathway
WT	Wild-type
YFP	Yellow fluorescent protein

ABSTRACT

Angelman syndrome (AS) is a genetic disorder caused by the absence of expression of the maternal copy of the *UBE3A* gene, that affects the central nervous system and triggers a generalized disruption of essential neurodevelopmental processes in the patients. This syndrome is very well-characterized and cardinal features include severe intellectual disability, lack of speech, movement impairments, sleeping problems, epilepsy and a unique behavioural phenotype that comprises bouts of excessive laughter, stereotypies, and hyperactivity.

Like many other neurodevelopmental disorders (NDDs), AS greatly impacts the quality of life of the affected individual, places a substantial burden on caregivers and has a significant economic strain, consequence of the patient's increased demand for healthcare services and interventions (Wheeler, Sacco, & Cabo, 2017)(Lamsal & Zwicker, 2017).

Since there are no mechanism-based therapies for AS, current treatments are very limited and directed to symptomatic management such as using anti-seizure medication to control epilepsy, prescription of melatonin to improve sleep and carry out physical, behaviour and speech therapies, amongst others. Nevertheless, there have been recent discoveries that accelerated the development of multiple mechanism-based approaches with the potential of treating AS. These approaches, extensively discussed in **Chapter 2**, are a major breakthrough, but also a challenge since the optimal therapeutic window for AS is still a mystery. How can one successfully apply treatments directed at functional correction of AS if the critical developmental window, during which UBE3A reinstatement can potentially rescue the most adverse phenotypes, is still unknown? Are the phenotypes manifested by the patients linked to a strict critical window? When should the treatments start and until when must UBE3A be present – during brain development only or also during adulthood?

In addition to the aforementioned questions, we must also address another important issue. While murine models of AS have been crucial for research, the rate of successful translation from animal models to clinical trials has been relatively low. This is exemplified by the unsuccessful Minocycline (trial register *NCT01531582* and *NCT02056665*) (Joseph C Grieco et al., 2014) and Levodopa (trial register *NCT01281475*) (W. H. Tan et al., 2017) trials, highlighting the need for improved strategies to bridge the gap between preclinical studies and human clinical trials.

The overall aim of the studies presented in this thesis is to fill in the knowledge gap in the field and answer those important questions that are hampering the development of a possible therapy for Angelman syndrome.

The first study in this thesis (**Chapter 3**) is devoted to determining the ideal treatment window for *Ube3a* reinstatement in a conditional AS mouse model. The results obtained from this

study unequivocally demonstrate the central role of UBE3A in neurodevelopment, and the reactivation of this gene's expression is sufficient to improve the impairments observed in AS mice. However, the effectiveness of this therapeutic intervention critically depends on its timely application during the critical period for intervention.

Chapter 4 focuses on several key objectives that aim to advance our understanding of AS and lay the groundwork for potential therapeutic interventions.
Firstly, we aim to establish a robust murine behavioural protocol specifically designed for preclinical pharmacological studies. By creating a reliable and standardized platform, we can effectively evaluate the effects of various treatments on AS-related behaviours in mouse models. This will be crucial in identifying potential therapeutic options that can be translated to future clinical trials. In addition, we have utilized this established behavioural test battery to assess the efficacy of Minocycline and Levodopa in AS. The preclinical studies in mice showed promising results for these drugs, leading to their approval for clinical trials with AS patients. However, the outcomes of the clinical trials with AS patients did not show positive effects.
Following the development of our behavioural test battery, we conducted experiments with Minocycline and Levodopa, which provided results that predicted the effects seen in the clinical trials. This discrepancy in outcomes suggests that the tests used in other laboratories might have been inadequate due to underpowered experiments. The findings from these experiments highlight the importance of using a robust and well-validated behavioural test battery for preclinical studies to better predict clinical outcomes, thus making significant strides towards improving the lives of individuals affected by AS.

In the **Chapter 5** of this thesis, we present work that reinforces the crucial role of UBE3A expression during brain development and its significance in adulthood. Our results, combined with those from Chapter 3, indicate that a transient UBE3A reinstatement during a critical early developmental window is likely to prevent most adverse AS phenotypes. This is supported by the fact that *Ube3a* gene deletion at 3 or 12 weeks of age in conditional mouse models did not manifest all AS phenotypes previously identified. However, deletion of the *Ube3a* gene during early development resulted in conditional mice behaving similarly to AS mice.

These findings provide a critical piece of information necessary for the proper design of future clinical trials focused on *UBE3A* gene expression reactivation as a treatment for AS.

RESUMO

A Síndrome de Angelman (SA) é uma doença genética causada pela ausência de expressão da cópia materna do gene *UBE3A,* que afeta o sistema nervoso central e desencadeia uma perturbação generalizada de processos essenciais do neurodesenvolvimento nos pacientes. Esta síndrome está bem caracterizada, e as principais características incluem deficiência intelectual grave, ausência de fala, perturbações motoras, problemas de sono, epilepsia e um fenótipo comportamental único que abrange episódios de riso excessivo, estereotipias e hiperatividade.

Assim como muitos outros distúrbios do neurodesenvolvimento, a SA tem um impacto significativo na qualidade de vida do indivíduo afetado, coloca uma carga substancial sobre os cuidadores e acarreta um peso económico significativo, consequência da maior procura por serviços de saúde e intervenções por parte do paciente (Wheeler, Sacco, & Cabo, 2017) (Lamsal & Zwicker, 2017).

Dado que não existem terapias baseadas em mecanismos para a SA, os tratamentos atuais são bastante limitados e focados no manejo sintomático, incluindo a utilização de medicação anticonvulsivante para controlar a epilepsia, a prescrição de melatonina para melhorar a qualidade do sono e a realização de terapias físicas, comportamentais e de fala, entre outras. Contudo, descobertas recentes têm impulsionado o desenvolvimento de várias abordagens baseadas em mecanismos com potencial para tratar a SA. Estas abordagens, detalhadamente discutidas no **Capítulo 2**, representam um avanço significativo, mas também um desafio, uma vez que a janela terapêutica ideal para melhorar os sintomas da SA continua a ser um enigma.

Como será viável implementar com sucesso tratamentos direcionados para a correção funcional da SA se a janela crítica de desenvolvimento, na qual a restauração do gene UBE3A pode potencialmente aliviar os fenótipos mais adversos, permanece desconhecida? Será que os fenótipos apresentados pelos pacientes estão vinculados a uma janela crítica estrita?

Qual é o momento mais adequado para iniciar os tratamentos nos pacientes com AS e até quando o gene *UBE3A* deve estar ativo? - apenas durante o desenvolvimento cerebral ou também na idade adulta, após a maturação do sistema nervoso central?

Além das questões previamente mencionadas, é importante abordar outro tópico relevante. Apesar de os modelos murinos de SA terem sido cruciais para a investigação, a taxa de sucesso na tradução destes modelos para ensaios clínicos tem sido relativamente baixa. Tal é evidenciado pelo insucesso nos ensaios clínicos com Minociclina (registo do ensaio NCT01531582 e NCT02056665) (Grieco et al., 2014) e Levodopa (registo do ensaio NCT01281475) (Wen-Hann Tan et al., 2018), realçando a importância de desenvolver

estratégias mais eficazes de modo a melhorar e potenciar a capacidade de previsão entre os resultados dos estudos pré-clínicos e os ensaios clínicos em seres humanos.

O objetivo geral dos estudos apresentados nesta tese é preencher as lacunas de conhecimento nesta área científica e responder a estas questões cruciais que têm dificultado o avanço em direção ao desenvolvimento de uma possível terapia para a Síndrome de Angelman.
O primeiro estudo abordado nesta tese (**Capítulo 3**) é dedicado a determinar a janela de tratamento ideal para a restauração do *Ube3a* num modelo murino condicional de SA.
Os resultados deste estudo demonstram inequivocamente o papel central do UBE3A no neuro desenvolvimento e que a reativação da expressão deste gene é suficiente para melhorar as deficiências observadas em murganhos com SA. No entanto, a eficácia desta intervenção terapêutica depende criticamente da sua aplicação atempada durante o período crítico de intervenção.

O **Capítulo 4** foca-se em diversos objetivos fundamentais que visam aprofundar a nossa compreensão da SA e estabelecer as bases para possíveis intervenções terapêuticas. Em primeiro lugar, procuramos estabelecer um protocolo robusto para avaliação comportamental de murinos, especialmente concebido para estudos farmacológicos pré-clínicos.
Ao criar uma plataforma confiável e padronizada, conseguimos avaliar de forma eficaz os efeitos de diversas abordagens terapêuticas em comportamentos relacionados com a SA nestes modelos animais. Esta abordagem revela-se essencial para identificar potenciais opções terapêuticas que possam ser posteriormente aplicadas em futuros ensaios clínicos.
Adicionalmente, utilizamos esta bateria de testes comportamentais para avaliar a eficácia da Minociclina e Levodopa na SA. Estudos pré-clínicos em murganhos apresentaram anteriormente resultados promissores para estes medicamentos, o que conduziu à sua aprovação para ensaios clínicos com pacientes com SA. Contudo, os resultados dos ensaios clínicos com pacientes com SA não demonstraram efeitos positivos.
Após o desenvolvimento da nossa bateria de testes comportamentais, realizamos experiências com Minociclina e Levodopa, cujos resultados previram os efeitos observados nos ensaios clínicos. Esta discrepância nos resultados sugere que os testes utilizados noutros laboratórios poderão ter sido inadequados devido a experiências subdimensionadas. Os resultados destas experiências sublinham a importância da utilização de uma bateria de testes comportamentais robusta e bem validada para estudos pré-clínicos, de forma a prever de forma mais eficaz os resultados clínicos e a fazer progressos significativos na melhoria da vida das pessoas afetadas pela SA.

No **Capítulo 5** desta tese, apresentamos um trabalho que reforça o papel crucial da expressão do *UBE3A* durante o desenvolvimento cerebral e a sua importância na idade adulta. Os nossos resultados, juntamente com os do Capítulo 3, indicam que uma restauração transitória de UBE3A durante uma janela crítica de desenvolvimento precoce é provavelmente capaz de prevenir a maioria dos fenótipos adversos da SA. Isto é suportado pelo facto de a deleção do gene *Ube3a* às 3 ou 12 semanas de idade em modelos murinos condicionais não manifestarem todos os fenótipos de SA previamente identificados. No entanto, a deleção do gene *Ube3a* durante o desenvolvimento precoce resultou em ratos condicionais a comportarem-se de forma semelhante aos murganhos com SA.

Estes resultados fornecem informações críticas necessárias para o design adequado de futuros ensaios clínicos focados na reativação da expressão do gene *UBE3A* como tratamento para a SA.

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Host Institution: The research reported in this thesis was conducted at the laboratory Molecular and cellular mechanisms underlying neurodevelopmental disorders, Department of Neuroscience at the Erasmus MC in Rotterdam (The Netherlands).

PUBLICATIONS IN SUPPORT OF THE THESIS

The author of this thesis declares that it was actively involved in the design and execution of the experimental work, in the interpretation of the results as well as in the writing of the following published articles:

1. **Silva-Santos S**, Van Woerden GM, Bruinsma CF, Mientjes E, Jolfaei MA, Distel B, et al. *Ube3a* reinstatement identifies distinct developmental windows in a murine Angelman syndrome model. J Clin Invest. (2015) doi: 10.1172/JCI8055

2. Sonzogni, M., Wallaard, I., **Santos, S.S**. et al. A behavioral test battery for mouse models of Angelman syndrome: a powerful tool for testing drugs and novel *Ube3a* mutants. Molecular Autism 9, 47 (2018) doi:10.1186/s13229-018-0231-7

3. Sonzogni, M., Hakonen, J., Bernabé Kleijn, M, **Silva-Santos S**. et al. Delayed loss of UBE3A reduces the expression of Angelman syndrome-associated phenotypes. Molecular Autism 10, 23 (2019) doi:10.1186/s13229-019-0277-1

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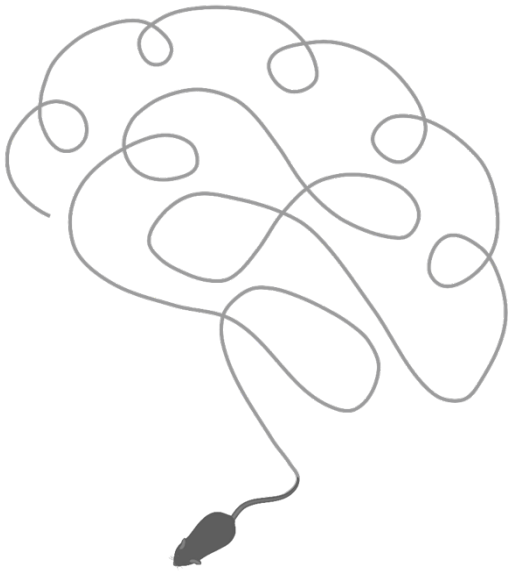
CHAPTER 1

SCOPE OF THE THESIS



SCOPE OF THE THESIS

1.1 **AIM**

The primary objective of the work contained in this thesis is to provide a comprehensive understanding of the optimal timeframe for therapeutic intervention in Angelman syndrome (*Online Mendelian Inheritance in Man* (OMIM) 105830). This is achieved by investigating the specific boundaries of the therapeutic window in Angelman syndrome (AS) mouse models.

Significant advancements in our knowledge of AS have emerged in recent years, leading to the development of various mechanism-based therapies for the condition. However, before these potential treatments can be applied to AS patients, it is crucial to establish the critical period during development when neurological symptoms of AS can be reversed. To address this research question, we utilized *Ube3a*-inducible AS mouse models and made valuable contributions to identifying the critical timeframe for therapeutic intervention in AS.
Nevertheless, the discovery of distinct critical periods during neurodevelopment, in which *Ube3a* expression is vital to prevent the onset of AS, has raised another question: Is sustained *Ube3a* expression necessary for brain function solely during these critical periods or throughout an individual's lifespan? Investigating the importance of maintaining *Ube3a* expression beyond normal brain development has become an additional focus of this thesis.

A recurrent challenge in the field is the low success rate in translating preclinical studies into clinical trials for AS. Therefore, we have also made it one of our objectives to propose a solution that enhances the translation of preclinical findings into human clinical trials.
This is achieved by developing a robust and standardised mouse behavioural paradigm. This paradigm should be replicable over time and across different laboratories, enabling researchers to assess the potential efficacy and off-target effects of new therapeutic compounds or therapies for AS. Additionally, it can serve as a means to phenotype novel *Ube3a* mutants. In this thesis, we provide a clear framework for evaluating specific behaviours that researchers can employ as a benchmark to assess the effectiveness of potential AS treatments, thereby addressing this recurring challenge.

1.2 **OUTLINE**

This thesis begins with an introduction outlining the primary objectives and significance of the research conducted (**Chapter 1**). It also provides an overview of the rationale and current state-of-the-art in Angelman syndrome (AS) research (**Chapter 2**).

Chapter 3 presents the findings of a systematic study aimed at investigating the potential for neurocognitive rescue through *Ube3a* reinstatement during different neurodevelopmental windows. By examining these windows in a temporal manner, we not only clarify which AS-related phenotypes can be rescued by reactivating *Ube3a* expression but also shed light on the critical treatment periods during which *Ube3a* reactivation can ameliorate AS-like phenotypes. This information is of paramount importance when considering therapeutic strategies involving UBE3A expression reinstatement in individuals with Angelman syndrome.

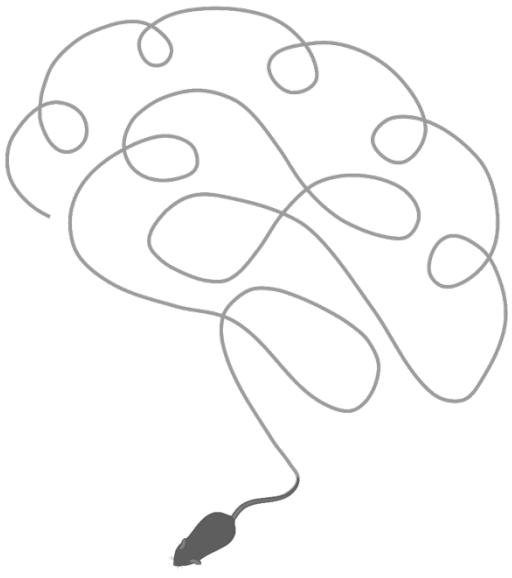
In **Chapter 4**, we assess the robustness of various behavioural paradigms using a standardized behavioural test battery. We present a reliable mouse behavioural paradigm that enhances the translatability of preclinical studies for AS. Additionally, this chapter evaluates the effectiveness of Minocycline and Levodopa, two drugs recently tested in clinical trials for AS.

Chapter 5 demonstrates the significant role of UBE3A in early brain development and its limited role in adulthood. Using a conditional mouse model, we selectively deleted the *Ube3a* gene at three different ages corresponding to distinct stages of brain maturation. When combined with the findings from Chapters 3 and 4, Chapter 5 offers crucial insights for upcoming clinical trials involving transient reactivation of *UBE3A* gene expression.

Finally, the thesis concludes with **Chapter 6**, which critically analyses and discusses the contribution of this research to the field, addressing the primary aims outlined earlier. Additionally, in **Chapter 7**, we explore and highlight potential future perspectives that may arise from the findings presented in this thesis.

CHAPTER 2

GENERAL INTRODUCTION



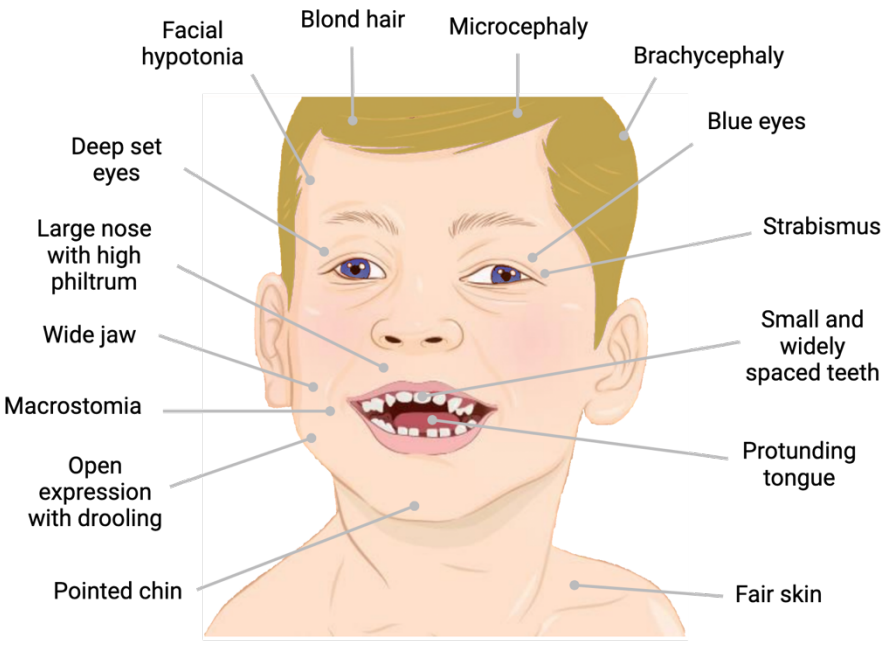
GENERAL INTRODUCTION

2.1 ANGELMAN SYNDROME (AS)

Angelman syndrome (AS) is a complex and severe genetic disorder primarily affecting the central nervous system (CNS). It was initially described by Dr. Henry Angelman in 1965 and has an estimated incidence of 1 in 20,000 individuals in the general population, showing no geographic clustering and affecting both males and females equally across different races (Angelman, 1965a)(Mertz et al., 2013; C. A. Williams et al., 1995)(Petersen, Brøndum-Nielsen, Hansen, & Wulff, 1995). AS is characterized by profound cognitive delay, speech impairment, ataxia, susceptibility to seizures, and hyper-excitable behaviours such as excessive laughter and hand flapping (Angelman, 1965b)(C. A. Williams et al., 2006). Usually, there is no report of an atypical prenatal history, and since the distinct clinical features of AS are not fully evident at birth, the syndrome is typically not diagnosed until the age of one year. Concerns are typically raised by caregivers and/or healthcare professionals when the child fails to reach developmental milestones, such as babbling and crawling, or when an epileptic condition arises (C. A. Williams et al., 1995)(Bindels-de Heus et al., 2020). Psychometric testing suggests that individuals with AS experience a peak period of developmental progress between two to three years of age (Peters et al., 2004).

While the behavioural features are the most consistent clinical manifestations in AS, many patients also exhibit subtle facial dysmorphisms (**Figure 1A-B**) and a distinctive ataxic gait while standing/walking (Clayton-Smith & Laan, 2003)(Van Buggenhout & Fryns, 2009)(Figure 1C).

A)



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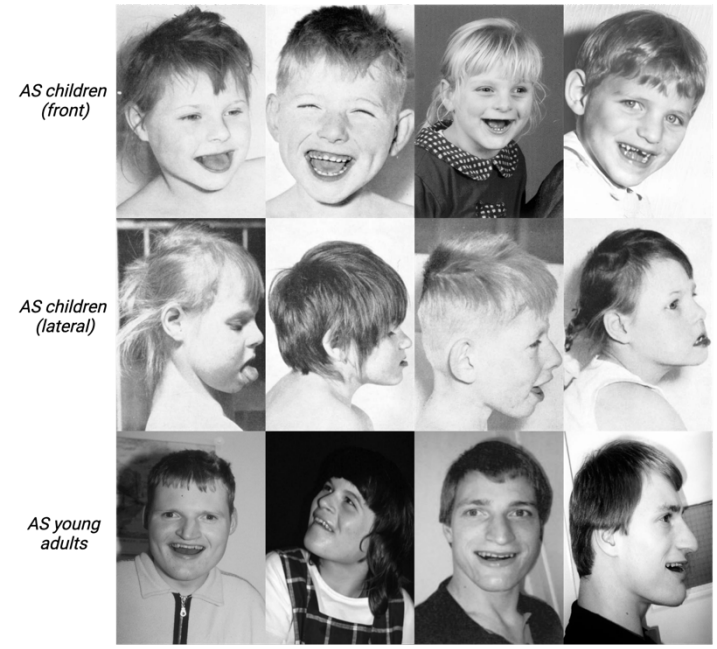
B)



C)

(Continued from the previous page)

Figure 1- Physical characteristics in AS. (**A**) Illustration summarizing the craniofacial dysmorphism frequently observed in diagnosed AS patients. The drawing is adapted from *sehatq.com.* (**B**) A composite photograph showcases the facial appearance of AS patients from infancy to early adulthood. It is evident that these individuals exhibit distinct features, such as a happy expression, macrostomia, a protruding tongue with widely spaced teeth, and brachycephaly. (C) In 10% of AS cases, patients are unable to walk, while those who possess the ability to do so present an unstable, jerky gait accompanied by uplifted arms. The photographs are adapted from *(Clayton-Smith & Pembrey, 1992).*

From a clinical perspective, while some features observed in individuals with Angelman syndrome, such as abnormal sleep patterns, epilepsy, and ataxia, have physiological origins, the majority of AS manifestations are behavioural in nature. The presence of traits like heightened sensitivity to heat, attraction to water, and episodes of uncontrollable laughter suggests impairments in sensory and emotional processing. Overall, both primary and secondary clinical findings, encompassing characteristics ranging from microbrachycephaly to ataxia, severely limited or absent speech abilities, and abnormal electroencephalogram (EEG) patterns, collectively indicate disorganization and dysfunction within the central nervous system (CNS) (Walz & Baranek, 2006). For a comprehensive overview of deficits and characteristic behavioural phenotypes displayed by individuals affected by this congenital disorder, please refer to **Table 1**.

Frequency	Characteristics	Description
Consistent (100%)	Developmental delay	Severe; peak developmental potential around 2-3 years old *(Peters et al., 2004)*.
	Speech impairment	No or minimal use of words; receptive and non-verbal communication higher than verbal *(Pearson, Wilde, Heald, Royston, & Oliver, 2019)*.
	Ataxia	Movement or balance disorder with ataxic gait and tremulous of limbs *(Clayton-Smith & Laan, 2003)*.
	Behavioural uniqueness	Frequent laughter; apparent happy demeanour with excessive smiling; easily excitable personality; short attention span; hand flapping; hypermotoric behaviour *(Angelman, 1965b)*.
Frequent (>80%)	Microcephaly	Delayed growth in head circumference by the age of 2 years *(Clayton-Smith & Laan, 2003) (Singhmar & Kumar, 2011)*.
	Abnormal EEG	With large-amplitude, slow spike waves and triphasic waves *(Boyd, Harden, & Patton, 1988)(Dan & Boyd, 2003)*.
	Seizures	Onset before the age of 3 years old and frequently refractory to medication *(Thibert, Larson, Hsieh, Raby, & Thiele, 2013)(Thibert et al., 2009)*.
Associated (20-80%)	Flat occiput	-
	Feeding problems during early infancy	May result from poor sucking abilities and swallowing difficulties *(Zori et al., 1992)*.
	Prognathism	Upper or lower jaw misalignment resulting in “underbite” or “overbite”(de Queiroz et al., 2013).
	Diastemas	Wide mouth with wide-spaced teeth *(de Queiroz et al., 2013)*.
	Protruding tongue	Tongue thrusting results in frequent drooling *(Angelman, 1965b)*.
	Strabismus	Most frequently exotropia *(Michieletto et al., 2020)*.
	Characteristic gait	Uplifted, flexed arm position, accompanied with jerky movements *(C. a. Williams, 2010)*.
	Increased heat sensitivity	May be related hypothalamic dysfunction for thermoregulation *(Yiş et al., 2008)*.
	Attraction to water and reflective surfaces	Possibly related to sensory processing abnormalities *(Walz & Baranek, 2006)*.
	Sleep disturbance	Decreased sleep, prolonged sleep latency and abnormal sleep-wake cycles *(Pelc, Cheron, Boyd, & Dan, 2008)*.
	Hypopigmentation	Lightness of skin, hair and eyes when compared with family; Only observed in deletion cases. *(J. Yoon, Song, & Choi, 2010)*.

Table 1– Summary of Developmental and Physical Findings in Angelman Syndrome. This table summarizes the developmental and physical findings of Angelman Syndrome according to their frequencies, based on consensus criteria for diagnosis by Williams *(C. A. Williams et al., 2006)*.

In terms of the life expectancy of individuals with Angelman syndrome (AS), there is a lack of population-based studies available. However, despite the presence of severe cognitive impairments and epilepsy, which are known risk factors for decreased life expectancy, it appears that the lifespan of AS patients is not significantly shortened (Eyman, Grossman, Chaney, & Call, 1990)(Gaitatzis, Carroll, Majeed, & Sander, 2004)(Clayton-Smith & Laan, 2003)(C. A. Williams, Driscoll, & Dagli, 2010). It has been known since 1997 that AS is attributed to a disruption in the expression of the *UBE3A* gene, which encodes the ubiquitin protein ligase E3A (Kishino, Lalande, & Wagstaff, 1997)(Matsuura et al., 1997). However, the underlying pathophysiology of AS remains unclear, and currently, there is no specific therapy available for this condition. For the time being, only management of symptoms (e.g. anti-epileptic drugs for seizure control and melatonin supplementation to maximize sleep) and physiotherapies can be offered to patients (W. Tan & Bird, 2016).

2.2 *UBE3A* (UBIQUITIN-PROTEIN LIGASE E3A) GENE AND ITS EXPRESSION PATTERN IN NEURONS

The *UBE3A* gene is situated within the chromosomal region 15q11-q13, spanning approximately 6 Mb in length. This region is subject to genomic imprinting, a relatively uncommon but normal epigenetic mechanism that governs gene expression in a parent-of-origin-specific manner among eutherian mammals (Sato, 2017)(Tucci et al., 2019) (Thamban, Agarwaal, & Khosla, 2020). Genomic imprinting, which affects only a small subset of human genes (approximately 160 genes, constituting less than 1% of the total), is considered an important regulatory process since almost all recognized imprinting abnormalities are associated with pathological states (Charalambous et al., 2012)(Soellner et al., 2017). Of particular interest is the role of genomic imprinting in the nervous system, as approximately half of the known imprinted genes exhibit parent-of-origin-specific expression in the brain (Davies, Isles, & Wilkinson, 2005). *UBE3A* is one such imprinted gene in the brain. Spanning 100 kb, this gene is typically biallelically expressed in most tissues, but it demonstrates exclusive maternal expression in neurons (**Figure 2**) (Albrecht et al., 1997)(Rougeulle, Cardoso, Fontés, Colleaux, & Lalande, 1998).

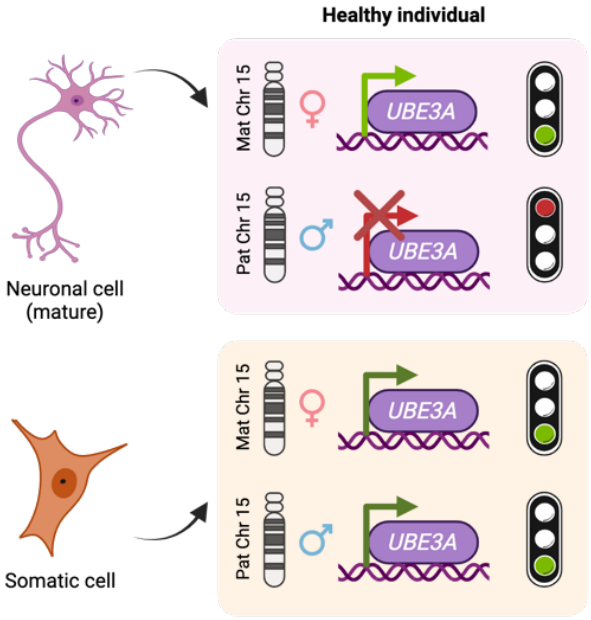


Figure 2 - Imprinting of *UBE3A* in neuronal cells. The *UBE3A* gene is subjected to genomic imprinting in neuronal cells, resulting in the solo expression of the maternal allele. In non-neuronal cells this gene is biallelically expressed.

Imprinting control regions (ICRs) play a pivotal role in governing the parental allele-specific expression of imprinted genes. In the chromosomal region 15q11–13, this regulation is mediated by a bipartite imprinting centre comprised of the Prader-Willi syndrome imprinting centre (PWS-IC) and the Angelman syndrome imprinting centre (AS-IC) (Matsubara et al., 2019).

The PWS-IC, spanning 4.1 kb, encompasses the major promoter and exon 1 of the SNURF-SNRPN gene. On the other hand, the AS-IC is believed to exert its influence by transcription-mediated DNA methylation, suppressing the PWS-IC specifically in the female germline. This silencing event renders the PWS-IC inactive, leading to the repression of paternally expressed genes on the future maternal allele (E. Y. Smith, Futtner, Chamberlain, Johnstone, & Resnick, 2011)(Rabinovitz, Kaufman, Ludwig, Razin, & Shemer, 2012)(Chamberlain, 2013). **Figure 3** provides a visual representation of the map of this chromosomal region.

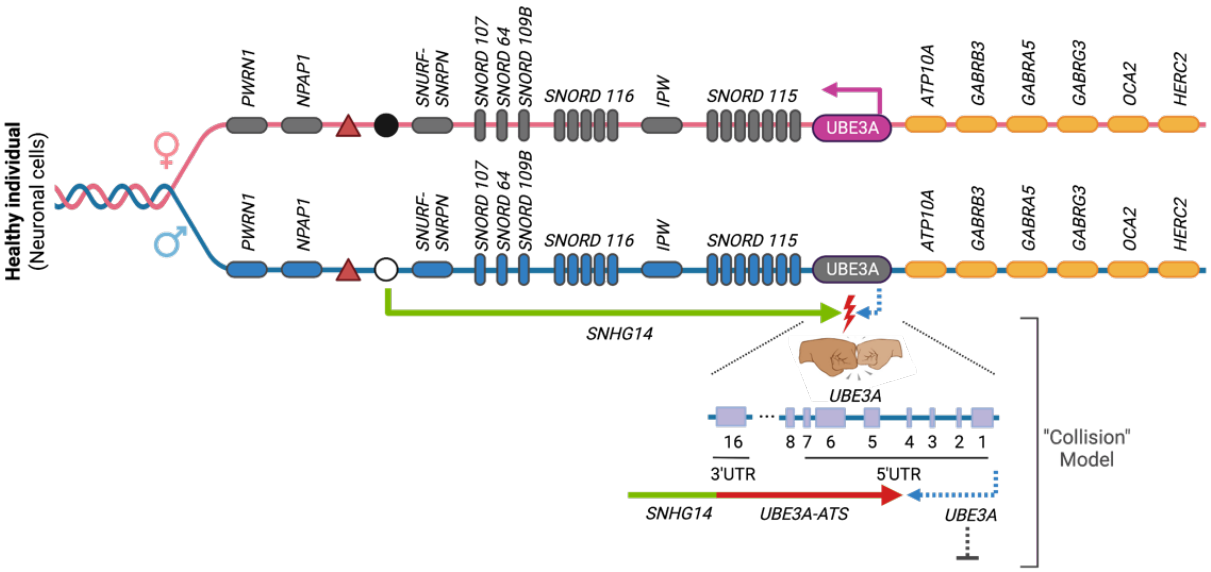


Figure 3 - Organization of the 15q11-q13 region in neurons. This diagram portrays a schematic representation of the human chromosome region 15q11-q13 in neurons, in relation to the maternal (♀) and paternal (♂) alleles. The Prader-Willi syndrome imprinting centre (PWS-IC) is depicted as a black circle, where methylation of the maternal allele leads to the repression of surrounding genes (*depicted as grey boxes*). Conversely, the absence of methylation (*white circle*) in the paternal allele allows for the expression of paternally expressed genes (*represented by blue boxes*) and the transcription of *SNHG14/UBE3A-ATS* (*indicated by green and red arrows*). The "Collision" model suggests that the *UBE3A-ATS* overlaps with the paternal *UBE3A* transcript (*represented by the blue dashed arrow*), resulting in its repression (*shown as a grey truncated dashed line*). Biallelic expressed genes are denoted by yellow boxes, and the Angelman syndrome imprinting centre (AS-IC) is represented by red triangles. Please refer to the "Abbreviation List" for the complete names of the genes depicted.

In a healthy individual, the 300 bp segment of the PWS-IC, which is inherited from the mother, undergoes methylation during oogenesis. Conversely, the same region on the paternal copy remains unmethylated during spermatogenesis. This differential methylation pattern allows for the transcription of genes regulated by the SNRPN promoter (Lossie et al., 2001) (Zeschnigk et al., 1997). These distinct methylation patterns observed in the gametes are faithfully maintained in preimplantation embryos and throughout embryonic development (Geuns, 2003).

The absence of methylation on the paternal PWS-IC region enables the transcription of the *SNHG14/UBE3A-ATS*, which extends beyond the *SNRPN* gene. Instead of terminating at *IPW*, as seen in immature neurons and non-neuronal cells, a change in chromatin structure occurs, allowing *SNHG14* to continue transcription until exons 4-5 of *UBE3A*. At this point, it collides with the transcript originating from the *UBE3A* promoter, resulting in the repression of the paternal *UBE3A* gene. This occurs because the incomplete *UBE3A* transcript will be

degraded. This mechanism, known as the "Collision model" provides insights into the regulatory mechanisms of the *UBE3A* gene (Yamasaki et al., 2003) (Numata, Kohama, Abe, & Kiyosawa, 2011)(Meng, Person, & Beaudet, 2012).

Several studies in mice have shown that the imprinting of *Ube3a* is established during neuronal maturation, resulting in silencing of the paternal *Ube3a* gene in mature neurons. This process occurs gradually, with a decrease in paternal *Ube3a* expression after E14 and complete cessation by P7, indicating the full establishment of imprinting on the paternal allele. (M. C. Judson, Sosa-Pagan, Del Cid, Han, & Philpot, 2014)(Sonzogni, Zhai, Mientjes, Van Woerden, & Elgersma, 2020). Additionally, as neurons mature, there is a shift in the subcellular localization of UBE3A, with an increasing nuclear presence of the protein. (M. C. Judson et al., 2014)(Munshi, Trezza, Sonzogni, Ballarino, & Smeeks, n.d.)(Avagliano Trezza et al., 2019). It is important to note that despite the role of genomic imprinting in transcriptional dosage control, the imprinting of *Ube3a* does not aim to reduce the overall amount of the gene. Instead, compensatory mechanisms come into play, leading to an increase in maternal *Ube3a* expression proportional to the imprinting of the paternal allele, thereby maintaining consistent levels of total UBE3A protein throughout development. (Hillman et al., 2017)(Sonzogni et al., 2020).

Although disruptions in maternal *Ube3a* expression result in the complete absence of UBE3A in mature neurons, leading to AS, recent experiments conducted on *Ube3a*$^{m+/p-}$ mice have provided promising results. These studies demonstrate that maintaining 50% *Ube3a* expression during embryonic development is sufficient to support normal prenatal brain development and behaviour (Sonzogni et al., 2020).These findings offer hope that future therapeutic interventions aimed at restoring *UBE3A* expression in patients, even if not fully reinstated, could still yield positive outcomes.

2.3 **GENETIC CAUSE OF ANGELMAN SYNDROME**

There are several genetic defects that can create an imbalance in the appropriate maternal expression of *UBE3A* in neuronal cells, thus causing AS. These genetic anomalies encompass microdeletion, mutations of the *UBE3A* gene, uniparental disomy (UPD), and imprinting defects (ID) (**Figure 4**)(Buiting, Williams, & Horsthemke, 2016)(Bindels-de Heus et al., 2020).

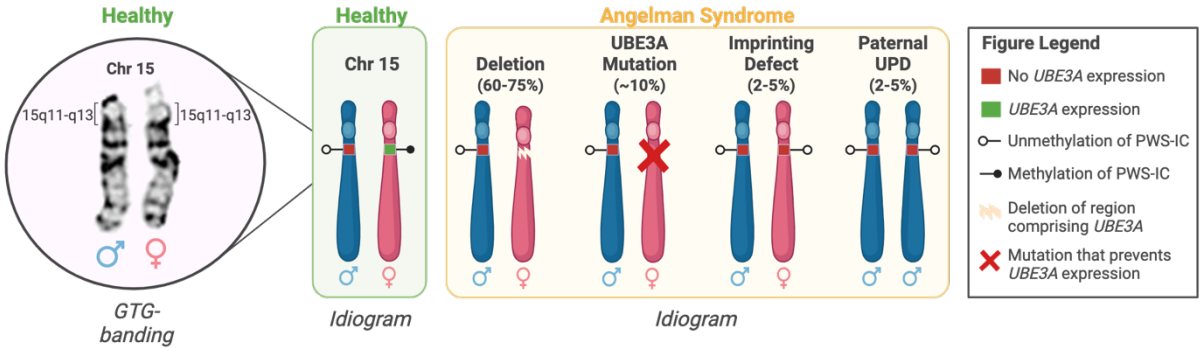


Figure 4 - Ideogram depicting the genetic abnormalities that affect chromosome 15 and result in Angelman syndrome. AS is attributed to the loss of maternal expression of the 15q11-q13 region, which includes the *UBE3A* gene. The absence of maternal expression can arise from various genetic factors, such as deletion of the region, inactivation due to a mutation or imprinting defect, or the presence of two paternal copies of chromosome 15, known as paternal uniparental disomy (UPD). In the figure, the paternal chromosome is depicted in blue, while the maternal chromosome is represented in pink. The green band signifies normal *UBE3A* expression, whereas the red band indicates the absence of *UBE3A* expression. An open circle denotes the lack of methylation of PWS-IC, while a full circle signifies the methylation of PWS-IC. The percentages shown in the figure align with the findings presented in Bindels-de Heus et. al. study *(Bindels-de Heus et al., 2020)*.

As depicted in Figure 4, the majority of AS patients (approximately 60%) exhibit large de novo interstitial deletions (~6 Mb) on the maternal chromosome 15q11-q13 region, resulting in a more severe phenotype compared to patients in other genetic classes.(Varela, Kok, Otto, & Koiffmann, 2004)(C. A. Williams et al., 2010). This increased severity can be attributed to the loss of not only the *UBE3A* gene but also additional non-imprinted genes within the 15q11.2-13 locus, including *Herc2* and one of the three genes encoding gamma-aminobutyric acid type A (*GABAA*) receptor subunits (*GABRB3, GABRA5 and GABRG3*). These genetic alterations have been documented in several studies (Keute et al., 2020) (T. M. DeLorey et al., 1998)(Harlalka et al., 2013)(Puffenberger et al., 2012) and are depicted in **Figure 5**.

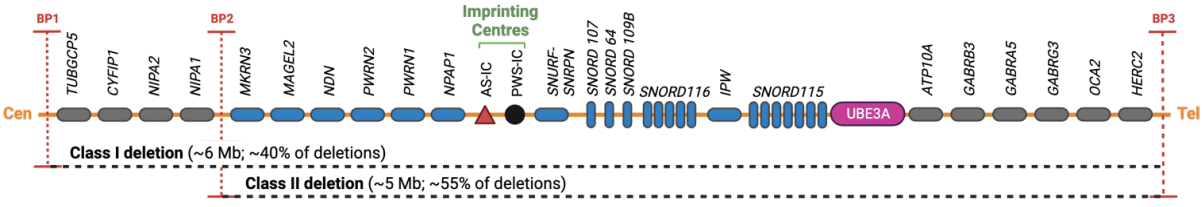


Figure 5 - Ideogram of chromosome 15q11-q13 highlighting the common deletion breakpoints observed in AS. Maternally expressed gene is depicted in pink, while paternally expressed genes are

shown in blue. The grey boxes represent non-imprinted genes. Imprinting centres are represented by a circle (PWS-IC) and a triangle (AS-IC). Deletions in this chromosomal region occur between specific breakpoints, indicated by dashed red lines (BP). Approximately 40% of deletion AS patients exhibit a Class I type of deletion (BP1-BP3), which includes an additional set of four genes near the centromere (Cen) compared to Class II (BP2-BP3) deletions. Class II deletions are the most common genetic finding among deletion patients, accounting for approximately 55% of cases.

Deletions in this genomic region occur between specific break points (BPs), leading to differential gene expression and varying impacts on the phenotypes of AS patients. Based on these breakpoints, the deletion cases can be classified into two groups: Class I, characterized by breakpoints at BP1 (proximal) and BP3 (distal), and Class II, characterized by breakpoints at BP2 (proximal) and BP3 (distal). This classification has been reported in several studies (Varela et al., 2004)(Valente et al., 2006)(Valente et al., 2013)(Dagli, Buiting, & Williams, 2012).

Approximately 20% of individuals with Angelman syndrome (AS) exhibit non-truncating missense mutations in the *UBE3A* gene, resulting in amino acid sequence alterations of the UBE3A protein (Buiting et al., 2016)(Geerts-Haages et al., 2020)(Cooper, Hudson, Amos, Wagstaff, & Howley, 2004). Some of these mutations lead to a loss of UBE3A's catalytic activity but, by studying the functional consequence of 28 previously reported and 3 new AS linked *UBE3A* mutations, Bossuyt and colleagues established that the pathogenicity of *UBE3A* missense mutations primarily stems from changes in the subcellular localization of UBE3A within neurons (Cooper et al., 2004)(Bossuyt et al., 2021). A detailed explanation of the role of the UBE3A protein in cells can be found in subchapter 2.5, while subchapter 2.6 elucidates UBE3A's subcellular localization.

Imprinting defects account for 4% of AS cases and are associated with abnormalities in the imprinting control centre (Bindels-de Heus et al., 2020)(Buiting et al., 2016)(Butler, 2020). These defects, which occur sporadically, result in the failure to establish or maintain the imprinting of genes during oogenesis. In this class of patients, the absence of DNA methylation at the maternal *SNRPN* promoter leads to the expression of paternal repressed genes, including the large antisense transcript *SNRPN* sense/UBE3A antisense transcript (*SNHG14*). Consequently, the maternal *UBE3A* gene is silenced, contributing to the core behavioural and physiological features observed in AS (Beygo et al., 2020).

Some individuals with imprinting defects that result in AS, have maternally inherited microdeletions affecting the AS-IC (Buiting et al., 1995)(Saitoh et al., 1996), the critical element for allele identity.
Additionally, reports indicate that AS patients without IC (Imprinting Centre) deletions may possess a 1-1.5 Mb inversion that separates the AS-IC and PWS-IC, suggesting that the proximity and proper orientation of these two IC elements are crucial for establishing a maternal imprinting (Buiting et al., 2001).

Paternal uniparental disomy of chromosome 15 (UPD15) can be classified into two types: heterodisomy, typically arising from a meiosis I error and potentially involving Robertsonian translocation, and isodisomy, resulting from a meiosis II error or post-zygotic chromosomal duplications. Both types lead to the inheritance of two identical copies of a gene or chromosome from one parent (Figure 4).
In AS, isodisomy is the more prevalent scenario, characterized by the presence of identical copies inherited from the father's chromosome 15 (Fridman & Koiffmann, 2000).

The AS phenotype in UPD15 patients is generally considered milder than in deletion cases. UPD15 patients tend to show improved physical growth, a reduced frequency of seizures, lower incidence of microcephaly, decreased ataxia, and higher cognitive abilities (A. Smith, Marks, Haan, Dixon, & Trent, 1997)(Gillessen-Kaesbach, Albrecht, Passarge, & Horsthemke, 1995)(Lossie et al., 2001). However, it is important to note that there are also documented cases of UPD15 individuals who present a phenotype as severe as that observed in deletion cases, with no clear explanation for this variability (Prasad & Wagstaff, 1997)(Poyatos et al., 2002).

2.4 **15Q11-Q13 INTEGRITY AND ASSOCIATED NEURODEVELOPMENTAL DISORDERS**

While this thesis primarily focuses on Angelman syndrome (AS) and the *UBE3A* gene, it is noteworthy to mention that disruption of any exclusively paternally expressed genes (genes depicted in blue in **Figures 3** and **5**) leads to Prader-Willi syndrome (PWS) as well (Angulo, Butler, & Cataletto, 2015). In contrast to AS, PWS is characterized by excessive eating leading to obesity, hypotonia, and hypogonadism. Individuals with PWS typically exhibit delayed motor and language development, along with some degree of cognitive impairment, although not as severe as seen in AS (Roof et al., 2000)(Cassidy, Dykens, & Williams, 2000). Due to their shared genetic basis, despite being clinically distinct disorders, AS and PWS are sometimes referred to as "Sister Disorders."

Another neurodevelopmental disorder associated with the 15q11-q13 region is 15q duplication syndrome (Dup15q which accounts for approximately 3% of autism cases (E. H. Cook et al., 1997)(Scoles, Urraca, Chadwick, Reiter, & LaSalle, 2011). This syndrome arises from duplications or even triplications of the region or the presence of an extra isodicentric chromosome 15, leading to severe autism, epilepsy, hypotonia, cognitive disabilities, and characteristic facial features (Battaglia, 2005)(Urraca et al., 2013). While Dup15q has complete penetrance in individuals with maternal duplications, paternal Dup15q has low penetrance, and individuals are typically unaffected or present milder symptoms compared to those with maternal Dup15q (E. H. Cook et al., 1997)(Elamin et al., 2022). This observation has led to the hypothesis that the causative gene for this syndrome is *UBE3A* since it is the only imprinted gene expressed exclusively from the maternal allele in mature neurons. Thus, a maternal duplication of this region would increase the gene dosage of *UBE3A* (Chamberlain & Lalande, 2010)(Browne et al., 1997)(Urraca et al., 2013). However, the mouse model of 15q duplication syndrome demonstrates an autism-like phenotype when the duplicated allele is paternally transmitted (Nakatani et al., 2009). A recent study has also shown that duplications of the paternally expressed gene *Ndn* in mice result in the same autism spectrum disorder (ASD)-related phenotypes observed in mice with paternal 15q duplications. This suggests that further investigations are necessary to clarify the contributions and importance of both *UBE3A* and paternally expressed genes to the pathogenicity of ASD (Tamada et al., 2021). With the current knowledge, it remains unclear whether maternal and paternal Dup15q represent equivalent syndromes or not. Nevertheless, all available data suggests that maintaining tightly regulated expression levels of genes within the 15q11-q13 region is crucial for normal brain function, and *UBE3A* plays a key role in neurodevelopment (Battaglia, 2008). This is particularly relevant when considering treatments aimed at restoring *UBE3A* expression in the brain to address AS, as any imbalance in UBE3A protein levels could potentially lead to a distinct neurodevelopmental syndrome.

2.5 **ROLE OF UBE3A PROTEIN IN CELLS: THE VERSATILE FACES OF UBE3A**

The ubiquitin-proteasome pathway (UPP) is a crucial post-translational modification pathway in eukaryotes that plays a pivotal role in regulating cellular processes. In the later stage of the UPP (Ligation), one or more ubiquitin molecules are attached to a specific protein, thereby dictating its fate within the cell. This process influences protein degradation, stability, interactions, and signalling pathways, contributing to the intricate control of cellular functions (Shang & Taylor, 2011).

Figure 6 illustrates the key components of the UPP, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). These components work in concert to facilitate the post-translational modification of target proteins with ubiquitin, as depicted in the figure (Humphreys, Smith, Chen, Fouad, & D'Angiolella, 2021).

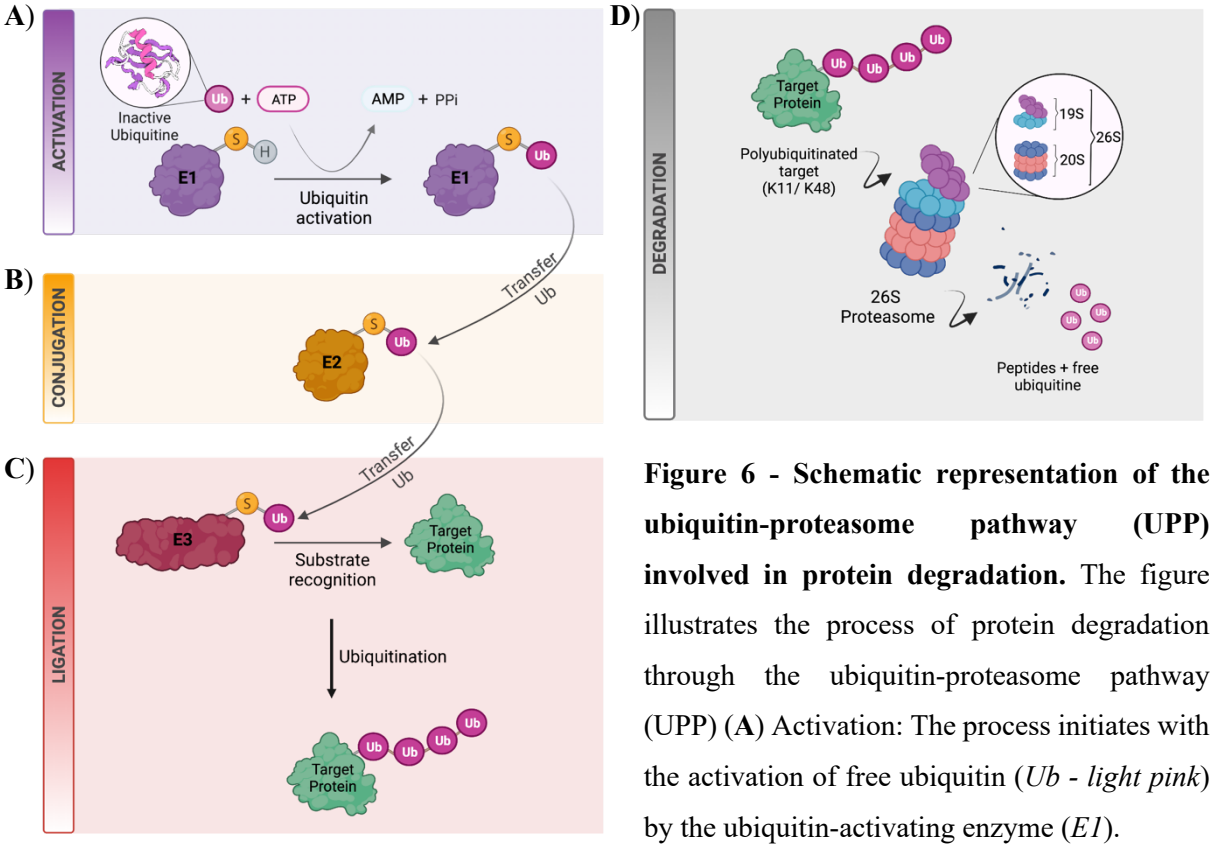


Figure 6 - Schematic representation of the ubiquitin-proteasome pathway (UPP) involved in protein degradation. The figure illustrates the process of protein degradation through the ubiquitin-proteasome pathway (UPP) (**A**) Activation: The process initiates with the activation of free ubiquitin (*Ub - light pink*) by the ubiquitin-activating enzyme (*E1*). ATP-dependent reaction forms a thioester bond between the C-terminus glycine residue of ubiquitin and the active cysteine of E1. (**B**) Conjugation: The activated ubiquitin (Ub - dark pink) is then transferred to the active cysteine side of the ubiquitin-conjugating enzyme (E2). (**C**) Ligation: Ubiquitin ligase (E3) facilitates the interaction between the E2-Ub intermediate and the target protein. This interaction results in the transfer of one (monoubiquitination) or multiple (polyubiquitination) ubiquitin molecules to specific lysine residues (K) on the substrate protein. (**D**) Degradation: Polyubiquitination of target proteins, where ubiquitin chains are assembled at lysine residues, particularly K11 or K48, signals proteasomal degradation. The 26S proteasome, a multi-catalytic ATP-dependent protease complex, degrades the tagged protein with the subsequent release of free ubiquitin molecules and digested peptides. This polyubiquitination serves as a signal for proteasomal degradation. The 26S proteasome, a multi-catalytic ATP-dependent protease complex, recognizes the tagged protein and proceeds to degrade it. This degradation process liberates free ubiquitin molecules and digested peptides.

The *UBE3A* gene is responsible for encoding a 100 kDa E3 ubiquitin-protein ligase, which plays a crucial role in the ubiquitin-proteasome pathway (UPP) (Scheffner, Huibregtse, Vierstra, & Howley, 1993) (refer to **Figure 6C**).
As an E3 ligase, UBE3A's primary role is to recognize specific substrates and mediate the transfer of ubiquitin from the E2 enzyme to the target protein.

E3 ligases can be classified into four families based on their C-terminal domains and the mechanism by which they transfer ubiquitin to the target substrate: RING (really interesting new gene), HECT (Homologous to the E6-AP Carboxyl Terminus), RBR (RING-BetweenRING-RING) and RCR (RING-Cys-Relay) (Humphreys et al., 2021) (see **Figure 7A-D**).

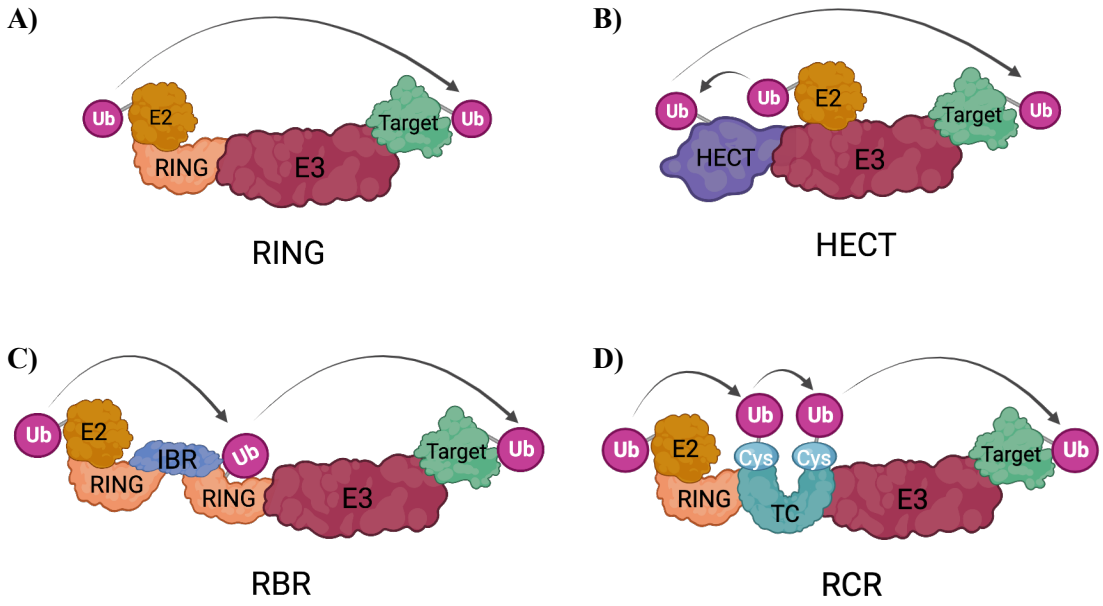


Figure 7 - Mechanism of action of E3 ubiquitin ligases families. (**A**) RING (Really Interesting New Gene) E3 ligases facilitate the direct transfer of ubiquitin from the ubiquitin-conjugated E2 enzyme to the target protein by binding to both. This interaction allows for the attachment of ubiquitin to a specific lysine residue on the target protein. (**B**) HECT (Homologous to E6-AP Carboxyl Terminus) E3 ligases carry out a two-step process. Initially, ubiquitin is transferred to a cysteine residue on the HECT domain of the E3 through a thioester bond with the C-terminal glycine residue of ubiquitin. Subsequently, the ubiquitin molecule is transferred from the HECT domain to a specific lysine residue on the target substrate. (**C**) RBR (RING-BetweenRING-RING) ligases consist of two RING domains connected by an IBR (in-between-RING) domain. The first step involves the transfer of ubiquitin to the cysteine residue of the second RING domain. Subsequently, the ubiquitin molecule is attached to the target

protein. (**D**) RCR (RING-Cys-Relay) ligases employ two catalytic cysteines to facilitate intramolecular ubiquitin transfer. Figure adapted from *(Humphreys et al., 2021)*.

The RING family of E3 ligases plays a prominent role in the direct transfer of ubiquitin from the E2 enzyme to the substrate recognized by the E3 (Metzger, Hristova, & Weissman, 2012) (refer to **Figure 7A**). However, UBE3A stands out as a founding member of the HECT domain family. As a HECT ligase, UBE3A recruits E2 ubiquitin-conjugating enzymes loaded with activated ubiquitin and actively transfers this signal to a specific substrate attached to UBE3A's N-terminal domain (Huibregtse, Scheffner, Beaudenon, & Howley, 1995) (**Figure 7B**)

The ligation process mediated by E3 ligases can lead to various types of ubiquitination on the substrate (**Figure 8A**). Monoubiquitinylation represents the simplest form, where a single ubiquitin moiety is covalently attached to a lysine residue on the target protein (B. B. Chen & Mallampalli, 2009). However, isopeptide-linked ubiquitin chains can also be formed at one of the seven lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, and K63), or even at the N-terminus's terminal methionine 1 (Met1). The fate of the target protein depends on the specific lysine residue involved in the formation of the polyubiquitination chain (Scheffner et al., 1993) (**Figure 8B**). The most common types of polyubiquitination chains are K11 and K48-linked chains, both serving as recognition signals for the 26S proteasome, leading to the proteolysis of the tagged substrate (see **Figure 6D**). UBE3A is recognized for catalysing the assembly of polyubiquitin chains through the K48 residue of ubiquitin, thereby providing a signal for proteasomal degradation. Interestingly, UBE3A not only targets several substrate proteins for proteasomal degradation but also undergoes self-ubiquitination (Scheffner et al., 1993) (Kumar, Talis, & Howley, 1999) (Carmody et al., 2017).

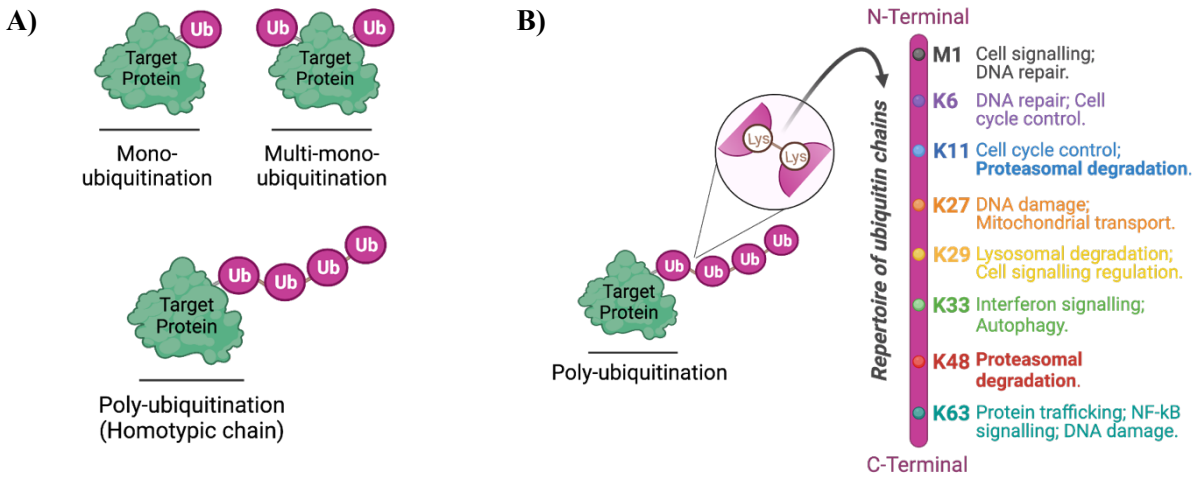


Figure 8 - Representation of possible ubiquitin chain topology and fates of polyubiquitinated targets. (**A**) Target proteins can undergo mono-ubiquitination (a single ubiquitin molecule attached to

the substrate), multi-mono-ubiquitination (a single ubiquitin conjugated to multiple lysine residues in the substrate), or polyubiquitination (multiple ubiquitin molecules binding to the same lysine residue in the target substrate). For simplicity, we will omit the additional complexity arising from the formation of heterotypic chains. (**B**) Polyubiquitination chains are formed by ligating an ubiquitin molecule to one of eight possible sites, including seven internal lysine residues (*K*) or the methionine at position 1 (*M1*) of the previous ubiquitin molecule. The site where the ubiquitin chain is formed determines the fate of the target protein.

Due to the intricate regulatory role of *UBE3A* in neurons, it has been postulated that the severe phenotypes observed in Angelman syndrome (AS) may arise from the toxic accumulation of UBE3A's substrates and/or other indirect targets. This hypothesis will be further explored in the subchapter "Therapeutic approaches for AS". Supporting this notion, studies have demonstrated promising rescue effects in certain AS mouse models by targeting aberrant downstream cell signalling pathways (van Woerden et al., 2007)(Kaphzan et al., 2012).
While a complete three-dimensional structure of the full-length UBE3A protein remains elusive, researchers have reported structures for several UBE3A domains (refer to **Figure 9**).

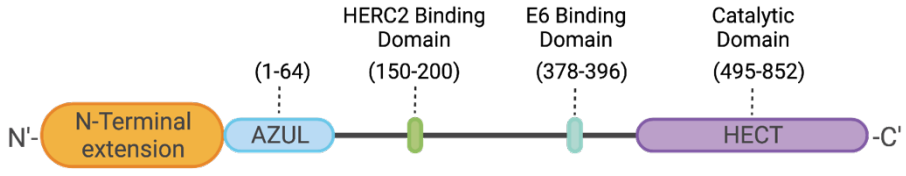


Figure 9 - Schematic representation of UBE3A protein (isoform 1) and its functional domains. These domains encompass the N-terminal Zn-binding AZUL (amino-terminal zinc-binding domain of ubiquitin E3a ligase) that binds to Rpn10 in the proteasome *(Kühnle et al., 2018)* and is required for UBE3A's nuclear location *(Avagliano Trezza et al., 2019).* Additionally, the HERC2 binding domain is essential for the association with the partner protein HERC2. The figure also highlights the E6 Binding domain, responsible for binding the HPV E6 oncoprotein, along with the catalytic HECT domain. The numbers in parentheses indicate the corresponding amino acid residue of UBE3A isoform 1. This figure has been adapted from the work of *(Owais, Mishra, & Kiyokawa, 2020)*.

Numerous point mutations associated with Angelman syndrome (AS) have been identified within the carboxyl-terminal HECT domain of UBE3A, leading to disruption of its catalytic activity. Interestingly, there have also been reports of point mutations affecting the N-terminus of UBE3A in AS patients (Malzac et al., 1998).

A recent study demonstrated that variants outside the HECT domain can also impair the catalytic potential of UBE3A, suggesting that the N-terminus domain has the ability to influence the enzyme's catalytic activity. Importantly, this study revealed that while UBE3A mutations affecting its ligase activity are frequent, they are not the primary cause of UBE3A dysfunction in AS. Instead, most AS-linked UBE3A mutations affect the subcellular distribution of the protein, which is influenced by the expression of different UBE3A isoforms (Bossuyt et al., 2021).

Initially, UBE3A was identified as a protein that forms a complex with the cancer-associated E6 oncoprotein of human papillomaviruses (HPV). This complex leads to the ubiquitin-mediated degradation of the tumour suppressor p53 by the proteasome, and it is implicated in HPV-positive cancers (Huibregtse, Scheffner, & Howley, 1991) (Scheffner et al., 1993). However, it is now known that UBE3A has diverse roles beyond its ubiquitin-ligase function. It directly interacts with the proteasome through its association with PSMD4 (Rpn10/S5a), acts as a transcriptional co-activator by binding to receptors and targeting transcription factors, and plays a critical role in the normal development and function of the nervous system (Kühnle et al., 2018) (Buel et al., 2020)(Buiting et al., 1995) (Nawaz et al., 1999)(Gossan et al., 2014b). Collectively, the accumulating evidence suggests that *UBE3A* functions as a central neuronal "housekeeping" gene involved in regulating protein homeostasis through protein ubiquitination, which serves both degradative and non-degradative functions. UBE3A is also implicated in proteasome inhibition and acts as a transcriptional coactivator for several genes, including various steroid hormone receptors (Avagliano Trezza et al., 2021)(Ferdousy et al., 2011)(Godavarthi, Dey, Maheshwari, & Jana, 2012)(Krishnan et al., 2017). The diverse functions of UBE3A are thought to be linked to the expression of different isoforms, which determine its subcellular distribution and cellular roles.

2.6 **UBE3A ISOFORMS AND SUBCELLULAR LOCALIZATION**

The human *UBE3A* gene undergoes alternative splicing of its first eight exons, giving rise to three mRNA transcript variants that encode three protein isoforms: isoform 1, isoform 2, and isoform 3. These isoforms exhibit differences in their extreme amino-termini (refer to **Figure 10**). Notably, all human UBE3A isoforms share a common feature, which is the presence of the catalytic HECT domain located at the carboxyl-terminus (C-terminus) spanning residues 495 to 852. Consequently, all human UBE3A isoforms possess the capacity to function as an E3 ligase, facilitating the transfer of ubiquitin molecules (Owais et al., 2020).

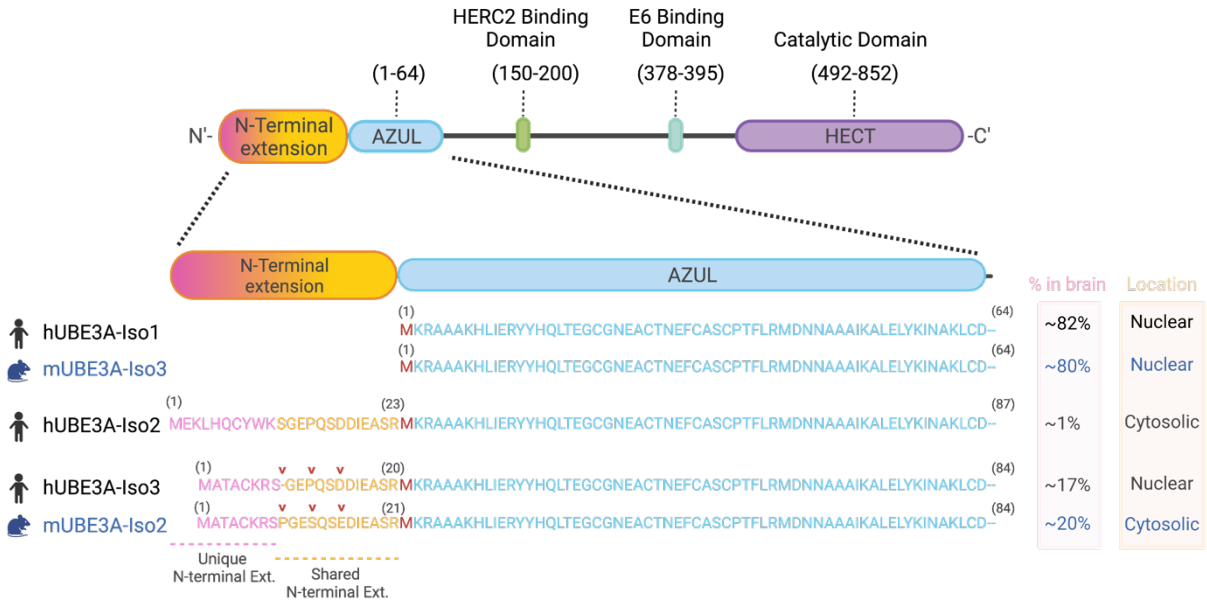


Figure 10 – Schematic representation of UBE3A protein functional domains (top) and its human (black) and murine (blue) isoforms (bottom). The catalytic domain (HECT domain) is shown in purple, while the AZUL domain, necessary for binding to PSMD4 and the proteasome, is depicted in blue. The N-terminal extension is illustrated with an orange and pink gradient, where the orange portion represents the shared N-terminal extension, and the pink portion represents the unique N-terminal extension. The numbers in parentheses indicate the corresponding amino acid residues. Within the protein sequence, the red "M" indicates the translational start sites, while "V" denotes the amino acid sequence differences between hUBE3A-Iso3 and mUBE3A-Iso2. These differences are responsible for the distinct subcellular location of each isoform. Information on the abundance and subcellular location of each isoform in the brain can be found on the right side of the figure. The image is adapted from a study by *(Buiting et al., 1995)*.

The abundance and subcellular localization of UBE3A isoforms have mainly been investigated in mice, revealing fundamental differences compared to human isoforms. To start, isoform nomenclature can be a bit confusing since three UBE3A isoforms have also been identified in mice, but they have been numbered differently and at least one isoform is unique to either mouse or human (an analogous form of human isoform 2 does not exist in the mouse). In contrast to human isoforms, only 2 murine isoforms (mouse isoform 2 and 3) retain ubiquitin ligase activity. Unlike human isoforms, only two murine isoforms (mouse isoform 2 and 3) retain ubiquitin ligase activity. Mouse isoform 1 (mUBE3A-iso 1), on the other hand, lacks enzymatic activity and exhibits low expression levels (Buiting et al., 1995). Despite suggestions of a role in brain development, recent evidence revealed that mUBE3A-iso 1 is a noncoding transcript with barely detectable expression levels, resulting in its removal from the

NCBI (National Centre for Biotechnology Information) database (Avagliano Trezza et al., 2019).

Mouse isoform 2 (mUBE3A-iso 2), the longer isoform with a 21 amino-acid extension in its N-terminus, predominantly localizes in the cytosol of neurons and is analogous to human isoform 3 (hUBE3A-Iso 3). The major difference between the two species lies in this isoform. While they are highly similar and homologous, a modification of three positions in the N-terminus extension (see the lower panel of **Figure 10**) leads to the nuclear localization of hUBE3A-Iso 3, while mUBE3A-iso 2 remains in the cytosol. This shared alteration, observed in humans and other higher primates, appears to affect protein folding and interactions, resulting in the nuclear retention of UBE3A (Zampeta et al., 2020).
Mouse isoform 3 (mUBE3A-iso 3), corresponding to human isoform 1 (hUBE3A-Iso 1), is the shortest protein isoform but exhibits the highest abundance in the nucleus of both murine and human neurons, constituting approximately 80% of the total protein (Buiting et al., 1995). These findings align with a study by Sirois et al., demonstrating that hUBE3A-Iso 1 is the most abundant isoform in human neurons, accounting for nearly 90% of total UBE3A.(Sirois et al., 2020).

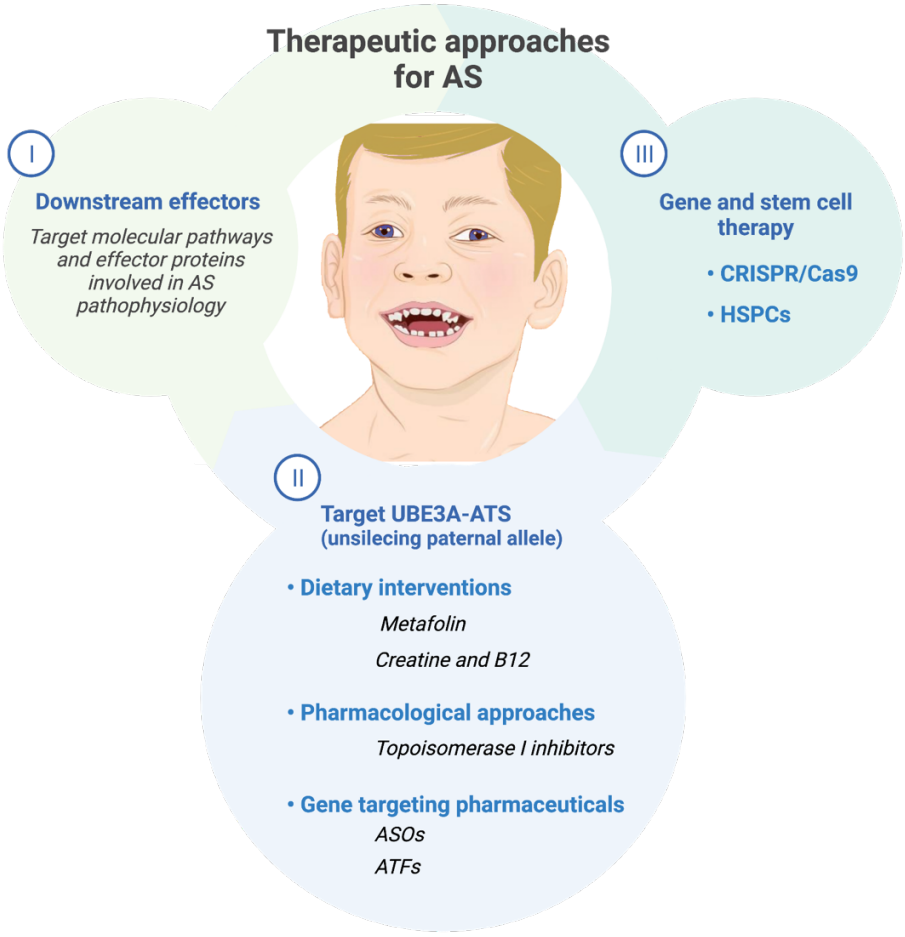
Avagliano et al. employed quantitative polymerase chain reaction (qPCR) to investigate the expression ratios of UBE3A short and long isoforms in the mouse cortex at embryonic stages (E15.5 and E17.5) and postnatal stages (P0 and P7), finding a ratio of ~4:1. In contrast, the expression ratio of short/long isoforms in the human cortex was ~3:1 (Avagliano Trezza et al., 2019). Furthermore, *Judson et al.* conducted RNA sequencing (RNA-seq) analysis to explore the expression ratios of UBE3A isoforms in the mouse and human brain across development and into adulthood. They observed that while the ratio of human UBE3A isoforms remained relatively consistent throughout brain development, the ratio of mouse Ube3a isoforms decreased to close to 1:1 in adulthood (M. C. Judson et al., 2021).

In summary, UBE3A is expressed throughout neurons in both species, and the subcellular localization is influenced by the isoform expression pattern. Bossuyt et al. demonstrated that missense mutations leading to the exclusive loss of the nuclear isoform of UBE3A play a significant role in the pathogenesis of Angelman syndrome (AS) (Bossuyt et al., 2021). These findings are supported by studies conducted in mouse models and human embryonic stem cell (hESC)-derived neurons, showing that the exclusive loss of the short, nuclear isoform mUBE3A-iso 3/hUBE3A-Iso 1 results in AS-like phenotypes, while mice lacking the long cytoplasmic isoform mUBE3A-Iso2 exhibit no behavioural abnormalities (Sadhwani et al., 2018)(Avagliano Trezza et al., 2019)(Sirois et al., 2020)(Bossuyt et al., 2021). Collectively,

these findings suggest a predominant nuclear role for UBE3A in the context of AS, although the underlying mechanism remains elusive.

2.7 **THERAPEUTIC APPROACHES FOR AS**

As mentioned earlier in this thesis, the current treatment options for Angelman syndrome (AS) primarily focus on managing symptoms through the use of medications to control seizures and improve sleep, as well as employing physical, communicative, and behavioural therapies. However, recent advancements have significantly advanced our understanding of AS, leading to the emergence of several mechanism-based approaches for therapeutic interventions. These approaches can be broadly classified into three strategies: developing therapeutic interventions that *target downstream effectors of UBE3A* to correct specific disrupted cellular pathways resulting from its absence; reactivating the dormant paternal UBE3A through *targeting the UBE3A-ATS*; and utilizing traditional gene therapy to introduce a functional copy of *UBE3A* or enzyme replacement therapy (refer to **Figure 11**, on the following page).
These emerging strategies offer new hope for the development of effective treatments for AS, moving beyond symptomatic management and targeting the core molecular defects.



(Figure legend on the next page.)

Figure 11 - Summary of potential therapeutic approaches for Angelman syndrome. Three types of strategies are currently being explored in preclinical settings to treat AS: (I) development of therapeutic interventions targeting downstream effectors of UBE3A to correct specific cellular pathways, (II) reactivation of the dormant paternal *UBE3A* by targeting the *UBE3A-ATS*, and (III) application of traditional gene therapy through direct introduction of a functional copy of *UBE3A* or enzyme replacement in the brain of patients.

2.7.1 **THERAPEUTIC INTERVENTIONS OF DOWNSTREAM EFFECTORS**

Throughout the research, various proteins have been proposed as potential targets of UBE3A, including MAPK1, RPN10, RING1B, and BMAL1, among others (Filonova, Trotter, Banko, & Weeber, 2014)(Jacobson, MacFadden, Wu, Peng, & Liu, 2014)(Zaaroor-Regev et al., 2010)(Gossan et al., 2014a). However, none of these brain-specific targets have been found to fully explain the spectrum of symptoms observed in Angelman Syndrome (AS). Network analysis of UBE3A indicates that multiple molecular pathways may contribute to the pathogenesis of AS (Martínez-Noël et al., 2018). It is worth noting that UBE3A not only functions as an E3 ligase, directing its protein substrates for proteasomal degradation, but also influences the proteolytic activity of the 26S proteasome through its interaction with PSMD4 (Rpn10/S5a). Consequently, this leads to the dysregulated upregulation of polyubiquitinated proteins, in addition to its direct substrates (Avagliano Trezza et al., 2019)(Avagliano Trezza et al., 2021). Given the complexity and interconnectivity of these pathways, targeting all of them simultaneously in a single AS patient seems impractical from a therapeutic perspective. Instead, a more promising approach to treating AS may involve addressing the upstream event responsible for the syndrome, namely the lack of *UBE3A* expression in neuronal cells. The most efficient way of accomplishing this could be by targeting the long antisense transcript responsible for the epigenetic silencing of the paternal *UBE3A* allele or through gene therapy.

2.7.2 **APPROACHES TO TARGET *UBE3A-ATS***

Regardless the genetic defect that results in the lack of expression of the maternal UBE3A gene, it has been observed that in all cases of AS, there is at least one intact copy of the paternal *UBE3A* gene (see **Figure 4**). The unique epigenetic regulation of the *UBE3A* gene suggests the possibility of reactivating the dormant paternal *Ube3a* allele in mouse neurons, where the maternal contribution is deficient due to mutations or deletions in this allele, thereby providing a potential treatment for the syndrome. The feasibility of this approach was demonstrated in 2013 by *Meng et al.*, who engineered an AS mouse model with a knocked-out maternal *Ube3a* gene, along with a poly(A) cassette (also known as a Stop cassette)

inserted between *Snord115* and the *Ube3a* gene on the paternal allele. This cassette induced a termination of *Ube3a-ATS* transcription downstream of the insertion site, while leaving the transcription of other noncoding RNAs located upstream of *Snord115* unaffected. By halting *Ube3a-ATS* transcription, the previously silenced paternal gene was reactivated, leading to the production of the UBE3A protein at levels reaching up to 70% of wild-type (WT) levels in the neocortex, 60% in the hippocampus, and 50% in the cerebellum (Meng et al., 2013). Building upon this proof of concept, subsequent studies have employed various approaches to target the expression of *Ube3a-ATS*, aiming to unsilence the paternal *Ube3a* allele in vivo.

2.7.2.1 ***DIETARY INTERVENTIONS***

Previous efforts have explored dietary interventions as a potential approach to treat Angelman syndrome (AS) by targeting DNA methylation. The underlying idea was that hypermethylation of the paternal *SNURF-SNRPN* locus could potentially reduce the expression of *UBE3A-ATS*, leading to an upregulation of UBE3A and potentially ameliorating the symptoms of AS.

To investigate this possibility, Bird et al. conducted a randomized double-blind placebo-controlled clinical trial. They evaluated the impact of methylation-promoting dietary supplements, such as betaine, 5-methyltetrahydrofolate (Metafolin), creatine, and vitamin B12, on individuals with AS. The aim was to increase the availability of methyl donors for DNA methylation. However, these attempts did not yield statistically significant changes in developmental performance when comparing the treatment group with the placebo group (Bird et al., 2011)(Han et al., 2019).

2.7.2.2 ***PHARMACOLOGICAL APPROACHES***

The first large scale screening study aimed at identifying compounds capable of reducing the expression of *UBE3A-ATS* was performed in 2012 by Huang et al.. Through in vitro experiments, they found that treatment with topoisomerase I inhibitors, such as Topotecan and Irinotecan, successfully downregulated the expression of the *Ube3a* antisense transcript, which overlaps with the paternal copy of *Ube3a*. Subsequently, in an in vivo model, the administration of Topotecan through unilateral intracerebroventricular infusion resulted in the unsilencing of the paternal *Ube3a* gene in specific brain regions, including the hippocampus, striatum, and cerebral cortex of the infused hemisphere. Notably, this unsilencing effect was achieved without affecting genomic methylation at the imprinting centre. The success of topoisomerase inhibitors in reducing *Ube3a-ATS* expression was attributed to their selective inhibition of the nuclear enzyme DNA topoisomerase, type I (Huang et al., 2012)(H. M. Lee et al., 2018).

A later study by King, et. al. demonstrated that pharmacological inhibition of topoisomerases results in the reduced expression of particularly long genes (> 200 kb) in which, not only the long antisense transcript responsible for the silencing of the paternal copy of *Ube3a* is included, but also numerous long genes associated with synaptic function and ASD (King et al., 2013)(Mabb et al., 2014). These findings suggest that topoisomerase inhibitors have the potential to rescue molecular, cellular, and perhaps behavioural deficits associated with loss of not only UBE3A but also ASD. However, before considering human clinical trials, further preclinical studies are required to evaluate whether topoisomerase inhibitors can alleviate the AS phenotype in animal models, as well as the assessment of the potential off-target effects (unintended repression of many different long genes and possibly overexpression of the shorter genes) of these drugs. This highlights the need for the development of strong behavioural test batteries, that would provide a set of well characterized AS phenotypes, allowing for the detection of any beneficial effects.

2.7.2.3 *GENE-TARGETING PHARMACEUTICALS*

The lack of specificity exhibited by Topoisomerase I inhibitor drugs (King et al., 2013) poses a significant limitation to their use as a treatment for Angelman syndrome (AS), since it can cause unknown consequences throughout the genome of patients, culminating in a fundamental need of a serious risk–benefit assessment. To address these concerns, a more desirable approach would involve a sequence-specific knockdown of the antisense transcript. Fortunately, such a therapy exists and has been utilized in experimental treatments for various systemic diseases and single-gene neurologic conditions, including spinal muscular atrophy (SMA), Duchenne's muscular dystrophy (DMD), and hereditary transthyretin amyloidosis (hATTR) (Porensky & Burghes, 2013)(Chiriboga et al., 2016)(Charleston et al., 2018)(Benson et al., 2018)(Mathew & Wang, 2019). The successful application of antisense oligonucleotides (ASOs) in these contexts instils confidence in the safety and potential of this technology. This tool consists in the application of antisense oligonucleotides (ASOs) to modulate gene expression in vivo. ASOs are short synthetic strands of nucleic acid capable of modulating expression of specific genes by binding to complementary mRNA in a sequence-specific manner. In the case of AS, *Meng et al.* developed an ASO composed of ten deoxynucleotides flanked by five 2'-O-methoxyethyl (2'-MOE) modified nucleotides on each end, forming a structure known as a "gapmer." This ASO promotes the degradation of the *Ube3a* antisense transcript through RNase H-mediated mechanisms (Meng et al., 2015). Since ASOs primarily interact with RNA rather than DNA, they are classified as gene-targeting pharmaceuticals rather than gene therapy.

Recently, it was shown that single intracerebroventricular (ICV) injection of ASOs at postnatal day 1 (P1) in AS mice resulted in specific UBE3A reinstatement in the brain, leading to the complete rescue of various AS phenotypes, including sensitivity to audiogenic seizures, open field and forced swim test behaviours, and improved performance on the reversed rotarod task (Milazzo et al., 2021). However, it was observed that UBE3A levels gradually declined in the brains of treated mice from 70% to 22-27% over the subsequent 8 weeks, underscoring the need for continued treatment, potentially throughout the lifespan of patients.
In addition to ASOs, artificial transcription factors (ATFs) have also been explored as a potential treatment for AS. ATFs consist of a zinc finger domain that binds directly upstream of *Snrpn*, fused with the Kruppel-associated box (KRAB) transcriptional repressor and a cell-penetrating protein TAT. Studies claim that ATFs can cross the blood-brain barrier and partially increase *Ube3a* expression in the brain of adult mouse models of AS. However, the results obtained thus far are not robust (Bailus et al., 2016).

2.7.3 GENETIC INTERVENTIONS

Genetic interventions offer potential strategies for addressing Angelman syndrome (AS), either by introducing a missing copy of the *UBE3A* gene or by manipulating the paternal *UBE3A* expression.

In 2011, *Daily et al*. showed for the first time the feasibility of using an adeno-associated virus (AAV) to deliver an exogenous murine *Ube3a* gene into the hippocampus of AS mice. Through direct injections of AAV serotype 9 (AAV-9) into the hippocampus, adult AS mice exhibited an improvement in memory and associative learning eight weeks after treatment. However, it is important to note that complete rescue of hippocampal long-term potentiation (LTP) was not achieved, and there was no significant amelioration of motor deficits. One major limitation of this study was the inadequate level of *Ube3a* transgene transduction, as there was no expression observed in the cerebellum, and data regarding expression in other brain regions outside the hippocampus were not provided. Additionally, it remained unclear which specific isoform(s) of UBE3A were transduced in this study (Daily et al., 2011).
In recent advancements, *Judson et al.* employed a recombinant AAV9-derived PHP.B vector to deliver a codon-optimized human *UBE3A* (hUBE3Aopt) transgene in neonatal AS model mice (M. C. Judson et al., 2021).
This approach addressed the limitations from the 2011 study, from *Daily and colleagues*, since it allowed for the reinstatement of both a short (hUBE3A-iso1) and a long (hUBE3A-iso3) isoform expression of human UBE3A (hUBE3A), at a near-endogenous ratio (Avagliano Trezza et al., 2019). Although the expression of hUBE3A was suboptimal in certain brain

regions, such as the cerebellum and inner half of the dentate gyrus granule cell layer, the restoration of both UBE3A isoforms in the developing brain led to the rescue of Nest building and Rotarod phenotypes and conferred resilience to epileptogenesis in the treated mice (M. C. Judson et al., 2021).

Another avenue under investigation for AS treatment involves targeting the *UBE3A-ATS* through genetic manipulation. Specifically, the CRISPR/Cas9 (short for clustered regularly interspaced short palindromic repeats CRISPR associated protein 9) mediated gene disruption technology to target regions within *UBE3A*-ATS. This approach aims to overcome the limitations associated with treatments using Topoisomerase type 1 inhibitor drugs and ASOs, which necessitate lifelong and repeated invasive injections in patients.
In 2020, Wolter and colleagues treated AS embryos (E15.5) with adeno-associated virus containing a short Cas9 variant and guide RNA that target about 75 *Snord115* genes. This treatment was repeated after birth (P1). Ninety days following transduction, the levels of paternal UBE3A protein in the cerebral cortex, hippocampus, and spinal cord of AS mice were restored to approximately 40% of wild-type levels, but no significant change was observed in the cerebellum.
This approach successfully unsilenced paternal *Ube3a* throughout the brain for at least 17 months after transfection and rescued some behavioural impairments in AS mice, such as the hindlimb clasping phenotype and open field (increase in the time spent at the centre of the arena). Also, rotarod test improvement was evident at 2 months of age and endured at 7 months of age (Wolter et al., 2020).

A recent study detected catalytically active UBE3A protein within cerebrospinal fluid (CSF) and hippocampal extracellular space of wild-type rats, suggesting that UBE3A may have a novel role outside of neurons (Dodge et al., 2021). This possibility opened a new therapeutic strategy for AS based on enzyme replacement (ERT) and/or cell therapy (hematopoietic progenitor cell gene therapy - HSC-GT). ERT involves delivering a purified form of the missing or non-functional UBE3A protein into the extracellular space and is currently in preclinical development. On the other hand, HSC-GT takes a step further and utilizes a lentiviral vector to deliver ex vivo modified autologous hematopoietic stem and progenitor cells (HSPCs), which, after transplantation, differentiate into microglia.
These gene-corrected cells secrete UBE3A, which is taken up by surrounding neuronal cells, thereby restoring the ligase function through a process known as "cross-correction (Adhikari et al., 2021). HSC-GT was performed in a novel immunodeficient *Ube3a*$^{mat-/pat+}$ *IL-2RG*$^{-/y}$ mouse model of AS and rescue of the AS phenotype in RR, OF, Balance beam, Digigait and Novel object recognition was achieved in neonatal treated AS mice. Surprisingly, this study

claims to have obtained the same phenotypic rescue in treated adult AS C57BL/6 mice, being the first study to do so. (Adhikari et al., 2021).

2.8 **AVAILABLE MURINE MODELS OF AS**

The murine syntenic region of chromosome 7C displays conserved genomic organization and imprinting regulation similar to the human 15q11-q13 region (Chamberlain, 2013). Also in mice, the neuronal expression of *SNHG14/Ube3a-ATS* is sufficient to silence the expression of the paternal *Ube3a* allele, since studies showed that interfering with the murine *Ube3a-ATS* results in paternal *Ube3a* expression (Huang et al., 2012)(Meng et al., 2013). Given its genetic and physiological similarities with human, the mouse (Mus musculus) is the primary animal model used to study Angelman syndrome.
Understanding the limitations and potential of each model is crucial for accurate preclinical assessment of novel therapeutic agents (Tadenev & Burgess, 2019)(Chadman, Yang, & Crawley, 2009).

Several murine models of AS have been created by diverse strategies that ultimately result in the inactivation of the maternal *Ube3a* gene in the brain. These models have greatly contributed to the exploration of *UBE3A's* function (D. Rotaru, Mientjes, & Elgersma, 2020)(see **Figure 12**).

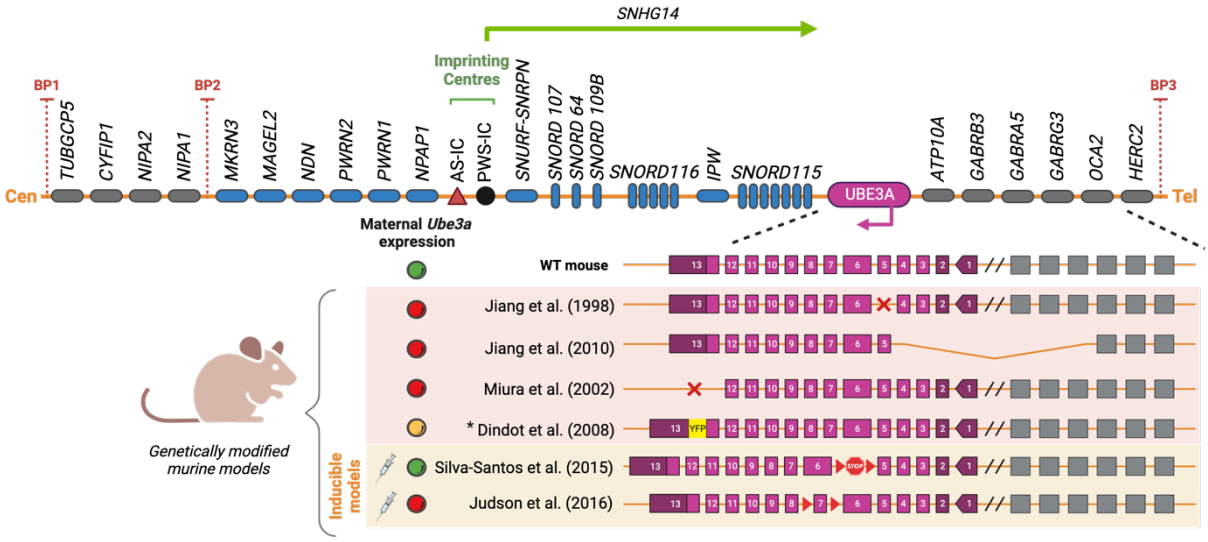


Figure 12 - Overview of genetically modified murine models commonly used to study AS. The top panel of the figure provides a schematic representation of the 15q11-q13 region, displaying the paternally expressed genes in blue and the maternally expressed gene in pink. Non-imprinted genes are depicted as grey boxes, while imprinting centres are represented by a circle (PWS-IC) and a triangle (AS-IC). Moving to the lower panel, the figure illustrates the commonly utilized transgenic murine

models for Angelman syndrome (AS) and their corresponding genomic alterations. Deletions are denoted by red crosses, and loxP sites are indicated by red triangles. (*) The *Ube3a-YFP* model is not primarily an AS model but rather a reporter model. Furthermore, the figure highlights the conditional models that have been developed up to the present time. Within this panel, circles provide specific information about *Ube3a* gene expression in certain models, with colours indicating the expression status of the *Ube3a* gene: A green circle indicates that the *Ube3a* gene is actively expressed; A yellow circle represents models in which the *Ube3a* gene is fused to a fluorescent yellow reporter; A red circle signifies models in which the *Ube3a* gene is not expressed. Additionally, the presence of a syringe symbol next to these circles indicates the response to tamoxifen treatment, where the model can either express or halt the expression of the *Ube3a* gene upon treatment. This figure legend has been adapted from the work of Rotaru et al. *(D. Rotaru et al., 2020)*.

In 1998, the first *Ube3a* knockout mice was developed, which quickly became the most extensively utilized model for studying AS. This mouse model was created by specifically deleting exon 5 of the *Ube3a* gene in mice. This deletion introduced a shift in the reading frame, effectively preventing the production of a functionally active UBE3A protein and all potential isoforms through a process called nonsense-mediated degradation of the *Ube3a* transcript (Jiang et al., 1998). Over the years, this particular mouse model has significantly contributed to our understanding of the pathogenic mechanisms underlying AS and has served as a valuable tool for identifying molecular targets associated with UBE3A.

An additional mouse model was generated with a targeted deletion in the C-terminal tail of UBE3A (Miura et al., 2002). This model exhibited similar impairments to those ones observed in the Jiang et al. model (such as impaired motor function, defective context-dependent fear learning, hippocampal LTP deficits, abnormal EEGs, sound induced seizures, and several behavioural abnormalities), with additional phenotypes related to sleep disturbances and abnormal oscillation induced by enhanced Purkinje cell rhythmicity and hypersynchrony (Colas, Wagstaff, Fort, Salvert, & Sarda, 2005)(Cheron, Servais, Wagstaff, & Dan, 2005).

Nonetheless, a substantial proportion of individuals with AS harbour a large deletion that includes but is not limited to *UBE3A* (Bird, 2014). While the maternal heterozygous *Ube3a* null mutation mouse model has proven invaluable in studying the consequences of *Ube3a* loss during development, it was not be the most suitable model for investigating the role of other deleted genes in AS pathogenesis. In 2010, the same research group responsible for generating the first *Ube3a* knockout mice, addressed this need by generating a mutant mouse model with a 1.6-Mb chromosomal deletion spanning from *Ube3a* to *Gabrb3*. This deletion resulted in the inactivation of the *Ube3a* and *Gabrb3* genes and the deletion of the *Atp10a* gene (refer to **Figure 5**) (Jiang et al., 2010). As predicted, this model presented typical AS phenotypes (such as abnormal motor function, learning and memory impairments, atypical

EEG, etc.) but also had increased spontaneous seizure activity and manifested irregular ultrasonic vocalizations. The maternal deletion from *Ube3a* to *Gabrb3* genes make this murine model more representative of most human AS patients than the previous models.

Imprinting defects in AS predominantly occur in the AS-IC, a region that remains unidentified in mice. To enhance our understanding of the AS-PWS imprinting centre and its regulatory mechanisms, it was essential to develop models that would replicate these imprinting defects. Such models would contribute to advancing our knowledge and increasing the efficacy of future therapies.

Two mouse mutations, resulting in defects similar to that seen in AS patients with deletion of the AS-IC, were reported by Wu et al. and Lewis et al.. In the first model, an insertion of 6-kb genomic DNA fragment upstream *Snrpn's* exon 1 was added, resulting in lack of methylation of the maternal allele at the *Snrpn* promoter (suggesting that the maternal chromosome assumed a paternal expression pattern), while in the other murine model a transcriptional STOP upstream PWS-IC resulted in the same change in parent-of-origin-specific gene expression (Lewis, Vargas-Franco, Morse, & Resnick, 2019) (Wu, Jiang, Zhai, Beaudet, & Wu, 2012).
The *Ube3a-YFP* mouse, developed in 2008 (Dindot, Antalffy, Bhattacharjee, & Beaudet, 2008), is another significant murine model that has made immense contributions to the field. While it is not specifically designed as an AS model, this mouse harbours a knock-in fluorescent reporter allele YFP (yellow fluorescent protein) fused to the carboxyl-terminus of UBE3A. This unique feature enables researchers to investigate the individual contributions of each parental *Ube3a* allele, track and visualize allele-specific *Ube3a* expression in various cells and cellular compartments, determine spatial and temporal changes in gene expression during brain development, and assess the effectiveness of specific treatments in unsilencing the paternal *Ube3a* (Dindot et al., 2008)(Meng et al. 2013)(Judson et al. 2014)(Huang et al. 2011)(Hillman et al. 2017)(Sonzogni et al., 2020). Despite not being a dedicated AS model, this recombinant transgenic mouse is extensively utilized in research focused on unsilencing the paternal *Ube3a* allele, warranting a mention in this subchapter (Lewis et al., 2019)(Wu et al., 2012).

While numerous murine models for Angelman syndrome (AS) exist, the aforementioned models have been particularly instrumental in key studies that significantly advanced our understanding of this syndrome. However, it is important to acknowledge that although these models are valuable, they do not provide insights into the critical treatment windows where *Ube3a* re-activation can effectively alleviate AS-like phenotypes. Understanding these

windows is crucial for developing therapeutic approaches aimed at restoring UBE3A expression.

To address this knowledge gap, the utilization of inducible AS mouse models, enabling Cre-dependent initiation or deletion of the maternal *Ube3a* allele at specific time periods, becomes indispensable. Currently, two conditional mouse models have been generated to facilitate such investigations. The first model was employed to investigate the impacts of *Ube3a* reinstatement on AS-like murine symptoms, facilitating the identification of distinct developmental windows relevant to AS (Silva-Santos et al., 2015). In this model, a floxed Stop cassette was inserted into intron 5 of the *Ube3a* gene, enabling its expression upon tamoxifen treatment. This model was conceptualized and characterized by the author of this thesis, and the results are presented in Chapter 3. Additionally, it allowed for the examination of the effects of cerebellar *Ube3a* reinstatement on cerebellar learning and locomotor impairments (Bruinsma et al., 2015).
The second inducible model incorporates a floxed exon 7 of *Ube3a* and was generated to analyse the consequences of selective *Ube3a* loss from either GABAergic or glutamatergic neurons (M. C. C. Judson et al., 2016). Its application has elucidated that GABAergic *Ube3a* loss, but not glutamatergic loss, is the primary cause of the seizures and EEG abnormalities observed in AS. Since this model permitted the deletion of the *Ube3a* gene upon tamoxifen treatment, we employed it in Chapter 5 of this thesis to evaluate the consequences of *Ube3a* deletion at various developmental stages.
These inducible AS mouse models play a pivotal role in exploring treatment windows and investigating the specific effects of *Ube3a* loss in distinct neuronal populations, providing valuable insights into the underlying mechanisms of AS pathogenesis.

2.9 **VALIDATY OF MURINE MODELS IN THE CONTEXT OF AS**

In order for genetically engineered mice to be considered viable models of Angelman syndrome (AS) and valuable for the development of targeted therapies, they must undergo rigorous assessment using external validation criteria that optimize the model's validity (Chadman et al., 2009). The three commonly used criteria for validating animal models are construct validity, face validity, and predictive validity (Willner, 1984).
Although it would be ideal for mouse models to exhibit validity in all three criteria, such models are rare.

In the context of AS, construct validity is evident as genetically engineered mice accurately replicate the genotypic features observed in patients. However, achieving face validity is more

challenging. This difficulty arises from the fact that certain distinct features of AS are not clearly replicated in the mouse model, or they may be subtle and easily overlooked without standardized behavioural paradigms. This discrepancy explains the conflicting reports regarding behaviour and the effects of potential treatments in AS mice across different laboratories (Daily et al., 2011)(M. C. Judson et al., 2021)(Allensworth, Saha, Reiter, & Heck, 2011)(Stoppel & Anderson, 2017).

Therefore, when evaluating treatments and therapies for AS, it is crucial to test their effectiveness in a preclinical trial setting while minimizing type I errors (false positives). This can be achieved through the use of standardized behavioural protocols that robustly capture behavioural phenotypes in AS mouse models, irrespective of the laboratory or experimenter involved. By doing so, the study outcomes will truly reflect the effectiveness of treatments in AS patients, ensuring a successful translation to clinical trials.

Predictive validity of animal models refers to the extent to which the model's response to an independent variable correlates with the response exhibited by humans exposed to the same variable (Belzung & Lemoine, 2011). This predictive ability is essential for the development of novel therapeutics for AS, as it allows for predictions regarding the effects of specific drugs or procedures on patients.

Developing a comprehensive and robust behavioural test battery for AS mouse models, capable of detecting subtle phenotypes that may otherwise go unnoticed, would significantly enhance the predictive validity of both existing and novel AS murine models. The contribution of behavioural testing in increasing our understanding of underlying pathology and in preclinical evaluation of potential treatments is undeniable (Saré, Lemons, & Smith, 2021).

Robust phenotypes observed in mouse models hold great promise as translational tools for discovering effective treatments for various disorders (Silverman, Yang, Lord, & Crawley, 2010).

Test batteries are effective in detecting behavioural changes induced by genetic manipulation of a species. Over the years, behavioural neuroscience has contributed a wide range of behavioural paradigms to assess murine behaviour and evaluate traits such as motor function, sensory processing, cognition, anxiety-like behaviours, and more (Brown, Stanford, & Schellinck, 2000).

2.10 **BEHAVIORAL PARADIGMS**

The study of animal behaviour provides valuable insights into the functioning of the nervous system, particularly when examining genetically modified mice. By dissecting a set of behaviours exhibited by these mice, researchers can uncover the role of the mutated gene and gain a better understanding of the underlying pathology (Saré et al., 2021). This is

essential for conducting preclinical testing of potential treatments and advancing our knowledge in the field. Robust phenotypes observed in mouse models hold great promise as powerful tools for translational research, enabling the discovery of effective treatments for various disorders (Silverman et al., 2010).

Behavioural test batteries have proven effective in detecting behavioural changes resulting from genetic manipulations in different species. Over the years, behavioural neuroscience has contributed a wide range of behavioural paradigms that evaluate various traits, including motor function, sensory processing, cognition, and anxiety-like behaviours, among others (Brown et al., 2000). However, it is possible for a single test to encompass multiple categories (for example the Open Field test which can be used to assess the motor function of the mice but can also be categorized as an anxiety-related test, depending on the parameters measured) (Kraeuter, Guest, & Sarnyai, 2019). This example highlights the complexity and interconnectedness of different behavioural domains in mice.

Some of the most commonly used and well-validated behavioural tests are summarized in **Figure 13**.

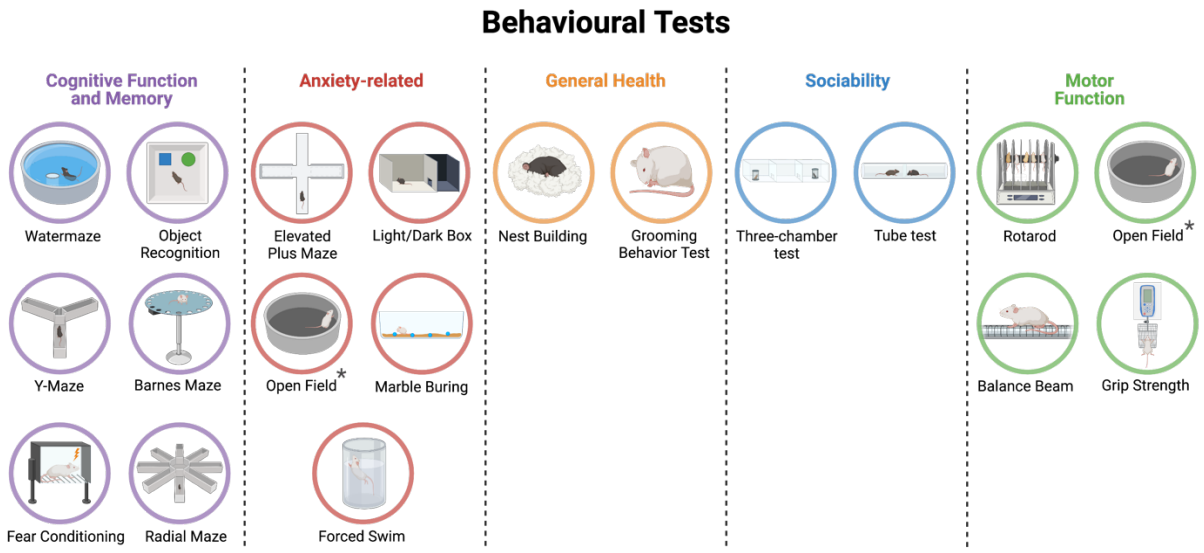


Figure 13 - Compilation of the most frequently employed and extensively validated behavioural tests conducted in mice. While categorizing some tests may pose challenges, they can be broadly grouped into the following categories: cognitive function and memory, anxiety-related behaviour, assessment of general health/innate behaviours, sociability, and motor function. An asterisk (*) denotes tests that belong to multiple categories, indicating their multifaceted nature.

In order to ensure reliable and interpretable results, it is important to follow a systematic approach when conducting behavioural tests. This often involves starting with an assessment

of overall general health to rule out any confounding factors and ensure that observed deficits are indeed a result of the genetic manipulation rather than a consequence of poor health or frailty. Additionally, the order of testing should progress from less stressful to more stressful tasks, considering that some tests may be influenced by prior experiences (Chadman et al., 2009).

The selection of specific behavioural tests and the experimental design depend on the research question at hand. In our study, we aimed to develop a robust tool for preclinical testing, as outlined in Chapters 3 and 5 of this thesis and validated in Chapter 4. To achieve this, we performed a series of behavioural tests in AS mice and their wild-type littermates. Only the tests that showed significant differences between the two groups were included in our behavioural test battery. Therefore, for the purpose of this general introduction, we will focus exclusively on these carefully chosen tests, namely the Accelerating Rotarod (RR), Open Field (OF), Marble Burying (MB), Nest Building (NB), Forced Swim (FST) tests, as well as susceptibility to audiogenic seizure tests.

2.10.1 **ACCELERATING ROTAROD TEST**

The Accelerating Rotarod (RR) test is a widely utilized method for evaluating motor coordination and balance in rodents (Rustay, Wahlsten, & Crabbe, 2003)(Bohlen, Cameron, Metten, Crabbe, & Wahlsten, 2009). This test involves an accelerating cylindrical rod with a diameter significantly smaller than the mouse's body length (see **Figure 14**) (Buitrago, 2004).

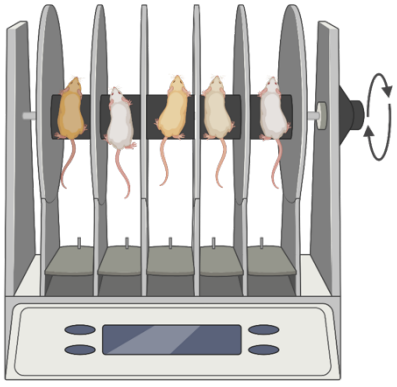


Figure 14 - Accelerated Rotarod test.

It comprises multiple trials conducted over several days to assess the mouse's ability to maintain balance, while subtle differences in motor learning can be observed by recording the time it takes for the animal to fall from the rotating rod (Jakkamsetti et al., 2021).

The key feature of the accelerating RR test is its ability to assess the coordination capability of animals, rather than merely measuring endurance. The acceleration aspect of the test

involves gradually increasing the speed of rotation from 4 to 40 rotations per minute over a 5-minute period (Deacon, 2013). To facilitate simultaneous testing of multiple mice, vertical barriers can be employed to divide the rod.

Compared to other behavioural tests evaluating motor performance, such as beam-walk and beam-balance, the RR test has demonstrated superior sensitivity in detecting motor deficits in rodents following mild to moderate central fluid percussion brain injury (Hamm, Pike, O'Dell, Lyeth, & Jenkins, 1994). This test is particularly effective in detecting cerebellar dysfunction in mice, given the cerebellum's role in regulating complex animal behaviours, including motor control and coordination, through communication with various brain regions (Sakayori et al., 2019) (Bohlen et al., 2009) (Shiotsuki et al., 2010)(Hamm et al., 1994). Studies have shown that cerebellar ablation negatively affects mouse performance in the RR test, further supporting its sensitivity to cerebellar function (Caston, Jones, & Stelz, 1995). Additionally, Nakamura et al. observed robust expression of c-Fos and jun-B (indirect markers of neuronal activity) in the cerebellar vermis and hemisphere of wild-type mice during days 1 and 5 of the accelerating rotarod test, indicating cerebellar involvement (Nakamura, Sato, Kitsukawa, Sasaoka, & Yamamori, 2015).

2.10.2 **OPEN FIELD TEST**

The Open Field (OF) test is a behavioural assessment that involves placing mice in a circular, wall-enclosed area with a central open space, creating an environment that elicits a sense of openness in the centre of the maze (see **Figure 15**) (Seibenhener & Wooten, 2015).

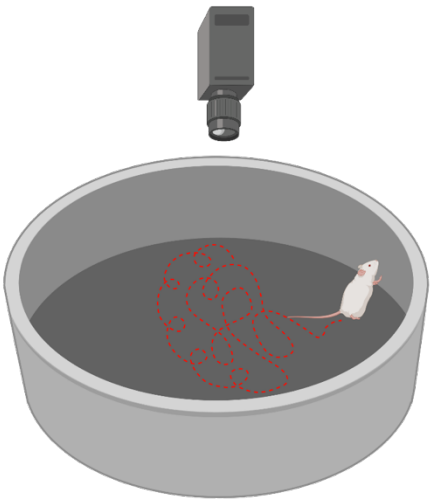


Figure 15 - Open Field test.

Automated tracking systems allow for the measurement of various parameters during the 10-minute test, including cumulative distance travelled, locomotor speed, and the time spent

exploring the centre versus other areas of the arena (Seibenhener & Wooten, 2015). These parameters offer a rapid assessment of overall activity and gross locomotor behaviour in the tested mice. Furthermore, they provide insights into the conflicting drives of mice to explore new environments while avoiding bright or exposed areas (Sturman, Germain, & Bohacek, 2018). Analysing the exploration patterns of genetically modified mice in the open field allows for the evaluation of their emotional reactivity to novel and open environments, serving as an indicator of anxiety-related behaviours (Gould, 2009).

Research suggests that the regulation of this behaviour involves the basolateral amygdala, as exposure to an open-field arena leads to increased expression of c-Fos, a marker of neuronal activity, in this specific brain region (Hale et al., 2008). Additionally, the hippocampus is believed to play a role in suppressing exploratory behaviour in response to anxiety-provoking (Sturman et al., 2018). In wild-type mice, exploration of a novel environment triggers an increase in c-Fos expression in the reward circuitry and the hippocampus (Bourgeois et al., 2012).

2.10.3 **MARBLE BURYING TEST**

The Marble Burying (MB) test involves placing 20 glass marbles in a polycarbonate cage with 4 cm of bedding material, arranged evenly in five rows of four (see **Figure 16 A-B**).

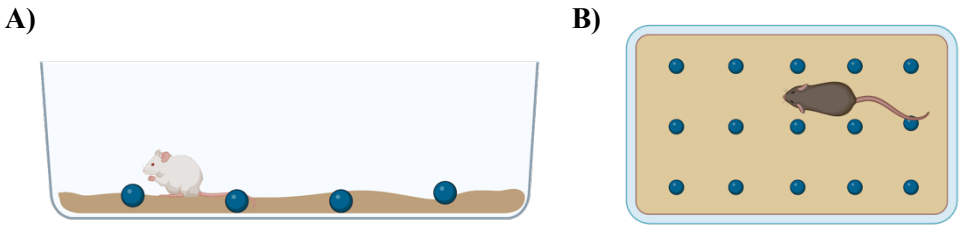


Figure 16 - Marble Burying test. (**A**) Lateral view. (**B**) Top view.

The mouse is placed in the cage, and after a 30-minute period, the number of marbles covered by at least 50% is quantified (Angoa-Pérez, Kane, Briggs, Francescutti, & Kuhn, 2013).

The marble burying test was originally proposed as a test to assess anxiety and compulsive-like behaviours, since the burying behaviour is inhibited, in a dose dependent manner, by anti-anxiolytic agents (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986)(Treit, Pinel, & Fibiger, 1981) and anticompulsive/SSRIs antidepressant drugs (Uday, Pravinkumar, Manish, & Sudhir, 2007)(Egashira et al., 2018).

While some studies still consider the MB test as an indicator of anxiety (Nicolas, Kolb, & Prinssen, 2006)(Jarrar et al., 2022), relying on the premise that mice perceived the marbles as an aversive stimulus, eliciting “defensive burying”, there has been ongoing debate

regarding the anxiety-like interpretation of marble-burying behaviour (Wolmarans, Stein, & Harvey, 2016)(Thomas et al., 2009)(Jimenez-Gomez, Osentoski, & Woods, 2011).
The MB behaviour is thought to reflect the functioning of the hippocampus and cortical regions (Deacon, 2006b)(Deacon, Croucher, & Rawlins, 2002)(Deacon & Rawlins, 2005)(Teissier et al., 2020) and it was shown that decreased frontal network excitability could contribute to decreased performance in this test (Dasilva et al., 2020). The involvement of the endocannabinoid system, via activation of CB1 (Cannabinoid receptor type 1) receptor, has also been implicated in this behaviour (Gomes, Casarotto, Resstel, & Guimarães, 2011).

2.10.4 **NEST BUILDING TEST**

The Nest Building (NB) test involves providing individually housed mice with pre-weighed pressed cotton squares just before the start of the dark cycle (Deacon, 2006a) The next morning, the unused material is weighed, serving as a measure of the mouse's nesting ability (see **Figure 17**) (Gaskill, Karas, Garner, & Pritchett-Corning, 2013)(Neely, Pedemonte, Boggs, & Flinn, 2019).

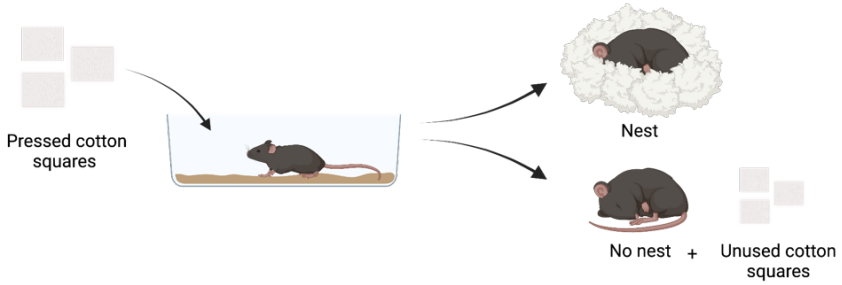


Figure 17- Nest Building test.

The ability to build a nest is crucial for the survival of feral mice as it provides protection against predators, aids in heat conservation, and serves as a safe shelter for their offspring (Latham & Mason, 2004)(Gaskill et al., 2013). Even in a relatively safe laboratory environment, mice exhibit a strong motivation to build nests when provided with appropriate materials (Hess et al., 2008). Since nest building is an innate behaviour, alterations in nesting behaviour can indicate changes in well-being or compromised biological functioning (Gaskill et al., 2013)(Neely et al., 2019). Nest building deficits have been observed in numerous models of neuropsychiatric disorders (Thompson et al., 2019)(Timothy M DeLorey, Sahbaie, Hashemi, Homanics, & Clark, 2008)(Gavrilovici et al., 2021) and these deficits are correlated with lesions in the Medial Prefrontal Cortex (mPFC) and hippocampus (Deacon et al., 2002)(Deacon, Penny, & Rawlins, 2003)(Cunningham et al., 2003) in the absence of any motor impairments.

2.10.5 **FORCED SWIM TEST**

In the Forced swim test (FST), mice are placed in a cylindrical transparent tank filed with water from which they cannot escape nor touch the bottom with their hind paws or tail (Porsolt, Bertin, & Jalfre, 1977) (see **Figure 18**).

Figure 18 - Forced Swim test.

The duration of active swimming or movement necessary to keep their head above water (termed immobility time) is measured. The FST has been utilized to assess depression-like behaviours in mouse models and mice exposed to conditions that contribute to depression in humans. These studies have observed increased immobility time compared to healthy, neurotypical mice, and the administration of antidepressants has been found to delay the onset of immobility. This has led to the assumption that immobility in the FST reflects a failure of persistence in escape-directed behaviour (Bagot et al., 2015)(Hao, Ge, Sun, & Gao, 2019)(Yankelevitch-Yahav, Franko, Huly, & Doron, 2015)(Cryan, Markou, & Lucki, 2002)(Commons, Cholanians, Babb, & Ehlinger, 2017). Despite ongoing debates surrounding this interpretation, as factors like age and long-term handling have been shown to influence immobility even in the absence of drug administration (Anyan & Amir, 2018)(Stanford, 2020)(de Kloet & Molendijk, 2016)(Gorman-Sandler & Hollis, 2022), the FST remains widely used for preclinical assessments of antidepressant drug efficacy. Furthermore, the FST has also shown relevance to other stress-related disorders, such as autism. A rodent model of autism exhibited increased immobility time, which was found to result from a hyperactive mesocortical dopamine system. Notably, AS mice also exhibit increased dopamine release in the mesolimbic pathway (Riday et al., 2012).

2.10.6 **SUSCEPTIBILITY TO AUDIOGENIC SEIZURES**

Audiogenic seizures are classified as reflex seizures that are triggered in susceptible animals by high-intensity sound stimuli, typically exceeding 100 decibels (Lazarini-Lopes, Do Val-da Silva, da Silva-Júnior, Cunha, & Garcia-Cairasco, 2021)(see **Figure 19**).

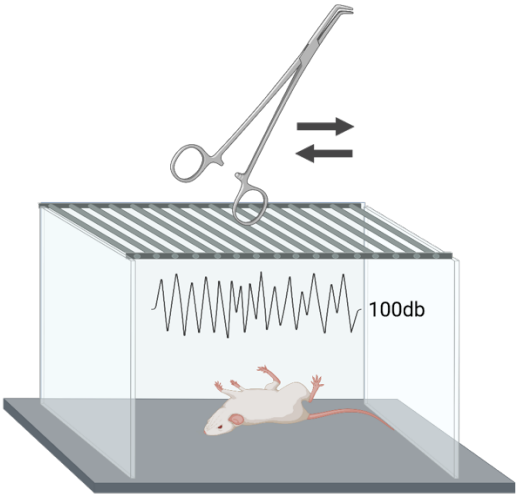


Figure 19 - Susceptibility to audiogenic seizures test.

In rodents, these seizures usually begin with wild running, after the sound onset, which could be regarded as an intense panic reaction progressing to a tonic–clonic phase mimicking generalized seizures in humans (Fedotova, Surina, Nikolaev, Revishchin, & Poletaeva, 2021). During the tonic-clonic seizures, observable behaviours include a series of back arching tonus with piloerection, opened jaws, and subsequent partial and generalized clonic seizures accompanied by severe clonic spasms and vocalization (N. Garcia-Cairasco, Terra, & Doretto, 1993). In more severe cases, the generalized myoclonus and tonic phase may be accompanied by respiratory arrest leading to death (Musumeci et al., 2000).

Research by *Garcia-Cairasco et al.* demonstrated that the afferent pathway for audiogenic seizures involves cochlear activation by sound, followed by hyperactivation of brainstem auditory structures, resulting in a disruption of sensory-motor integration (N. Garcia-Cairasco et al., 1993). While several forebrain structures such as the cortex, amygdala, and hippocampus appear to be involved in the susceptibility to audiogenic seizures (Lazarini-Lopes et al., 2021) the inferior colliculus (IC) circuits play a central role in the genesis and maintenance of sound-induced seizures (Norberto Garcia-Cairasco, 2002) (N. Garcia-Cairasco et al., 1993). Being the main structure involved in the development of seizures in the audiogenic models, the IC is considered the epileptogenic nucleus.
Numerous studies have provided evidence for the involvement of GABAergic systems and abnormalities in GABAergic neurotransmission in audiogenic seizures (Ribak, 2017)(Faingold,

Gehlbach, & Caspary, 1986), which could partially explain the audiogenic seizure susceptibility in Angelman syndrome mouse models (M. C. C. Judson et al., 2016).
It has been observed that mice of certain genetic backgrounds display a higher susceptibility to generalized audiogenic seizures compared to other genetic backgrounds (Zhu, Chen, Lin, & Liu, 2022)(Seyfried, Yu, & Glaser, 1980)(Jawahar et al., 2011). Similar findings have been reported in AS mice (Jiang et al., 1998)(van Woerden et al., 2007)(M. C. Judson et al., 2014).

The study by *Zhu et al.* suggests that differences in the expression profiles of mRNA and long noncoding RNA (lncRNA) in the brainstem of these mice could potentially explain the varying susceptibility to audiogenic seizures observed among different genetic strains (Zhu et al., 2022). However, it is worth noting that the exploration of this aspect in the context of Angelman syndrome remains unexplored.

2.11 **MURINE CRITICAL PERIODS IN DEVELOPMENT**

Critical periods (CP) refer to specific and limited time windows during brain development when heightened plasticity occurs. During these periods, environmental input, sensory experiences, and gene expression play crucial roles in the proper formation of brain circuits. If these factors are absent or impaired, it can lead to permanent compromises in brain function (White, Hutka, Williams, & Moreno, 2013). The irreversible changes in brain function and structure observed during critical periods are manifested in the development of specific behaviours.
The closure of a critical period is facilitated by molecular mechanisms that limit plasticity and promote the permanent consolidation of brain structures. Once the critical period is closed, even in the presence of relevant stimuli, further alterations or corrections are prevented.
Our understanding of critical periods primarily comes from studies on the developing visual system in animal models. In these models, the eyes can be sutured, depriving them of normal visual experience during a specific early postnatal period. This alteration leads to irreversible changes in neuronal connections in the visual cortex. However, if the same procedure is performed in adult animals, beyond the critical period, it has no effect on the responses of neurons in the visual cortex. This phenomenon, known as ocular dominance plasticity, represents the most extensively studied and well-characterized model of critical period plasticity in the mammalian brain (Wiesel & Hubel, 1965)(Levelt & Hübener, 2012).

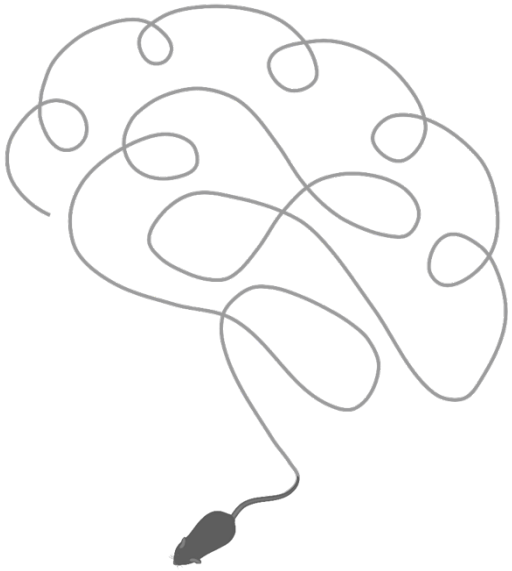
In the case of Angelman syndrome, the absence of UBE3A is known to cause the condition, but it remains unclear during which stages of neurodevelopment this gene needs to be present to prevent the emergence of abnormal behavioural manifestations. It is also uncertain whether UBE3A is required for brain function throughout life.

By identifying the critical windows during development that contribute to the establishment of Angelman syndrome-related behaviours, we can gain insights into the role of UBE3A in brain development. This knowledge will contribute to the design of future clinical trials aimed at developing treatments for this debilitating disorder.

CHAPTER 3

UBE3A REINSTATEMENT IDENTIFIES DISTINCT DEVELOPMENTAL WINDOWS IN A MURINE ANGELMAN SYNDROME MODEL

(J Clin Invest. 2015 May 1; 125(5): 2069–2076.)



UBE3A REINSTATEMENT IDENTIFIES DISTINCT DEVELOPMENTAL WINDOWS IN A MURINE ANGELMAN SYNDROME MODEL

In preclinical studies, therapeutic interventions such as administration of Topoisomerase I inhibitor drugs, antisense oligonucleotides (ASOs), artificial transcription factors (ATFs), and adeno-associated virus (AAV)-mediated delivery of exogenous *Ube3a* gene have successfully reinstated *Ube3a* expression. However, the key question remains: *Can reinstating Ube3a expression lead to improvements in AS symptoms?*

Therefore, the following study, published in the Journal of Clinical Investigation (JCI), aims to determine the effectiveness of reinstating *Ube3a* expression in neurons as a potential treatment for AS.

Specific objectives of this study include:

- Identify the developmental stages where reinstating *Ube3a* is most effective in improving motor deficits, anxiety, repetitive behaviour, and epilepsy associated with AS.
- Investigate whether there are critical windows during development for reversing cellular-level plasticity deficits.
- Assess the efficacy of targeting downstream mechanisms, such as antiepileptic drugs (AEDs), compared to postnatal *Ube3a* gene reactivation in preventing seizures.

The study findings hold promise for advancing our understanding of AS and guiding future clinical trials to improve treatments for individuals with this condition.

UBE3A REINSTATEMENT IDENTIFIES DISTINCT TREATMENT WINDOWS IN ANGELMAN SYNDROME MODEL MICE

J Clin Invest. 2015 May 1; 125(5): 2069–2076

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

Angelman syndrome (AS) is a severe neurodevelopmental disorder, caused by the lack of maternal *Ube3a* gene expression. Previous studies have demonstrated the feasibility of therapeutic strategies to activate the paternal *Ube3a* allele, which holds great promise for developing treatments. However, a recent study showed that adult pharmacological *Ube3a* gene reactivation fails to rescue the vast majority of neurocognitive phenotypes in AS model mice. Here we performed a systematic study to investigate the possibility to achieve neurocognitive rescue by reinstating *Ube3a* at different time points in development. Using temporally-controlled reactivation of the *Ube3a* gene in a novel AS mouse model, we reveal distinct treatment windows for rescuing AS-relevant phenotypes. For example, motor deficits can be rescued by gene reinstatement in adolescent mice, whereas anxiety, repetitive behaviour, and epilepsy can only be rescued by gene reinstatement during early life. These findings are critical for guiding future clinical trials and suggest that early *Ube3a* reinstatement may be necessary to prevent or rescue most AS phenotypes.

Introduction

Children with AS are typically diagnosed within the first year of life, due to developmental delay. The most prominent symptoms include motor impairments, epilepsy, intellectual disability, and absence of speech (1). AS is caused by loss of function of the maternally-inherited *UBE3A* allele. In neurons, the maternally-inherited *UBE3A* allele is the only active allele, since the paternally-inherited *UBE3A* allele is silenced through cell-type specific imprinting. This imprinting results in the allele-specific and neuronally-restricted expression of

a large antisense RNA transcript (*UBE3A-ATS*), which selectively interferes with paternal *UBE3A* transcription through a cis-acting mechanism (2-6).

There is currently no effective treatment for AS, but the unique silencing mechanism of the paternal *UBE3A* allele holds great promise for developing novel therapeutic strategies. Two recent studies have shown that the paternal *UBE3A* allele can be pharmacologically reactivated (7,8), which offers a unique molecular target with high clinical potential for the treatment of AS.

Notably however, activation of the paternal *Ube3a* gene in adulthood appears insufficient to rescue the majority of neurocognitive phenotypes in the AS mouse model. The failure of phenotypic rescue in adult AS mice might have resulted from the incomplete reinstatement of UBE3A expression (35-47% of wild-type levels), or alternatively, because a neurocognitive rescue by UBE3A reinstatement requires early therapeutic intervention (8). Hence, for such a treatment strategy to be successful in the clinic, it is imperative to know whether there is a critical time window during which a disease-modifying therapy would be effective. This is particularly relevant for early-onset disorders, such as AS, whose causative gene is highly expressed in developing neural circuits.

Despite extensive knowledge of critical periods for the development of sensorimotor networks, much less is known about the critical periods for complex behaviours in neurodevelopmental disorders (9,10). An inducible mouse model for Rett syndrome showed that adult activation of the *Mecp2* gene could rescue the behavioural alterations and synaptic plasticity deficits, suggesting a broad window of therapeutic opportunity (11). In contrast, adult reactivation of the *Syngap1* gene in a mouse model of intellectual disability and autism did not reverse any of the core behavioural deficits related to anxiety and behavioural flexibility (12). However, no previous study has ever been performed to systematically investigate the influence of developmental critical periods on the ability to rescue disease-relevant behavioural phenotypes. Here, we explore the effect of gene reactivation across multiple developmental windows in a novel mouse model for AS. Our results demonstrate an essential role for *Ube3a* in neurodevelopment and define a critical period for therapeutic intervention during which *Ube3a* gene reactivation ameliorates the neurocognitive impairments of AS model mice.

Results

Generation and characterization of a conditional Ube3a mutant.

To examine whether the therapeutic benefit of *Ube3a* gene reactivation is dependent upon the developmental stage at which gene expression is restored, we generated a conditional AS mouse model to allow temporally-controlled reactivation of the *Ube3a* gene upon Cre-mediated deletion of a floxed transcriptional stop cassette inserted within intron 3 by

homologous recombination (*Ube3a*$^{Stop/p+}$) (**Supplemental methods** and **Supplemental Figure 1**).

We first investigated the efficiency of the transcriptional stop-cassette to block *Ube3a* expression. Female *Ube3a*$^{Stop/p+}$ mice were crossed to a constitutive Cre-expressing line with an early embryonic onset of recombination (13) (**Figure 1A).**

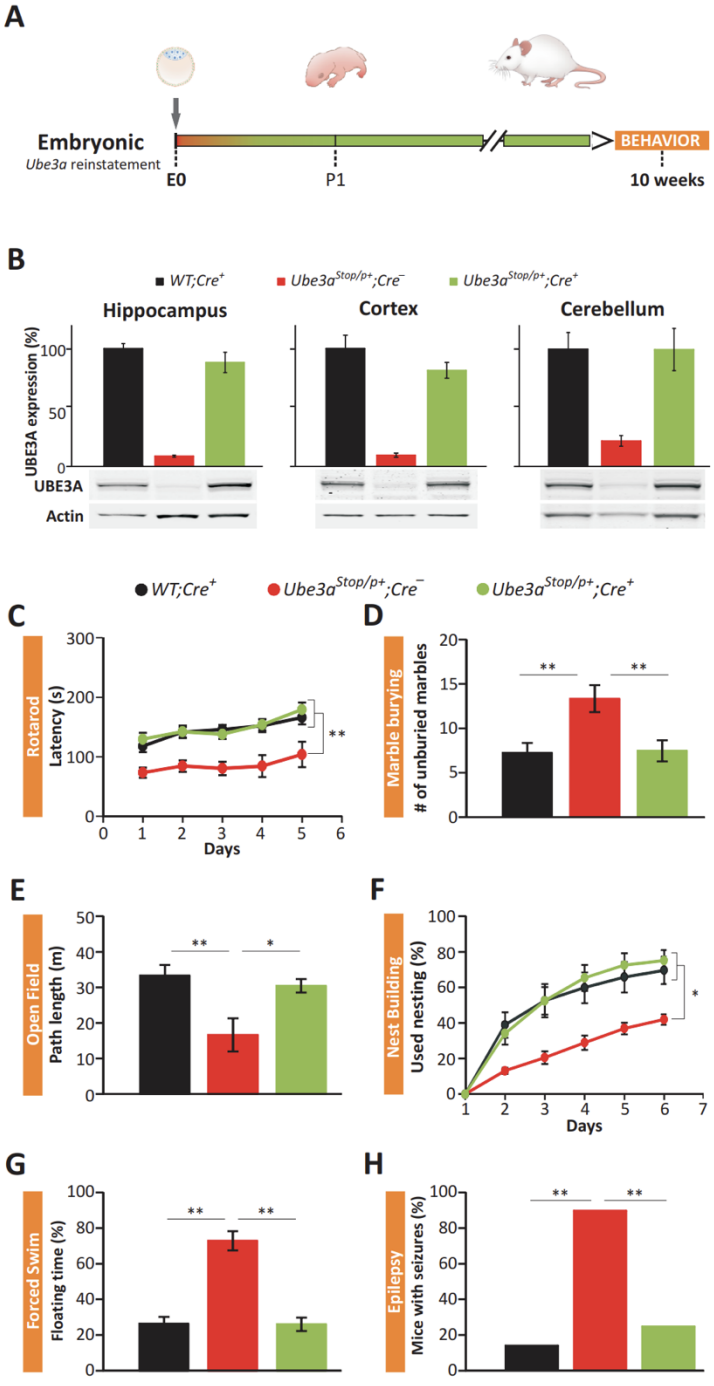


Figure 1. Embryonic reactivation of *Ube3a* expression rescues AS-like behavioural phenotypes. (**A**) Schematic representation of *Ube3a* reactivation (indicated by the grey arrow) during mouse embryonic development and time point of behavioural testing. (**B**) Western blot analysis of hippocampus (n=4 per genotype), cortex (n=5) and cerebellum (n=5) from *Ube3a*$^{Stop/p+}$ and wild-type

(WT) littermates crossed with an embryonically active cre-line. (**C-H**) *Ube3a*$^{Stop/p+}$*;Cre*$^{-}$ mice show robust behavioural AS-relevant phenotypes, which can be fully rescued by embryonic reactivation of the *Ube3a* gene in the *Ube3a*$^{Stop/p+}$*;Cre*$^{+}$ mice (see Supplemental Methods for the exact number of mice used for each behavioural paradigm). All data represent mean ± S.E.M. An ANOVA with genotype as independent variable was used for statistical comparisons. A significant effect of genotype was identified in all behavioural tests (See **Supplemental Table S1**). Asterisks denote Bonferroni's post-hoc analysis: * P<0.05; **P<0.01, ***P<0.001.

Because the paternal *Ube3a* allele is epigenetically silenced, mice with a maternally inherited stop-cassette without Cre expression (*Ube3a*$^{Stop/p+}$*;Cre*$^{-}$ mice) showed a severe loss of UBE3A protein (also known as E6-Associated Protein, E6AP), comparable to the reduction in UBE3A expression observed in the traditional AS mouse model with a maternally-inherited deletion of *Ube3a* (*Ube3a*$^{m-/p+}$) (**Figure 1B**, see also **Figure 2D** for a comparison to *Ube3a*$^{m-/p+}$ mice). Immunohistochemical staining of *Ube3a*$^{Stop/p+}$*;Cre*$^{-}$ brain slices was indistinguishable from *Ube3a*$^{m-/p+}$ mice (**Supplemental Figure 1B**). These results confirm that the floxed stop-cassette is highly effective in blocking transcription of the maternal *Ube3a* allele, while preserving the normal epigenetic silencing of the paternal allele.
Next, we investigated the efficiency of *Ube3a* reactivation upon Cre-mediated deletion of the floxed stop cassette. UBE3A protein levels were reinstated in *Ube3a*$^{Stop/p+}$*;Cre*$^{+}$ mice to 89% of wild-type (WT) levels in the hippocampus, 82% in the cerebral cortex, and 99% of WT levels in the cerebellum. Furthermore, the subcellular distribution of UBE3A was indistinguishable between *Ube3a*$^{Stop/p+}$*;Cre*$^{+}$ and WT*;Cre*$^{+}$ mice, thereby validating the functionality of the *Ube3a* reactivation method (**Figure 1B** and **Supplemental Figure 1B**).

Early embryonic gene reactivation prevents the manifestation of AS phenotypes.
Impaired motor coordination, autistic traits, anxiety, and epilepsy are hallmarks of AS patients, for which analogous phenotypes are well-established in AS model mice (3,14-16). As expected based on the loss of *Ube3a* expression, *Ube3a*$^{Stop/p+}$*;Cre*$^{-}$ mice exhibited significant alterations in rotarod performance (**Figure 1C**), marble-burying (**Figure 1D**), open field exploration (**Figure 1E**), nest building (**Figure 1F**), and the audiogenic seizure threshold (**Figure 1H**), all of which are also present in the classical *Ube3a*$^{m-/p+}$ mouse model of AS (3,14). In addition, we identified a novel and highly robust phenotype in the forced swim test present in both the classical *Ube3a*$^{m-/p+}$ mutant (data not shown) and the conditional *Ube3a*$^{Stop/p+}$ AS mouse model (**Figure 1G**).
Consistent with the therapeutic potential of *Ube3a* gene reactivation, *Ube3a*$^{Stop/p+}$*;Cre*$^{+}$ mice exhibited a full rescue of all of these neurological and behavioural abnormalities, thereby confirming that embryonic reactivation of UBE3A protein expression is sufficient to prevent the

manifestation of AS phenotypes across multiple domains (**Figure 1** and **Supplemental Table S1**).

Gene reactivation in juvenile, adolescent, and adult animals reveal the presence of distinct critical periods.

We next crossed the *Ube3a*$^{Stop/p+}$ mice with a tamoxifen-inducible *Cre*$^{ERT+}$ mouse line (17) to determine the efficacy of *Ube3a* reactivation at later stages of postnatal development. In particular, we induced *Ube3a* gene reactivation at 3 weeks ('juvenile mice'), 6 weeks ('adolescent mice'), and 14 weeks of age ('adult mice'), with behavioural testing performed at a mean age of 16 weeks, 22 weeks and 28 weeks respectively (**Figure 2A**).

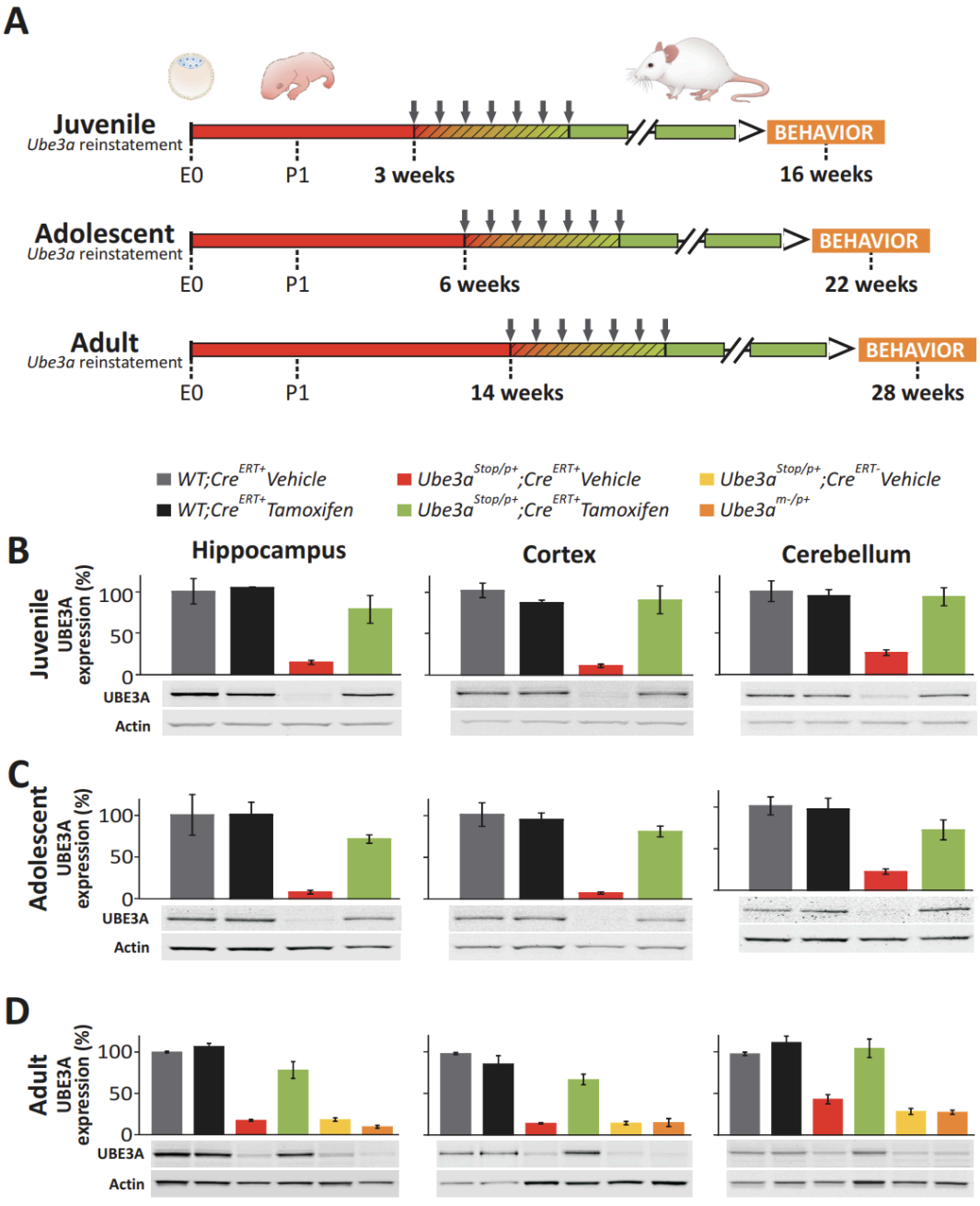


Figure 2. Molecular analysis of *Ube3a*$^{Stop/p+}$;*Cre*$^{ERT+}$ mice reveals successful reactivation of the maternal *Ube3a* gene upon tamoxifen induction. (**A**) Schematics representing *Ube3a* reactivation achieved by tamoxifen treatment (grey arrows) in each experimental group. (**B-D**) *Ube3A*$^{Stop/p+}$;*Cre*$^{ERT+}$ mice with postnatally induced gene reactivation express UBE3A at levels comparable to early embryonic gene reactivation in hippocampus (Juvenile n=3 per genotype; Adolescent n=4 per genotype; Adult n=4 per genotype), cortex (Juvenile n=3 per genotype; Adolescent n=4 per genotype; Adult n=3-4 per genotype) and cerebellum (Juvenile n=3 per genotype; Adolescent n=4 per genotype; Adult n=5 per genotype). Data represent mean ± S.E.M.

Across these developmental time points, UBE3A protein levels in tamoxifen-treated *Ube3a*$^{Stop/p+}$;*Cre*$^{ERT+}$ mice were reinstated to 70-100% of wild-type levels, which is comparable to those achieved by early embryonic reactivation (**Figure 2B-D**, **Supplemental Figure 2** and **Figure 1B**). Importantly, UBE3A expression in vehicle-treated *Ube3a*$^{Stop/p+}$;*Cre*$^{ERT+}$ mice were also similar to those observed in *Ube3a*$^{Stop/p+}$;*Cre*$^{-}$ and *Ube3a*$^{m-/p+}$ mice (**Figure 2D**), thereby demonstrating the tight control of gene reactivation.

Juvenile reactivation of *Ube3*a resulted in a full rescue of the motor coordination deficit. In contrast, adolescent reactivation only partially rescued motor coordination, while no improvement was observed with adult reactivation (**Figure 3A**). Together, these findings identify a critical period for *Ube3a*-dependent motor development, which closes between 3 and 6 weeks postnatally.

The critical window for rescuing motor coordination deficits was distinct from the window for rescuing autism- and anxiety-related phenotypes such as the marble burying task, open field test, nest building test and forced swim test, which could be rescued by embryonic reactivation (**Figure 1D-G** and **Supplemental Table S1**) but not upon juvenile, adolescent, or adult reactivation (**Figure 3B-E** and **Supplemental Table S2**).

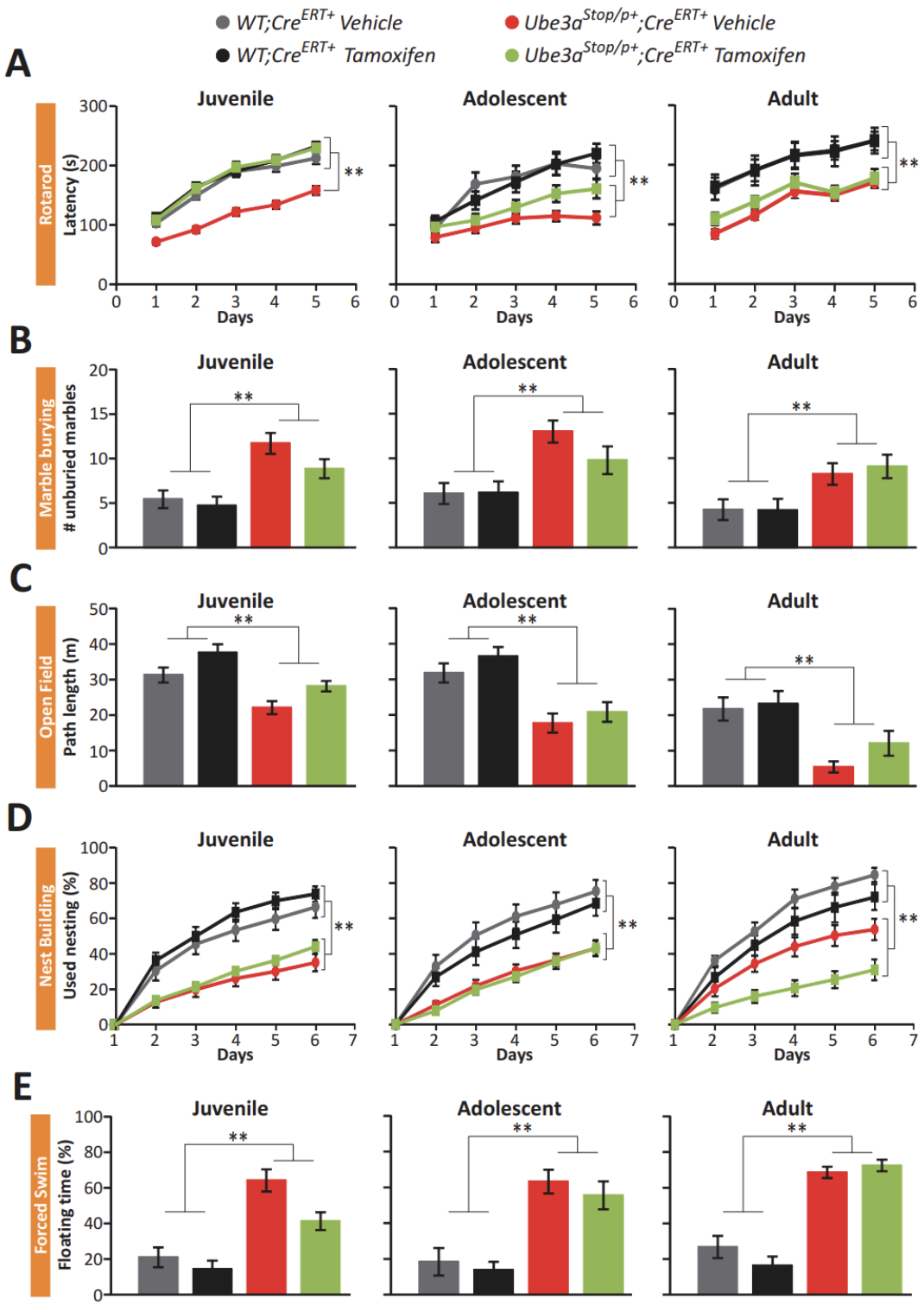
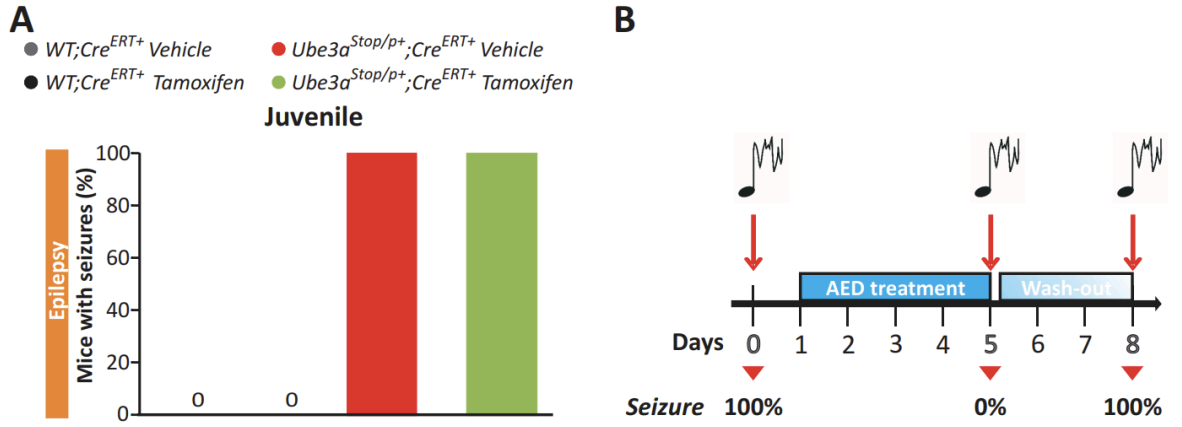


Figure 3. Postnatal reactivation of *Ube3a* expression reveals a critical period for behavioural rescue. (**A-E**) Behavioural testing of *Ube3a*$^{Stop/p+}$ and WT littermates treated with either vehicle or tamoxifen shows distinct critical periods for recovery of the behavioural deficits (see Supplemental Methods for the exact number of mice used for each behavioural paradigm). All data represent mean ± S.E.M. A two-way ANOVA or a repeated measures two-way ANOVA with genotype and treatment as independent variables was used for statistical comparisons. A significant effect of genotype was

identified in all behavioural tests (See **Supplemental Table S2**). Asterisks denote genotype significance: * P<0.05; **P<0.01.

The epilepsy phenotype was also refractory to postnatal *Ube3a* reactivation, as seizures persisted despite gene reactivation at a juvenile age (**Figure 4A**). Next, we sought to confirm whether the epilepsy phenotype in AS mice is differentially responsive to treatment with anti-epileptics (AEDs) in mice with *Ube3a* reactivation. Adult (>8 weeks) *Ube3a*$^{m-/p+}$ and *Ube3a*$^{Stop/p+}$;*Cre*$^{ERT-}$ mice were treated for 5 days with either valproate or clonazepam using a within-subjects design. Prior to treatment, every AS mouse examined showed audiogenic seizures (**Figure 4B**). However, after 5 days of treatment with either of these AEDs, seizures were completely prevented in all mice. Moreover, three days after the cessation of AED treatment (wash-out period), all AS mice again showed audiogenic seizures (**Figure 4B**). These data confirm that seizures can be successfully treated in adult AS mice using conventional AEDs, but that they are nevertheless insensitive to postnatal *Ube3a* reactivation. To investigate the extent to which postnatal *Ube3a* reactivation is able to rescue electrophysiological phenotypes, we measured hippocampal long-term potentiation (LTP), a form of synaptic plasticity required for experience-dependent neurodevelopment. Intriguingly, we observed full recovery of hippocampal long-term potentiation (LTP) following *Ube3a* gene reactivation at all time points examined (**Figure 4C-D** and **Supplemental Table S2**), indicating the absence of a critical period window for rescue of this important cellular phenotype.



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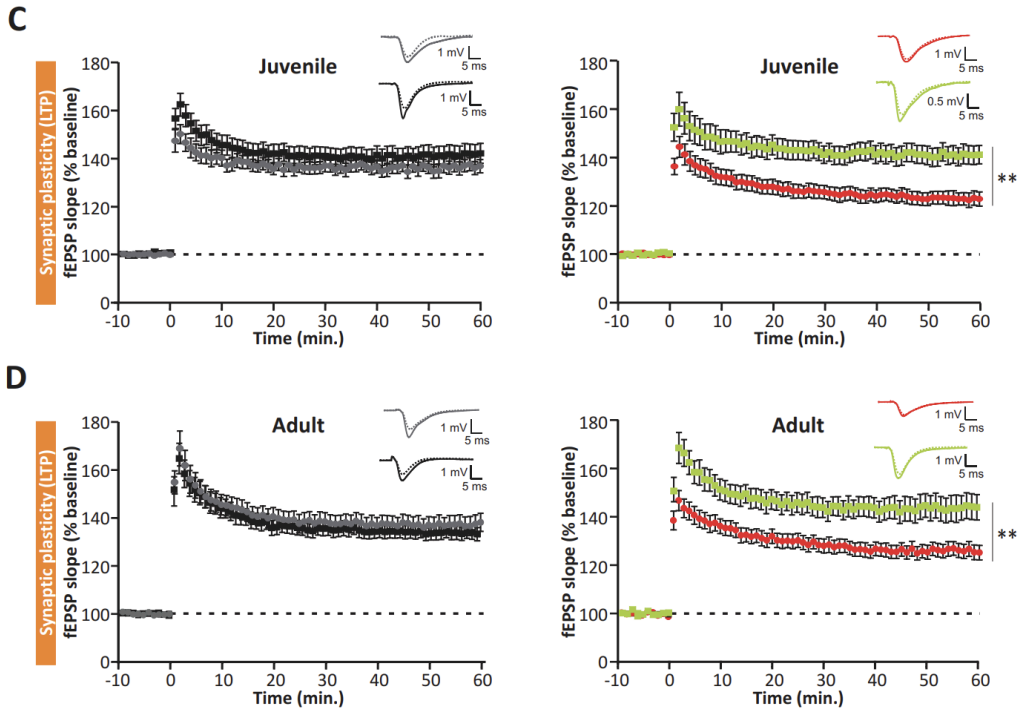

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Figure 4. *Ube3a* reactivation in juvenile animals does not recover epilepsy susceptibility but Schaffer collateral-CA1 LTP is fully recovered. (**A**) Epilepsy susceptibility in *Ube3A*$^{Stop/p+}$;*Cre*$^{ERT+}$ mice persists after *Ube3a* gene reactivation at a juvenile age. (**B**) The induction of tonic-clonic seizures induced by audiogenic stimulation (indicated by the red arrows) is efficiently treated by administration of antiepileptic drugs (AED; blue rectangle illustrates treatment administration and wash-out period) in adult *Ube3a*$^{Stop/p+}$;*Cre*$^{-}$ (n=2) and *Ube3a*$^{m-/p+}$ (n=12) mice. Seizures reappeared 3 days after cessation of treatment. Percentage indicates the number of mutant mice that developed seizures upon audiogenic stimulation (see **Supplemental Methods** for more experimental details). (**C**) Hippocampal plasticity deficit as measured by Long-term Potentiation (LTP) in mutant mice is ameliorated upon gene reactivation at both juvenile and (**D**) adult ages. Data represent mean ± S.E.M. A two-way ANOVA with genotype and treatment as independent variables was used for statistical testing. All tests showed a significant effect of genotype (see also **Supplementary Table 2** for statistical comparisons and **Supplemental Methods** for the exact number of slices and mice used in the electrophysiology experiments). * P<0.05; ** P<0.01.

Partial gene reactivation in newborn animals rescues the motor coordination and open field phenotype.

With the notable exception of motor coordination and hippocampal LTP, our data suggest that UBE3A is required during a critical period between early embryogenesis and the third postnatal week in order to prevent a wide spectrum of AS-like deficits. To further refine the critical period window, we next induced gene reactivation immediately following birth by

administering tamoxifen to lactating dams (**Figure 5A**). This method of tamoxifen administration resulted in a moderately reduced efficacy of *Ube3a* reactivation, yielding 44%, 34%, and 63% of WT UBE3A levels respectively in the hippocampus, cortex, and cerebellum (**Figure 5B**). Notably however, even with a reduced level of UBE3A reactivation, motor coordination was entirely rescued and performance in the open field test was significantly improved (**Figure 5C**). However, AS-like deficits persisted in the other behavioural paradigms, suggesting that the *Ube3a*-dependent neurodevelopmental critical period for autism-related phenotypes in AS might not extend significantly beyond birth. Alternatively, given that we achieved only partial reactivation during the neonatal period, it remains distinctly possible that functional plasticity may extend beyond three weeks of age, but is only evident with a higher efficiency of *Ube3a* reactivation.

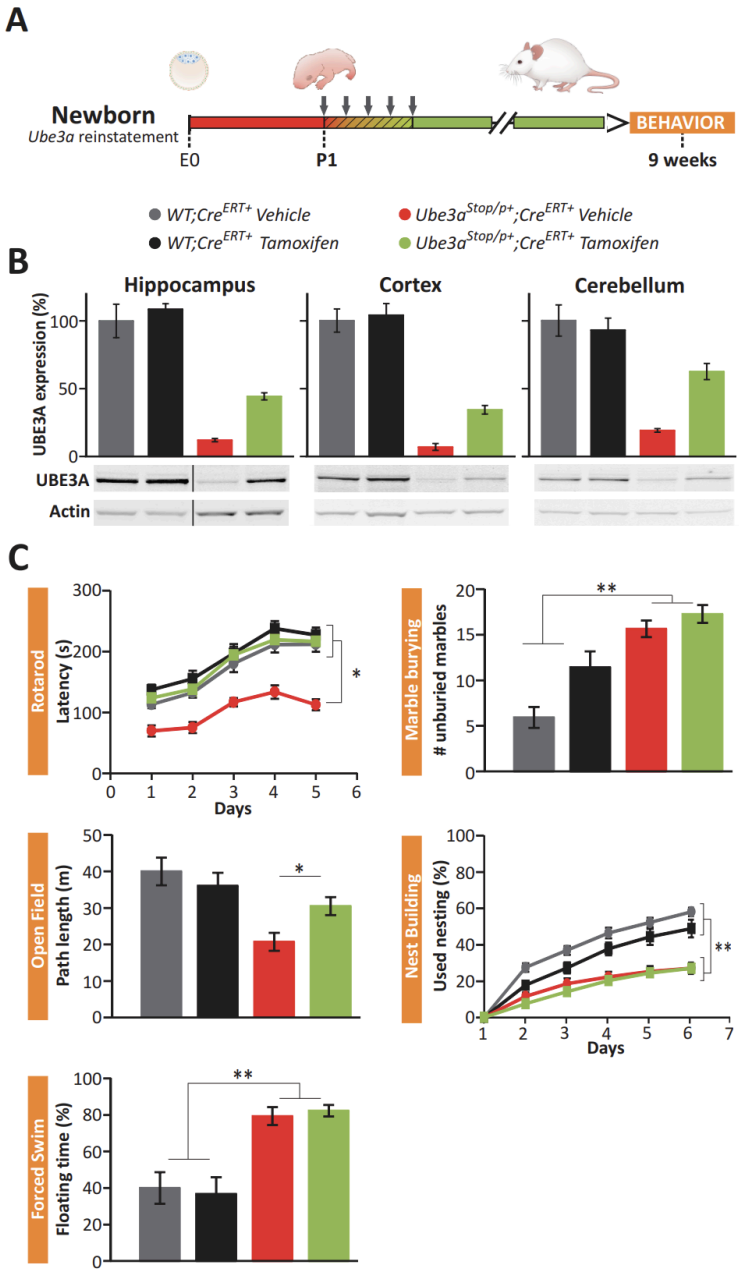

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Figure 5. Partial reactivation of *Ube3a* expression during the first postnatal week attenuates the motor coordination and the open field deficits. (**A**) Schematics representing *Ube3a* reactivation achieved by tamoxifen administration (grey arrows) to the lactating dams starting at the day of delivery. (**B**) Western blot analysis of UBE3A expression in hippocampal (n=4 per genotype), cortical (n=5) and cerebellar (n=5) tissues of mutant mice and their WT littermates. The thin black line on the hippocampus WB indicates non-contiguous samples run on the same gel. (**C**) Rescue of the accelerating rotarod and open field impairments (see **Supplemental Methods** for the exact number of mice used for each behavioural paradigm). All data represent mean ± S.E.M. A two-way ANOVA or a repeated measures two-way ANOVA with genotype and treatment as independent variables was used for statistical comparisons. A significant effect of genotype was identified in all behavioural tests (see **Supplemental Table S2** for statistical comparisons). * P<0.05; ** P<0.01.

Discussion

Our results demonstrate an essential role for *Ube3a* in neurodevelopment and define critical periods during which *Ube3a* gene reactivation can ameliorate AS-like phenotypes. In particular, the window for improving motor coordination extends furthest into postnatal development, whereas the autism and anxiety-related phenotypes appear to be established much earlier. In contrast, at the cellular level, there appears to be no critical window for reversing plasticity deficits. The finding that LTP could be fully recovered at all ages is consistent with previous findings showing that the hippocampal LTP deficit in adult AS mice is reversible upon acute pharmacological treatment with an ErbB inhibitor or with Ampakine cognitive enhancers (18,19). However, expression of UBE3A in adult *Ube3a*$^{m-/p+}$ mice through a viral-mediated approach, only partially recovered synaptic plasticity (20). This apparent discrepancy likely reflects the limited efficiency of *in vivo* virally-mediated neuronal transduction, compared with the more homogeneous biodistribution of systemically-administered pharmacological compounds.

Importantly, and consistent with our findings, no studies in AS mice have ever demonstrated the successful rescue of the behavioural phenotypes related to anxiety and repetitive behaviour upon adult treatment, despite multiple efforts using a variety of different interventions. In addition, it is notable that the behavioural deficits related to anxiety and behavioural flexibility in the Syngap mouse model for intellectual disability were also not rescued by adult reactivation of *Syngap1* gene expression (12). These findings could suggest that the window during which gene activation can ameliorate autism-related phenotypes, closes early in neurodevelopment. Importantly however, these observations do not exclude the possibility that directly targeting downstream signalling pathways could have a broader window for therapeutic intervention (21). In fact, this possibility is very well demonstrated by the highly effective intervention of AEDs in preventing audiogenic seizures, in contrast to the

failure of postnatal gene reactivation to alter the susceptibility to audiogenic seizures. Therefore, at least for seizure susceptibility, restoration of the etiological loss of UBE3A was inferior to the therapeutic benefit achieved by targeting downstream mechanisms, in this case AEDs. This finding provides compelling evidence for the importance of understanding the targets of UBE3A and their downstream signalling pathways, as a complementary strategy for therapeutic drug discovery.

We believe that our results will be important for informing future AS clinical trials regarding the critical period for therapeutic intervention. However, there are two important limitations of our study. First, although our behavioural experiments were performed in an isogenic F1 hybrid background of 129/Sv and C57BL/6 mice, we cannot exclude an effect of the many heterozygous mutations that are contributed by each these two inbred strains, such as the *Disc1* mutation which is common to all 129/Sv sub-strains (22,23). Such mutations may interact with the *Ube3a* mutation and interfere with the ability to obtain a behavioural rescue. To minimize such confounding effects, we included matched littermate control groups for all experiments performed. Moreover, we only selected those behaviours which consistently exhibited a robust and reliably reproducible phenotype across all experiments, and for which we have demonstrated that a full rescue could be obtained upon early embryonic gene reactivation. A second translational limitation of our study is the obviously profound difference in brain development and systems-level functioning between mice and humans. Whereas a three-week old mouse can take care for itself, and adult maturity is complete by six to eight weeks of age, humans have a very extended childhood even compared to other primates. Therefore, it remains highly uncertain of how and to what extent the precise critical period windows we have identified can be translated to humans. A recent comprehensive comparative study of early brain maturation across multiple mammalian species estimated that the extent of brain maturation observed in a three week-old mouse pup is comparable to a two year-old human infant (24). Regarding critical period windows, among the most well-studied examples is ocular dominance plasticity. In mice, the critical period for acquiring binocular vision closes by four weeks of age. However, in humans this extends until approximately seven years of age (25). Therefore, the window of therapeutic opportunity in human AS patients is likely to be much broader than in mice and may offer some reason for optimism that gene reactivation could be more effective in humans than we have observed in mice. However, regardless of the precise conversion of the developmental time scales, our studies suggest that early intervention is very likely to determine the extent to which gene reactivation is therapeutically effective.

In addition to demonstrating an important developmental role for UBE3A, our study provides a notable contrast to a similar study of gene reactivation therapy in Rett syndrome (11), another neurodevelopmental imprinting disorder that is clinically reminiscent of AS. Whereas

we demonstrate that adult reactivation of *Ube3a* is only minimally efficacious as a therapeutic intervention in AS, adult reactivation of *Mecp2* appears to be highly effective for the treatment of Rett syndrome (11). This distinction not only emphasizes the unique neurodevelopmental requirements for *Ube3a* and *Mecp2*, but also illustrates the importance of systematically investigating disease-specific preclinical models, no matter how phenotypically similar, when the goal is to accurately inform therapeutic discovery and human clinical trials.

Methods

Further details are provided in the Supplemental Methods.

Mice. For all behavioural experiments except epilepsy test, we crossed female *Ube3a*$^{stop/p+}$ (in the 129S2/SvPasCrl background (Charles River)) with either *TgCAG-cre mice* (13) *(*hereafter referred as Cre^{+} mice*)* or with *Tg (CAG-cre/Esr1*)5Amc/J* (Jackson) (17) (hereafter referred a*s Cre*$^{ERT+}$), both kept in the C57BL/6J background (Charles River), to generate heterozygous *Ube3a*$^{stop/p+}$ *;Cre*$^{+}$ and *Ube3a*$^{stop/p+}$*;Cre*$^{ERT+}$ mutants and littermate controls in the F1 hybrid 129S2-C57BL/6 background.

Tamoxifen treatment. One day to eight-month-old *Ube3a*$^{Stop/p+}$ mice and their WT littermates (both genders) were used in this study. *Ube3a*$^{stop/p+}$*;Cre*$^{ERT+}$ mutants and WT mice were given tamoxifen to induce Cre-mediated deletion of the Stop-cassette. Tamoxifen (Sigma-Aldrich) was diluted in sunflower oil at a concentration of 20mg/ml. Each mouse received 0.10 mg tamoxifen per gram body weight, by daily intraperitoneal (i.p.) injection. The control groups were treated with daily i.p. injections of sunflower oil (vehicle). The Newborn group received tamoxifen through the milk of the mother, who received daily i.p. injections of tamoxifen for 5 consecutive days starting at the day of delivery. Juvenile, adolescent and adult groups received 7 daily i.p. injections of tamoxifen.

Behavioural analysis. All behavioural experiments were performed during the light period of the cycle. The experimenter remained blind to the genotype and treatment until final statistical analysis. For the accelerating Rotarod test, mice were given two trials per day with a 45-60 min inter-trial interval for 5 consecutive days. Maximum duration of a trial was 5 min. For the marble burying test, clean macrylon cages (50x26x18 cm) were filled with 4 cm of bedding material and 20 glass marbles, which were arranged in an equidistant 5 x 4 grid. Animals were given access to the marbles for 30 minutes. Marbles covered for more than 50% by bedding were scored as buried. For the Open Field test mice were placed in a brightly lit 120 cm diameter circular open field for 10 minutes. For the nest building test, mice were single housed for a period of 5 to 7 days before the starting of the experiment. Subsequently 12 grams of extra-thick filter paper (Bio-rad©) and the unused nesting material was determined for 5 consecutive days. For the forced swim test, mice were placed for 6 min in a cylindrical transparent tank (18cm diameter) with water (at 26±1 degrees Celsius). The duration of immobility was assessed during the last 4 min of the test. For the epilepsy test we used mice in the 129/Sv background, since epilepsy susceptibility in AS mice is dependent on the genetic background (16). Audiogenic seizures were induced by vigorously screeching scissors across the metal grating of the cage lid. This was done for 20s or shorter if a tonic-clonic seizure developed before that time.

Electrophysiology. After the behavioural tests, animals were sacrificed, and hippocampal sagittal slices (400 µm) were obtained using vibratome. Extracellular field recordings were

obtained in a submerged recording chamber and perfused continuously with ACSF. LTP was evoked using the 10 Theta burst protocol (10 trains of 4 stimuli at 100Hz, 200ms apart), performed at two-third of the maximum fEPSP.

Western blots. Blotted nitrocellulose membranes were probed with antibodies directed against E6AP (E8655 Sigma-Aldrich®; 1:1,000) and Actin (MAB1501R Millipore©; 1:20,000). A fluorophore-conjugated Goat anti-mouse antibody (Westburg©, IRDye 800CW 1:15,000) was used as secondary antibody and the amount of protein was quantified using a Li-cor® Odyssey Scanner and Odyssey 3.0 software (Li-cor® Biosciences).

Immunohistochemistry. 40µm thick frozen sections were subjected to a hydrogen peroxidase (H_2O_2) treatment, placed in blocking solution (10% normal horse serum (NHS), 0.5% Triton X-100) for 1h and incubated overnight with the primary antibody (mouse α-E6AP (E8655 Sigma-Aldrich, 1:2000) in 2% of NHS, 0.5% Triton X-100). The next day the slices were incubated with the secondary antibody (α-mouse HRP (Dako©; 1:200)), which was detected by 3,3'-diaminobenzidine (DAB) as the chromogen.

Data analysis and statistics. All the data was statistically analysed using the IBM® SPSS software and P-values of <0.05 were considered significant. Statistical analysis was performed using a one-way analysis of variance (ANOVA) or a two-way ANOVA with Bonferroni's post hoc comparison.

Study approval. All animal experiments were approved by the Dutch Ethical Committee and in accordance with Dutch animal care and use laws.

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SUPPLEMENTAL DATA INDEX

SUPPLEMENTAL METHODS

Generation and breeding of the Ube3a$^{stop/+}$ line. The *Ube3a*$^{stop/p+}$ mouse was generated as follows: the *Ube3a* genomic sequence (ENSMUSG00000025326) was obtained from Ensembl and used to design the primers for the targeting constructs. PCR fragments encompassing exon 3 using 5′ primer: 5′-CCGCGGGCTCCACTAGTCAATTTC-3′ and 3′ primer: 5′- GCGGCCGCACCACAGTCCCTGGAGTTC-3′ (4.9 kb; exon denotation according to ENSMUSG00000025326) and exon 4 using 5′ primer: 5′-GGCCGGCCGGAACTACCATATCCTGTTTTAC-3′ and 3′ primer: 5′-GCGGCCGCAGCCGATCTAGGTATTC′ (4.6 kb) were amplified using High Fidelity Taq Polymerase (Roche) on ES cell genomic DNA and cloned on either side of a Neomycin-stop cassette flanked by loxP sites (1). Exon 3 and 4 were sequenced to verify that no other mutations were introduced. For counter selection, the diphtheria toxin chain A (DTA) gene was inserted at the 5′ of the targeting construct. The targeting construct was linearized and electroporated into embryonic day 14 (E14) ES cells (derived from 129P2 mice). Cells were cultured in BRL cell- conditioned medium in the presence of leukemia inhibitory factor. After selection with G418 (200 µg/ml), targeted clones were identified by PCR (long-range PCR from neomycin resistance gene to the region flanking the targeted sequence). A clone with verified karyotype was injected into blastocysts of C57BL/6 mice. Male chimeras were crossed with female 129S2/SvPasCrl mice. The resulting heterozygous offspring was used for subsequent breedings. The *Ube3a*$^{stop/p+}$ was maintained by breeding heterozygous males with wild-type 129S2/SvPasCrl mice (Charles River). For all behavioural experiments except epilepsy tests, we crossed female *Ube3a*$^{stop/p+}$ with either *TgCAG-cre mice* (2) *(hereafter referred as Cre*$^{+}$ mice*)* or with *Tg(CAG- cre/Esr1*)5Amc/J* (Jackson) (3) (here after referred as *Cre*$^{ERT+}$) kept in the in C57BL/6J background (Charles River) to generate F1 heterozygous *Ube3a*$^{stop/p+}$*;Cre*$^{+}$ and *Ube3a*$^{stop/p+}$*:Cre*$^{ERT+}$ mutants and littermate controls in the F1 hybrid 129S2-C57BL/6 background. For the epilepsy test, both Cre lines were crossed 5 times into

129S2/SvPasCrl and subsequently crossed with *Ube3a*$^{stop/p+}$ mice to obtain *Ube3a*$^{stop/p+}$*;Cre*$^{+}$ and *Ube3a*$^{stop/p+}$ *;Cre*$^{ERT+}$ mutants and littermate controls in the 129S2 background.

Mice were genotyped when they were 7-10 days, and re-genotyped at the moment the mice were sacrificed. Genotyping records were obtained and kept by a technician not involved in the experimental design, performance and analysis. All animals were kept at 22±2ºC with 12 hours dark and light cycle and were provided with food and water *ad libitum*. Mice were preferably group-housed (2-4) cage, unless when fighting between males was observed.

Tamoxifen treatment and randomization. Cages were semi-randomly (alternatingly) assigned to either treat all mice within the cage with vehicle or with tamoxifen. Both male and female mice were used. The alternating randomization was adjusted if there was an imbalance of genotype or sex.

One day to eight-month-old *Ube3a*$^{Stop/p+}$ mice and their WT littermates (both males and females) were used in this study. The group of *Ube3a*$^{Stop/p+}$ and WT mice crossed with *Cre*$^{ERT+}$ transgenic mice were divided into 4 different experimental groups (classified as Newborn, Juvenile, Adolescent and Adult) based on the age of Tamoxifen administration to induce Cre-mediated deletion of the Stop-cassette. Tamoxifen (Sigma-Aldrich) was diluted in sunflower oil at a concentration of 20mg/ml. Each mouse received 0.10 mg Tamoxifen per gram body weight, by daily intraperitoneal (i.p.) injection. The control groups were treated with daily i.p. injections of sunflower oil (vehicle). The Newborn group received Tamoxifen through the milk of the mother, who received daily intraperitoneal (i.p.) injections of Tamoxifen for 5 consecutive days starting at the day of delivery. Tamoxifen treatment was initiated between 21-23 days of age in the Juvenile group; at 6 weeks of age in the Adolescent group; and at 14 weeks in the Adult group. These last 3 groups received 7 daily i.p. injections of Tamoxifen. The group of *Ube3a*$^{Stop/p+}$ and WT mice crossed with the embryonic active *Cre*$^{+}$ transgenic mice ('Embryonic') received 3 vehicle injections when they were 6-8 weeks old.

Behavioral analysis. All behavioral experiments were performed during the light period of the cycle. All animal experiments were approved by the Dutch Ethical Committee and in accordance with Dutch animal care and use laws. The experimenter remained blind to the genotype and treatment until final statistical analysis. Both male and female mice were used for the experiments. All behavioural assays and scoring were done by an

experimenter blind to genotype and treatment. Behavioral tests were typically run in the order as presented below. However the marble burying and nest building test were added later to the battery, and a new cohort of adolescent and adult mice were used for these tests. Since the mice for the epilepsy test required a different genetic background (see above) we used a separate cohort for this test.

Accelerating Rotarod. Motor function was tested using the accelerating rotarod (4-40 rpm, in 5 minutes; model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). Mice were given two trials per day with a 45-60 min inter-trial interval for 5 consecutive days. For each day we calculated the average of the time spent on the rotarod, or the time until the mouse made 3 consecutive rotations on the rotarod. Maximum duration of a trial was 5 min. Number of mice used in this task: Number of mice used: Embryonic: Wt;Cre+ (n=14), Ube3a$^{Stop/p+}$;Cre– (n=8), Ube3a$^{Stop/p+}$;Cre+ (n=17), Inducible: Adult – WT;Cre^{ERT+} Veh. (n=11), WT;Cre^{ERT+} Tamox. (n=9), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=13); Adolescent – WT;Cre^{ERT+} Veh. (n=11), WT;Cre^{ERT+} Tamox. (n=11), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=10), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11); Juvenile – WT;Cre^{ERT+} Veh. (n=22), WT;Cre^{ERT+} Tamox. (n=20), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=22), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=22); Newborn – WT;Cre^{ERT+} Veh. (n=14), WT;Cre^{ERT+} Tamox. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11).

Marble burying test. Clean open makrolon (polycarbonate) cages (50x26x18 cm) were filled with 4 cm of bedding material. On top of the bedding material 20 blue glass marbles were arranged in an equidistant 5 x 4 grid and the animals were given access to the marbles for 30 minutes. After the test the mice were gently removed from the cage. Marbles which were covered for more than 50% by bedding were scored as buried. Occasionally, a mouse managed to escape out of the cage during the test, and was excluded. Number of mice used in this task: Embryonic: Wt;Cre+ (n=24), Ube3a$^{Stop/p+}$;Cre– (n=18), Ube3a$^{Stop/p+}$;Cre+ (n=28), Inducible: Adult – WT;Cre^{ERT+} Veh. (n=13), WT;Cre^{ERT+} Tamox. (n=11), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=15); Adolescent – WT;Cre^{ERT+} Veh. (n=20), WT;Cre^{ERT+} Tamox. (n=20), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=21), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=23); Juvenile – WT;Cre^{ERT+} Veh. (n=21), WT;Cre^{ERT+} Tamox. (n=20), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=20), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=20); Newborn – WT;Cre^{ERT+} Veh. (n=14), WT;Cre^{ERT+} Tamox. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11).

Open Field test. To test locomotor activity and anxiety, the mice were individually placed in a brightly lit 120 cm diameter circular open field. The total distance travelled was

recorded for 10 minutes (SMART software, Panlab, Barcelona). Number of mice used in this task: Embryonic: Wt;Cre+ (n=14), Ube3a$^{Stop/p+}$;Cre– (n=8), Ube3a$^{Stop/p+}$;Cre+ (n=17), Inducible: Adult – WT;Cre^{ERT+} Veh. (n=10), WT;Cre^{ERT+} Tamox. (n=8), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=9), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=10); Adolescent – WT;Cre^{ERT+} Veh. (n=11), WT;Cre^{ERT+} Tamox. (n=11), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=10), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11); Juvenile – WT;Cre^{ERT+} Veh. (n=21), WT;Cre^{ERT+} Tamox. (n=21), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=22), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=22); Newborn – WT;Cre^{ERT+} Veh. (n=14), WT;Cre^{ERT+} Tamox. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11).

Nest Building test. To measure nest building, mice were single housed for a period of 5 to 7 days before the starting the experiment. Subsequently 12 grams (12±1) of compressed extra-thick blot filter paper (Bio-rad©) was added to the cage and cages were put back in the rack and undisturbed for 24h. For 5 consecutive days and approximately at the same time of the day, the unused nesting material was carefully cleaned, dried and weighed to determine the amount used for nestbuilding. Number of mice used in this task: Embryonic: Wt;Cre+ (n=7), Ube3a$^{Stop/p+}$;Cre– (n=7), Ube3a$^{Stop/p+}$;Cre+ (n=7), Inducible: Adult – WT;Cre^{ERT+} Veh. (n=9), WT;Cre^{ERT+} Tamox. (n=8), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=9), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=8); Adolescent – WT;Cre^{ERT+} Veh. (n=14), WT;Cre^{ERT+} Tamox. (n=13), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=16), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=17); Juvenile – WT;Cre^{ERT+} Veh. (n=12), WT;Cre^{ERT+} Tamox. (n=13), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=13); Newborn – WT;Cre^{ERT+} Veh. (n=12), WT;Cre^{ERT+} Tamox. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11).

Forced swim test. Mice were placed for 6 min in a cylindrical transparent tank (27cm high and 18cm diameter), filled with water (kept at 26±1 degrees Celsius) 15 cm deep. Since little or no immobility is observed during the first 2 min after the mouse has been placed in the water, the duration of immobility was only assessed during the last 4 min of the test. The mouse was considered to be immobile when he ceased to move altogether, making only movements necessary to keep its head above water. Number of mice used in this task: Embryonic: Wt;Cre+ (n=14), Ube3a$^{Stop/p+}$;Cre– (n=8), Ube3a$^{Stop/p+}$;Cre+ (n=17), Inducible: Adult – WT;Cre^{ERT+} Veh. (n=11), WT;Cre^{ERT+} Tamox. (n=8), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=9), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=9); Adolescent – WT;Cre^{ERT+} Veh. (n=11), WT;Cre^{ERT+} Tamox. (n=11), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=10), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11); Juvenile – WT;Cre^{ERT+} Veh. (n=17), WT;Cre^{ERT+} Tamox. (n=19), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=18), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=19); Newborn – WT;Cre^{ERT+} Veh. (n=14), WT;Cre^{ERT+} Tamox.

(n=12), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=14), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=11).

Epilepsy test. Since epilepsy susceptibility in AS mice is dependent on the genetic background, these experiments were performed in a 129/sv background. For this test, mice were taken from their home cage and placed in a clean cage. To assess seizure susceptibility, audiogenic seizures were induced by producing a loud and continuous noise, achieved by vigorously screeching scissors across the metal grating of the cage lid. This was done for 20s or shorter if a tonic-clonic seizure developed before that time. Number of mice used in this task: Embryonic: Wt;Cre+ (n=7), Ube3a$^{Stop/p+}$;Cre– (n=10), Ube3a$^{Stop/p+}$;Cre+ (n=8), Inducible: Juvenile – WT;Cre^{ERT+} Veh. (n=8), WT;Cre^{ERT+} Tamox. (n=8), Ube3a$^{Stop/p+}$;Cre^{ERT+} Veh. (n=8), Ube3a$^{Stop/p+}$;Cre^{ERT+} Tamox. (n=8).

Mean age of the mice used in the epilepsy susceptibility test was the following: Embryonic, 10 weeks; Juvenile, 9 weeks.

Antiepileptic drug (AED) treatment. Mutant Ube3aStop/p+;Cre– (n=2), Ube3am-/p+ (n=12) and WT littermates (n=3) in the 129sv background were used for these experiments.

Tonic-clonic seizures were verified in all mutant mice upon audiogenic stimulation. No seizues were observed in WT mice. Mutant mice were randomized into two treatment groups (sodium valproate and clonazepam), and drug administration started 24hrs after the first epilepsy test.

1 Ube3a$^{Stop/p+}$;Cre^{-}, 7 Ube3a$^{m-/p+}$ and 2 WT mice were treated with 5 daily i.p. injections of sodium valproate (Alliance Healthcare™) at a concentration of 200mg/kg. The valproate was dispensed in sterilized water through agitation and syringes were filled while stirring. Valproate was freshly prepared daily.

The second treatment group composed of 1 *Ube3a$^{Stop/p+}$;Cre–, 5 Ube3a$^{m-/p+}$* and 1 WT mouse, which were treated with 5 daily i.p. injections of 0.05mg/kg Clonazepam (Roche) dispensed in PBS-methylcellulose. Clonazepam was prepared fresh every two days.

Two re-test experiments were performed in both treatment groups; the first re-test was performed 30 min after the last injection, on the fifth day of treatment, and the second re-test was done 3 days after treatment cessation.

Electrophysiology. After the behavioural tests, animals have been sacrificed, sagittal slices (400 µm) were made and submerged in ice-cold artificial CSF (ACSF) using a

vibratome, and hippocampi were dissected out. These sagittal hippocampal slices were maintained at room temperature for at least 1.5 h to recover before experiments were initiated. Then they were placed in a submerged recording chamber and perfused continuously at a rate of 2 ml/min with ACSF equilibrated with 95% O_2, 5% CO_2 at 31°C. ACSF contained the following (in mM): 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose.

Extracellular recording of field EPSP (fEPSPs) were made in CA1 stratum radiatum with platinum/iridium electrodes (Frederick Haer). A bipolar Pt/Ir was used to stimulate Schaffer- collateral/commissural afferents with a stimulus duration of 100 μs. LTP was evoked using the 10 Theta burst protocol (10 trains of 4 stimuli at 100Hz, 200ms apart), performed at two-third of the maximum fEPSP. fEPSP measurements were done once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. Only stable recordings were included, and this judgment was made blind to genotype. Average LTP was defined as the mean last 10 min of each protocol. Recordings showing unstable baselines were excluded from the experiment.

Number of slices/mice used: Juvenile- WT;Cre^{ERT+} Veh. (n=16/4), WT;Cre^{ERT+} Tamox. (n=25/4), $Ube3a^{Stop/p+}$;Cre^{ERT+} Veh. (n=22/4), $Ube3a^{Stop/p+}$;Cre^{ERT+} Tamox. (n=22/5); Adult - WT;Cre^{ERT+} Veh. (n=18/6), WT;Cre^{ERT+} Tamox. (n=37/8), $Ube3a^{Stop/p+}$;Cre^{ERT+} Veh. (n=23/4), $Ube3a^{Stop/p+}$;Cre^{ERT+} Tamox. (n=15/4).

Western Blots. To collect tissue for Western blot analysis, hippocampus, cortex and cerebellum were dissected and immediately frozen in liquid nitrogen. The lysates were prepared by adding lysis buffer (10mM Tris-HCL pH 6.8, 2.5% SDS) supplemented with protease inhibitor cocktail (Sigma-Aldrich®) to the tissue and homogenization was achieved by sonication. After centrifugation (6000 rpm for 5 min) supernatants were collected. The protein concentration of the supernatants was determined using a BCA kit (Pierce, Thermo Scientific). A total of 20μg of each sample was loaded on the gel and a wet transfer was performed. The blotted nitrocellulose membrane was probed with antibodies directed against E6AP (E8655 Sigma-Aldrich®; 1:1,000) and Actin (MAB1501R Millipore©; 1:20,000). A fluorophore-conjugated secondary Goat anti-mouse antibody (Westburg©, IRDye 800CW 1:15,000) was used and the protein was detected using Li-cor® Odyssey Scanner system. Quantification was done using Odyssey 3.0 software (Li-cor® Biosciences). Number of samples used for immunoblot analysis range from 2 to 5 per genotype/brain area.

Immunohistochemistry. Brains from adult mice were fixed by transcardial perfusion with

4% paraformaldehyde. Immunocytochemistry was performed on 40µm thick frozen sections. The sections were subjected to a hydrogen peroxidase (H_2O_2) treatment, placed in blocking solution (10% normal horse serum (NHS), 0.5% Triton X-100) for 1h and incubated overnight with the primary antibody (mouse α-E6AP (E8655 Sigma-Aldrich, 1:2000) in 2% of NHS, 0.5% Triton X-100). The next day the slices were incubated with the secondary antibody (α-mouse HRP (Dako©; 1:200), which was detected by 3,3'-diaminobenzidine (DAB) as the chromogen. DAB sections were analysed and photographed using a Leica© DM-RB microscope and a Leica DFC450 digital camera. For overview pictures of the slices a Zeiss Stemi SV6 was used.

Data analysis and statistics. Values are represented as means ± S.E.M. All the data was statistically analyzed using the IBM® SPSS software and P-values of <0.05 were considered significant. Statistical analysis was performed using a one-way analysis of variance (ANOVA) or a two-way ANOVA with Bonferroni's post hoc comparison.

SUPPLEMENTAL REFERENCES

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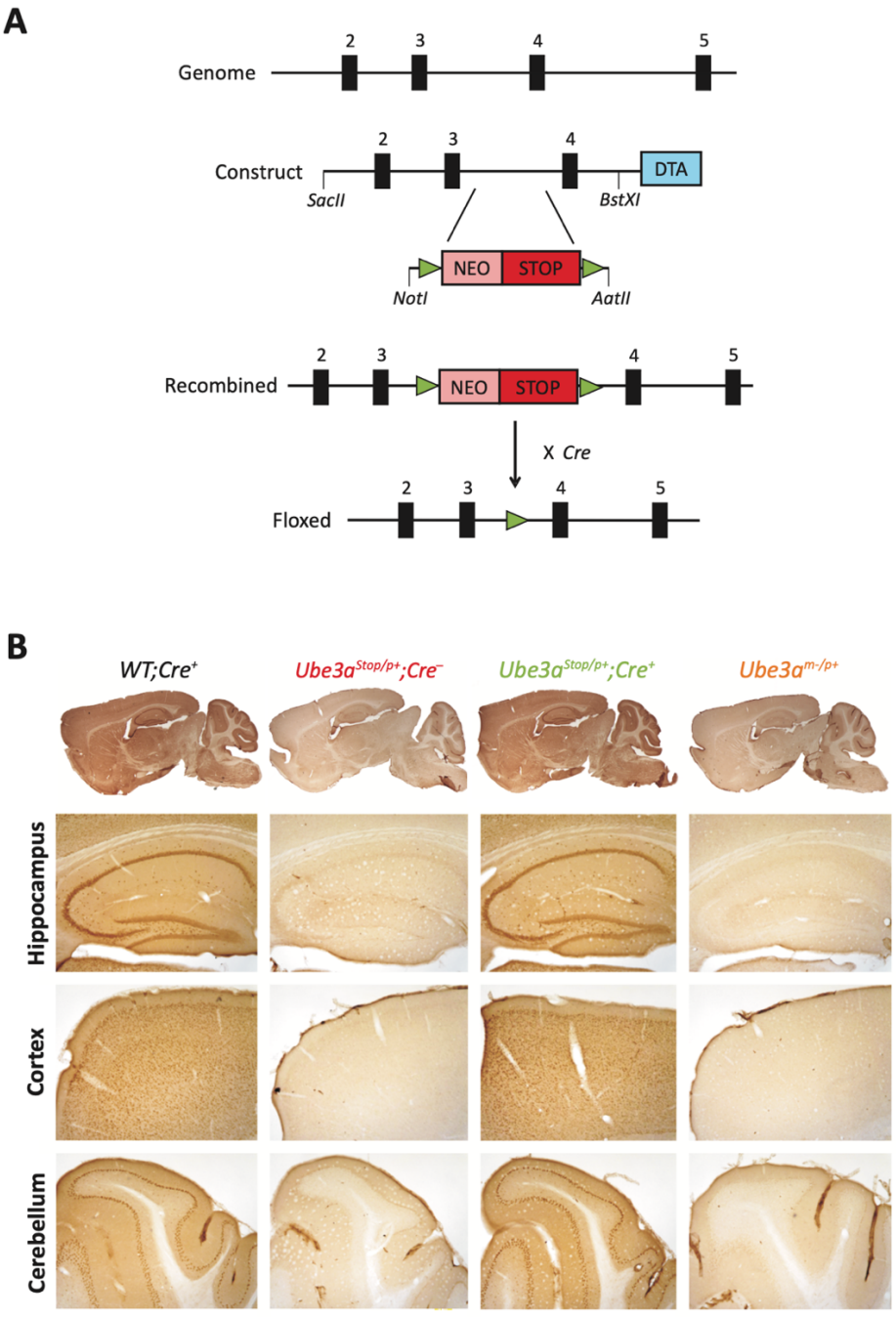
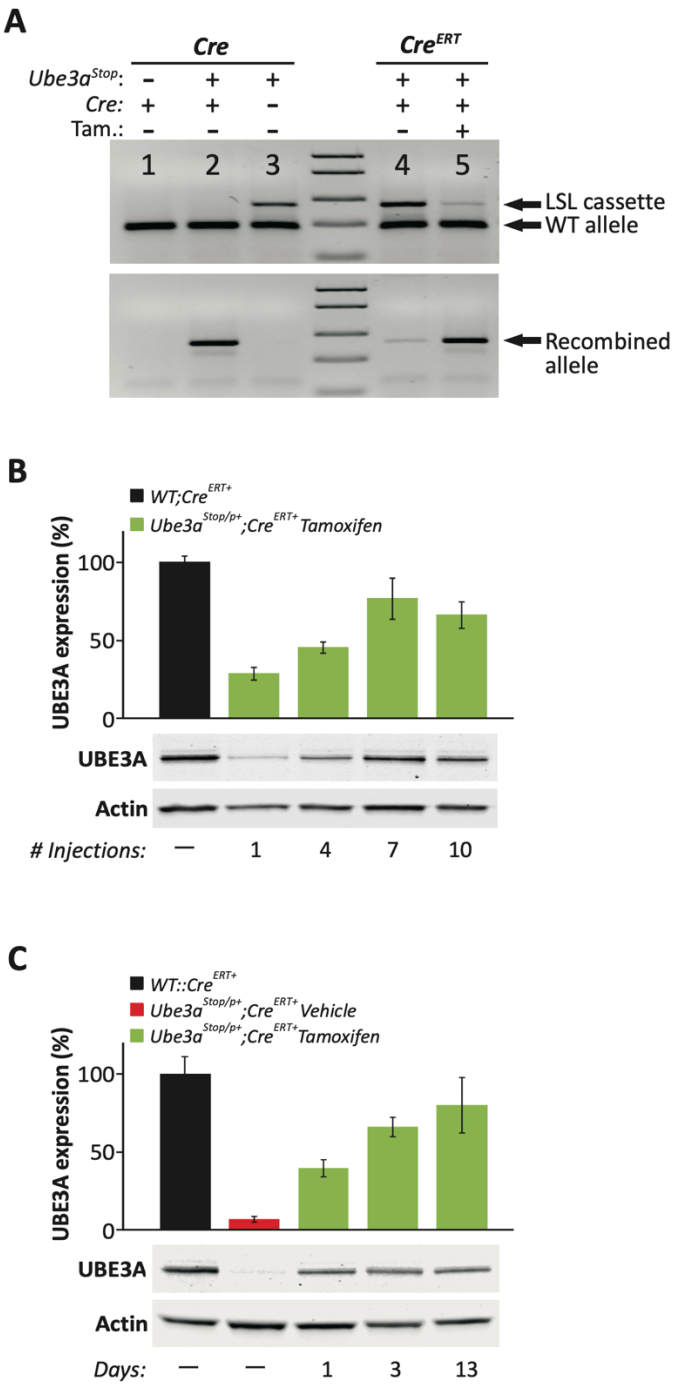
3. Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol*. 2002;244(2):305–318.

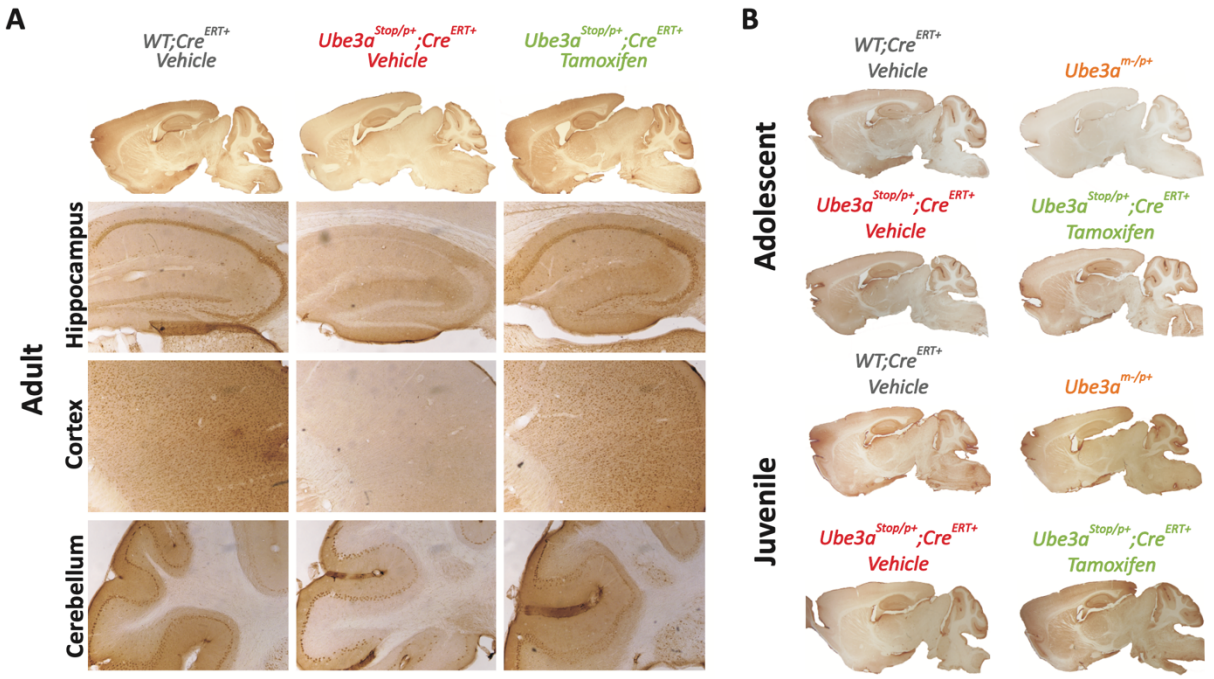
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Generation and histological validation of *Ube3a*$^{Stop/p+}$ knock-in mice. (**A**) Schematics depicting the generation of the inducible mouse model by the insertion of a floxed stop cassette into intron 3 of *Ube3a*, for which cre-mediated recombination leads to reinstatement of *Ube3a* gene expression. Black boxes correspond to *Ube3a* coding exons and green triangles represent the LoxP sites. (**B**) Immunohistochemical UBE3A stainings from *Ube3a*$^{m-/p+}$ knock-out mice and *Ube3a*$^{Stop/p+}$ and WT littermates crossed with a embryonically active cre-line. Brain overviews magnification = 1.6x (upper); zoomed-in pictures magnification = 5x (lower).



Supplemental Figure 2. High level of recombination is achieved by embryonically expressed cre ('Cre') and tamoxifen inducible cre expression ('Cre^{ERT}'). (**A**) Polymerase chain reaction (PCR) of hippocampal tissue reveals successful recombination in mutant mice expressing Cag-Cre and in *Ube3a*$^{Stop/p+}$;Cre^{ERT+} mutant mice injected 7 times with tamoxifen (Tam.). (**B**) Representative Western blot and corresponding quantification illustrates hippocampal UBE3A reinstatement achieved by successive tamoxifen injections in the *Ube3a*$^{Stop/p+}$;Cre^{ERT+} mutant mice. (**C**) Time-course of UBE3A expression following gene reinstatement (days following the last seventh injection of tamoxifen). All data are represented as mean ± S.E.M..



Supplemental Figure 3. Histological analysis of *Ube3A$^{Stop/p+}$;Cre^{ERT+}* mice reveals successful reactivation of the maternal *Ube3a* gene upon tamoxifen induction. (**A-B**) Hippocampal, cortical, and cerebellar sections demonstrate brain-wide reactivation of *Ube3a* expression in tamoxifen-treated *Ube3A$^{Stop/p+}$;Cre^{ERT+}* mice. Brain overviews magnification = 1.6x; zoomed-in pictures magnification = 5x.

SUPPLEMENTAL TABLES

	Paradigm	Dependent variable	Statistical test	Independent variable	p	Post-hoc test		p
Embryonic *Ube3a* gene reinstatement	RR	Latency to fall (s)	Repeated measures one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.01
	Marble	Number unburied marbles	Univariate one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.01
	OF	Path length (m)	Univariate one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.05
	NB	Used nesting material (%)	Repeated measures one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.01
	FST	Floating time (%)	Univariate one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.01
	Epilepsy	Presence of seizures	Univariate one-way ANOVA	Genotype	p<0.01	$Ube3a^{Stop/p+};Cre^{+}$	$WT;Cre^{+}$	p= 1.000
							$Ube3a^{Stop/p+};Cre^{-}$	p<0.01

Supplemental Table S1 Summary of the statistical tests used for each behavioral paradigm performed on the early embryonic reactivation group and statistical outcomes obtained. Statistical significance ($P < 0.05$) is indicated by green shading.

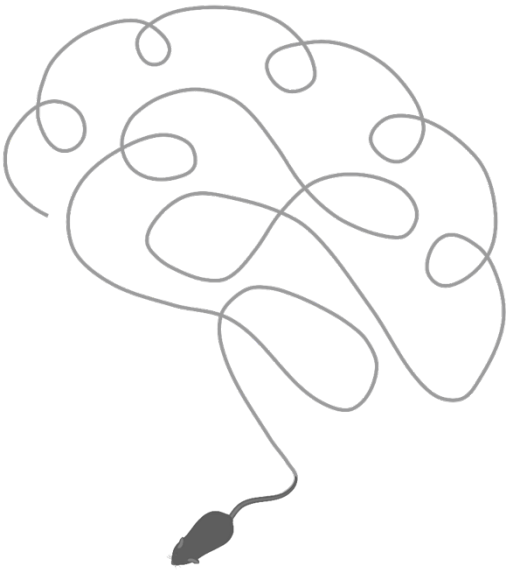
		Paradigm	Dependent variable	Statistical test	Independent variable	p
Adult *Ube3a* gene reinstatement	Induction at 14 weeks	**RR**	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.602
					Genotype*Treatment	p= 0.613
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.751
					Genotype*Treatment	p= 0.722
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.203
					Genotype*Treatment	p= 0.411
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.377
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.499
					Genotype*Treatment	p= 0.154
		LTP	fEPSP slope (% from baseline)	Univariate two-way ANOVA	Genotype	p= 0.849
					Treatment	p= 0.071
					Genotype*Treatment	p<0.01
Adolescent *Ube3a* gene reinstatement	Induction at 6 weeks	**RR**	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.284
					Genotype*Treatment	p= 0.284
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.249
					Genotype*Treatment	p= 0.221
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.240
					Genotype*Treatment	p= 0.413
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.308
					Genotype*Treatment	p= 0.530
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.374
					Genotype*Treatment	p= 0.822
Juvenile *Ube3a* gene reinstatement	Induction at P21-23	**RR**	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p<0.01
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.080
					Genotype*Treatment	p= 0.537
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p= 0.948
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.173
					Genotype*Treatment	p= 0.708
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p= 0.135
		LTP	fEPSP slope (% from baseline)	Univariate two-way ANOVA	Genotype	p= 0.088
					Treatment	p<0.01
					Genotype*Treatment	p=0.055
Newborn *Ube3a* gene reinstatement	Induction at P1	**RR**	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p<0.05
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.124
		OF	Path length (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.365
					Genotype*Treatment	p<0.05
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.208
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.987
					Genotype*Treatment	p= 0.663

Supplemental Table S2 Summary of the statistical tests applied for each behavioural paradigm performed on the postnatal reactivation groups. Statistical significance ($P < 0.05$) is indicated by green shading.

CHAPTER 4

A BEHAVIOURAL TEST BATTERY FOR MOUSE MODELS OF ANGELMAN SYNDROME: A POWERFUL TOOL FOR TESTING DRUGS AND NOVEL UBE3A MUTANTS

(Mol Autism 2018 Sep 14;9:47.)



A BEHAVIORAL TEST BATTERY FOR MOUSE MODELS OF ANGELMAN SYNDROME: A POWERFUL TOOL FOR TESTING DRUGS AND NOVEL UBE3A MUTANTS

The scientific literature presents numerous instances that highlight the presence of a subtle phenotype in AS mice compared to human patients, which creates challenges in assessing the effectiveness of drugs for treating AS. This ambiguity hampers the translation of mouse studies into preclinical trials, emphasizing the necessity for robust and standardized testing methods that yield consistent outcomes across diverse research laboratories worldwide. Therefore, the question arises: *Is it feasible to develop a sensitive and consistent behavioural test battery capable of evaluating the efficacy of drugs for AS and characterizing novel Ube3a mouse models*?

Therefore, the main objectives of the following study, published in the journal Molecular Autism, can be summarized as follows:

- Develop a comprehensive behavioural test battery specifically designed to evaluate the effectiveness of drugs in treating AS and characterize *Ube3a* mutants in mouse models.
- Assess the ability of the behavioural test battery to measure relevant clinical phenotypes of AS, including motor performance, anxiety, innate behaviour, and seizure susceptibility.
- Determine the statistical power of the tests to minimize type I errors (false positives) and evaluate potential confounding factors such as sex and animal weight.
- Compare *Ube3a* mutants in different genetic backgrounds and independently derived *Ube3a* mutant lines.
- Assess the translational value of the behavioural test battery by revaluating the efficacy of Minocycline and Levodopa, previously tested in clinical trials for AS.
- Explore the possibility of conducting experiments with a single cohort of mice to reduce costs and the number of animals required, while also considering the potential for retesting the same animals to enable a within-subject testing design.

The study findings are expected to enhance our understanding of the behavioural manifestations in AS and provide valuable information for the development of effective therapeutic interventions for individuals affected by this syndrome.

A BEHAVIORAL TEST BATTERY FOR MOUSE MODELS OF ANGELMAN SYNDROME: A POWERFUL TOOL FOR TESTING DRUGS AND NOVEL UBE3A MUTANTS

Mol Autism 2018 Sep 14;9:47.

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Keywords

Angelman Syndrome; UBE3A; Mouse-model; Behaviour; Drug screening

Abstract

Background: Angelman Syndrome (AS) is a neurodevelopmental disorder caused by mutations affecting UBE3A function. AS is characterized by intellectual disability, impaired motor coordination, epilepsy and behavioural abnormalities including autism spectrum disorder features. The development of treatments for AS heavily relies on the ability to test the efficacy of drugs in mouse models that show reliable, and preferably clinically relevant, phenotypes. We previously described a number of behavioural paradigms that assess phenotypes in the domains of motor performance, repetitive behaviour, anxiety, and seizure susceptibility. Here we set out to evaluate the robustness of these phenotypes when tested in a standardized test battery. We then used this behavioural test battery to assess the efficacy of Minocycline and Levodopa, which were recently tested in clinical trials of AS.

Methods: We combined data of eight independent experiments involving 111 *Ube3a* mice and 120 wild-type littermate control mice. Using a meta-analysis, we determined the statistical power of the subtests, and the effect of putative confounding factors, such as the effect of sex and of animal weight on rotarod performance. We further assessed the robustness of these phenotypes by comparing *Ube3a* mutants in different genetic backgrounds, and by comparing the behavioural phenotypes of independently derived *Ube3a* mutant lines. In addition, we investigated if the test battery allowed retesting the same animals, which would allow a within-subject testing design.

Results: We find that the test battery is robust across different *Ube3a* mutant lines but confirm and extend earlier studies that several phenotypes are very sensitive to genetic background. We further found that the audiogenic seizure susceptibility phenotype is fully reversible upon pharmacological treatment and highly suitable for dose finding studies. In agreement with the clinical trial results, we found that Minocycline and Levodopa treatment of *Ube3a* mice did not show any sign of improved performance in our test battery.
Conclusions: Our study provides a useful tool for preclinical drug testing to identify treatments for Angelman Syndrome. Since the phenotypes are observed in several independently derived *Ube3a* lines, the test battery can also be employed to investigate the effect of specific *Ube3a* mutations on these phenotypes.

Introduction

Angelman Syndrome (AS) is a neurodevelopmental disorder first described in 1965 by Harry Angelman, with a birth incidence of approximately 1:20,000 [1]. AS is caused by the functional loss of the maternal allele encoding an E3 ubiquitin protein ligase (UBE3A) [2]. Loss of functional UBE3A results in the core phenotypes of severe intellectual disability, motor coordination deficits, absence of speech and abnormal EEG, as well as in high comorbidity of sleep abnormalities, epilepsy and phenotypes related to autism spectrum (C. A. Williams et al., 2006).

Currently, only symptomatic treatments are available for AS, primarily aimed at reducing seizures and improving sleep (W.-H. H. Tan & Bird, 2016). The development of targeted treatments for AS heavily relies on the ability to test the efficacy of treatments in mouse models of the disorder. The success of such translational studies depends on three critical factors (Katz et al., 2012): (1) high construct validity, (2) high face validity and (3) robustness of the behavioural phenotypes. First, the construct validity (shared underlying aetiology between mouse models and patients) of the AS mouse model is very good, since AS mouse models recapitulate the patient genetics by carrying a mutated *Ube3a* gene specifically at the maternal allele. However, it should be noted that the majority of the AS patients carry a large deletion (15q11-15q13) which encompasses also other genes besides the *UBE3A* gene, and which may contribute to a more severe phenotype (Gentile et al., 2010). Second, with respect to face validity (*i.e.* similarity of phenotypes between patient and the mouse model), the AS mouse model captures many neurological key features of the disorder really well (*e.g.* epilepsy, motor deficits, abnormal EEG), as well as some of the behavioural abnormalities (abnormal sleep patterns, increased anxiety, repetitive behaviour)(Allensworth et al., 2011; Born et al., 2017; Huang et al., 2013; Jiang et al., 1998, 2010; Miura et al., 2002). Robustness of the behavioural

phenotypes is the third important aspect to identify novel treatments, as it allows experiments to be sufficiently powered to detect the effect of the treatment, and meanwhile minimizes a Type I error in which a drug is declared effective whereas it is not. Robustness, as well as face validity, also takes into account the sensitivity to genetic background and the extent in which a phenotype is also observed in independently derived mouse models. Notably, almost all behavioural testing described in literature has been performed using the original *Ube3a*^{tm1Alb} mouse strain generated in the Beaudet lab (Born et al., 2017; Huang et al., 2013; Jiang et al., 1998), hence it is unknown to what extent the reported phenotypes are actually specific to this mouse line.

We previously developed a series of behavioural paradigms in the domains of motor performance, anxiety, repetitive behaviour and seizure susceptibility, for testing the effect of *Ube3a* gene reinstatement in the inducible *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) mice (Silva-Santos et al., 2015). Here we used these paradigms in a highly standardized way, to assess phenotypes in the independently derived *Ube3a*^{tm1Alb} and *Ube3a*$^{mE113X/p+}$ (*Ube3a*^{tm2Yelg}) maternal knock-out strains. We combined data of eight independent experiments across five experimenters and involving 111 *Ube3a*^{tm1Alb} and 120 wild-type littermate control mice. Using a meta-analysis, we determined the statistical power of the different behavioural tests, and the effect of putative confounding factors, such as the effect of sex differences. We further assessed the robustness of these phenotypes by comparing *Ube3a* mutants in different genetic backgrounds. Finally, we employed this behavioural test battery to reassess the efficacy of Minocycline and Levodopa in the AS mouse model. These drugs were previously tested in the AS mouse model and based on the favourable outcome of these preclinical experiments, three clinical trials were performed (J C Grieco et al., 2014; Ruiz-Antorán et al., 2015; W. H. Tan et al., 2017). Unfortunately, none of these drugs showed a significant improvement in AS patients.

Methods

Mouse husbandry and breeding

For this study, we used *Ube3a*$^{m-/p+}$ mice (*Ube3a*^{tm1Alb}; MGI 2181811) [7] and *Ube3a*$^{mE113X/p+}$ mutants (*Ube3a*^{tm2Yelg}; MGI5911277) as previously described [17]. *Ube3a*^{tm1Alb} mice were maintained (>40 generations) in the 129S2 background (full name: 129S2/SvPasCrl) by crossing male *Ube3a*$^{m+/p-}$ mice with female 129S2 wild-type mice. *Ube3a*^{tm2Yelg} mice were maintained (>20 generations) in the C57BL/6J (Charles River) background by crossing male *Ube3a*$^{m+/pE113X}$ mice with female C57BL/6J wild-type mice. For the seizure susceptibility experiments with *Ube3a*$^{mE113X/p+}$ animals, this line was backcrossed 8 times in 129S2 by crossing *Ube3a*$^{pE113X/m+}$ males with 129S2 wild-type females.

For behavioural experiments, female *Ube3a*^{tm1Alb} (*Ube3a*$^{m+/p-}$) mice were bred to yield *Ube3a*$^{m-/p+}$ mice in two different backgrounds: *Ube3a*$^{m-/p+}$ (AS) mice and their WT littermates in the F1 hybrid 129S2-C57BL/6J background (WT=120, AS=111) and in the 129S2 background (WT=11, AS=16). *Ube3a*$^{mE113X/p+}$ mice and their WT littermates were generated in the same manner in the F1 hybrid 129S2-C57BL/6J background (WT=10, *Ube3a*$^{mE113X/p+}$ =10) and in C57BL/6J background (WT=15, *Ube3a*$^{mE113X/p+}$ =16).

For the seizure susceptibility test we used *Ube3a*$^{m-/p+}$ (WT=45, AS=114) and *Ube3a*$^{mE113X/p+}$ mice (WT=4, AS=8) in the 129S2 background.

Mice were housed in individually ventilated cages (IVC; 1145T cages from Techniplast) in a barrier facility. Mice were genotyped when they were 4-7 days old, and re-genotyped at the completion the experiments. All animals were kept at 22±2ºC with a 12 hours dark and light cycle, and were tested in the light period, provided with mouse chow (801727CRM(P) from Special Dietary Service) and water *ad libitum.* During behavioural testing, mice were group-housed (2-4), except when fighting between males was observed and during the nest building and forced swim test. All animal experiments were conducted in accordance with the European Commission Council Directive 2010/63/EU (CCD approval AVD101002016791).

Behavioural analysis

The weight of the animals was determined a few of days before the start of the behavioural analysis. Prior to each test, mice were acclimatized to the testing room for 30 minutes.

All behavioural experiments were performed during the light period of the light/dark cycle. Both male and female mice at the age of 8-12 weeks were used for the experiments.

All behavioural testing and scoring were performed by experimenters who were blind to genotype and treatment. Behavioural tests were always run in the following order and with a minimal number of days between tests: 1) accelerating rotarod test for 5 consecutive days performed at the same hour every day; 2) 2 days of pause; 3) open field test; 4) 1 day of

pause; 5) marble burying test; 6) between 5 to 7 days of pause to allow adaptation to being single caged; 7) nest building test for 5 consecutive days, in which the weight of the nest was assessed at the same hour every day; 8) 2 days of pause; 9) forced swim test.

Accelerating rotarod. Motor function was tested using the accelerating rotarod (4-40 rpm, in 5 minutes; model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). Mice were given two trials per day with a 45-60 min inter-trial interval for 5 consecutive days (same hour every day). For each day, the average time spent on the rotarod was calculated, or the time until the mouse made 3 consecutive wrapping / passive rotations on the rotarod (latency in seconds). Maximum duration of a trial was 5 min.

Open Field test. To test locomotor activity and anxiety, mice were individually placed in a brightly lit 110 cm diameter circular open field and allowed to explore for 10 min. The total distance moved by each mouse in the open arena was recorded by an infrared camera (Noldus® Wageningen, NL) connected to the EthoVision® software (Noldus® Wageningen, NL), and the final outcome is indicated as distance moved in meters. For some groups we also analyzed the time spent in the inner zone (IZ), middle zone (MZ) and outer zone (OZ) (IZ r=25cm, MZ r=40cm, OZ r=55cm).

Marble burying test. Open makrolon (polycarbonate) cages (50x26x18 cm) were filled with 4 cm of bedding material (Lignocel® Hygenic Animal Bedding, JRS). On top of the bedding material 20 blue glass marbles were arranged in an equidistant 5 x 4 grid and the animals were given access to the marbles for 30 minutes. After the test the mice were gently removed from the cage. Marbles covered for more than 50% by bedding were scored as buried and the outcome measured is the number of buried marbles.

Nest Building test. To measure nest building, mice were single housed for a period of 5 to 7 days before the start of the experiment. Subsequently used nesting material was replaced and 11 grams (11±1) of compressed extra-thick blot filter paper (Bio-rad©) was added to the cage. The amount of the unused nest material was weighed and noted every day for a consecutive of 5 days, each day at the same hour.

Forced swim test. Mice were placed for 6 min in a cylindrical transparent tank (27cm high and 18cm diameter), filled with water (kept at 26±1 degrees Celsius) 15 cm deep. The mouse was first left in the cylinder for 2 minutes to habituate. The duration of immobility was only assessed during the last 4 min of the test. The mouse was considered to be immobile when he ceased

to move altogether, making only movements necessary to keep its head above water. The outcome measured is the time in seconds in which the mouse was immobile.

Susceptibility to audiogenic seizures. Because of the different genetic background requirements, an independent cohort of mice was used to test susceptibility to audiogenic seizures. Mice were placed in makrolon (polycarbonate) cages (50x26x18 cm) and audiogenic seizures were induced by vigorously scraping scissors across the metal grating of the cage lid (which creates approximately a 100 dB sound). This noise was generated for 20 seconds, or less if a tonic-clonic seizure developed before that time. Susceptible mice responded with wild running and leaping followed by a tonic-clonic seizure, which typically lasted 10–20 seconds.

Drug administration

Vehicle treatment.

All animals used for the meta-analysis were treated with vehicle, either by IP (Intraperitoneal) injection, (max. volume 10ul/g, Hypodermic-needle 25G x 16 mm (Sterican®/B-Braun)), Oral gavage (max 10ul/g, Stainless steel animal feeding tubes 20G x 38 mm (Instech Laboratories)) or by adding to the drinking water.

Minocycline treatment.

The adult-treated group consisted of 8–10-week-old *Ube3a*$^{m-/p+}$ (n=11 saline; 11 Minocycline) and WT (n=9 saline; 10 Minocycline) littermate control mice in F1 hybrid 129S2-C57BL/6J background. Due to space limitations, only 6 animals per group were used for nest building. Mice were assigned to two groups in such a way that both groups had a comparable distribution of males and females and mutant and wild-type mice. Mice were subjected to daily minocycline or vehicle IP injections (Minocycline hydrochloride, Sigma-Aldrich 45 mg/kg in saline solution), starting three weeks prior to commencing behavioural testing, as previously described (J C Grieco et al., 2014). Behavioural testing was started 1.5 hours post-injection, based on the half-life of Minocycline (~ 2h in plasma) and the peak brain levels are reached about 2h after injection (Andes & Craig, 2002).

For the postnatal-treated group, cages with *Ube3a*$^{m-/p+}$ and WT pups in F1 hybrid 129S2-C57BL/6J background were split in two groups in such a way that both groups had a comparable distribution of males and females and mutant and wild-type mice: WT/AS treated with minocycline (n=33/22) and WT/AS treated with vehicle mice (n=21/17). The treatment group received minocycline via the lactating dam, which received minocycline through the drinking water (0.2 mg minocycline/ml, supplemented with 1 mg/ml aspartame to counteract

the bitter taste and shielded for light), as described previously (Bilousova et al., 2009). Once the mice were weaned, they were supplied with the same concentration of minocycline in their drinking water. Under the assumption that mice drink 5 ml/day, with an average weight of 25 g/mouse, the average amount of minocycline the mice received is 40 mg/kg/day. The drinking water was refreshed every other day. Treatment continued until all behavioural experiments were completed. The control group received water with aspartame.

Levodopa/Carbidopa treatment

Cages containing *Ube3a*$^{m-/p+}$ and wild-type littermate control mice (8-12 weeks old) in the F1 hybrid 129S2-C57BL/6J background were assigned to two groups in such a way that both groups had 15 wild-type and 15 mutants and a comparable distribution of males and females. Mice in the treatment group received 15 mg/kg Levodopa and 3.75 mg/kg Carbidopa dissolved in saline (Levodopa, Sigma-Aldrich; Carbidopa, Sigma-Aldrich) by IP injection with an injection volume of 10 ul/g. The untreated group received vehicle injection by IP as described by Tan et al. (W. H. Tan et al., 2017). The mice were injected 1 hour prior to carrying out the behavioural tasks, during the entire period while partaking in these tests.

Levetiracetam treatment

Ube3a$^{m-/p+}$ mice in the 129S2 background were tested for audiogenic seizure susceptibility after IP injection of Levetiracetam (Sigma-Aldrich). The injection volume used is 5 ml/kg and the drug was dissolved in 1% Tween-80 (Sigma-Aldrich) in Milli-Q water as previously described (Florek-Luszczki, Wlaz, & Luszczki, 2014). Increasing doses of Levetiracetam (0-0.5-1-2-10-15 mg/kg) were administered one hour before testing.

Data analysis

Data was analysed using Excel 2010 (Microsoft) and IBM SPSS software (NY, USA). The open field, marble burying and forced swim test data were analysed using an unpaired T-Test in the untreated experimental groups, and a 2-way ANOVA in Minocycline and Levodopa treated animals (in which we assessed a genotype-treatment interaction). Rotarod and nest building were measured with a repeated measures ANOVA in the untreated experimental groups, or with a multivariate repeated measures ANOVA (assessing significance of interaction of time, genotype and treatment) in the Minocycline and Levodopa experimental groups. We used a *Bonferroni's post hoc test* to detect significant differences in male and female groups. For the within subject experiment, we used a paired T-Test for open field, marble burying and forced swim test, while we used a repeated measures factorial ANOVA when analyzing the rotarod and the nest building test. For the audiogenic seizure analysis, a Fisher's exact test was used. The correlation between body weight and maximal performance

on the rotarod test was assessed with a Pearson's correlation test. For the power calculation we performed a priori analysis using G*Power 3.1 software (Kiel, Faul, Erdfelder, Lang, & Buchner, 2007) with α=0.05 and power (1-β)=0.95; 0.90 or 0.80. Data is presented as mean $\pm$ SEM in all figures. For all tests, statistical significance was denoted by $p \leq 0.05$(*), $p < 0.01$(**), $p < 0.001$ (***).

Results

Robust behavioural phenotypes in *Ube3a*$^{m-/p+}$ mice in the F1 hybrid 129S2-C57BL/6J background

We recently developed a number of behavioural tests for testing the effect of gene reinstatement in inducible *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) mice (Silva-Santos et al., 2015). These tests can be applied in successive order to assess phenotypes in the domains of motor performance, anxiety, and repetitive behaviour. Here we set out to assess the robustness of these phenotypes in an independently derived mouse model of AS, by using F1 hybrid 129S2-C57BL/6J *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice (Jiang et al., 1998), which is the *Ube3a* mouse mutant used for nearly all behavioural studies. We have frequently used this strain to test the efficacy of novel treatments, and combined all data obtained from vehicle treated *Ube3a*$^{m-/p+}$ and wild-type littermate controls in the F1 hybrid 129S2-C57BL/6J background to perform a meta-analysis. In total, this constitutes the combined data of 8 experiments, carried out by 5 experimenters and totalling 111 *Ube3a*$^{m-/p+}$ and 120 wild-type littermate controls (**Table 1; Figure 1**).

Exp.#	Person	WT/MUT (n)	Rotarod (time(s)) WT mean (sd) Mut mean (sd)	Open field (distance(m)) WT mean (sd) Mut mean (sd)	Marble burying (# marbles buried) WT mean (sd) Mut mean (sd)	Nest building (% material used) WT mean (sd) Mut mean (sd)	Forced swim test (% floating) WT mean (sd) Mut mean (sd)
1	A	15/13	128 (42) 96 (32)	41 (14) 22 (11)	11 (4) 4 (3)	14 (25) 79 (18)	53 (23) 83 (7)
2	A	15/13	142 (43) 80 (32)	49 (10) 32 (12)	8 (4) 2 (3)	36 (23) 79 (14)	44 (24) 81 (7)
3	A	15/13	133 (42) 92 (46)	40 (12) 29 (8)	11 (3) 2 (2)	27 (18) 70 (18)	41 (19) 73 (14)
4	B	21/17[1)]	159 (60) 102 (36)	31 (12) 19 (11)	14(4) 4(5)	10 (11) 48 (25)	24 (22) 63 (18)
5	C	9/11[2)]	163 (49) 91 (37)	25 (6) 10 (7)	12 (5) 4(5)	48 (27) 69 (12)	28 (24) 76 (8)
6	D	15/14[3)]	107 (44) 74 (26)	44 (7) 29 (13)	12 (3) 3 (3)	40 (18) 79 (12)	14 (20) 60(31)
7	E	15/15	196 (57) 126(52)	45(10) 35 (7)	11 (5) 6 (3)	63 (20) 74 (14)	47 (20) 67 (14)
8	A	15/15[4]	162 (49) 95 (35)	49 (9) 33 (13)	10 (3) 2 (3)	N/A	47 (20) 88 (9)

Table 1. Overview of experiments used for the meta-analysis. All experiments were performed using *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. For all tests shown in this table, we found a significant effect of genotype ($p<0.05$), except for the nest building test of experiment 8, which was not performed. The table indicates the individual that performed the test battery, the number of wild-type and mutant mice used for each test, and the mean and standard deviation of the outcomes obtained. For rotarod we indicated the average performance over the 5 days, while for the nest building, we provided the data as measured at day 5. Note that for some of the tests we used a different number of mice (mice were not properly tracked, or a smaller cohort was used for nest building because of space limitations). The adapted n for these experiments is: 1): nest building 13/12, forced swim test 20/17; 2): nest building 6/7; 3): open field 13/14; 4): open field 10/10, nest building not performed.

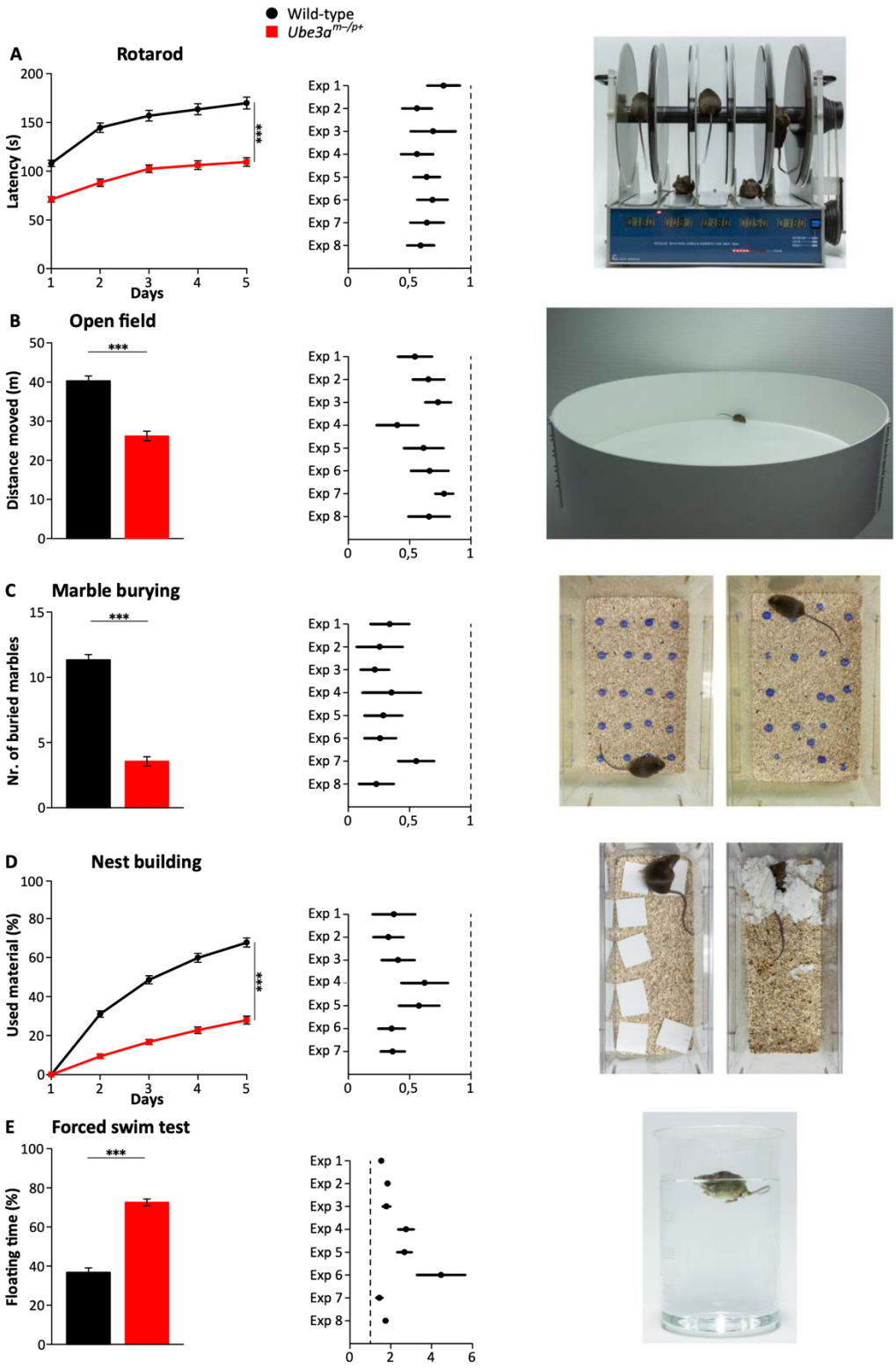

Figure 1. Behavioural testing of *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. For each behavioural paradigm, the pooled (raw) data of all experiments is presented on the left panel, whereas the Forrest plots in the middle panel show the normalized data of the individual experiments (in which the data of each experiment is normalized against wild-type; represented by a dashed line), as well as the 95% confidence interval. The picture on the right panel shows the behavioural set-up used

for our experiments. For the marble burying test and nest building test the picture shows the onset and finish of a behavioural experiment. **A** Accelerating rotarod in wild-type (WT) and *Ube3a*$^{m-/p+}$ mice (n=120,111). **B** Open field test in WT and *Ube3a*$^{m-/p+}$ mice (n=113,106). **C** Marble burying test in WT and *Ube3a*$^{m-/p+}$ mice (n=120,111). **D** Nest building test in WT and *Ube3a*$^{m-/p+}$ mice (n=94,86). **E** Forced swim test in WT and *Ube3a*$^{m-/p+}$ mice (n=120,111). All data represent mean ± SEM. A repeated measures ANOVA or t-test was used for statistical comparison of the non-normalized data. All tests show a significance effect of genotype (***p<0.001).

Individuals with Angelman syndrome show clear motor impairments, and impaired performance on the accelerating rotarod is the most frequently described phenotype in *Ube3a* mice. Indeed, our meta-analysis shows a very robust significant difference between the two genotypes (p<0,001; **Figure 1A**). A power analysis with α=0.05; (1-β)=0.95 showed that this task requires 14 animals per genotype **(Table 2).**

	Wildtype (mean±SD)	Ube3a (mean ±SD)	Test	Achieved effect size	Sample size per group β = 0.95	Sample size per group β = 0.90	Sample size per group β = 0.80
Rotarod Time on machine (s)	149±55	95±40	ANOVA	0.56	14	11	9
Open Field Distance moved (m)	40 ±13	26 ±13	T test	1.17	21	17	13
Marble burying (# Marbles buried)	11±4	4 ±4	T test	2.26	7	6	5
Nest building (% used nesting material)	68±23	28 ±19	T test	1.95	8	7	6
Forced swim test (% floating time)	37±25	73 ±18	T test	1.73	10	9	7
Susceptibility to audiogenic seizure (% of animals)	7	98	T test	4.55	3	3	3

Table 2. Achieved power for each behavioural test of the behavioural test battery. Data provided is based on the experiments using *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. The table provides the obtained effect size, number of mice needed per genotype for each behavioural test (with power equal to 0.95, 0.90, 0.80) and statistical test used. For rotarod calculations we used the average performance over the 5 days, while for the nest building, we used the data of the last test day.

Following 2 days of rest, the same mice were then tested in the open field test. This paradigm is commonly used to assess anxiety in mice. Increased anxiety is commonly observed in individuals with AS (Pelc, Cheron, & Dan, 2008), as well as individuals with autism spectrum disorder. In this test, we place the mice in an open arena situated in a brightly lit room and record the distance the mice travel during a 10-minute time span. The measurements of the

distance moved in the open arena indicated that AS mice moved significantly less (WT: 40.3±1.3m; AS: 26.2 ±1.3m; p<0,001; **Figure 1B**). A power analysis (α=0.05; (1-β)=0.95) showed that this task requires a minimum number of 21 mice per genotype, which makes this test a relative weak test **(Table 2).** Previous studies reported no significant difference observed **between genotypes** in the time spent in the (Born et al., 2017; Huang et al., 2013) inner zone of the open field, which is another measure of anxiety. Our meta-analysis revealed a significant difference between genotypes (p<0.005), but this difference was small (WT: 1.1% versus mutant 0.7% time in inner zone), and a significant effect was only observed in 4 out of the 8 individual experiments (data not shown).

After one day of rest, the same mice were then analysed in the marble burying test, a test used to assess repetitive and perseverative behaviour as well as anxiety (Angoa-Pérez et al., 2013; Kedia & Chattarji, 2014). When exposed to marbles, AS mice show a strongly impaired marble burying behaviour compared to WT mice (WT: 11.3±0.4; AS: 3.6±0.3; p<0.001; **Figure 1C**). A power analysis (α=0.05; 1-β=0.95) showed that 7 animals/group are sufficient for this test, indicating a very robust phenotype (**Table 2**).

After the marble burying task, all mice were single housed for 5-7 days and then analysed for 5 consecutive days while performing the nest building test. The nest building test assesses the innate behaviour of mice to create a nest to maintain body temperature and to find shelter (Jirkof, 2014). AS mice showed a clear phenotype compared to their WT control littermates (p<0.001; **Figure 1D**). As indicated in **Table 2**, the nest building phenotype is quite robust, since it only requires 8 mice (α=0.05; 1-β=0.95) per group if analysed over the last day.

Following 2 days of pause, the animals were finally subjected to the forced swim test, in which the mouse is placed in a beaker filled with water, from which the mouse will try to escape by swimming. This test is typically used to test depressive-like behaviour in mice (Can et al., 2011). AS mice showed significant more time floating (instead of swimming) compared to WT mice (WT: 36.8±2.3; AS: 72.6±1.7; p<0.001; **Figure 1E**). The power analysis test showed that this task requires a minimum of 10 mice (α=0.05; 1-β=0.95).

Taken together, the data indicates that this test battery yields a series of robust behavioural phenotypes that can be obtained in a relative quick manner using a single cohort of mice.

The dependence of sex on the behavioural phenotypes

Angelman syndrome affects both males and females, with no known differences between the sexes. To assess if this is also the case for the *Ube3a* mouse phenotypes described above, we analysed if there were any significant sex differences. An effect of sex was noted on the rotarod, in which female wild-type and *Ube3a* mice performed significantly better than male wild-type and *Ube3a* mice (p<0.001; **Figure 2A**). Since male mice are heavier than female

mice and since *Ube3a*$^{m-/p+}$ mutants show increased weight (**Figure 2F**)(Huang et al., 2013; Meng et al., 2013), we investigated if the impaired rotarod performance as seen in *Ube3a*$^{m-/p+}$ mutants could be attributed to their increased weight. Hence, we performed a correlation analysis between body weight and time on the rotarod (as measured on the last training day). As shown in **Figure 2G,** no meaningful correlation is observed between body weight and latency to fall in both WT mice and AS mice (WT males Pearson r=0.08; AS males Pearson r=–0.21; WT females Pearson r=0.35; AS females Pearson r=0.02). These results suggest that the motor activities measured on the rotarod are not caused by the increased body weight observed in *Ube3a*$^{m-/p+}$ mutants, but truly reflect differences in motor performance.

We also observed a small effect of sex for the nest building task in which female *Ube3a*$^{m-/p+}$ mutants outperformed the male *Ube3a*$^{m-/p+}$ mutants ($p<0.05$). A similar tendency was also observed in wild-type mice, but this effect was not significant (**Figure 2B**). Despite the slightly better performance of female *Ube3a*$^{m-/p+}$ mutants, female *Ube3a*$^{m-/p+}$ mutants were still significantly different from wild-type mice ($p<0.001$).

We observed no significant effect of sex in the open field test ($p=0.25$), marble burying test ($p=0.06$) and forced swim test ($p=0.27$; **Figure 2C-E**). Overall, these data suggest that the set of behavioural phenotypes observed in AS mice are robust and are not markedly influenced by the sex of the animal. However, given the decreased performance of male mice on the rotarod, mixed cohorts used for rotarod testing should be well balanced with respect to sex to obtain a reliable phenotype.

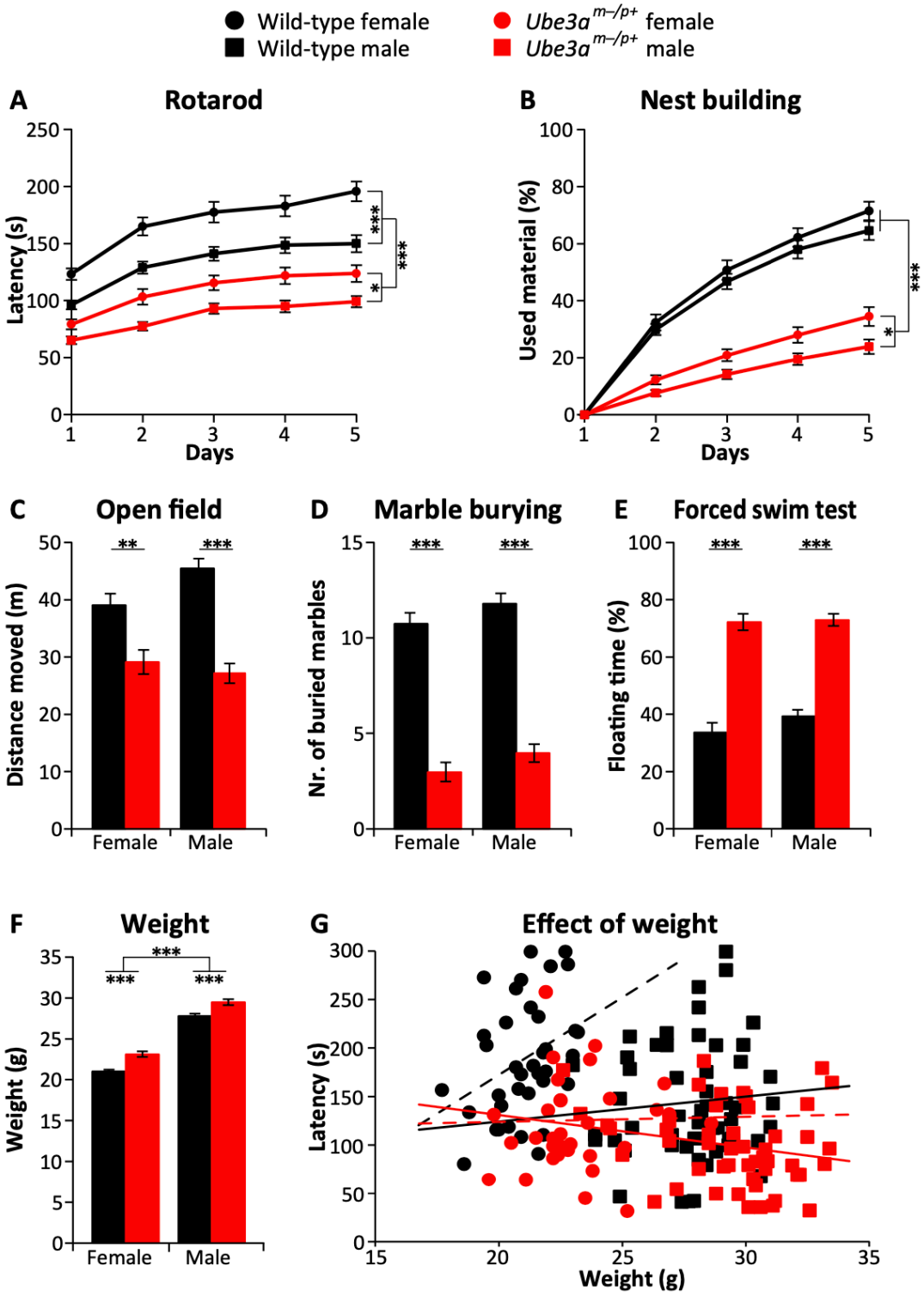

Figure 2. Effect of sex on the behavioural phenotypes of *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. A Accelerating rotarod in WT and *Ube3a*$^{m-/p+}$ female mice (n=52,46) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=68,65). **B** Nest building test in WT and *Ube3a*$^{m-/p+}$ female mice (n=42,33) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=52,53). **C** Open field test in WT and *Ube3a*$^{m-/p+}$ female mice (n=47,41) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=66,65). **D** Marble burying test in WT and *Ube3a*$^{m-/p+}$ female mice (n=52,46) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=68,65). **E** Forced swim

test in WT and *Ube3a*$^{m-/p+}$ female mice (n=52,46) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=68,65). **F** Body weight in WT and *Ube3a*$^{m-/p+}$ female mice (n=37,33) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=53,50). **G** Pearson correlation test between body weight and latency to fall at day 5 in WT and *Ube3a*$^{m-/p+}$ female mice (n=37,33) and in WT and *Ube3a*$^{m-/p+}$ male mice (n=53,50). Multivariate repeated ANOVA or a 2-way ANOVA was used for statistical comparison. A *Bonferroni's post hoc test* was used to detect significant differences in behavioural phenotypes of male and female groups. All data represent mean ± SEM. Significant effects of genotype or sex are indicated as *p<0.05, **p<0.01, ***p<0.001.

The behavioural test battery is suitable for within-subject testing design

A within-subject testing design is a powerful design for drug testing purposes, as it allows assessing the efficacy of a drug with considerable fewer animals. Therefore, we investigated whether the behavioural test battery allowed retesting the same animals while maintaining a similar phenotype, which is a prerequisite for applying a within subject design. We subjected 15 *Ube3a*$^{m-/p+}$ mice (*Ube3a*^{tm1Alb}) and 15 WT littermates in the F1 hybrid 129S2-C57BL/6 background to the behavioural test battery and repeated the test battery after a pause of 4 weeks. As shown in **Figure 3**, performance on the rotarod test, nest building test and forced-swim test was highly similar when the initial test data were compared to the re-testing data. However, performance in the open field test as well as nest building test was significantly different when this test was performed for the second time (open field: wild-type initial vs retest p<0.001; *Ube3a*$^{m-/p+}$ initial vs retest p<0.001. Marble burying: wild-type initial vs retest p<0.001; *Ube3a*$^{m-/p+}$ initial vs retest p<0.001; Paired T-test). These differences upon retesting are likely due to the decreased anxiety levels and or habituation of the mice upon re-testing in these paradigms. Importantly, *Ube3a*$^{m-/p+}$ mice remained significant different from wild-type littermates when tested for a second time, with the exception of the marble burying test, which no longer yielded a phenotype upon re-testing (p=0.13). Hence, we conclude that most tests of the behavioural test battery are suitable for a within-subject design to test the efficacy of a drug.

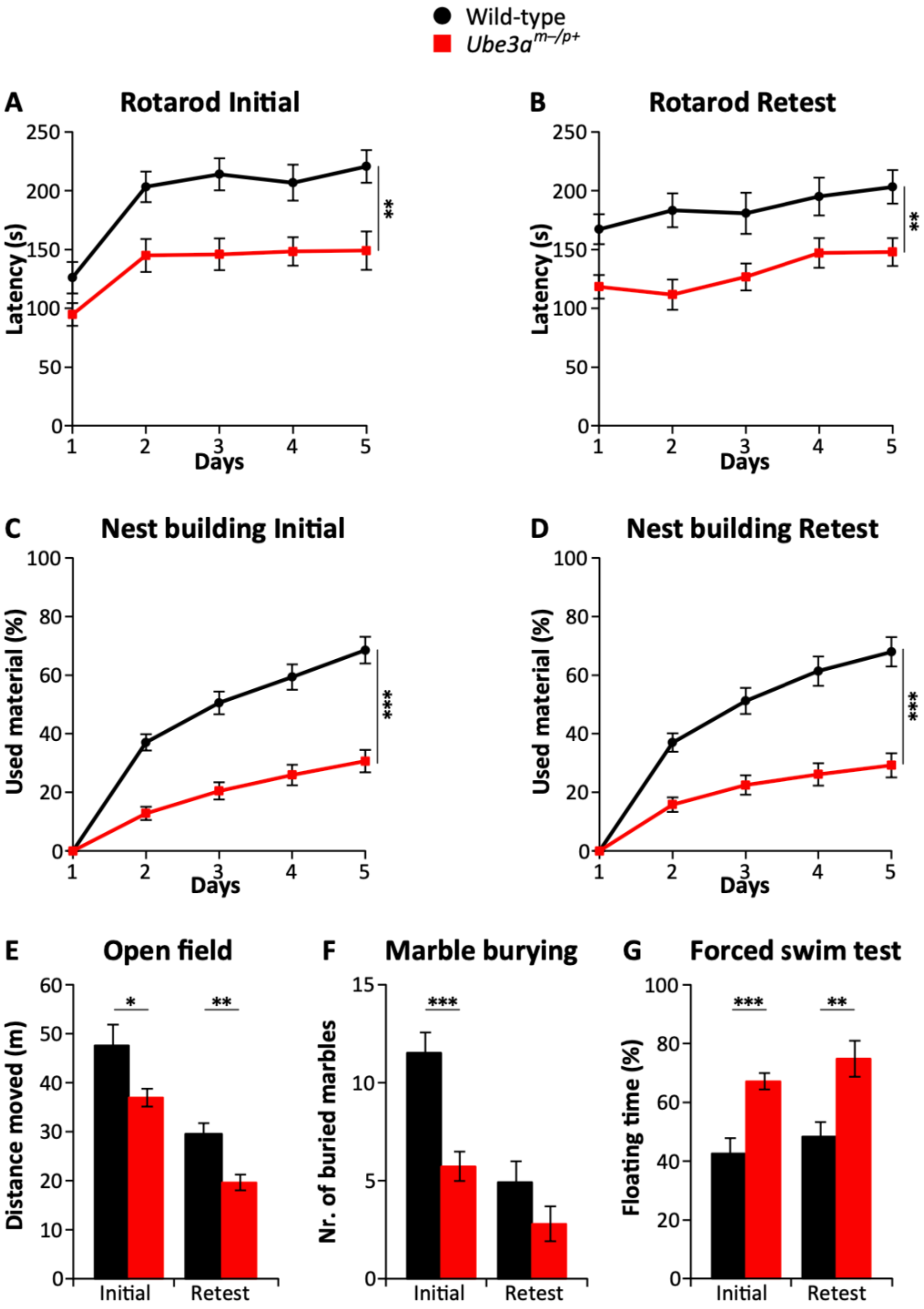

Figure 3. Most behavioural phenotypes are stable upon re-testing *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. A,C,E,F,G WT and *Ube3a*$^{m-/p+}$ mice at initial testing and (**B,D,E,F,G**) upon re-testing. A single cohort of 15 wild-type (8 females, 7 males) and 15 *Ube3a*^{tm1Alb} (8 females, 7 males) was used for all experiments. A repeated measures ANOVA or T-Test was used for statistical comparison of genotypes, as described in legend of **Figure 1**. All data represent mean ± SEM. Significant effects of genotype are indicated as *p<0.05, **p<0.01, ***p<0.001 for genotype significance.

Behavioural phenotypes are also observed in the *Ube3a*^{E113X} mouse model

The results above indicate that the behavioural test battery gives robust phenotypes in the *Ube3a*^{tm1Alb} line as well as in the previously published *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) line. In order to test the robustness of the battery in a third independently derived *Ube3a* mutant strain, we used the *Ube3a*$^{mE113X/p+}$ (*Ube3a*^{tm2Yelg}) strain, which we recently described (Wang, van Woerden, Elgersma, & Borst, 2017). As shown in **Figure 4** the *Ube3a*$^{mE113X/p+}$ mutant mice in the F1 129S2-C57BL/6J background, showed again clear impairments on the rotarod ($p<0.001$), open field test ($p<0.01$), marble burying test ($p<0.05$), nest building test ($p<0.01$), and forced swim test ($p<0.001$). Taken together, these data suggest that the identified set of behavioural phenotypes in this test battery is present in 3 independently derived *Ube3a* mutant lines.

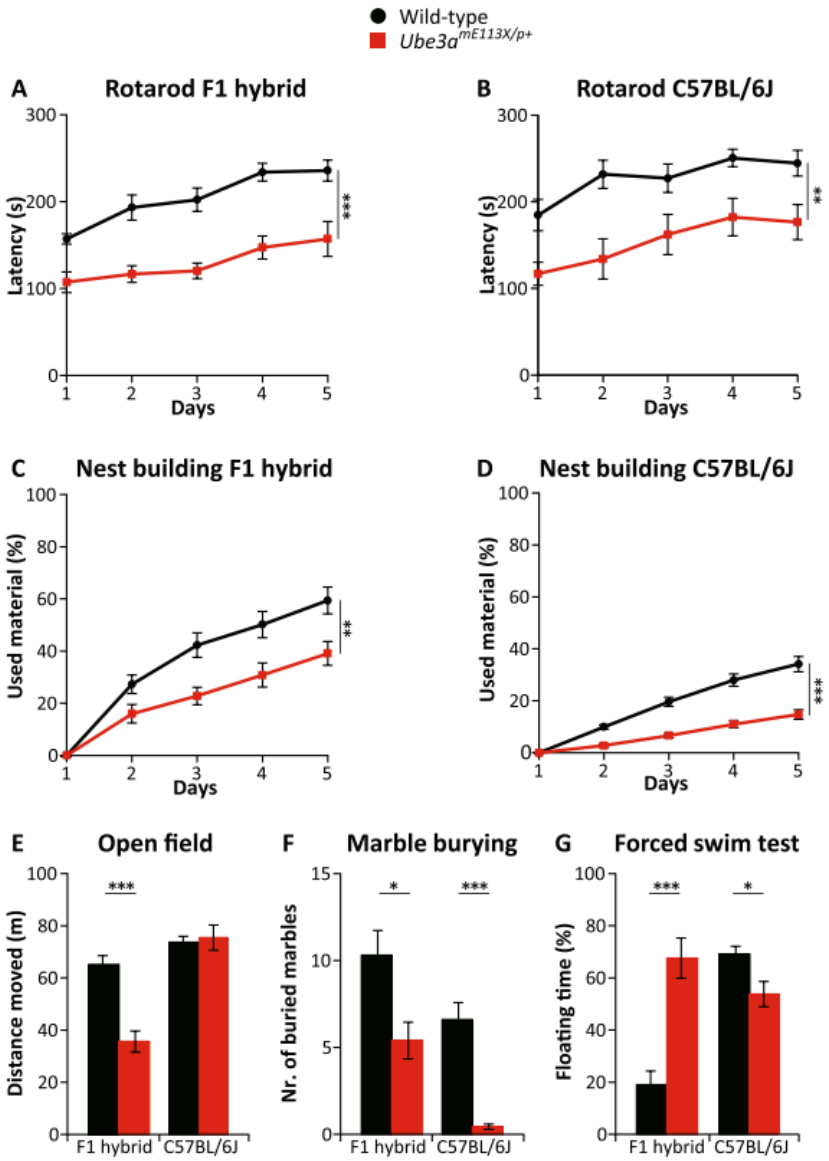

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Figure 4 Behavioural testing of Ube3a$^{mE113X/p+}$ (Ube3a^{tm2Yelg}) mice in the F1 hybrid 129S2-C57BL/6J and the C57BL/6J background. a, b Accelerating rotarod in WT and Ube3a$^{mE113X/p+}$ mice in F1 hybrid 129S2-C57BL/6J and C57BL/6J background. **c, d** Nest building test in WT and Ube3a$^{mE113X/p+}$ mice in F1 hybrid 129S2-C57BL/6J and C57BL/6J background. **e–g** Open field, marble burying, and forced swim tests in WT and Ube3a$^{mE113X/p+}$ mice in F1 hybrid 129S2-C57BL/6J and C57BL/6J background. For all behavioural tests, we used a single cohort of 10 wild-type (1 female, 9 males) and 10 Ube3a$^{mE113X/p+}$ mice (6 females, 4 males) in F1 hybrid 129S2-C57BL/6J, and 15 wild-type (11 females, 4 males) and 16 Ube3a$^{mE113X/p+}$ (Ube3a^{tm2Yelg}) (13 females, 4 males) mice in C57BL/6J background. All data represent mean ± SEM. A repeated measures ANOVA or T test was used for statistical comparison of genotypes, as described in the legend of **Figure 1**. Significant effects of genotype are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001

Mouse genetic background affects many of the identified AS phenotypes

Previous studies have indicated the importance of the genetic background for certain *Ube3a* phenotypes (Born et al., 2017; Huang et al., 2013). To test the importance of the genetic background on the behavioural test battery, we performed the test battery on AS mice on a pure C57BL/6J and 129S2 background instead of the F1 hybrid background. *Ube3a*$^{mE113X/p+}$ mice in C57BL/6J background showed a similar phenotype as *Ube3a*$^{mE113X/p+}$ mutants in the F1 hybrid 129S2-C57BL/6J background with respect to rotarod ($p<0.001$), marble burying test ($p<0.001$) and nest building test ($p<0.001$) (**Figure 5**).

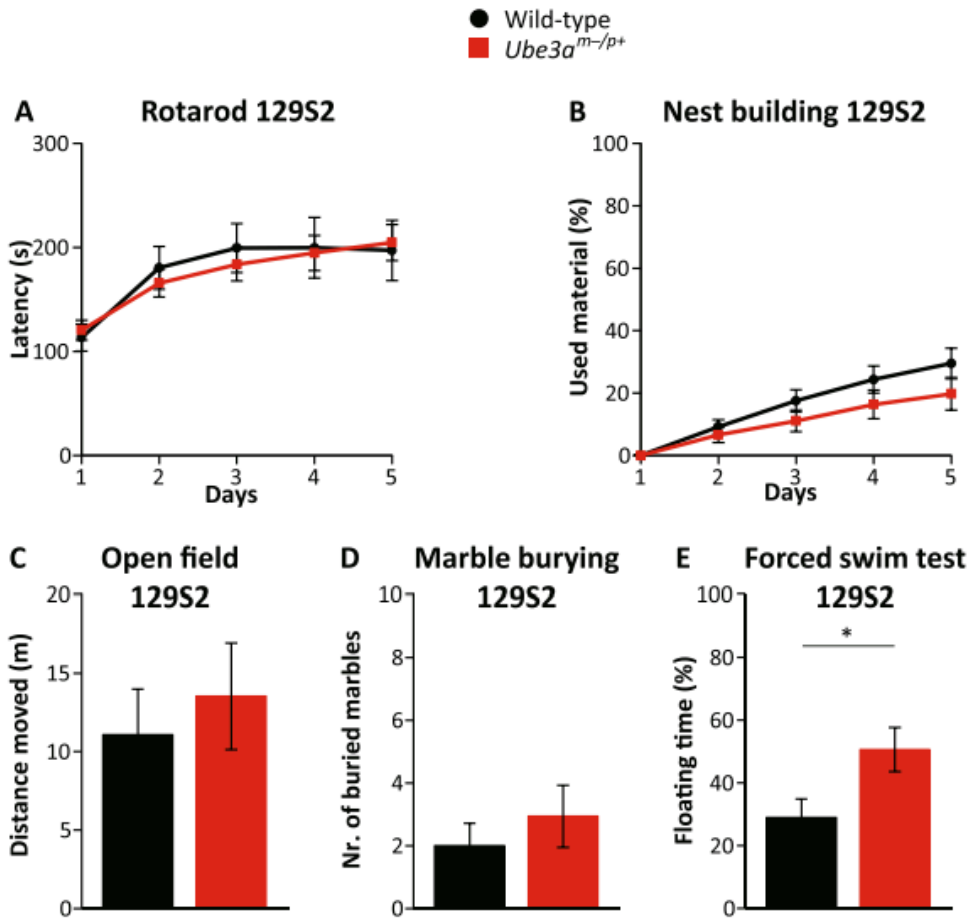


Figure 5 Behavioural testing of *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice in the 129S2/SvPasCrl background. **A-E** Accelerating rotarod, nest building, open field, marble burying, and forced swim test in wild-type and Ube3at^{m1Alb} mice in 129S2/SvPasCrl background (n = 11, 16) (WT = 5 females, 6 males) (Ube3a$^{m-/p+}$ = 8 females, 8 males). A repeated measures ANOVA or T test was used for statistical comparison of genotypes, as described in the legend of Figure 1. Significant effects of genotype are indicated as *p < 0.05.

No deficit was observed in the open field test (p=0.75). Notably, the *Ube3a*$^{mE113X/p+}$ mice in C57BL/6J background showed a significant phenotype in the forced swim test (p<0.05), however in the opposite direction compared to AS mice in F1 hybrid 129S2-C57BL/6J background.

The test battery was also performed using *Ube3a*^{tm1Alb} mice in the inbred 129S2 background. *Ube3a*^{tm1Alb} mice in the 129S2 background did not show any of the phenotypes observed in *Ube3a*^{tm1Alb} mice in the F1 hybrid background, with the exception of the forced swim test (p<0.05), which yielded a similar result as obtained in mice in the F1 hybrid background. Taken together these data confirm and extend previous studies that most AS mouse phenotypes are strongly dependent on the genetic background.

Susceptibility to audiogenic seizures

Epilepsy is a common feature of individuals with AS (Fiumara, Pittalà, Cocuzza, & Sorge, 2010). We previously showed that *Ube3a*^{tm1Alb} mice as well as *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) mice are highly susceptible to audiogenic seizures, a phenotype that is specifically observed in mice in the 129S2 background (Jiang et al., 1998). To investigate the strength of this test in more detail, we performed a meta-analysis of 5 independent experiments with a total of 114 *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice and 45 wild-type littermates in the 129S2 background. This analysis showed that this is a very robust phenotype with seizures observed in 98% of *Ube3a*$^{m-/p+}$ mice and in 7% of the wild-type littermates ($p<0.001$). The robustness of this test was further confirmed by a power calculation analysis (**Table 2**).

We tested whether seizures were also present in the *Ube3a*$^{mE113X/p+}$ (*Ube3a*^{tm2Yelg}) line. To that end, we crossed *Ube3a*$^{pE113X/m+}$ females (back-crossed 8 times in 129S2) with 129S2 males. As shown in **Figure 6**, an audiogenic seizure could be provoked in all *Ube3a*$^{mE113X/p+}$ mutants tested ($p<0.001$), indicating that this phenotype is observed across 3 independently derived *Ube3a* mutant lines.

We previously demonstrated that the sensitivity to audiogenic seizures can be reversed upon acute treatment with anti-epileptic drugs (Silva-Santos et al., 2015). Given the high power of this assay, we investigated if this assay is suitable to determine the effective dose of a treatment. To that end, we treated mice with Levetiracetam, a compound that acts as ligand of the synaptic vesicle protein 2A, which is a commonly used anti-epileptic drug for both partial and generalized seizures and which is also often prescribed to individuals with AS (Thibert et al., 2009; Weber, 2010). *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice in 129S2 background were first assessed for their sensitivity to audiogenically evoked seizures without treatment. After establishing that all mice were sensitive, we treated these mice using increasing doses of Levetiracetam and tested the mice 1 hour after IP injection. As shown in **Figure 6D**, a good dose-response curve could be obtained, in which 2 mg/kg Levetiracetam yielded approximately 60% of mice to be resistant to audiogenic seizures. This indicates that this test is highly suitable for quickly determining the effective dose of a treatment.

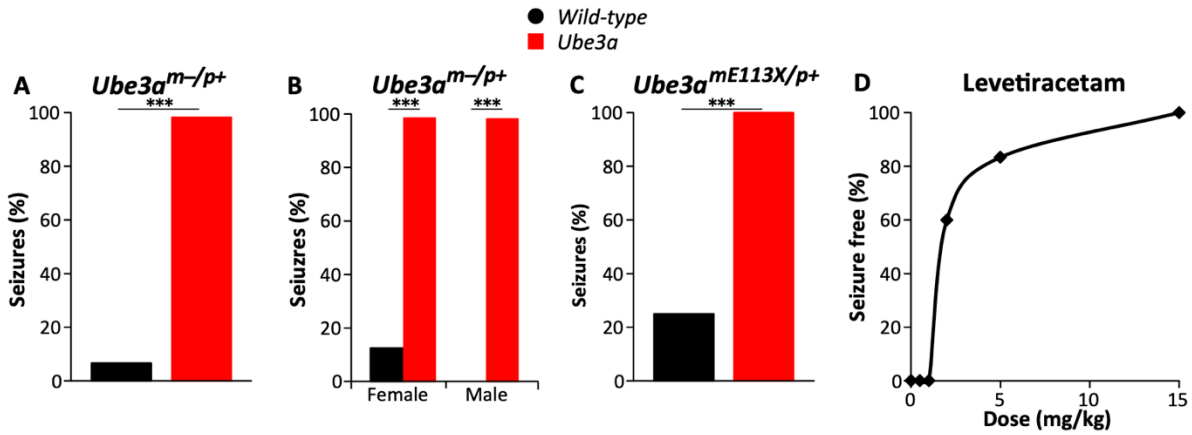


Figure 6 Audiogenic seizure susceptibility in *Ube3a*$^{m-/p+}$ and *Ube3a*$^{mE113X/p+}$ mice in the 129S2/SvPasCrl background. A Audiogenic seizure susceptibility in WT and *Ube3a*$^{m-/p+}$ mice (n=45,114). **B** Effect of sex on seizure susceptibility in wild-type and *Ube3a*$^{m-/p+}$ mice (females n=24,62; males n=21,52). **C** Seizure susceptibility in wild-type and *Ube3a*$^{mE113X/p+}$ mice (n=4,8). **D** Effect of increasing doses of Levetiracetam on epilepsy susceptibility of *Ube3a*$^{m-/p+}$ mice (0 mg/kg, n=12; 0.5mg/kg, n=6; 1 mg/kg, n=6; 2mg/kg, n=30; 5mg/kg, n=30; 15mg/kg, n=30). Fisher's exact test was used for statistical comparison. ***denotes $p<0.001$ for genotype significance.

Minocycline treatment does not improve behavioural phenotypes of *Ube3a* mice

It has previously been reported that Minocycline treatment of *Ube3a* animals improves synaptic plasticity as well as motor coordination, which was the basis for an open label study with minocycline in individuals with AS (trial register NCT01531582 and (J C Grieco et al., 2014)), as well as a randomized controlled trial (NCT02056665), (Ruiz-Antorán et al., 2015). Unfortunately, the randomized trial showed no difference between placebo and Minocycline treated individuals (Ruiz-Antorán et al., 2015). To test if Minocycline ameliorated the *Ube3a* mutant phenotypes in our behavioural test battery, we subjected the animals to the same treatment protocol as used for the initial mouse study (J C Grieco et al., 2014). Adult-treated *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice and littermates controls (8-12 weeks of age) in the F1 hybrid 129S2-C57BL/6J background, received daily Minocycline (45 mg/kg) or control saline IP injections starting three weeks prior to behavioural testing. After 3 weeks of daily injections, the mice were sequentially subjected to the behavioural test battery as described above. In contrast to the previous finding (trial register NCT01531582) we did not observe a rescue on the rotarod. We also observed no effect of Minocycline on any of the other tests of the behavioural battery (**Figure 7**; 2-way ANOVA, genotype/treatment interaction $p>0.08$ in all tests). Notably, prolonged exposure to daily Minocycline injections resulted in yellow deposits over the organs and dullness of the liver (data not shown), confirming previous studies that IP administration of Minocycline is not the best choice of administration (Fagan et al., 2004).

Minocycline has also been used to reverse the behavioural deficits of a mouse model of Fragile X, possibly by its ability to reduce MMP9 activity, which synthesis is increased in FMRP mice (Bilousova et al., 2009; Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012). Notably, in these studies Minocycline treatment was initiated immediately after birth and provided though the drinking water. Since we previously showed that a behavioural rescue of *Ube3a* mice may also depend on the timing of treatment initiation (Silva-Santos et al., 2015), we decided to treat *Ube3a* animals immediately after birth, using the same protocol as described for FMRP mice (Bilousova et al., 2009). However, also this prolonged postnatal treatment regimen did not yield a significant behavioural improvement, as none of these tests showed a significant interaction of genotype and treatment (2-way ANOVA, genotype/treatment interaction $p>0.16$ in all tests) (**Figure 7**).

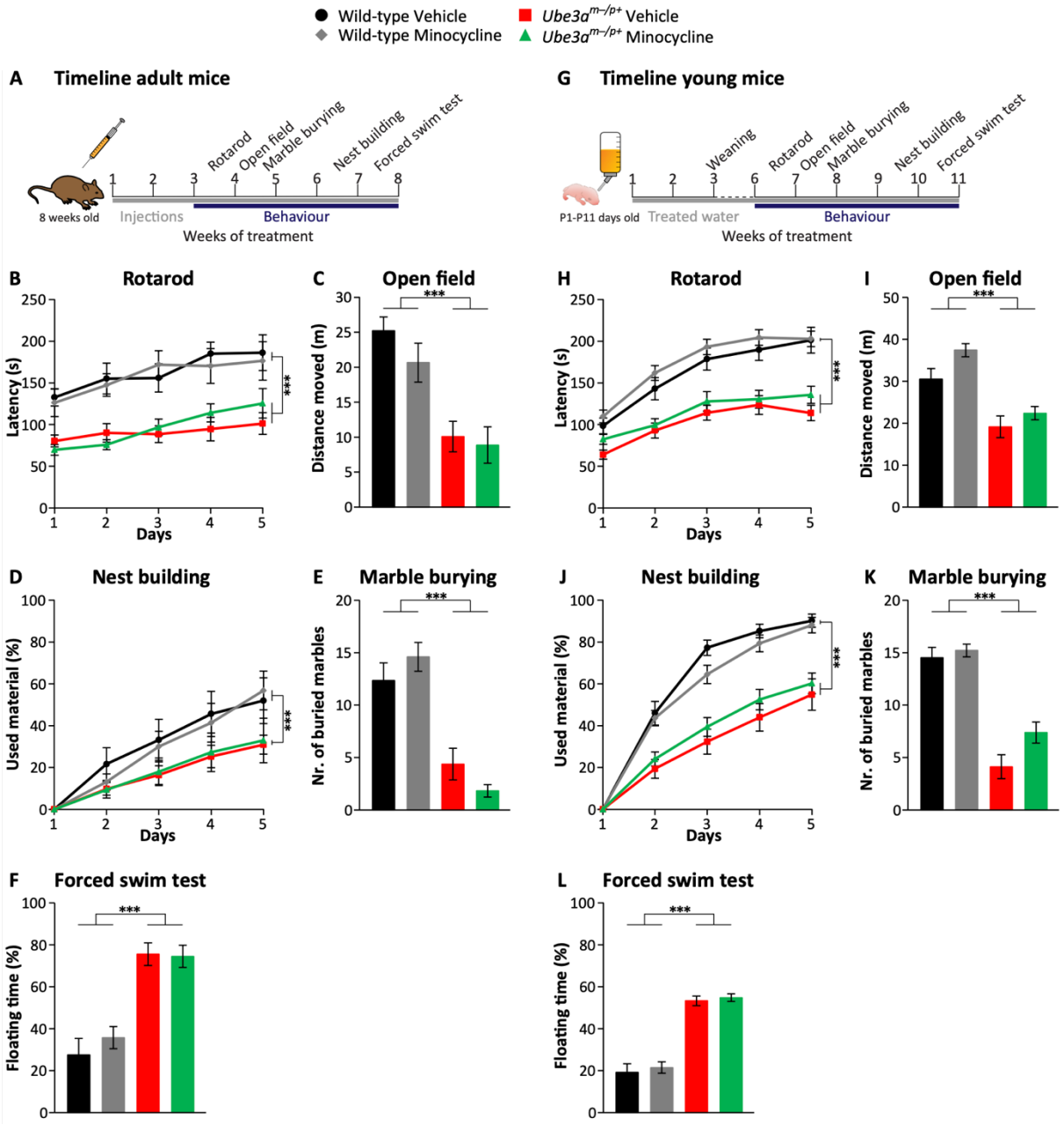
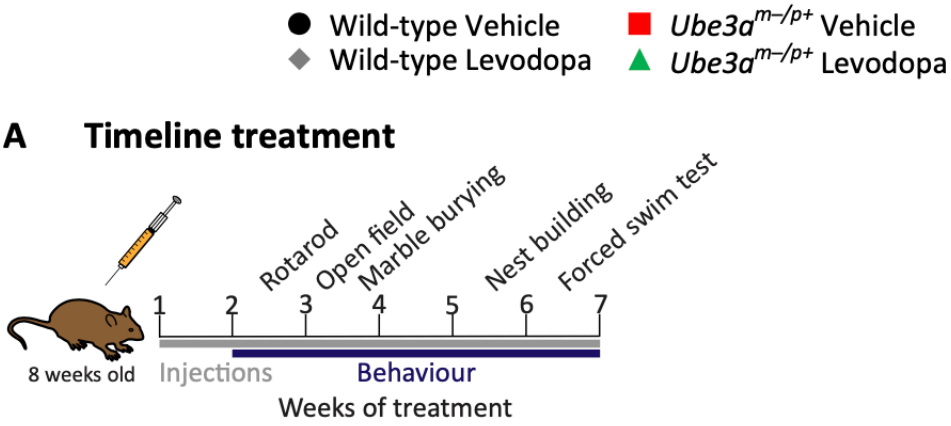


Figure 7 Effect of Minocycline treatment on adult and young *Ube3a*^{tm1Alb} mice in F1 hybrid 129S2-C57BL/6J background. A Timeline representing Minocycline treatment and behavioural phenotyping of adult *Ube3a*$^{m-/p+}$ mice. **B-F** Effect of Minocycline on adult *Ube3a*^{tm1Alb} mice on the behavioural test battery**.** Wild-type and *Ube3a*$^{m-/p+}$ *(Ube3a*^{tm1Alb}*)* vehicle treated adult mice: n= 9,11, with the exception of the nest building (n=6,7). Minocycline treated wild-type and *Ube3a*$^{m-/p+}$ *(Ube3a*^{tm1Alb}*)* adult mice: n=10,11 mice, with the exception of the nest building (n=6,6). **G** Timeline representing Minocycline treatment and behavioural phenotyping of young *Ube3a*$^{m-/p+}$ mice. **H-L** Effect of Minocycline on young *Ube3a*^{tm1Alb} mice on the behavioural test battery. Wild-type and *Ube3a*$^{m-/p+}$ *(Ube3a*^{tm1Alb}*)* vehicle treated young mice: n= 21,17, with the exception of the nest building (n=13,12) and the forced swim test (20,17). Minocycline treated wild-type and *Ube3a*$^{m-/p+}$ *(Ube3a*^{tm1Alb}*)* young mice: n=33,22 mice, with

the exception of the open field (33,21), the marble burying (33,21) and the nest building (n=16,17). A multivariate repeated ANOVA or a 2-way ANOVA was used for statistical comparison in behavioural phenotypes. ***p<0.001 indicates effect of genotype. In none of the tests we observed an interaction of genotype and treatment.

Levodopa/Carbidopa treatment does not improve behavioural phenotypes of *Ube3a* mice

A recent study showed that treatment of *Ube3a* mice with Levodopa resulted in improvement of their motor skills compared to untreated *Ube3a* mice (W. H. Tan et al., 2017). Based on this preclinical observation, a placebo-controlled trial of Levodopa was initiated in 55 children between 4 and 12 years diagnosed with AS. Unfortunately, no significant improvement was observed on any the outcomes measured following a 1-year treatment (trial register NCT01281475 and (W. H. Tan et al., 2017)). To test to what extent Levodopa ameliorated the phenotypes of *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) mice in our behavioural battery, we subjected the animals to the same treatment protocol as used for the initial mouse study (W. H. Tan et al., 2017). *Ube3a*$^{m-/p+}$ and wild-type littermates (8-12 weeks of age) in F1 hybrid 129S2-C57BL/6J background received daily Levodopa/Carbidopa (15 mg/kg Levodopa and 3.75 mg/kg Carbidopa) or control saline IP injections, starting 1 hour prior to behavioural testing. In contrast to the earlier finding (W. H. Tan et al., 2017), we did not observe a rescue on the rotarod. We also observed no effect of Levodopa treatment on any of the other tests of the behavioural battery (2-way ANOVA, genotype/treatment interaction p>0.17 in all tests) **(Figure 8).**



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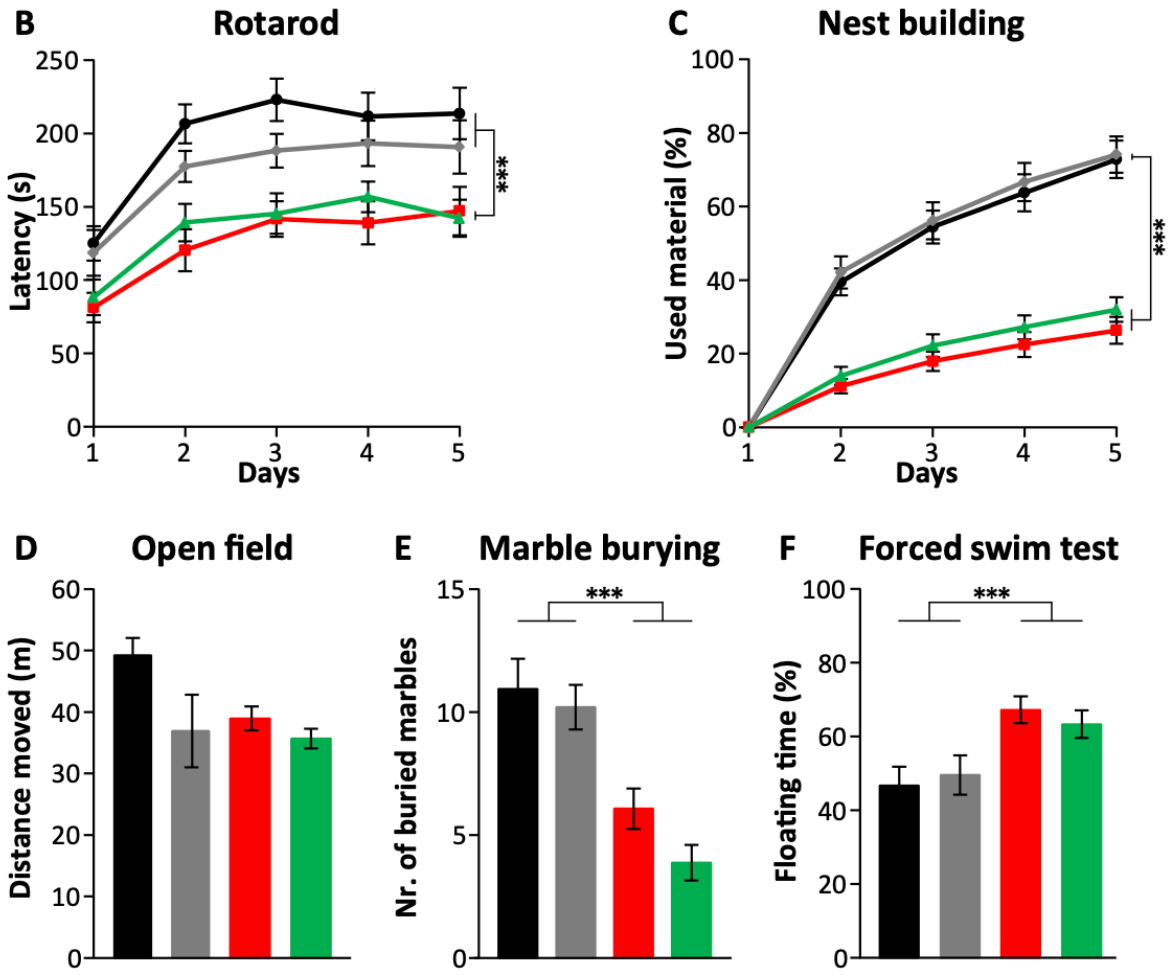

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Figure 8 Effect of Levodopa treatment on *Ube3a^{tm1Alb}* mice in F1 hybrid 129S2-C57BL/6J background. A Timeline representing Levodopa treatment and behavioural phenotyping of *Ube3a$^{m-/p+}$* mice. **B-F** Effect of Levodopa on the behavioural test battery. Wild-type and *Ube3a$^{m-/p+}$ (Ube3a^{tm1Alb})* vehicle treated mice: n= 15,15. Levodopa treated wild-type and *Ube3a$^{m-/p+}$ (Ube3a^{tm1Alb})* mice: n=15,15 mice. A multivariate repeated ANOVA or a 2-way ANOVA was used for statistical comparison in behavioural phenotypes. ***indicates significant effect of genotype $p<0.001$. No effect of genotype was observed in the open field test, since Levodopa-treated wild-type mice were similar to *Ube3a* mice. In none of the tests we observed an interaction of genotype and treatment.

Discussion

Robust behavioural phenotypes with high construct and face validity in mouse models of disease, are critical for the identification of novel treatments, and the successful translation of these therapies to clinical trials. These preclinical studies may give us important information about the therapeutic dose, optimal age of treatment and the best outcome measures to be used in a clinical trial. Given the high failure rate of clinical trials aimed at improving cognitive function (van der Vaart, Overwater, Oostenbrink, Moll, & Elgersma, 2015), it is absolutely

critical that the preclinical data is robust (reproducible results across different mutant lines and different experimenters), and that the animal studies have high construct and face validity.

In this study we investigated the robustness of a number of behavioural phenotypes, which we previously described using the inducible *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) mice (Silva-Santos et al., 2015). These phenotypes were assessed in two independently derived *Ube3a* lines: in the commonly used *Ube3a*^{tm1Alb} line (Jiang et al., 1998), and the recently generated *Ube3a*$^{mE113X/p+}$ (*Ube3a*^{tm2Yelg}) line (Silva-Santos et al., 2015). Recently we have tested 2 additional novel *Ube3a* lines in this test battery with the same results; the *Ube3a*$^{tm1.1Bdph}$ line; (MGI:5882092) and a novel (unpublished) *Ube3a* line (*Ube3a*^{em1Yelg}). Thus, taken together a total of five independently derived *Ube3a* lines show phenotypes on all the behavioural tests of the test battery described in this study. In all cases, we used heterozygous *Ube3a* mice in which the mutation was located on the maternally inherited *Ube3a* allele. Therefore, we conclude that construct validity is very high. However, since the majority of individuals with AS carries a large chromosomal deletion of the AS critical region (15q11-q13) which encompasses also other genes besides *Ube3a* and which may contribute to a more severe phenotype (Gentile et al., 2010), it would be of interest to test a mouse model of AS with large maternal deletion (Jiang et al., 2010) in our behavioural test battery.

In terms of face validity, we used behavioural paradigms in the domains of motor performance, anxiety, repetitive behaviour, and seizure susceptibility, which are all relevant clinical phenotypes of AS. However, an important clinical feature of AS that is lacking in our behavioural test battery, is a paradigm that assesses cognitive function. Despite profound cognitive impairments in individuals with AS, learning deficits in the AS mouse model are rather mild. We and others, have reported learning deficits in AS mice by using the Morris water maze (Daily et al., 2011; Huang et al., 2013; Woerden et al., 2007). However, this paradigm is very labour intensive and hence less suitable for drug testing. Moreover, we found that a large number of mice are needed to detect significant differences and results varied strongly among experimenters (data not shown). A good learning paradigm that is highly suitable for drug testing is fear conditioning, in which animals are subjected to a single training session in which they are trained to associate a context (training chamber) or cue (tone) with a foot shock. However, we have not been able to get consistent results across experiments and experimenters (data not shown), and varying results are published in literature, with some studies showing a specific deficit in context conditioning (Hethorn et al., 2015; Jiang et al., 1998) and others a specific deficit in cued conditioning (Huang et al., 2013) or both (Baudry et al., 2012; Sun et al., 2016, 2015). Notably, the two studies that investigated the behavioural deficits of *Ube3a* mice across strains in great detail, showed no context conditioning deficit in *Ube3a* mice in the F1 hybrid 129-C57BL/6J background and C57BL/6 background, and either normal (Born et al., 2017) or impaired (Huang et al., 2013) cued fear conditioning in *Ube3a*

mice in the C57BL/6J background. Collectively, these studies indicate that this phenotype is rather weak, and hence results obtained with these tests should be interpreted with care.

By combining the data of 8 independent experiments performed by 5 different experimenters, we were able to perform a meta-analysis of 111 *Ube3a*$^{m-/p+}$ (*Ube3a*^{tm1Alb}) and 120 WT littermate mice in the F1 hybrid 129S2-C57BL/6 background and determine the robustness of the phenotypes. In all 8 experiments, we replicated *Ube3a* phenotypes observed on the rotarod, open field test and marble burying test, nest building test and the forced swim test. Deficits of *Ube3a* mice in rotarod performance, open field behaviour and marble burying have been reported by many other investigators, and hence our results confirm the robustness of these tests. Impaired nest building behaviour and impaired performance in the forced swim test of *Ube3a* mice have not yet been reported by other laboratories, but our study shows that these deficits are also very robust. In fact, a power analysis showed that these tests are among the most robust tests of the behavioural test battery. The open field paradigm was found to have the weakest power.

Our meta-analysis further shows that there is no major effect of sex on the behavioural phenotypes, which is in line with the general notion that such differences are also not present in AS patients. We did however find that female wild-type and mutant mice outperformed male wild-type and mutant mice on the rotarod. Improved performance of female mice on the rotarod has also been reported previously (Kovács & Pearce, 2013), and emphasizes the need of using well-matched groups when groups of both sexes of *Ube3a* mice are tested on the rotarod. Given that male mice are heavier than female mice, we investigated if the impaired performance of *Ube3a* mice on the rotarod can be attributed to the increased weight of these mutants. However, we found no correlation between weight of the animal and performance on the rotarod. This observation is in line with other studies (M. N. Cook, Bolivar, McFadyen, & Flaherty, 2002; Kovács & Pearce, 2013; McFadyen, Kusek, Bolivar, & Flaherty, 2003) and indicates that the reduced performance of *Ube3a* mice on the rotarod represents a *bona fide* impairment in motor performance.

Besides the reproducibility of the observed phenotypes and the high face and construct validity, there are two additional features that make the behavioural test battery for *Ube3a* mice highly useful for drug testing. We show that with the exception of the epilepsy test, all behavioural experiments can be performed with a single cohort of mice, which greatly reduces costs as well as the number of mice needed. In addition, we found that with the exception of the marble burying task, the behavioural test battery can be performed twice with the same cohort while maintaining a phenotype. This makes it possible to test the efficacy of a drug using a within-subject design.

We confirmed previous studies that the audiogenic seizure phenotype is a very powerful test to investigate seizure susceptibility in *Ube3a* mice (Jiang et al., 1998; Silva-Santos et al., 2015;

Woerden et al., 2007). With this study, this phenotype is now also confirmed in 3 independently derived lines: the commonly used *Ube3a*^{tm1Alb} line (Jiang et al., 1998), the *Ube3a*$^{mSTOP/p+}$ (*Ube3a*^{tm1Yelg}) line (Silva-Santos et al., 2015) and the recently generated *Ube3a*$^{mE113X/p+}$ (*Ube3a*^{tm2Yelg}) line (Wang et al., 2017). Since nearly all *Ube3a* mice show this phenotype compared to less than 10% of wild-type animals, this test has very high power. Moreover, we showed that the phenotype is readily reversible with the anti-epileptic drug Levetiracetam, and that the test is highly suitable for dose finding. The only disadvantage of the audiogenic seizure test, is that it cannot be performed on the same animals as used in the behavioural test battery, since the sensitivity to audiogenic seizures is exclusively observed in *Ube3a* mice in the 129S2 genetic background.

We also observed an effect of genetic background on the tests of the behavioural test battery. *Ube3a* mice in the C57BL/6J background showed a significant phenotype in the rotarod, nest building and marble burying test, but no effect of genotype was observed in the open field test. A significant effect of genotype was found in the forced-swim test, but remarkably, this was in the opposite direction. In contrast, *Ube3a* mice in the 129S2 genetic background showed only a significant deficit in the forced swim test (in the same direction as F1 hybrid mice) and no phenotype on any of the other tests of the behavioural battery. This confirms previous reports that many of the *Ube3a* phenotypes are very sensitive to genetic background and not present in 129 lines (Born et al., 2017; Huang et al., 2013). There are however several common findings as well as a few discrepancies between these studies and our study. With respect to the rotarod (Born et al., 2017; Huang et al., 2013) and marble burying phenotype (Born et al., 2017), our findings that only *Ube3a*-C57BL/6J and *Ube3a*-F1 hybrid mice show a phenotype, are in full agreement with each other (Huang *et al* only tested *Ube3a*-C57BL/6J in the marble burying test). With respect to the open field test (distance travelled), the other two studies also found no phenotype in *Ube3a*-129 mice, but in contrast to our findings they both found a phenotype in *Ube3a*-C57BL/6J mice. One major difference between their and our experimental design, is the time the mice were placed in the open field. Indeed, when we left the *Ube3a*-C57BL/6J mice for 30 minutes in the open field (instead of the 10 minutes we used) we found a nearly significant phenotype in *Ube3a*-C57BL/6J mice ($p=0.06$; data not shown). With respect to percentage of time spent in the inner zone of the open field (which is another measure of anxiety), the other two studies showed no significant effect of genotype in any of the genetic backgrounds. Our meta-analysis did however reveal a significant difference between genotypes in F1 hybrid mice (WT: 1.1% versus mutant 0.7% time in inner zone; $p<0.005$), which further indicates that *Ube3a* mutant mice are more anxious. However, we note that the observed difference was small, and a significant effect was only observed in 4 out of the 8 individual experiments. Hence this measure is not very robust.

Taken all studies into consideration, it is clear that *Ube3a* mice in the F1 hybrid 129S2-C57BL/6 background shows the most robust phenotypes, with the notable exception of the audiogenic seizure susceptibility test, which is strictly seen in *Ube3a*-129S2 mice. The question arises whether the observed differences between *Ube3a* mice in different genetic backgrounds has any translational significance. The lack of phenotypes of *Ube3a*-129S2 mice in most tests could simply reflect the passive/hypoactive phenotype of these mice, resulting in a floor effect. However, it could also be that the AS phenotype is sensitive to genetic background, and that the changes that are observed between individuals with AS are in part caused by genetic modifiers, rather than the nature of the mutation. Detailed studies of individuals with recurrent or similar mutations could provide more insight in that question (Abaied et al., 2010).

To test the translational value of the behavioural test battery, we decided to re-evaluate the two drugs that previously were tested in clinical trials involving individuals with AS: Minocycline (trial register NCT01531582, (J C Grieco et al., 2014), and NCT02056665, (Ruiz-Antorán et al., 2015)) and Levodopa (trial register NCT01281475, (W. H. Tan et al., 2017)). Both drugs were previously shown to rescue the rotarod impairment of *Ube3a* mice (see NCT01531582 for minocycline, and (W. H. Tan et al., 2017) for Levodopa). In addition, Minocycline rescued the hippocampal LTP deficit of *Ube3a* mice (J C Grieco et al., 2014), whereas Levodopa rescued the increased phosphorylation of CaMK2 observed in *Ube3a* mice (W. H. Tan et al., 2017). We tested the effect of both drugs on all tests of our behavioural test battery, using the same drug administration protocols as used for the original studies. In addition, we also tested the effect of Minocycline when administered from birth, as previously published for the Fragile X mouse model (Bilousova et al., 2009). However, in line with the clinical trials, we did not observe any efficacy of these drugs when tested on *Ube3a* mice. Our finding that Minocycline and Levodopa are unable to improve performance on the rotarod is at odds with aforementioned previous preclinical studies. Failure of replication could be due to differences in strains or procedures, although there is full agreement between our labs with respect to performance of *Ube3a* mice on the rotarod and the effects of different genetic backgrounds on this performance (Born et al., 2017). We think it is more likely that the rotarod experiments used for the preclinical studies were underpowered, as our analysis showed that 14 mice per group are needed for a well-powered rotarod study using two groups. In the Levodopa study, the authors used 6 different treatment groups and only 6 mice per group (W. H. Tan et al., 2017). Such small sample sizes make the test underpowered, and also very vulnerable for the sex differences that we describe here. Since the details of the rotarod experiments of the Minocycline treatment were not provided (NCT01531582), we cannot comment on these discrepancies.

Conclusions

Here we provided a behavioural test battery with a robust set of well-characterized *Ube3a* phenotypes, which allows researchers to investigate the effects of pharmacological and genetic interventions involving *Ube3a* mice. A standardized set of tests, in combination with a well-defined genetic background, will also be very useful to compare data across laboratories. Moreover, using a standardized behavioural test battery may reduce selective reporting bias (Tsilidis et al., 2013). Future studies should reveal how well the results of this behavioural test battery can be replicated between different laboratories in which housing and testing environment is different (Flint et al., 1995; Mandillo et al., 2008; Mineur & Crusio, 2009; Richter, Garner, & Würbel, 2009). In addition, robust tests that capture phenotypes in the domain of cognitive function should be identified and added to this test battery.

Abbreviations

AS: Angelman Syndrome; UBE3A: Ubiquitin-protein ligase E3A; EEG: electroencephalography; WT: Wild-type; Mut: mutant; IP: intraperitoneal

Declarations

Ethics approval

All animal experiments were conducted in accordance with the European Commission Council Directive 2010/63/EU (CCD approval AVD101002016791).

Consent for publication

All authors have approved the final manuscript and consent for publication.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MS, IW, SSS, JK and DM performed the behavioural experiments. MS, IW, SSS and YE analysed and interpreted the data. GMW generated the *Ube3a*^{tm2Yelg} mouse model and setup the tracking system. IW made the figures. YE designed the study. MS and YE wrote the manuscript. All authors contributed intellectually to this study, and edited and approved the final manuscript.

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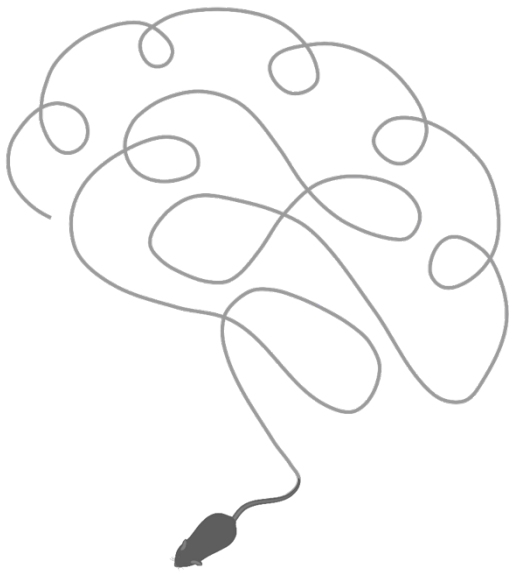
CHAPTER 5

DELAYED LOSS OF UBE3A REDUCES THE EXPRESSION OF ANGELMAN SYNDROME-ASSOCIATED PHENOTYPES

(Mol Autism. 2019; 10: 23.)



DELAYED LOSS OF UBE3A REDUCES THE EXPRESSION OF ANGELMAN SYNDROME ASSOCIATED PHENOTYPES

One of the most promising therapies proposed to improve Angelman syndrome (AS) is the use of antisense oligonucleotides (ASOs) that target the *UBE3A* antisense transcript (*UBE3A-ATS)*. However, the need for repeated dosing due to the relatively short half-life of the UBE3A protein, coupled with the invasive routes of administration (intracerebral/intrathecal), presents potential challenges.

Studies have consistently demonstrated that disease-modifying therapies are most effective in rescue AS phenotypes when initiated early in development. However, the long-term role of UBE3A in brain function beyond early development remains uncertain. This raises the question: Does sustained UBE3A expression beyond early brain development continue to be necessary for rescuing AS symptoms? In other words, *can the application of a treatment targeting UBE3A reinstatement after the critical period lead to a continued positive effect on AS manifestation,* or *is UBE3A expression required for normal brain function even after the therapeutic window of intervention has closed*?

The present study, published in the Journal of Molecular Autism, aims to address these critical questions.

The main objectives of this work are as follows:

- Assess the impact of *Ube3a* gene deletion at different ages during brain maturation on motor performance, anxiety, innate behaviours, and susceptibility to audiogenic seizures.
- Investigate the effect of *Ube3a* gene deletion at early embryonic stages and compare it with deletion at later ages to elucidate the critical periods during which UBE3A exerts its influence on AS-associated phenotypes.

The findings of this study underscore the crucial role of UBE3A in early brain development while highlighting its more limited contribution to adulthood. These insights will provide valuable knowledge for future clinical trials involving *UBE3A* gene reactivation and the potential therapeutic benefits for AS. The results will contribute to a deeper understanding of the syndrome and guide the design of clinical trials, emphasizing the critical window of early development and the potential need for sustained UBE3A expression into adulthood to achieve effective treatment for individuals with AS.

DELAYED LOSS OF UBE3A REDUCES THE EXPRESSION OF ANGELMAN SYNDROME ASSOCIATED PHENOTYPES

Mol Autism. 2019; 10: 23

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Keywords

Ube3a, Angelman syndrome, Mouse model, Seizure, Autism spectrum disorder, Phenotype

Abstract

Background: Angelman syndrome (AS) is a severe neurodevelopmental disorder caused by mutations affecting *UBE3A* gene expression. Previous studies in mice revealed distinct critical periods during neurodevelopment in which reactivation of *Ube3a* gene expression can prevent the onset of behavioural deficits. Whether UBE3A is required for brain function throughout life is unknown. Here, we address the importance of maintaining *UBE3A* expression after normal brain development.

Findings: Using a conditional mouse, we deleted the *Ube3a* gene at three ages spanning brain maturation. We assessed the consequences of *Ube3a* gene deletion by testing the mice in behavioural tasks previously shown to produce robust phenotypes in AS model mice. Early embryonic deletion of *Ube3a* recapitulated all behavioural deficits of AS mice. In contrast, *Ube3a* gene deletion at 3 or 12 weeks of age did not have a significant effect on most behavioural tasks and did not increase seizure sensitivity.

Conclusions: Taken together, these results emphasize that UBE3A critically impacts early brain development but plays a more limited role in adulthood. Our findings provide important considerations for upcoming clinical trials in which *UBE3A* gene expression is reactivated and suggest that even transient *UBE3A* reinstatement during a critical window of early

development is likely to prevent most adverse Angelman syndrome phenotypes. However, sustained UBE3A expression into adulthood is probably needed for optimal clinical benefit.

Introduction

Loss of the maternally inherited *UBE3A* allele results in Angelman syndrome (AS), a severe neurodevelopmental disorder, which is characterized by severe intellectual disability, motor coordination deficits, absence of speech, abnormal EEG, and behavioural deficits [1]. *UBE3A* gene dosage also appears to be critical with respect to autism spectrum disorder (ASD) [2]. Previous studies showed that there is up to 50% ASD comorbidity in AS individuals [3– 5], while overdosage of *UBE3A*, due to copy number variation of the 15q11–13 region, is among the highest genetic risk factors for ASD, accounting for up to 0.4% of all cases [6, 7]. Since duplication of the maternal locus is highly associated with pathogenicity [7–10], it is likely that *UBE3A* (the only maternally expressed gene in this locus) is the major effector of ASD outcome. Indeed, duplications of just the *UBE3A* gene, as well as a gain-of-function *UBE3A* point mutation that renders UBE3A enzymatically hyperactive, have been linked to severe forms of ASD [11–14].

One of the most promising approaches for developing a treatment for AS is based on the activation of the epigenetically silenced paternal *UBE3A* gene. Paternal *UBE3A* is silenced in neurons by a long non-coding *UBE3A-ATS* transcript, which can be activated by either using anti-sense oligonucleotides (ASOs) that target the degradation of this transcript or by topoisomerase inhibitors that interfere with the transcription of the *UBE3A-ATS* [15–17]. These *UBE3A* reinstatement approaches are particularly attractive since they restore UBE3A protein levels without risking over-expression.

Using an inducible *Ube3a* mouse model in which gene expression was genetically reinstated at different time points of brain development, we revealed distinct critical windows during brain development in which Ube3a needs to be reactivated to achieve an optimal behavioural rescue [18]. This suggests that early therapeutic intervention is needed for *UBE3A* reinstatement therapy to be fully effective. However, these results also pose a new question: to what extent is UBE3A expression required after brain development, and should treatment be continued after brain development has taken place?

To address this question, we made use of a conditional mouse model for AS that enabled us to delete the *Ube3a* gene at any desired time point. We found that early embryonic deletion of *Ube3a* recapitulated phenotypes that were previously described for AS mice [19]. In contrast, behavioural deficits were mostly absent when the *Ube3a* gene was deleted in young (3 weeks) or fully adult (12 weeks) mice. These results emphasize that most phenotypes observed in AS mice reflect developmental deficits. Continued UBE3A expression beyond the

completion of brain development may not be required for normal performance on most behavioural tasks.

Methods

Mouse breeding

We made use of the Ube3a^{flox} (Ube3a$^{tm1.1Bdph}$, MGI:58820 92) mice as previously described [20]. These mice were maintained in the C57BL/6 J (Charles River Laboratories) background by crossing male B6.Ube3a$^{m+/pflox}$ mice with C57BL/6 J females. For the behavioural experiments, we used mice in the B6129S2F1 background, which were generated as described below. To generate embryonic deletion of Ube3a, B6.Ube3a$^{m+/pflox}$ female mice were crossed with CAG (CMV early enhancer/chicken actin promoter) CRE-expressing male mice [MGI:2176435; Tg (CAG-cre)13Miya, in the manuscript referred to as Cre^{embryo}] in the 129S2/ SvPasCrl background (Charles River Laboratories) [21]. This breeding yielded four experimental groups in a B6129SF1 background: WT mice with and without CRE, and Ube3a$^{mflox/p+}$ mice with and without CRE.

To allow temporal control of Ube3a deletion at 3 and 12 weeks of age, B6.Ube3a$^{m+/pflox}$ female mice were crossed with homozygous 129S2Cre^{ERT} [MGI:2182767; Tg CAG-CRE/Esr1*)5Amc/J, also referred to as Tg (CAG-CRE- ERT2)] male mice [22]. This breeding yielded two experimental groups in a 129S2B6F1 background: WT mice with the CAGcre/Esr1 allele and Ube3a$^{mflox/p+}$ mice with the CAGcre/Esr1 allele.

For the seizure susceptibility experiments, we used mice in the 129S2/SvPasCrl background. To that end, B6.Ube3a$^{mflox/p+}$ mice were backcrossed for four to five generations in the 129S2/SvPasCrl background. Female 129S2.Ube3a$^{m+/pflox}$ mice were crossed with either Tg (CAG-CRE) or Cre^{ERT} male mice in the congenic 129S2/ SvPasCrl background (backcrossed > 20 generations).

Mouse husbandry

All mice were group-housed in a barrier facility, in cages that were individually ventilated (IVC; 1145 T cages from Techniplast). Mice were genotyped when they were 4–7 days old and re-genotyped at the completion of the experiments. All animals were kept at 22 ± 2 °C with a 12-h dark and light cycle and provided with mouse chow (801727CRM(P) from Special Dietary Service) and water ad libitum. During behavioural testing, mice remained group-housed, except during the nest building test and subsequent forced swim test.

Tamoxifen treatment and randomization

Three or 12-week-old Cre^{ERT};Ube3a$^{mflox/p+}$ transgenic mice and their wild-type littermates (both sexes) received tamoxifen to induce Cre-mediated deletion of the *Ube3a* gene. Tamoxifen

(Sigma-Aldrich) was diluted in vegetable (sunflower) oil at a concentration of 20 mg/ml as previously described [18, 22, 23] and as recommended by the Jackson Laboratories [24]. For five consecutive days, each mouse received 0.10 mg tamoxifen per gram body weight daily by intraperitoneal (IP) injection. The control group received daily IP injections of sunflower oil for five consecutive days (vehicle). Injection of either tamoxifen or vehicle was randomly assigned to the mice and the experimenter was blind to genotype.

Behavioural test battery

All behavioural experiments were performed during the light period of the light/dark cycle. Both male and female mice were used at the ages indicated in the text. Mice were acclimatized to the testing room for 30 min before each behavioural performance. All behavioural testing and scoring were performed by an experimenter blind to genotype. Behavioural tests were precisely performed as previously described [18, 19] and as listed below:

Accelerating rotarod

Motor capabilities were tested by placing the mice on the accelerating rotarod (4–40 rpm, in 5 min; model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). Mice were tested twice per day with a 45–60-min inter-trial interval for five consecutive days (same hour every day). For each day, the average time spent on the rotarod was calculated, or the time until the mouse made three consecutive wrapping/passive rotations on the rotarod (latency in seconds). The maximum duration of a trial was 5 min.

Open field test

In this test, which is useful to test locomotor activity and anxiety, mice were individually placed in a brightly lit 110-cm-diameter circular open field (25 lx in the middle of the arena) and allowed to explore the space for 10 min. The total distance moved by each mouse in the open arena was recorded by an infrared camera (Noldus® Wageningen, NL) connected to the EthoVision® software (Noldus® Wageningen, NL), and the final outcome is indicated as distance moved in centimetres.

Marble burying test

Open makrolon (polycarbonate) cages (50 × 26 × 18 cm) were provided with 4 cm of bedding material (Lignocel® Hygenic Animal Bedding, JRS). On top of the bedding material, 20 blue glass marbles were placed in an equidistant 5 × 4 grid and the animals were free to access to the marbles for 30 min. Once the time was run out, the mice were gently removed from the cage. The outcome measured is the number of buried marbles, which were scored as buried when covered more than 50% by bedding material.

Nest building test

Mice were single housed for a period of 5 to 7 days before the start of the experiment. Successively, the used nesting material was replaced with around 11 g (11 ± 1) of compressed extra-thick blot filter paper (Bio-rad©). The amount of the unused nest material was weighed and noted daily for a consecutive of 5 days, each day at the same hour.

Forced swim test

Mice were placed in a cylindrical transparent tank (27 cm high and 18 cm diameter), filled with water (26 ± 1 °C) 15 cm deep for 6 min. The outcome measured is the time in seconds in which the mouse was immobile. The latency of immobility was only assessed during the last 4 min of the test. The mouse was considered to be immobile when its topped moving, making only movements necessary to keep its head above water.

Susceptibility to audiogenic seizures

Mice were placed in makrolon (polycarbonate) cages (50 × 26 × 18 cm) and audiogenic seizures were induced by vigorously scraping scissors across the metal grating of the cage lid (which creates approximately a 100 dB sound). This noise was generated for 20 s, or less if a tonic-clonic seizure developed before that time. Susceptible mice responded with wild running and leaping followed by a tonic-clonic seizure, which typically lasted 10–20 s.

Western blot analysis and immunohistochemistry Mice were sacrificed at 20–25 weeks of age, for subsequent analysis. For Western blots analysis, approximately 20 µg of protein lysate were loaded on 4–12% SDS-PAGE gel (Bio-Rad) and transferred on nitrocellulose membranes to be then incubated with anti-UBE3A antibody (E8655 Sigma-Aldrich; 1:1000) and anti-actin antibody (MAB1501R, Millipore; 1: 20000). Briefly, membranes were blocked in 4% TBS milk solution for 1 h at room temperature and incubated at 4 °C overnight, rotating end over end, with the primary antibody dissolved in 2% TBS-T milk solution. The day after membranes were washed three times for 10 min with TBS-T and incubated with the secondary antibody, a fluorophore-conjugated goat anti-mouse antibody (IR Dye 800CW, Westburg; 1:15000), dissolved in 2% TBS-T milk solution for 1 h. At the end of the incubation, membranes were washed three times for 10 min with TBS and the resulting blots were analysed and quantified using a LI-COR Odyssey Scanner and Odyssey 3.0 software.

For immunohistochemistry, mice were sedated with 0.15 ml Nembutal (60 mg/kg), transcardially perfused and the brains were post-fixed with 4% paraformaldehyde in sodium phosphate buffer (PB) for 2 h. After incubation in 10% sucrose (in 0.1 M phosphate buffer) overnight, brains were embedded in a sucrose/gelatin mixture (10 and 12%, respectively). Brain sections were cut on a microtome (SM2000R; Leica Microsystems, Rijswijk,

Netherlands) at a thickness of 40 µm and treated with peroxidase (H_2O_2). The brain sections were then washed in PBS and were incubated for 1 h in blocking buffer containing 10% horse serum, 0.5% Triton X-100 in PBS. Subsequently, sections were incubated for 48–72 h in 2% normal horse serum, 0.5% Triton X-100 incubation buffer in PBS with primary antibody (mouse anti-E6AP, clone 3E5 Sigma–Aldrich; 1:750). The secondary antibody (anti-mouse HRP, P0447 Dako; 1:200) was detected by 3,3-diaminobenzidine DAB) as the chromogen, and DAB sections were analysed and photographed using a Nanozoomer scanner.

Statistics

All data were statistically analysed using IBM SPSS software, and P values less than 0.05 were considered significant. Statistical analysis was performed using univariate ANOVA (Kruskal-Wallis statistic test when data were non-normally distributed) or two way-repeated measures ANOVA with Bonferroni's and Dunnet/Mann-Whitney U test post hoc comparison (see the Additional files for more details).

Results

To elucidate the importance of continued *UBE3A* expression after early brain development, we took advantage of a conditional Ube3a$^{mflox/p+}$ mouse model [20] that enabled us to delete the maternal *Ube3a* gene at any desired time point. We first crossed female Ube3a$^{mflox/p+}$ mice with a constitutive Cre-expressing mouse line [21]. This resulted in full, early embryonic deletion of the maternal *Ube3a* allele, and a consequent depletion of neuronal UBE3A protein expression in cortex, hippocampus, striatum and cerebellum, similar to what has been observed for the Ube3a$^{m-/p+}$ AS mouse model [18] (**Figure 1, Additional file 1: Table S1, Additional file 2: Figure S1, Additional file 3: Table S2**).

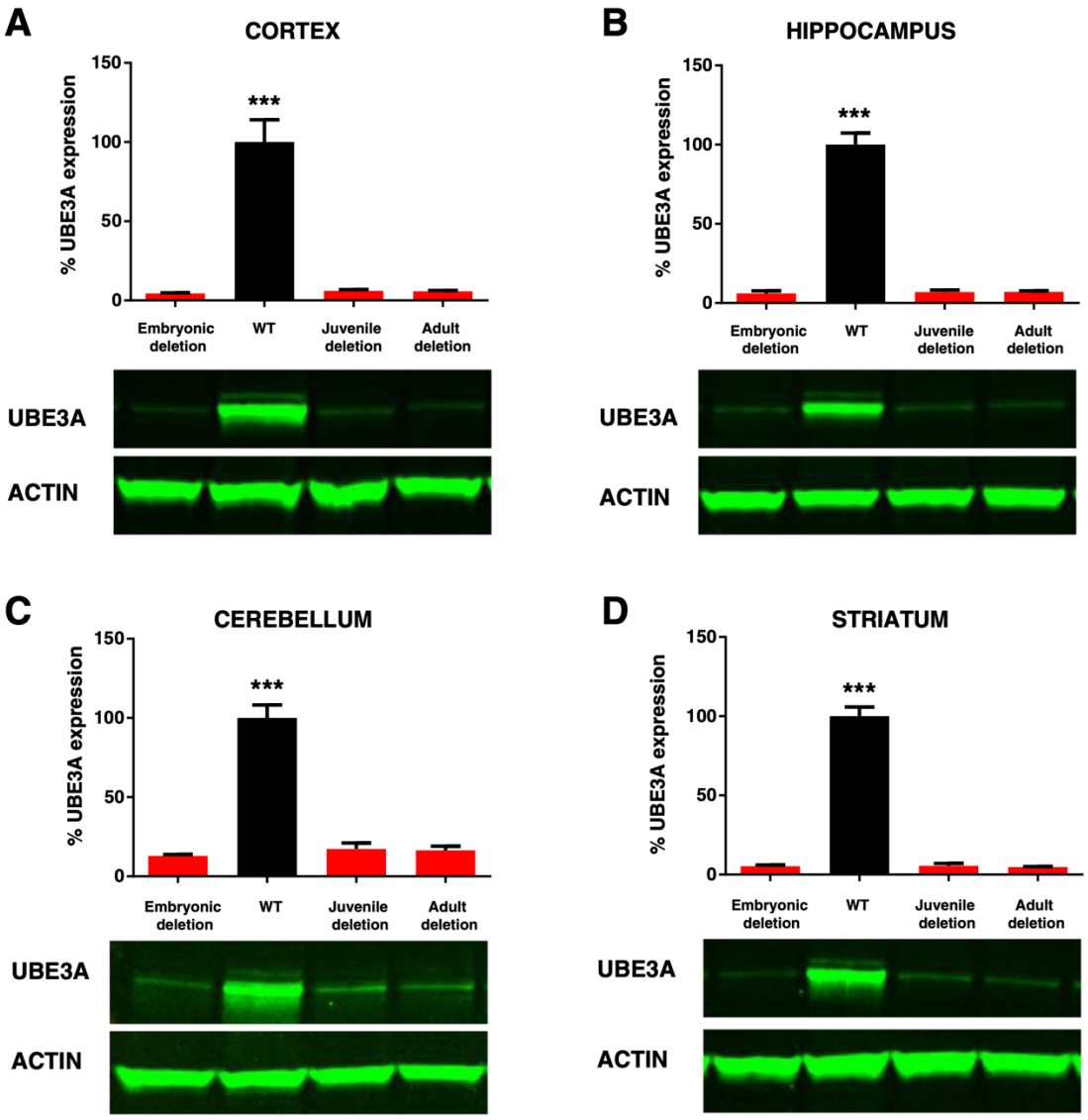

Figure 1 Loss of **UBE3A expression upon juvenile and adult *Ube3a* gene deletion.** UBE3A Western blot analysis of wild-type mice and mice in which the *Ube3a* gene deletion is induced at 3 weeks ('juvenile deletion') or at 12 weeks 'adult deletion'. Mice were sacrificed between 22 and 25 weeks of age. The analysis shows that loss of UBE3A expression in the cortex (**A**), hippocampus (**B**), cerebellum (**C**) and striatum (**D**) of these mice is comparable to mice in which the *Ube3a* gene is absent throughout development ('embryonic deletion') ($N = 3$ per genotype). Data shown are means with SEM (see methods and **Additional file 1** for statistical tests), ***p<0.001.

Next, we demonstrated that Cre^{embryo};$Ube3a^{mflox/p+}$ faithfully recapitulate the phenotypes that we previously established to be present in three independent AS mouse models [18, 19] (**Figure 2, Additional file 4: Table S3, Additional file 5: Figure S2, Additional file 6: Table S4**).

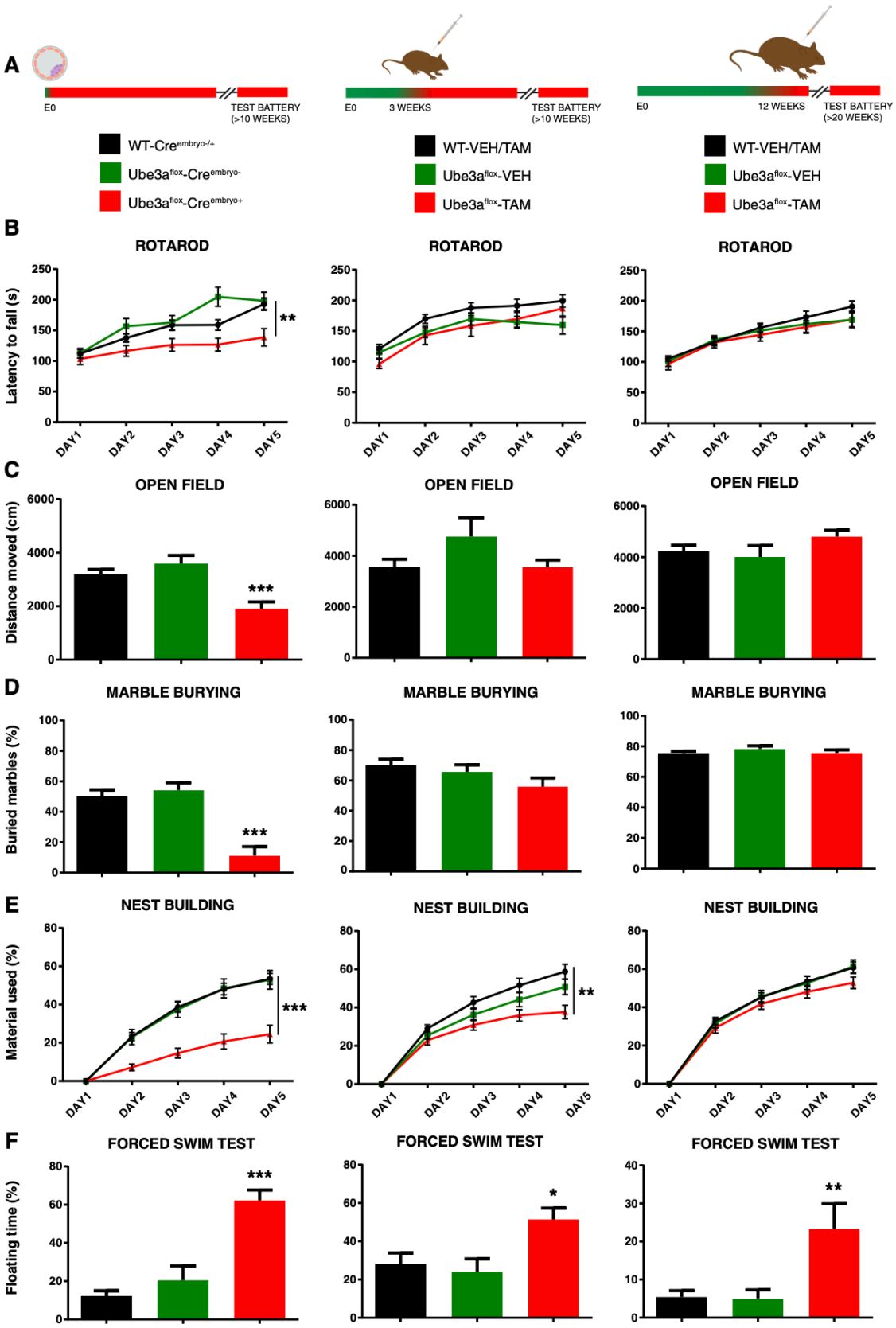

Figure 2 ***Ube3a*** **gene deletion in juvenile and adult mice does not recapitulate the phenotypes observed in embryonically deleted** ***Ube3a*** **mice. A** Schematic depicting *Ube3a* gene deletion at early embryonic age, juvenile age (3 weeks) and adult age (12 weeks). **B-F** Behavioural tasks performed with

Cre^{embryo};*Ube3a*$^{mflox/p+}$ and Cre^{ERT};*Ube3a*$^{mflox/p+}$ mice. Juvenile and adult *Ube3a* gene deletion results in deficits in the forced swim test. Asterisks indicate the effect of genotype. Wild-type (WT) mice in the Cre^{embryo};*Ube3a*$^{mflox/p}$ group represent combined data of Cre positive and Cre negative animals (embryonic deletion: N for WT-Cre$^{embryo-}$/ WT-Cre$^{embryo+}$ / *Ube3a*$^{mflox/p+}$-Cre^{embryo}–/ *Ube3a*$^{mflox/p+}$-Cre$^{embryo+}$ mice = 15/group). Wild-type mice (WT) in the juvenile and adult-treated gene deletion group represent combined data of tamoxifen and vehicle-treated wild-type mice (Juvenile deletion: N for WT-OIL/ WT-TAM / *Ube3a*$^{mflox/p+}$-VEH/ *Ube3a*$^{mflox/p+}$-TAM mice = 11, 13, 14, 16) (Adult deletion: N for WT-OIL/ WT-TAM / *Ube3a*$^{mflox/p+}$-VEH/ *Ube3a*$^{mflox/p+}$-TAM mice = 15/group). Data shown are means with SEM (see methods and **Additional file 4** for statistical tests). *p<0.05, ***p<0.001.

These behavioural phenotypes are in the domains of motor function, anxiety and repetitive behaviours and were selected to fulfil the following criteria: (1) large effect sizes to allow multiple comparisons within and between cohorts, (2) reproducible phenotypes across multiple AS lines, (3) phenotypic penetrance in AS mice of different ages and (4) tolerance of different experimenters. Moreover, these tests can all be performed in a single cohort of mice using a highly standardized (and optimized) method [19].

Having established that Ube3a$^{mflox/p+}$ mice phenocopy other AS lines tested in our behavioural test battery, we investigated the importance of continued UBE3A expression in these behavioural paradigms, after early brain development and into adulthood. To that end, we crossed female Ube3a$^{mflox/p+}$ mice with a tamoxifen inducible Cre line (Cre^{ERT}) [22]. *Ube3a* gene deletion was induced by IP injection of tamoxifen in juvenile mice at 3 weeks of age, and in adult mice at 12 weeks of age (**Figure 2**). Western blot analysis of mice sacrificed at approximately 20–25 weeks showed that overall UBE3A protein levels in cortex, hippocampus and striatum of Cre^{ERT};Ube3a$^{mflox/p+}$ mice closely resembled UBE3A levels of brains in which *Ube3a* was deleted embryonically (**Figure 1**; see also **Additional file 7**: **Figure S3, Additional file 8: Figure S4; Additional file 3: Table S2**). Immunohistochemistry confirmed tamoxifen-induced Ube3a gene deletion throughout the brain (**Additional file 7: Figure S3, Additional file 8: Figure S4**).

Having ascertained that tamoxifen efficiently deleted the maternal *Ube3a* gene, we assessed the behavioural phenotypes of these Cre^{ERT};Ube3a$^{mflox/p+}$ mice, minimally 7 weeks after gene deletion. This extended time period between gene deletion and testing not only allowed full clearance of UBE3A protein, but also allowed the neurons and neuronal networks to adapt to the loss of *UBE3A* expression, ensuring that any observed phenotypes were due to permanent consequences of UBE3A loss.

Maternal *Ube3a* gene deletion in adult (12 week old) mice resulted in impaired forced-swim test behaviour. Surprisingly however, performance in the accelerating rotarod, open field, nest building and marble burying paradigms was not affected (**Figure 2**; see also **Additional file**

5: **Figure S2**). This indicates that these behaviours and their supporting neural circuits do not depend on continued *UBE3A* expression during adulthood [18]. Importantly, the lack of phenotypic penetrance in these tests is not caused by the age of testing (approximately 20–22 weeks), since we have previously shown that these phenotypes are still clearly present in AS mice aged 28 weeks [18]. Moreover, a retrospective analysis of several studies in our laboratory in which mice aged > 20 weeks were included, showed a strong phenotype on all these tests (**Additional file 9**: **Figure S5**).

For experiments in which *Ube3a* gene deletion was induced in juvenile mice at 3 weeks of age, we chose again to delay behavioural testing for a minimum of 7 weeks to allow the brain to respond to the gene deletion. Consequently, the age of testing was similar between juvenile deletion and embryonic deletion mice (Cre^{embryo};$Ube3a^{mflox/p+}$), allowing for a direct comparison between these groups. Maternal *Ube3a* gene deletion in juvenile mice resulted in a significant impairment in the forced-swim test, highlighting once more the necessity of continued *UBE3A* expression for normal performance on this test. In addition, these mice also showed an impairment in the nest-building task. Since this phenotype was not present in mice in which *Ube3a* gene deletion was induced at 12 weeks of age, this result indicates that the neuronal network supporting performance in the nest-building task is not yet fully developed at 3 weeks of age. Surprisingly, none of the other tests revealed impairments, suggesting that by 3 weeks of age, the brain has already developed to such an extent that UBE3A protein is no longer required for normal performance of most behaviours.

Most individuals with AS suffer from epilepsy. We have previously shown that AS mice in the 129S2 background exhibit exaggerated susceptibility to audiogenic seizures, which can be suppressed by modulating CAMK2 activity as well as by anti-epileptic drugs [18, 19, 25]. This phenotype is not age dependent as it is readily observed in AS mice tested between 8 and 28 weeks old [18, 19]. We tested sensitivity to audiogenic seizures in 129S2-backcrossed $Ube3a^{mflox/p+}$ mice in which the *Ube3a* gene was deleted embryonically, at 3 weeks of age or in adulthood. Early embryonic deletion of the *Ube3a* gene rendered all (15/15) Cre^{embryo}; $Ube3a^{mflox/p+}$ mice susceptible to audiogenic seizures. In contrast, neither juvenile (0/8) nor adult *Ube3a* gene deletion (0/16) resulted in mice that were sensitive to audiogenic seizures. This indicates that the sensitivity to audiogenic seizure is exclusively dependent on the presence or absence of UBE3A during early brain development [18].

Discussion

The purpose of this study was to assess the role of the *Ube3a* gene in the mature brain, with the specific goal of gaining insight into whether *UBE3A* reinstatement therapies must be sustained throughout life for maximal efficacy in treating AS. In order to address this question, we took advantage of the conditional $Ube3a^{mflox/p+}$ mouse model [20], crossed with either a

constitutive Cre-expressing mouse line (Cre^{embryo}) [21] or with a tamoxifen-inducible Cre line (Cre^{ERT}) [22], to allow deletion of the *Ube3a* gene at distinct times during brain development By deleting *Ube3a* during early embryogenesis, we were able to reproduce all the behavioural deficits observed in various AS mouse models, highlighting the usefulness of this mouse model and the robustness of these phenotypes [18, 19]. These results further confirm the critical role of UBE3A during brain development, as we established previously [18]. In contrast, we observed limited phenotypic penetrance upon *Ube3a* deletion at 3 weeks or 12 weeks of age. We observed no deficits in motor coordination (rotarod), explorative behaviour and anxiety (open field), or repetitive behaviour and anxiety (marble burying). Nor did we evince a predisposition toward epilepsy as assessed by the audiogenic seizure provocation test. These results cannot be explained by the age of the mice at the time of testing: juvenile deletion mice were the same age at testing as Cre^{embryo};$Ube3a^{mflox/p+}$ mice in which the gene was deleted embryonically. Moreover, AS mice older than 20 weeks of age continue to exhibit robust phenotypes on these tasks, as we demonstrated both here and in a previous study [18].

Our results corroborate findings from our reciprocal *Ube3a* reinstatement studies [18], leading us to conclude that the circuits underlying these behaviours are brought online during the perinatal period, and are well established by weaning. In contrast, it appears that circuits supporting nest-building behaviour are not yet fully mature at 3 weeks of age, since deletion of Ube3a at this age (but not at 12 weeks) still results in a significant deficit. Notably, irrespective of the age of the mice, deletion of *Ube3a* always caused a deficit in the forced swim test paradigm, suggesting that the requisite circuits must sustain UBE3A expression for normal performance on this task.

Limitations

Our study has several limitations. First of all, it is possible that tamoxifen-induced postnatal Ube3a gene deletion does not occur in all cells. Our Western blot analysis shows no significant differences between UBE3A protein levels in mice with an embryonic *Ube3a* deletion compared to mice with a postnatal deletion of *Ube3a*. But this does not rule out the possibility that a small percentage of neurons did not undergo *Ube3a* gene deletion following tamoxifen treatment, and that *Ube3a* expression in a small subset of cells is sufficient to maintain normal behavioural function. A second limitation is that we did not assess behaviours related to learning and memory. Individuals with AS show severe intellectual deficits, but as discussed previously [19], AS mouse models do not show robust learning deficits in our hands. Hence, we cannot exclude that normal learning and memory requires UBE3A to be present at a time when learning takes place. Finally, the face validity of some of our behavioural tests is quite

limited (e.g. marble burying and nest building), as we do not know the underlying circuits and the relevance of these circuits to human AS phenotypes.

Conclusions

Our findings underscore the critical role of UBE3A for normal brain development and suggest that most AS behavioural phenotypes arise from the absence of UBE3A during embryonic or early postnatal development. Our results also demonstrate that while expression of UBE3A in the mature brain may not be required for the acquisition and performance of most tests investigated in this study, certain behaviours do depend on continued *UBE3A* expression. Hence, our study indicates that there is likely to be a clinical benefit by having enduring *UBE3A* reinstatement. Although we do not know how the first 3 weeks of postnatal brain development in mice translates to human brain development, our results suggest that even transient *UBE3A* reinstatement during a critical window of early development is likely to prevent most adverse Angelman syndrome phenotypes. Taken together these results emphasize the need to start *Ube3a* gene reactivation therapies early in life, and to sustain reactivation into adulthood for optimal effect.

Abbreviations

AS: Angelman syndrome; ASD: Autism Spectrum Disorder; ASO: Antisense oligonucleotides; CAMK2: Ca^{2+}/calmodulin-dependent protein kinase 2; UBE3A: Ubiquitin-protein ligase E3A; UBE3A-ATS: Ubiquitin-protein ligase E3A antisense transcript; WT: Wild-type

Declarations

Ethical approval and consent to participate

All animal experiments were conducted in accordance with the European Commission Council Directive 2010/63/EU (CCD approval AVD101002016791).

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Availability of data and materials

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

Authors' contributions

MS, JH, MBK and SSS performed behavioural, molecular and immunostaining experiments. MS analysed the data and performed statistical analysis. MCJ and BDP generated the floxed mice. GvW provided training and technical advice. YE and MS designed and coordinated the investigations. YE, MS, MCJ and BDP wrote the manuscript. The final version of the manuscript was approved by all authors.

Consent for publication Not applicable.

Competing interests

Y.E., B.D.P and M.C.J. have received money from pharmaceutical companies for consultation and/or drug testing studies regarding Angelman syndrome. The other authors declare that they have no competing interests.

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SUPPLEMENTAL DATA

Delayed loss of UBE3A reduces the expression of Angelman syndrome associated phenotypes

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ADDITIONAL FILES

Brain area	Genotype (n)	UBE3A (%)	Standard deviation	Independent variable	Test statistic	Test statistic value (df)	p value	Posthoc test (Bonferroni)		p value
Cortex	embryonic deletion (3)	4	1	genotype	Univariate ANOVA	F(3,8)=53.39	<0.001	WT	embryonic deletion	<0.001
	WT (3)	100	22						juvenile deletion	<0.001
	juvenile deletion (3)	6	2						adult deletion	<0.001
	adult deletion (3)	6	1							
Hippocampus	embryonic deletion (3)	6	3	genotype	Univariate ANOVA	F(3,8)=149.60	<0.001	WT	embryonic deletion	<0.001
	WT (3)	100	13						juvenile deletion	<0.001
	juvenile deletion (3)	7	2						adult deletion	<0.001
	adult deletion (3)	7	1							
Striatum	embryonic deletion (3)	5	1	genotype	Univariate ANOVA	F(3,8)=511.09	<0.001	WT	embryonic deletion	<0.001
	WT (3)	100	10						juvenile deletion	<0.001
	juvenile deletion (3)	7	2						adult deletion	<0.001
	adult deletion (3)	5	1							
Cerebellum	embryonic deletion (3)	13	2	genotype	Univariate ANOVA	F(3,8)=80.71	<0.001	WT	embryonic deletion	<0.001
	WT (3)	100	14						juvenile deletion	<0.001
	juvenile deletion (3)	17	6						adult deletion	<0.001
	adult deletion (3)	16	4							

Table S1. (referring to Figure 1). Summary of the statistical tests used for each Western blot analysis performed on each experimental group. Statistical significance (2-sided, $p < 0.05$) is indicated by green colour.

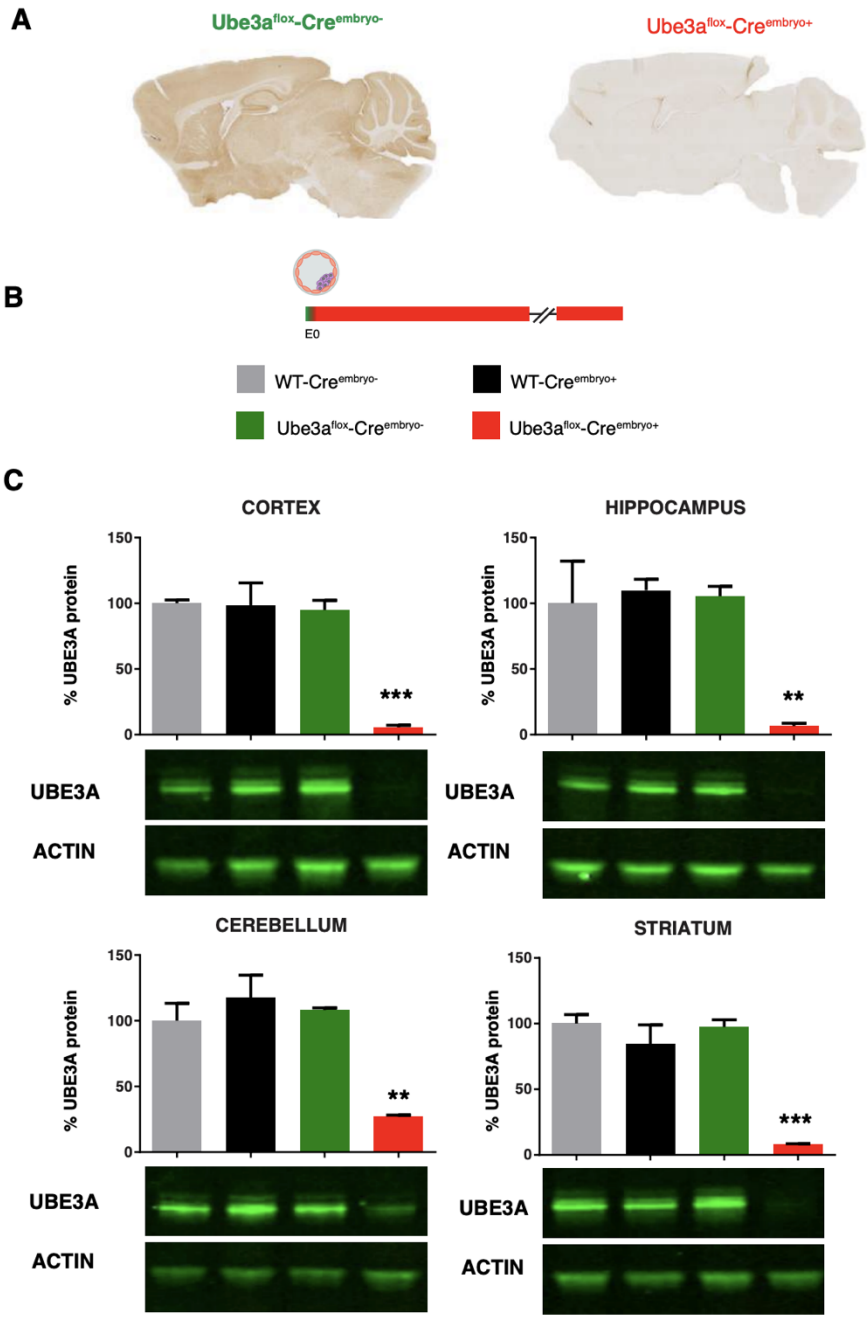


Figure S1. Deletion of UBE3A during embryogenesis. **A.** Whole brain immunohistochemical stainings indicate reduced UBE3A protein levels in *Ube3a*^{flox}-*Cre*$^{embryo+}$ mice compared to *Ube3a*^{flox}-*Cre*$^{embryo-}$ control mice. **B.** *Ube3a* gene deletion upon CRE activation driven by the Cag promoter during embryogenesis. **C.** Western blot data indicate reduced UBE3A protein levels in *Ube3a*^{flox}-*Cre*$^{embryo+}$ mice compared to control groups. Number of mice used for the Western blot analysis is $n = 3$ per genotype. Data shown are mean (±SEM). **p<0.01, ***p<0.001.See Additional file 3 (Table S2) for statistical analysis and the sample sizes.

UBE3A gene deletion	Brain area	Genotype	UBE3A % (Mean)	Standard deviation	Test statistic	Test statistic value (df)	p value	Posthoc test (Bonferroni) (N)		p value
Embryonic deletion	cortex	WT-Creembryo-	100	4	Univariate ANOVA	F(3,8)=24.03	<0.001	Ube3aflox-Creembryo+ (3)	WT-Creembryo- (3)	0.001
		WT-Creembryo+	99	29					WT-Creembryo+ (3)	0.001
		Ube3aflox-Creembryo-	95	13					Ube3aflox-Creembryo- (3)	0.001
		Ube3aflox-Creembryo+	6	2						
	hippocampus	WT-Creembryo-	100	56	Univariate ANOVA	F(3,8)=8.37	0.008	Ube3aflox-Creembryo+ (3)	WT-Creembryo- (3)	0.029
		WT-Creembryo+	110	14					WT-Creembryo+ (3)	0.016
		Ube3aflox-Creembryo-	106	13					Ube3aflox-Creembryo- (3)	0.021
		Ube3aflox-Creembryo+	7	4						
	striatum	WT-Creembryo-	100	12	Univariate ANOVA	F(3,8)=25.85	<0.001	Ube3aflox-Creembryo+ (3)	WT-Creembryo- (3)	<0.001
		WT-Creembryo+	84	24					WT-Creembryo+ (3)	0.001
		Ube3aflox-Creembryo-	97	9					Ube3aflox-Creembryo- (3)	<0.001
		Ube3aflox-Creembryo+	8	1						
	cerebellum	WT-Creembryo-	100	23	Univariate ANOVA	F(3,8)=14.75	0.001	Ube3aflox-Creembryo+ (3)	WT-Creembryo- (3)	0.008
		WT-Creembryo+	118	29					WT-Creembryo+ (3)	0.002
		Ube3aflox-Creembryo-	108	3					Ube3aflox-Creembryo- (3)	0.004
		Ube3aflox-Creembryo+	27	2						
Juvenile deletion	cortex	WT-OIL	100	11	Univariate ANOVA	F(3,12)=26.30	<0.001	Ube3aflox-TAM (4)	WT-VEH (4)	<0.001
		WT-TAM	99	18					WT-TAM (4)	<0.001
		Ube3aflox-VEH	118	33					Ube3aflox-VEH (4)	<0.001
		Ube3aflox-TAM	7	2						
	hippocampus	WT-OIL	100	3	Univariate ANOVA	F(3,8)=145.46	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	85	7					WT-TAM (3)	<0.001
		Ube3aflox-VEH	93	9					Ube3aflox-VEH (3)	<0.001
		Ube3aflox-TAM	7	3						
	striatum	WT-OIL	100	8	Univariate ANOVA	F(3,8)=23.90	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	78	21					WT-TAM (3)	0.002
		Ube3aflox-VEH	78	13					Ube3aflox-VEH (3)	0.002
		Ube3aflox-TAM	7	3						
	cerebellum	WT-OIL	100	7	Univariate ANOVA	F(3,8)=43.64	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	90	19					WT-TAM (3)	<0.001
		Ube3aflox-VEH	110	3					Ube3aflox-VEH (3)	<0.001
		Ube3aflox-TAM	21	1						
Adult deletion	cortex	WT-OIL	100	8	Univariate ANOVA	F(3,8)=48.44	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	96	20					WT-TAM (3)	<0.001
		Ube3aflox-VEH	106	10					Ube3aflox-VEH (3)	<0.001
		Ube3aflox-TAM	6	1						
	hippocampus	WT-OIL	100	7	Univariate ANOVA	F(3,8)=72.33	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	99	15					WT-TAM (3)	<0.001
		Ube3aflox-VEH	93	8					Ube3aflox-VEH (3)	<0.001
		Ube3aflox-TAM	7	1						
	striatum	WT-OIL	100	12	Univariate ANOVA	F(3,8)=33.43	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	<0.001
		WT-TAM	103	17					WT-TAM (3)	<0.001
		Ube3aflox-VEH	90	14					Ube3aflox-VEH (3)	<0.001
		Ube3aflox-TAM	14	3						
	cerebellum	WT-OIL	100	14	Univariate ANOVA	F(3,8)=23.22	<0.001	Ube3aflox-TAM (3)	WT-VEH (3)	0.001
		WT-TAM	96	21					WT-TAM (3)	0.001
		Ube3aflox-VEH	91	11					Ube3aflox-VEH (3)	0.001
		Ube3aflox-TAM	17	6						

Table S2. (referring to Additional file 2: Figure S1, Additional file 4: Figure S3, Additional file 8: Figure S4). Summary of the statistical tests used for each Western blot analysis performed on each experimental group. Statistical significance (2-sided, $p < 0.05$) is indicated by green colour.

UBE3A deletion	Behavioral test	Dependent variable	Test statistic	Directionality	Independent variable	Test statistic value (df)	p value F test	Post-hoc test (Bonferroni)		p-value	Genotype (N)
embryonic deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,56)=5.53	**p=0.006**	Ube3aflox-Creembryo+	WT	0.060	WT (30), Ube3a-cre embryo- (15), Ube3a-cre embryo+ (15)
									Ube3aflox-Creembryo-	0.006	
				2-sided	Time	F(3.19, 178. 85)=44.56	p<0.001				
				2-sided	Genotype*Time	F(6.387, 178,85)=4.47	p<0.001				
	open field	distance moved (cm)	Univariate ANOVA	2-sided	Genotype	F(2,56)=11.80	**p<0.001**	Ube3aflox-Creembryo+	WT	0.001	
									Ube3aflox-Creembryo-	p<0.001	
	marble burying	% buried marbles	Univariate ANOVA	2-sided	Genotype	F(2,56)=18.43	**P<0.001**	Ube3aflox-Creembryo+	WT	p<0.001	
									Ube3aflox-Creembryo-	p<0.001	
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,56)=17.08	**p<0.001**	Ube3aflox-Creembryo+	WT	p<0.001	WT (29), Ube3a-cre embryo- (15), Ube3a-cre embryo+ (15)
									Ube3aflox-Creembryo-	p<0.001	
				2-sided	Time	F(1.84,103.03)=224.98	P<0.001				
				2-sided	Genotype*Time	F(3.68, 103.03)=10.561	P<0.001				
	forced swim test	floating time (%)	kruskall-wallis	2-sided	Genotype	H(2)=23.85	**p<0.001**	Ube3aflox-Creembryo+	WT	p<0.001	
									Ube3aflox-Creembryo-	0.001	
juvenile deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,51)=2.53	p=0.089				WT (24), Ube3a-VEH(14), Ube3a-TAM (16)
				2-sided	Time	F(3.05,155.79)=43.51	p<0.001				
				2-sided	Genotype*Time	F(6.11,155.79)=1.295	p=0.262				
	open field	distance moved (cm)	univariate ANOVA	2-sided	Genotype	F(2,51)=2.17	p=0.125				
	marble burying	% buried marbles	univariate ANOVA	2-sided	Genotype	F(2,51)=2.31	p=0.109				
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,51)=5.38	**p=0.008**	Ube3aflox-TAM	WT	0.006	
									Ube3aflox-VEH	0.390	
				2-sided	Time	F(1.41,71.74)=352.86	p<0.001				
				2-sided	Genotype*Time	F(2.81, 71.74)=5.97	p<0.01				
	forced swim test	floating time (%)	kruskall-wallis	2-sided	Genotype	H(2)=9.22	**p=0.010**	Ube3aflox-TAM	WT	0.041	
									Ube3aflox-VEH	0.015	
adult deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,57)=0.705	p=0.50				WT (30), Ube3a-VEH (15), Ube3a-TAM (15)
				2-sided	Time	F(2.50,142.61)=60.01	p<0.001				
				2-sided	Genotype*Time	F(5,142.61)=0.737	p=0.597				
	open field	distance moved (cm)	Univariate ANOVA	2-sided	Genotype	F(2,57)=1.42	p=0.250				
	marble burying	% buried marbles	Univariate ANOVA	2-sided	Genotype	F(2,57)=0.765	P=0.470				
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2-sided	Genotype	F(2,57)=0.947	p=0.394				
				2-sided	Time	F(1.73,98.37)=588.35	p<0.001				
				2-sided	Genotype*Time	F(3.45,98.37)=1.08	p=0.368				
	forced swim test	floating time (%)	kruskall-wallis	2-sided	Genotype	H(2)=10.05	**p<0.01**	Ube3aflox-TAM	WT	0.014	
									Ube3aflox-VEH	0.016	

Table S3. (referring to Fig. 2). Summary of the statistical tests used for behavioural paradigms performed on each experimental group. Statistical significance (2-sided, $p < 0.05$) is indicated by green colour. The genotype is the independent variable of all the statistical tests.

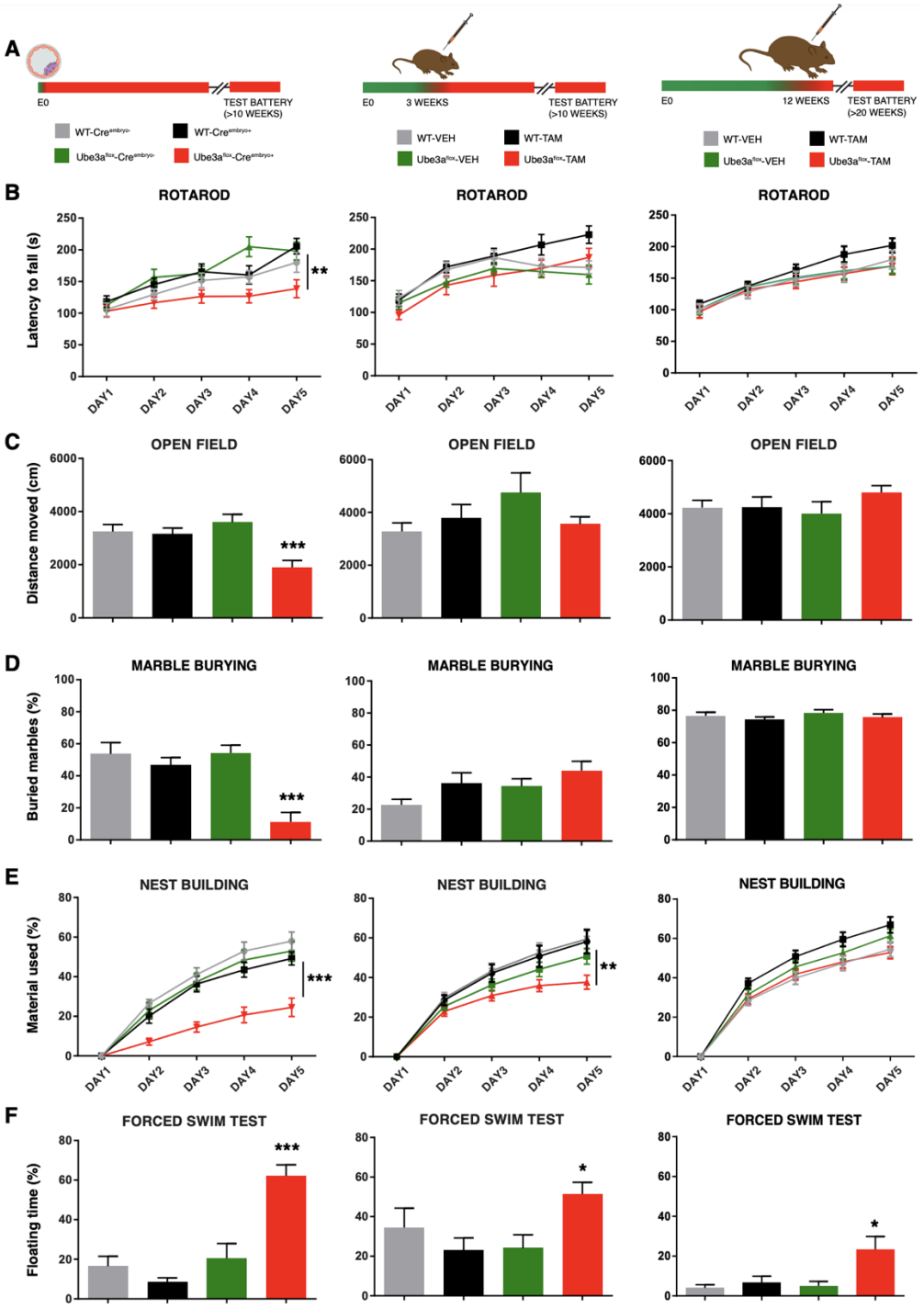


Figure S2. *Ube3a* gene deletion in juvenile and adult mice does not recapitulate the phenotypes observed in embryonically deleted *Ube3a* mice. **A.** Schematic depicting *Ube3a* gene deletion at early embryonic age, juvenile age (3 weeks) and adult age (12 weeks). **B-F.** Behavioural tasks performed with Cre^{embryo};$Ube3a^{mflox/p+}$ (N for WT-$Cre^{embryo-}$/ WT-$Cre^{embryo+}$ / $Ube3a^{mflox/p+}$-$Cre^{embryo-}$/ $Ube3a^{mflox/p+}$-$Cre^{embryo+}$ mice = 15/group) and Cre^{ERT};$Ube3a^{mflox/p+}$ mice (Juvenile deletion: N for WT-OIL/ WT-TAM / $Ube3a^{mflox/p+}$-VEH/ $Ube3a^{mflox/p+}$-TAM mice = 11, 13, 14, 16; Adult deletion: N for WT-OIL/ WT-TAM / $Ube3a^{mflox/p+}$-VEH/ $Ube3a^{mflox/p+}$-TAM mice = 15/group) . Juvenile and adult *Ube3a* gene deletion results in deficits in the forced swim test. Asterisks indicate the effect of genotype. Data shown are means with SEM. See methods and Additional file 6 for statistical tests and sample sizes. (*p<0.05, **p<0.01, ***p<0.001).

UBE3A deletion	Behavioral test	Dependent variable	Test statistic	Directionality	Independent variables	Test statistic value (df)	p value F test	post-hoc test for effect of genotype		p-value (Bonferroni)	p value (Dunnet/Mann-whitney U test)	Genotype (N)
Embryonic deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,56)=4,117	p<0.01	Ube3aflox-Creembryo+	WT-Creembryo-	0.623	0.243	WT-Cre embryo-(15), WT-Cre embryo+ (15), Ube3a-cre embryo- (15), Ube3a-cre embryo+ (15)
					Time	F(3.15,176.59)=51.29	p<0.001		WT-Creembryo+	0.057	0.025	
					Genotype*Time	F(9.46,176.59)=3.096	p<0.01		Ube3aflox-Crembryo-	0.011	0.005	
	open field	distance moved (cm)	univariate ANOVA	2 sided	Genotype	F(3,56)=7,867	p<0.001	Ube3aflox-Creembryo+	WT-Creembryo-	0.004	0.002	
									WT-Creembryo+	0.008	0.004	
									Ube3aflox-Crembryo-	0	0	
	marble burying	% buried marbles	univariate ANOVA	2 sided	Genotype	F(3,56)=12,827	p<0.001	Ube3aflox-Creembryo+	WT-Creembryo-	0	0	
									WT-Creembryo+	0	0	
									Ube3aflox-Crembryo-	0	0	
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,55)=12,324	p<0.001	Ube3aflox-Creembryo+	WT-Creembryo-	0	0	WT-Cre embryo-(15), WT-Cre embryo+ (14), Ube3a-cre embryo- (15), Ube3a-cre embryo+ (15)
					Time	F(1.84,101.45)=278.524	p<0.001		WT-Creembryo+	0.001	0	
					Genotype*time	F(5.53,101.45)=7.56	p<0.001		Ube3aflox-Crembryo-	0	0	
	forced swim test	floating time (%)	kruskall-wallis	2 sided	Genotype	H(3)=24.48	p<0.001	Ube3aflox-Creembryo+	WT-Creembryo-	0.001	0	
									WT-Creembryo+	0	0	
									Ube3aflox-Crembryo-	0.002	0.001	
juvenile deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,50)=2,163	p=0.104					WT OIL (11), WT TAM (13), Ube3a-VEH (14), Ube3a-TAM (16)
					Time	F(3.2,159.79)=48.36	p<0.001					
					Genotype*Time	F(9.59,159.79)=2.12	p<0.05					
	open field	distance moved (cm)	univariate ANOVA	2 sided	Genotype	F(3,50)=1,573	p=0.208					
	marble burying	% buried marbles	univariate ANOVA	2 sided	Genotype	F(3,50)=2,483	p=0.072					
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,50)=3,539	p<0.05	Ube3aflox-TAM	WT-OIL	0.043	0.02	
					Time	F(1.41,70.33)=393.86	p<0.001		WT-TAM	0.059	0.027	
					Genotype*Time	F(4.22,70.33)=3.92	p<0.01		Ube3aflox-VEH	0.802	0.313	
	forced swim test	floating time (%)	kruskall-wallis	2 sided	Genotype	H(3)=9.445	p<0.05	Ube3aflox-TAM	WT-OIL	0.471	0.175	
									WT-TAM	0.108	0.007	
									Ube3aflox-VEH	0.030	0.005	
adult deletion	rotarod	latency to fall (s)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,56)=1,098	p=0.358					WT OIL (15), WT TAM (15), Ube3a-VEH (15), Ube3a-TAM (15)
					Time	F(2.52,141)=71.15	p<0.001					
					Genotype*Time	F(7.55,141)=0.76	p=0.631					
	open field	distance moved (cm)	univariate ANOVA	2 sided	Genotype	F(3,56)=0,93	p=0.432					
	marble burying	% buried marbles	univariate ANOVA	2 sided	Genotype	F(3,56)=0,745	p=0.53					
	nest building	used nesting material (%)	2-way repeated measure ANOVA	2 sided	Genotype	F(3,56)=2,957	p<0.05	Ube3aflox-TAM	WT-OIL	1	1	
					Time	F(1.79,100.04)=705.38	p<0.001		WT-TAM	0.092	0.041	
					Genotype*Time	F(5.36,100.04)=2.21	p=0.055		Ube3aflox-VEH	1	0.536	
	forced swim test	floating time (%)	kruskal-wallis	2 sided	Genotype	H(3)=10,12	p<0.05	Ube3aflox-TAM	WT-OIL	0.058	0.011	
									WT-TAM	0.120	0.019	
									Ube3aflox-VEH	0.031	0.006	

Table S4. (referring to Additional file 5: Figure S2). Summary of the statistical tests used for behavioural paradigms performed on each experimental group. Statistical significance (2-sided, $p < 0.05$) is indicated by green colour. The genotype is the independent variable of all the statistical tests.

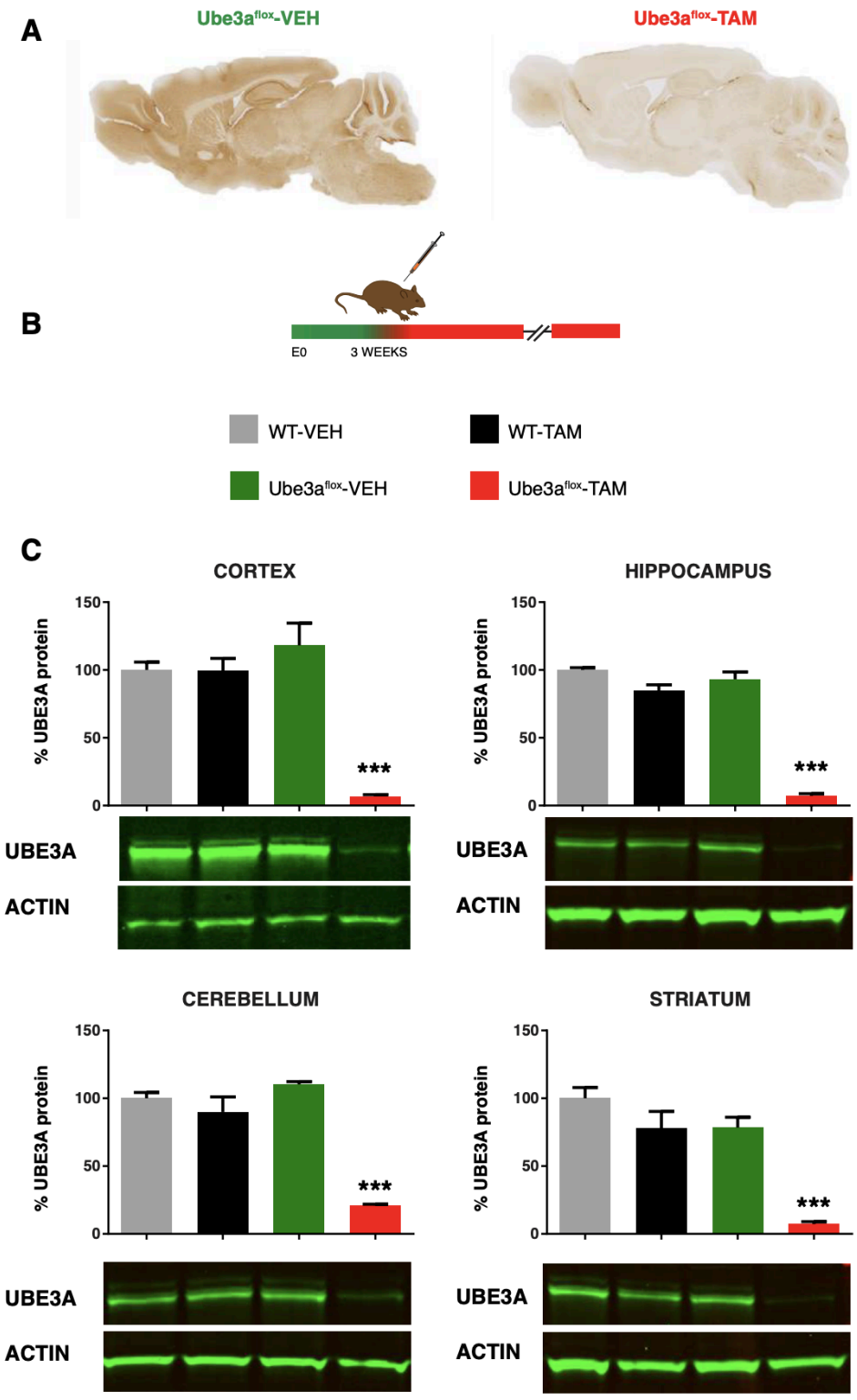


Figure S3. Deletion of UBE3A in young mice. **A.** Immunohistochemical staining indicate reduced UBE3A protein levels in *Ube3a*^{flox}-TAM mice compared to *Ube3a*^{flox}-VEH control group. **B.** *Ube3a* gene deletion induced at 3 weeks of age upon CRE activation by tamoxifen injection. **C.** Western blot data indicate reduced UBE3A protein levels in *Ube3a*^{flox}-*Cre*$^{embryo+}$ mice compared to control groups. Number of mice used for the Western blot analysis is $n = 3–4$ per genotype. Data shown

are mean (±SEM). See Additional file 3: Table S2 for statistical analysis and the sample sizes. ***p<0.001.

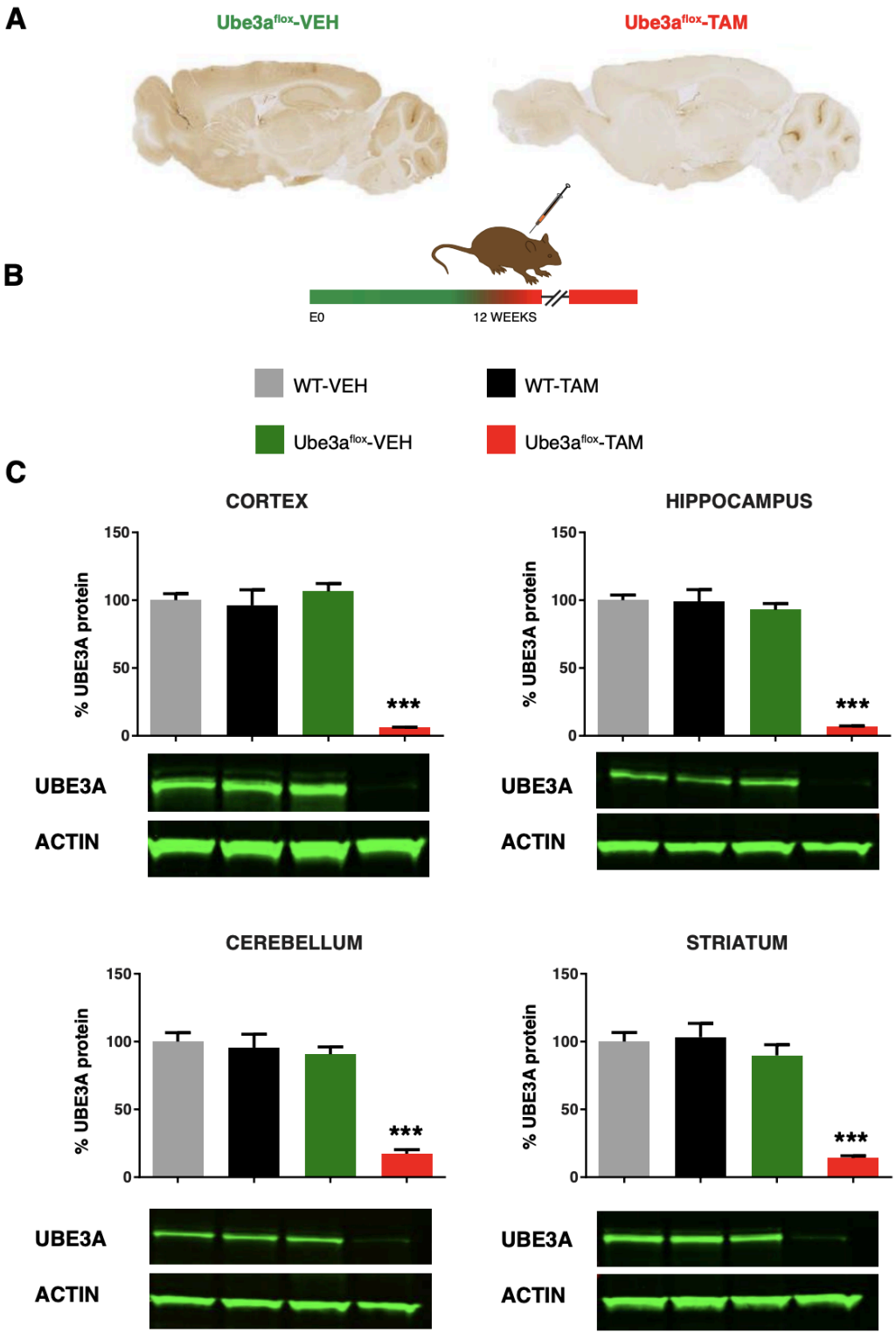


Figure S4. Deletion of UBE3A in adult mice. **A.** Immunohistochemical stainings indicate reduced protein levels of UBE3A in Ube3a^{flox}-TAM mice compared to Ube3a^{flox}-VEH control group. **B.** *Ube3a* gene deletion at 12 weeks of age upon CRE activation by tamoxifen injection **C.** Western blot data indicate reduced UBE3A protein levels in *Ube3a^{flox}-Cre$^{embryo+}$* mice compared to control groups.

Number of mice used for the Western blot analysis is $n = 3$/genotype. Data shown are mean (±SEM). See Additional file 3: Table S2 for statistical analysis and the sample sizes. ***p<0.001.

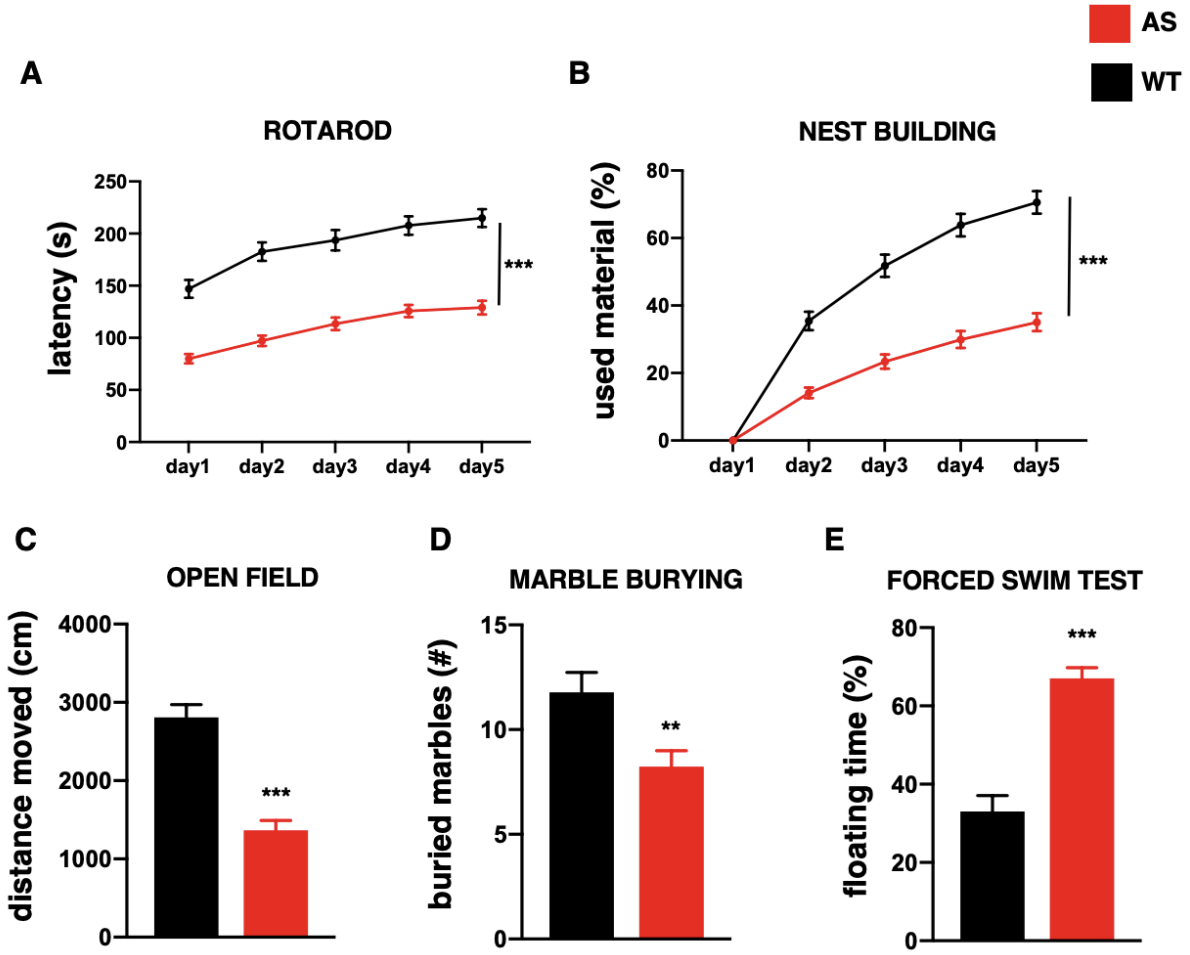
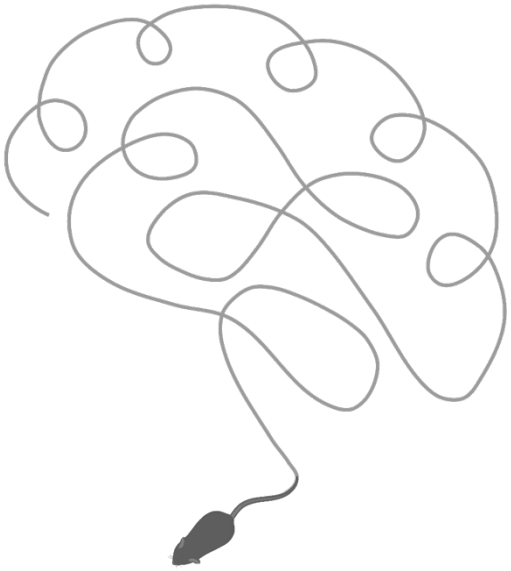


Figure S5. Behavioural test battery in mice older than 20 weeks of age. **A.** Accelerating rotarod in wild-type (WT) and AS mice ($n = 51, 67$). **B.** Nest building test in WT and AS mice ($n = 39, 45$). **C.** Open field test in WT and AS mice ($n = 36, 57$). **D.** Marble burying test in WT and AS mice ($n = 47, 62$). **E.** Forced swim test in WT and AS mice ($n = 37, 60$). All data represent mean ± SEM. A repeated measures ANOVA or t-test (or Mann Whitney *U* test for nonparametric data) was used for statistical comparison. All tests show a significance effect of genotype (**p<0.01,***p<0.001.***$p < 0.001$).

CHAPTER 6

GENERAL DISCUSSION



GENERAL DISCUSSION

The studies encompassed in this thesis have yielded significant findings that contribute to the overarching objective of treating Angelman syndrome (AS). If we envision the goal of treating AS as an intricate puzzle, the studies conducted by researchers in the field can be viewed as individual pieces that advance our understanding of this puzzle. In this context, our research has added crucial pieces to the puzzle, including the identification of the critical period for therapeutic intervention in AS, the development of a robust behavioural test battery for mouse models of AS, and insights into the necessity of sustained *Ube3a* expression into adulthood to achieve optimal clinical benefit.

However, it is important to acknowledge the limitations of our studies and the unanswered questions that persist. This chapter aims to address these unresolved aspects, discussing their implications and contextualizing our findings within the broader body of research that has been inspired by our work. By doing so, we aim to provide a comprehensive understanding of the data we have obtained and its significance in the larger scientific landscape.

6.1 **TIMING IS EVERYTHING: ESTABLISHMENT OF THE THERAPEUTIC WINDOW OF INTERVENTION FOR ANGELMAN SYNDROME IN A MOUSE MODEL**

In this thesis, we investigated the therapeutic window of intervention for Angelman Syndrome (AS) using a new inducible mouse model and a set of well characterized and robust behavioural paradigms. Specifically, we analysed the impact of *Ube3a* reinstatement at different developmental stages, and our findings suggest that timing plays a critical role in rescuing AS phenotypes.

In our first study "*Ube3a* reinstatement identifies distinct developmental windows in a murine Angelman syndrome model", presented in Chapter 3, we investigated the role of *Ube3a* in embryonic neurodevelopment and its potential for rescuing AS phenotypes in mice. We found that reinstating UBE3A levels to 80-100% to wild-type (WT) levels, after conception, prevented mice from developing any of the AS phenotypes extensively described in Chapter 4.

We further investigated the effect of reinstating *Ube3a* levels at different developmental stages, postnatally, ranging from 3 to 14 weeks of age. Our observations revealed that reinstating UBE3A to 70-100% of WT levels at postnatal week 3 (juvenile mice) resulted in a full rescue of motor deficits but only a partial recovery is achieved if *ube3a* was reinstated at postnatal week 6 (adolescent mice). Notably, no rescue of *Ube3a*-dependent motor deficits was observed upon the same levels of reinstatement in adult mice (14-week-old mice). The Rotarod data presented in Chapter 3 (**Figure 3A**) allowed us to conclude that it exists a critical

window for rescuing motor deficits in AS mice, and this window starts closing somewhere between postnatal weeks 3 and 6 and fully closes between postnatal week 6 and week 14.
We also investigated the effect of tamoxifen treatment on inducible AS mice at postnatal day 1 (P1) and found that reactivating UBE3A to 34-63% to WT levels led to a full rescue of motor deficits in AS mice, despite the lower levels of *Ube3a* expression. This allowed us to narrow down the optimal treatment window for complete reversal of motor deficits in AS mice from birth to between 3-6 weeks of age.

However, for behaviours like marble burying (MB), nest building (NB), and forced swim test (FST), the lack of rescue at all developmental stages studied (birth, juvenile, adolescence and adulthood), with the exception of post-conception reinstatement of UBE3A, led us to conclude that either the critical window for therapeutic intervention for these behaviours closes somewhere from conception to P1, or the window is larger, but we need higher levels of UBE3A expression (more than 30%) to achieve an improvement. Further experiments were needed to clarify this issue.
Two subsequent studies, namely Sonzogni et al. and Milazzo et al., have expanded upon our research (Sonzogni et al., 2020)(Milazzo et al., 2021).
In the Sonzogni et. al. study, researchers utilized a Nestin-Cre conditional AS mouse model, enabling the activation of maternal *Ube3a* expression around E12.5 and achieving WT levels by the final week of embryonic development (E15). Using the behavioural paradigms presented in Chapter 4 of this thesis, their data demonstrated a complete rescue of MB and NB phenotypes in these mice (Sonzogni et al., 2020).
Additionally, the researchers asserted that having only 50% of UBE3A is sufficient for normal embryonic brain development, as evidenced by the indistinguishable behaviour of *ube3a* $^{m+/p-}$ mice compared to WT mice. Furthermore, they found that complete silencing of the paternal ube3a allele in AS mice is not achieved until the first week after birth, which supports the findings of the Judson et al. study (M. C. Judson et al., 2014).
However, an alternative explanation should be considered for the normal behaviour of *Ube3a*$^{m+/p-}$ mice, which is comparable to that of WT mice. Instead of solely attributing this normal behaviour to the 50% UBE3A expression during embryonic development (as suggested by Sonzogni et al. (Sonzogni et al., 2020)) it is possible that the upregulation of the maternal allele during the critical period from P1 to P21 plays a role. During this period, the UBE3A levels in the maternal allele reach approximately 70-80% (see blue curve in **Figure 1**), which could contribute to the observed normalcy of behaviour in these mice. Therefore, to see a rescue in the Marble Burying (MB) and Nest Building (NB) phenotypes in our inducible mouse model, I would hypothesize that UBE3A protein expression would need to be increased from around 30% to 70-80% after birth.

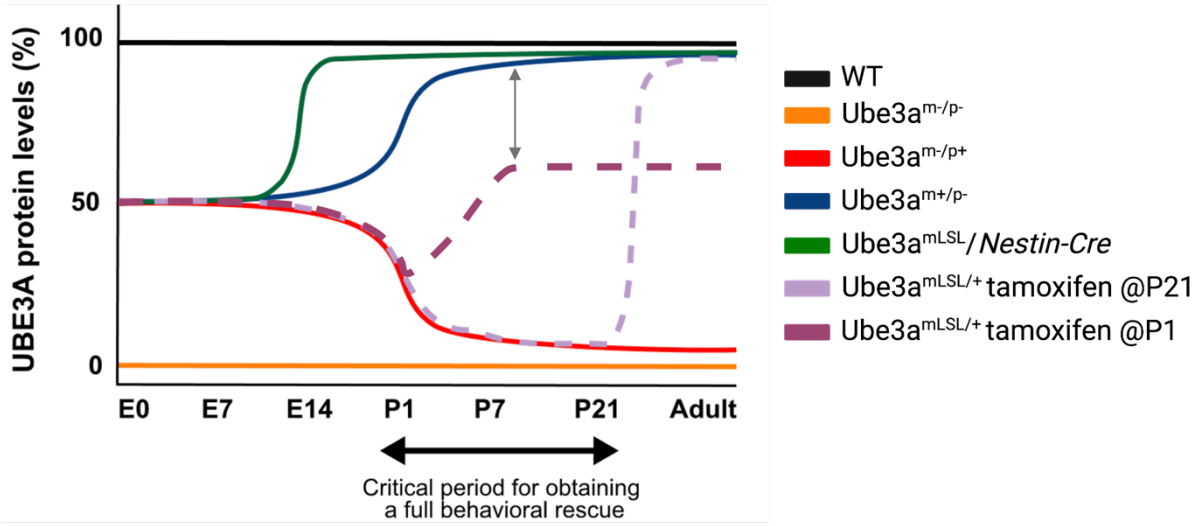


Figure 1 – Schematic representation of Ube3a lines used to study the critical period for rescuing behavioural deficits in AS mice by gene reinstatement. The critical window for therapeutic intervention lies around birth and P21. Each curve depicts the level of UBE3A protein in WT, *Ube3a*$^{m-/p-}$, *Ube3a*$^{m-/p+}$, *Ube3a*$^{m+/p-}$, *and Ube3a*^{mLSL}*/Nestin-Cre* mice (adapted from Sonzogni, 2020) over time. Additionally, the results of *Ube3a*^{mLSL} mice crossed with the inducible CreERT line, where gene reactivation was induced by tamoxifen injection at P21 and P1 (our study presented within this thesis), are included. If the *Ube3a* expression levels of *Ube3a*$^{mLSL/+}$ treated with tamoxifen at birth were to increase from approximately 30-60% (pink dashed line) to the same expression levels as *Ube3a*$^{m+/p-}$ mice (blue line), we hypothesize that a behavioural rescue would be observed in *Ube3a*$^{mLSL/+}$ mice treated with tamoxifen at birth. The hypothetical increase in behavioural rescue is represented by the grey arrows. The figure is adapted from the study by (Sonzogni et al., 2020).

In the study conducted by Milazzo et al., the administration of antisense oligonucleotides (ASOs) shortly after birth resulted in the reinstatement of UBE3A expression to the levels hypothesized to be necessary for rescuing the NB and MB phenotypes (Milazzo et al., 2021). However, they did not observe a rescue in both phenotypes.

One possible explanation for this lack of rescue is the instability of UBE3A expression levels upon ASO treatment, which decreased to around 60% at three weeks after treatment and 50% at six weeks after treatment (Milazzo et al., 2021).

In Chapter 5 of this thesis, it was shown that to achieve a rescue in the MB phenotype, continuous *Ube3a* expression is needed until three weeks postnatal, while for the NB phenotype, *Ube3a* expression beyond three weeks postnatal is required. Therefore, one could argue that to achieve a rescue in the MB phenotype, it is essential to maintain stable 70-80% UBE3A expression during the critical period from P1 to three weeks postnatal (P21). Similarly, for the NB phenotype, the same stable percentage of *Ube3a* reinstatement is necessary, but

the treatment leading to the gene's reinstatement should be delivered beyond 21 days after birth. Therefore, it can be inferred that the critical window for this behaviour closes shortly after the 21-day mark. These findings highlight the importance of timing and duration of *Ube3a* expression for the rescue of specific AS phenotypes.

To further investigate and support this hypothesis, additional experiments should be conducted.

For the FST behaviour the data presented in Chapter 4 of this thesis demonstrated that 30% reinstatement of UBE3A by P1 was insufficient to observe a rescue of this phenotype. However, Milazzo et al., by ASO application at P1 reinstated UBE3A to 70-80% and despite the slow decline of UBE3A levels to around 60% at three weeks after treatment, this was enough to obtain a rescue of the forced swim test (FST) phenotype in AS mice. Therefore, we can conclude that to effectively rescue the FST phenotype, UBE3A needs to be reinstated at the percentage shown by Milazzo et al. from P1, with the therapeutic window for this phenotype closing by P21.

Regarding the open field (OF) behaviour, our data showed that reinstating UBE3A at levels corresponding to 34-63% of WT expression successfully rescued this phenotype, when the reinstatement occurs during the first postnatal week. Our results strongly suggest that the critical window for therapeutic intervention in relation to the OF behaviour closes between birth and three weeks postnatally. Importantly, the study conducted by Milazzo et al. also supports and corroborates our data in this regard.

In the case of seizures in AS, we observed no rescue upon high levels of UBE3A reinstatement in juvenile mice, suggesting that the window for intervention closes somewhere shortly between conception and 21 days after birth. However, by following our established protocols, including the behavioural test battery we developed and published and using the same developmental groups, Milazzo et al. conducted a study in which they treated AS mice with ASOs and found that by P21, they had a rescue of seizures (Milazzo et al., 2021). Supplementary data presented in Chapter 3 of this thesis, **Supplementary Figure 2B**, demonstrates that when we treat P21 mice with Tamoxifen, we only achieve maximum Ube3a reinstatement in the hippocampus three days after the treatment stops, indicating that the maximum Ube3a expression is attained by P30. In contrast, Milazzo et al. shows that one week after ASO treatment, by P28, the treated mice had over 80% of Ube3a expression. Thus, the Milazzo et al. paper builds upon our study and allows us to conclude that the therapeutic window for intervention for epilepsy in AS mouse models closes between P21 and P30, during which time Ube3a expression is required to halt seizures.

Our data shows that the therapeutic window for hippocampal LTP remains open until adulthood, giving hope that therapeutic strategies targeting Ube3a reinstatement would show positive neurocognitive effects in AS patients even at older ages.

Given the complexity of the data from multiple studies exploring the relationship between the percentage of Ube3a reinstatement and the critical windows for therapeutic intervention to rescue AS phenotypes, a summary of the published data without incorporating specific hypotheses can be found in **Figure 2**. This figure provides an overview of the findings and allows for a visual representation of the relationship between these variables.

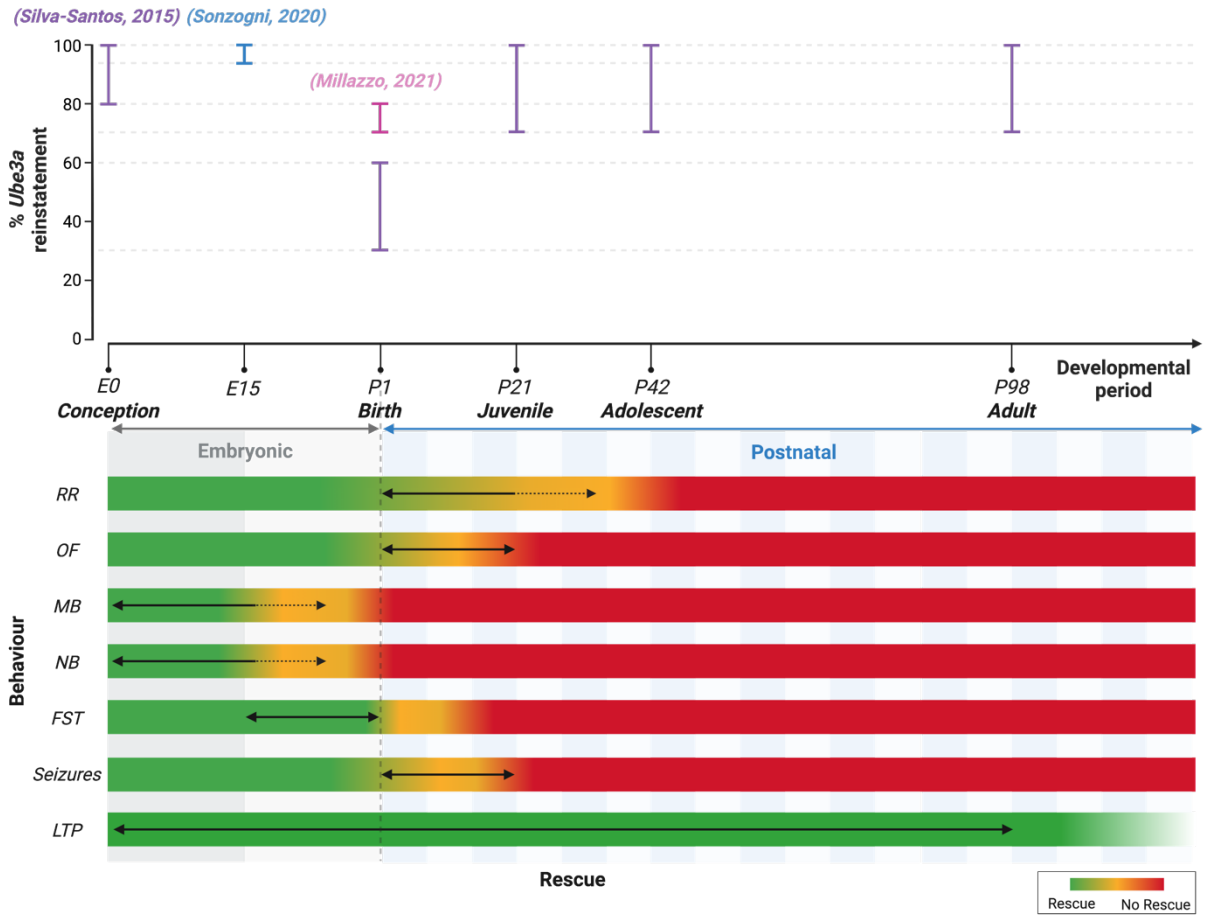


Figure 2 – Overview of Ube3a Reinstatement and Therapeutic Intervention Windows in AS Phenotypes. This figure provides a summary of the published data on the relationship between the percentage of Ube3a reinstatement and the critical windows for therapeutic intervention to rescue AS phenotypes. The color-coded legend indicates the degree of rescue, where green represents a full rescue, yellow indicates partial rescue, and red denotes the time point where the therapeutic window is closed. The arrows represent the time period during mouse developmental stages where intervention is still possible for rescuing AS-related phenotypes.

From the figure, it is evident that two key areas require further investigation to address the remaining questions. Firstly, there is a need to develop a technique that enables a higher and stable percentage of Ube3a reinstatement soon after birth. This would allow for more effective evaluation of the relationship between UBE3A expression levels and AS-related behaviours. Additionally, it would be valuable to refine the timeframe during which treatments are applied. The data suggests that significant changes occur between P1 and P21, indicating that the critical period for Ube3a reinstatement may be concentrated within the first and second week after birth. By reducing this window, further investigation can provide insights into the precise timing and duration of Ube3a expression required to effectively rescue AS phenotypes.

Despite the limitations of conducting our study in mouse models, where the neurodevelopmental processes differ from those in humans, we have observed that early therapeutic intervention is crucial for the successful implementation of UBE3A reinstatement therapy. While we cannot yet ascertain the critical windows for therapeutic intervention in human patients, our findings suggest that reinstatement of *Ube3a* beyond the earliest stages of postnatal development may not be fully effective in rescuing the AS phenotypes. Further exploration of the optimal timing for therapeutic intervention in human patients is necessary, and we will attempt to theorize on this later in subchapter 6.3 of this thesis discussion.

6.2 **CRITICAL TIMEFRAME: UNTIL WHEN *UBE3A* NEEDS TO BE EXPRESSED FOR NORMAL BRAIN FUNCTION?**

As previously mentioned in the introduction of this thesis, ASO injections are considered one of the most promising therapies for Angelman syndrome. However, due to the relatively short half-life of the ASO itself, it seems that this treatment needs to be continued to sustain high levels of UBE3A over time (Milazzo et al., 2021). In light of potential complications associated with ASO injections (Cohen-Pfeffer et al., 2017) and its neurotoxicity (Jia et al., 2023) it is critical to investigate whether UBE3A expression is necessary beyond brain development. This information will help us determine whether future treatments aimed at restoring UBE3A levels need to be continuously administered throughout life. To address this question, we designed a set of experiments presented in Chapter 5, where we used a novel conditional AS mouse model that enables temporal control for *Ube3a* deletion and deleted the gene in mice at 3 and 12 weeks old.

Our findings suggest that UBE3A expression is crucial in neuronal circuits involved in behaviours such as RR and MB from post-conception to 3 postnatal weeks. However, after this point, mice do not exhibit any deficits in these behaviours, even in the absence of UBE3A.

Regarding the OF behaviour, our studies, as well as the findings from the Milazzo paper (Milazzo et al., 2021), indicate that Ube3a expression is necessary from birth until the 3-week mark for normal OF behaviour.

The data from chapters 3 and 5 reveal that UBE3A is required for NB during embryonic development and extends slightly beyond three weeks postnatally. Specifically, the window of UBE3A requirement for NB behaviour closes between the 3-week and 12-week marks since the absence of UBE3A expression has no significant effect by 12 weeks.

For FST, however, continued UBE3A expression is necessary from birth until after the brain is fully mature.

In the previous subchapter, the hypothesis was proposed that the critical window for UBE3A expression in halting seizures and curing epilepsy in AS may be relatively short, potentially closing between P21 and P30. Further experiments are required to test this hypothesis and to also determine whether continuous treatment is necessary after this window. However, it is interesting to note that the data from Chapter 4 (**Figure 4B**) reveals that even if the window of intervention is short or the presence of UBE3A is required after this timepoint, antiepileptic drugs (AEDs) can be used as an alternative to continuous Ube3a reinstatement treatment, as they appear to have a beneficial effect in treating seizures in AS.

6.3 **ATTEMPT TO TRANSLATE THERAPEUTIC WINDOW OF INTERVENTION FROM MICE TO PATIENTS**

Our research was conducted on murine models of AS, making it challenging to translate the therapeutic intervention window defined in our studies to clinical applications. However, this limitation can be addressed in this discussion by drawing a tentative connection between murine and human development.

As extensively reviewed by Dutta et al., various methods have been used to establish a correlation between mouse age and human age (S. Dutta & Sengupta, 2016). One commonly employed calculation involves comparing the average lifespan of both species, which is approximately 80 years in humans and 24 months in mice. Based on this, we can determine the correlation factor: 1 mouse day corresponds to 40 human days (S. Dutta & Sengupta, 2016).

Applying this correlation to our data, we can conclude that the closing of the rescue window for AS motor impairments (established in mice to be between postnatal weeks 3 and 6) corresponds to around 2 years and 6 months to 4 years and 7 months of age in human age. Similarly, the full closing of this window in mice occurs somewhere between postnatal week 6 and week 14, which would mean that if treatment is applied to patients after the age of 10

years and 8 months, there would not be any noticeable improvement in motor deficits caused by the absence of Ube3a.

The Open Field (OF), Marble Burying (MB), Nest Building (NB), and Forced swim test (FST) are specifically designed tests to assess and measure behavioural responses in rodents, providing insights into AS-related behaviours. However, it is important to acknowledge that the direct applicability and relevance of these findings to human behaviour are not fully understood. That being said, based on the findings from mouse studies the window for intervention in behaviours such as Open Field (OF) and Marble Burying (MB), which was shown to be from birth to 3 weeks of age in mice, would roughly correspond to around 2 years and 6 months of age in human patients. This indicates that the optimal time for treatment in human patients is before this age range.
The same observation holds true for the NB and FST tests. However, in the case of NB, the intervention window in mice extends slightly beyond the 3-week mark, as the deletion of Ube3a at this timepoint leads to an AS phenotype in this test. On the other hand, for the FST test, Ube3a needs to be present even after the mouse reaches adulthood. This suggests that in human patients, when it comes to tasks involving similar neuropathways as the FST test in mice, long-term treatment may be necessary to achieve desired outcomes.
These findings indicate the importance of considering the specific aspects associated with each test and tailoring treatment strategies accordingly.
In terms of epilepsy in AS mouse models, the window for intervention closes between P21 to P30 in mice. By applying the correlation factor, we estimate that the treatment window for epilepsy in human patients would extend until approximately 3 years and 3 months of age.

However, I share the view that this simplistic approach may not adequately capture the complexities involved in correlating mouse and human age, and that it has certain limitations. Although it offers a general estimation based on average lifespans, it is important to acknowledge that mouse and human development differ in various aspects and cannot be perfectly equated. While mice and humans share main stages of postnatal development, such as weaning and adolescence, it is important to consider the differences in the pace of development between the two species. As mentioned by Dutta et al., mice undergo accelerated development, rapidly reaching key developmental milestones after birth, while human development progresses more gradually (S. Dutta & Sengupta, 2016).
This suggests that the therapeutic intervention window for AS may close later in humans than in mice. Nonetheless, whether the therapeutic window we identified in mice applies to humans can only be determined through clinical trials that involve restoring UBE3A in the nervous system of AS patients. While these estimations provide a framework for understanding the

potential treatment window, further validation and refinement through additional research and clinical studies are necessary to ensure their accuracy and applicability in human patients.
A positive finding from the LTP experiments presented in Chapter 3 of this thesis, as well as data from Rotaru et al. suggests that electrophysiological phenotypes observed in AS are reversible upon Ube3a reactivation, even when the brain is fully mature (D. C. Rotaru, van Woerden, Wallaard, & Elgersma, 2018).
This may indicate some level of cognitive improvements in older AS patients, even if the treatment starts later. To increase the chances of a positive outcome and to fully elucidate the optimal timing of intervention in humans, I would suggest that future clinical trials on AS patients would ideally commence with a cohort of patients no older than 2-3 years old in order to clarify some of the questions that remain open.

6.4 **CONSIDERATIONS ABOUT THE PHENOTYPICAL RESCUE OBTAINED IN ADULT ANGELMAN MOUSE MODELS IN OTHER PUBLISHED STUDIES**

The data presented in Chapters 3 and 5 revealed that to correct most of the behavioural phenotypes characteristic of AS, *Ube3a* must be reinstated at a young age. Nonetheless, some studies demonstrate some level of adult rescue in a subset of AS phenotypes (Meng et al., 2015)(D. Lee et al., 2023)(Adhikari et al., 2021)(Daily et al., 2011)(D. C. Rotaru et al., 2018). For instance, while Lee et al. observed rescue of sleep disturbances and EEG rhythms in adult AS mice upon *Ube3a* restoration (D. Lee et al., 2023), Rotaru and colleagues reported a rescue in the electrophysiological properties of medial prefrontal cortex (mPFC) neurons of AS mice (D. C. Rotaru et al., 2018). Both studies point to an attainable electrophysiological rescue in a fully mature AS brain upon *Ube3a* restoration, which is in line with the hippocampal (Schaffer collateral-CA1) LTP rescue we obtained, shown in Chapter 3.
However, the Daily et. al. study reported a rescue in contextual fear memory and partial rescue in the Morris water maze (Daily et al., 2011), which we were unable to replicate in our experiments (data not shown), preventing us from assessing whether these behaviours are rescued upon *Ube3a* reinstatement in adult AS mice.
The same argument applies to the study by Meng et al., where a rescue in contextual fear conditioning was reported upon treatment of adult AS mice (Meng et al., 2015). Despite being a valuable hippocampal dependent learning paradigm, like others (Wolter et al., 2020), we too were unable to obtain consistent results in contextual fear conditioning (data not shown) that would allow us to evaluate a possible rescue in cognitive deficits upon adult gene reactivation.

This leaves the study conducted by Adhikari et al., which proposes a therapeutic intervention for AS by genetically modifying hematopoietic stem cells (HSPCs) using a lentiviral vector expressing Ube3a. The modified HSPCs are expected to differentiate into microglia that can cross-correct Ube3a-deficient neurons and ultimately correct AS phenotypes in adult mice (Adhikari et al., 2021). The authors propose that their findings suggest the potential absence of a critical treatment window for AS. These results are unexpected and inconsistent with other studies, including our own experiments and those of others, such as Gu et al. (2019) (Gu et al., 2019), and Sidorov et al. (2018) (Sidorov et al., 2018), which consistently demonstrate the existence of a therapeutic window for intervention in AS.

Besides, we have some concerns regarding their conclusions. Firstly, the transplantation and engraftment of human CD34+ cells transduced with Ube3a occurred at an earlier stage when the mice were 4-5 weeks old (Adhikari et al., 2021). At this age, the brain is still undergoing significant maturation, with the transition from juvenile to adult state typically occurring around postnatal week 8 based on existing literature (Gutierrez-Castellanos, Sarra, Godinho, & Mainen, 2022)(Schneider, 2013). Therefore, any observations should be attributed to treatment during adolescence rather than adulthood, as claimed by the authors.

Another critical concern with this study is the lack of sufficient evidence to support the conclusion that cross-correction is responsible for the reported outcomes in the treated mice. Additionally, the study fails to propose a plausible mechanism by which secreted Ube3a enters the neurons, and it lacks appropriate controls to support its conclusions. The use of S5a as a non-specific model substrate for assessing the activities of various ubiquitin ligases (Uchiki et al., 2009), instead of a Ube3a-specific ubiquitination target such as RING1B or HERC2 (Kühnle, Mothes, Matentzoglu, & Scheffner, 2013), raises doubts about the validity of the positive control. The positive control also appears to be practically indistinguishable from background, which further undermines confidence in the ubiquitination test. The lack of robust evidence to support their conclusions warrants further investigations with appropriate controls and mechanisms of action.

In our view, the Adhikari et al. study does not provide sufficient evidence to support the claim that HSPCs treatment for AS in Ube3a deficient mice does not require a critical treatment window for certain behaviours such as RR and OF. However, for other behaviours like balance beam, where we did not detect a significant difference between WT and AS mice (data not shown), and for treadmill walking, which was not analysed in our experiments, we acknowledge the possibility of a wider therapeutic window for other AS phenotypes that may extend into adulthood. More research is needed to clarify these aspects.

6.5 **THE IMPORTANCE OF EARLY IDENTIFICATION AND INTERVENTION IN AS**

As mentioned in Chapter 2, AS is not generally diagnosed at birth mostly because the unique clinical features of AS are not fully manifested until the age of one year. Considering the findings presented in this thesis, one must recognize the advantage early diagnosis of AS would have in increasing the favourable outcome of the treatments reinstating UBE3A's expression.

Neonatal Bloodspot Screening (NBS) was initiated in Europe during the 1960s and the panel of screened disorders varies between different countries (Loeber et al., 2021), but several inherited conditions, from phenylketonuria to cystic fibrosis and lysosomal storage disorders, are routinely screened via this program.

The main objective of such program is to detect newborns with potential rare and genetic conditions in a timely and pre-symptomatic manner, enabling early intervention to prevent or ameliorate the long-term consequences of the condition. Currently, AS is not included in these screening programs, and the decision to include new conditions depends on medical and technical knowledge and/or the personal interest of scientists, clinicians and public health colleagues involved in the decision making process, together with the availability of funding (Loeber et al., 2021).

Based on the potential therapeutic benefits of early intervention for AS, as supported by the work contained in this thesis, it is prudent to initiate discussions on including AS in neonatal screening panels. This inclusion could significantly increase the success rate of treatments aimed at UBE3A reinstatement, particularly if future clinical trials reach similar conclusions.

Furthermore, from an economic perspective, the inclusion of AS in screening programs would be advantageous, as the cost of providing care for individuals with rare genetic disorders, including AS, is substantial (Andreu P, Karam J, Child C., & Chiesi G., 2022). Moreover, a recent study has shown that AS incurs the highest yearly costs per individual compared to several other disorders (Baker et al., 2021). In addition, this study also suggests that both affected families and Governments could benefit from a significant decrease in this economic impact if the diagnosis is made soon after birth.

6.6 **WIRED FOR LEARNING, NOT FOR BEHAVIOUR? - THE COMPLEXITIES OF *UBE3A* REINSTATEMENT IN AS**

The findings presented in Chapter 3 of this thesis provide evidence that the reinstatement of *Ube3a* expression during adulthood can correct deficits in hippocampal long-term potentiation (LTP). LTP is a crucial process in synaptic plasticity, which is essential for learning and

memory. Similarly, a recent study by Rotaru and colleagues showed that *Ube3a* reinstatement in the medial prefrontal cortex (mPFC) of adult mice can restore electrophysiological deficits in layer 5 neurons (Avagliano Trezza et al., 2019). These findings highlight the potential for *UBE3A*-targeted therapies in treating AS. However, despite the successful restoration of UBE3A expression, the behavioural deficits associated with AS were not completely corrected, revealing a dissociation between the rescue of electrophysiological properties and the behavioural deficits. This could indicate that the AS behavioural deficits we analysed involve neural circuits beyond the hippocampus and mPFC regions. While the hippocampus and mPFC are recognized as crucial regions for learning and memory processes, it is important to acknowledge that complex behaviours involve a network of brain regions. Thus, the reinstatement of UBE3A expression in these specific regions may not be sufficient to fully restore normal functioning in other brain areas affected by AS.

Interestingly, a growing body of evidence suggests that the loss of UBE3A has diverse effects and varying impacts on neurons across different brain regions, despite its expression in all neuronal types. For instance, a study by Wang et al. demonstrated changes in intrinsic membrane properties specifically in hippocampal pyramidal neurons and medial nucleus of the trapezoid body neurons (Wang et al., 2017), which were not observed by Kaphzan et al. in layer 2/3 neurons of the somatosensory cortex (Kaphzan, Buffington, Jung, Rasband, & Klann, 2011). This implies that the consequences of UBE3A loss can be region-specific and may manifest differently across different neuronal populations within the brain.

Furthermore, UBE3A is involved in the regulation of multiple cellular processes and signalling pathways, including protein degradation, ubiquitination, and gene expression. The restoration of UBE3A expression may correct deficits in certain cellular processes and signalling pathways but may not impact others involved in behaviour regulation. Therefore, it is necessary to conduct further research to comprehend the complex interactions between UBE3A expression, neural circuitry, and behaviour in the context of AS.

Altogether, these findings underline the need for a comprehensive approach to studying AS and the development of targeted therapies that address the multiple neural circuits affected in the disorder. It is essential to explore alternative therapeutic targets beyond UBE3A to develop more effective treatments for AS.

The data presented in Chapter 3, specifically in **Figure 4 A** and **B**, suggests that a therapy targeting Ube3a gene reactivation may be effective in rescuing certain phenotypes but may not fully rescue the epilepsy phenotype. However, seizures can be successfully rescued by the administration of anti-epileptic drugs, indicating that a combination approach of Ube3a reinstatement therapies along with treatments targeting downstream effectors may yield better outcomes.

Future studies should consider the intricate relationships between genetic, neural, and behavioural factors to achieve a comprehensive understanding of AS.

6.7 **NAVIGATING THE COMPLEXITY OF BEHAVIOURAL PHENOTYPES IN MOUSE MODELS OF ANGELMAN SYNDROME**

Animal models play a crucial role in biomedical research by providing a platform to investigate a variety of scientific questions. Mice, in particular, are highly suitable for research purposes, as they share many biological similarities with humans, including a large proportion of genes associated with diseases (Yue et al., 2014). Researchers attempt to draw correlations between observed behaviours in patients with certain disorders and the behavioural deficits seen in murine models of the disease. While genetically altered Fmr1 KO mice exhibit a perfect correspondence with Fragile X syndrome patients (Kazdoba, Leach, Silverman, & Crawley, 2014), the face validity of mice lacking the *Ube3a* gene for Angelman Syndrome (AS) is not as straightforward.

In contrast to AS patients, AS mice exhibit mild behavioural impairments that may be easily overlooked, with genetic background effects present for certain phenotypes (Born et al., 2017)(Huang et al., 2013). Inconsistencies and a lack of replication in AS mouse phenotypes may be attributed to differences in procedures, genetic backgrounds, or underpowered experiments (as we shown in Chapter 4), highlighting the need to identify reliable biomarkers for AS and a potential rescue of these phenotypes through experimental pharmacological treatments to enhance pre-clinical translational power.

In this thesis, we present a robust behavioural test battery for mouse models of AS. The battery comprises six tests designed to assess motor performance, repetitive behaviour, anxiety, and susceptibility to tonic-clonic seizures induced by audiogenic stimulation, namely, accelerating Rotarod (RR), Open field test (OF), Marble burying test (MB), Nest building test (NB), Forced swim test (FST), and audiogenic seizure susceptibility test. Although the behavioural battery we propose in this thesis has been shown to be consistent and reliable across multiple AS mouse strains and laboratories worldwide (Moreira-de-Sá et al., 2020)(Shi et al., 2022)(M. C. Judson et al., 2021)(Milazzo et al., 2021), their translational value remains unknown.

While the accelerating RR and OF tests are known to evaluate motor coordination and exploratory behaviour in mice, interpretation of tests such as MB, NB, and FST is more complex as the underlying brain circuits are not fully understood. Nonetheless, controlled assessment of such behaviour can provide insight into the specific brain areas and neuronal circuits involved in the final output of the central nervous system (Hånell & Marklund, 2014).

Although some c-Fos (immediate early gene used as a marker for neuronal activation) studies revealed that behavioural tests have an inherent complexity where distinct behavioural traits

may be mediated by the activation of different brain regions and measure different parameters (Hånell & Marklund, 2014)(X. Chen et al., 2021)(Chung, 2015)(Gallo, Katche, Morici, Medina, & Weisstaub, 2018), not much research has been done to clarify the correlation between the specific behaviours we explored in our battery and the brain areas involved. Despite these limitations, we will attempt to speculate on the meaning of these behaviours in the context of AS.

6.7.1 **THE MEANING OF A BEHAVIOUR: RR**

In the introductory chapter of this thesis, it was stated that the Rotarod (RR) is widely used as a preferred behavioural paradigm to assess cerebellar function (Bohlen et al., 2009) (Shiotsuki et al., 2010)(Hamm et al., 1994). However, the motor impairments detected in AS mice by the RR test do not appear to result from the loss of UBE3A in the cerebellum. This is because cerebellar reinstatement of UBE3A is sufficient to rescue cerebellar-specific tasks such as the Vestibulo-Ocular Reflex, but insufficient to rescue the RR phenotype (Bruinsma et al., 2015). These findings suggest that motor impairments detected in AS mice by the RR test result from UBE3A loss in other extracerebellar circuits than the cerebellum.

Indeed, the cerebellum is not the only brain structure involved in motor coordination. Bureau et al. observed upregulation of c-Fos in the motor cortex (M1 and M2), cingulate cortex (CG1 and CG2), and dorsal striatum of RR trained mice (Bureau, Carrier, Lebel, & Cyr, 2010). Using deformation-based morphometry to assess local volume and fractional anisotropy derived from diffusion MRI, Scholz and colleagues found that the hippocampus, frontal cortex, and amygdala are larger in rotarod-trained mice compared to controls who never performed this task. Interestingly, the cerebellum and white matter in the corpus callosum underlying the primary motor cortex are smaller after training (Scholz, Niibori, W Frankland, & P Lerch, 2015). It is known that learning and performance of sequential and coordinated movements, like the ones necessary to execute the RR test, are dependent on the uninterrupted communication between the cerebellum and several brain areas such as presupplementary motor area (pre-SMA), supplementary motor area (SMA), dorsal premotor cortex (PMd), primary motor cortex (M1), primary somatosensory cortex, superior parietal lobule, thalamus, basal ganglia (Ohbayashi, 2021)(Sakayori et al., 2019).

It is known that the major targets of the cerebellar projections are the thalamic regions, and selective elimination of neurons from the dentate nucleus in the cerebellum to central lateral thalamic nucleus impairs motor coordination in the RR test without hampering the mice's overground locomotion (Sakayori et al., 2019). Moreover, by using multimodal quantitative MR imaging analysis, Yoon and colleagues observed that, compared with controls, children with

AS exhibit significantly decreased functional connectivity in the thalamus and basal ganglia (H. M. Yoon et al., 2020), both areas that strongly interconnect with the cerebral cortex to enable proper motor control and balance. Not excluding the possibility that the RR phenotype observed in AS mice could arise from a failure of this interplay.

6.7.2 **THE MEANING OF A BEHAVIOUR: OF**

All Open Field (OF) experiments conducted within the context of this thesis indicate that hybrid AS mice exhibit reduced mobility in the test arena compared to WT mice. However, there was no significant difference observed in the time spent by both genotypes in different areas of the OF. This finding is consistent with other published research studies (Born et al., 2017)(Huang et al., 2013)(Tanas et al., 2022).

The short total distance travelled by the AS mice led to speculation that this phenotype may result from the motor impairments caused by the absence of UBE3A (R. Dutta & Crawley, 2020). However, the experiments presented in Chapter 3, which showed that postnatal reactivation of *Ube3a* expression at P21 rescued motor impairments in RR but did not change the OF phenotype, suggest that the hypoactivity of AS mice in the OF test is not caused by motor impairments but may depend on another factor.

In addition to assessing the exploratory locomotion, the OF can also be used as a readout of anxiety in mice. Typically, this is measured by the time mice spend in the centre versus other areas of the arena. Similar to previous studies, we did not observe a significant difference in this parameter (Tanas et al., 2022)(Syding et al., 2022). However, it is possible that AS mice have such high anxiety levels that they are hesitant to explore even the surrounding areas of the arena.

It is known that *Ube3a* regulates glucocorticoid receptor (GR) transactivation, and that the GR signalling pathway is disrupted in the brains of AS mice. Altered GR signalling in the hippocampus leads to hypothalamo-pituitary-adrenal axis hyperactivity, which increases anxiety in AS mice (Godavarthi et al., 2012). Several studies have observed a decrease in horizontal and vertical activity (R. Dutta & Crawley, 2020) and fewer rearing episodes (Born et al., 2017) in AS mice when compared with their WT littermates during the OF test.

Both of these exploratory behaviours are highly sensitive to environmental factors and are known to be suppressed under anxiety-provoking circumstances (Sturman et al., 2018). Godavarthi et al. also found an increased frequency of freezing in AS mice during the OF test (Godavarthi et al., 2012).

Taken together, these findings suggest that the hypoactivity observed in AS mice in the OF test may be an indication of anxiety, which could impact the innate exploratory drive in these mice.

6.7.3 **THE MEANING OF A BEHAVIOUR: MB AND NB**

Despite its apparent simplicity, the marble-burying (MB) behaviour is often accompanied by uncertainty in its interpretation. While many studies in the literature have suggested that this paradigm reflects the efficacy of anxiolytic and psychoactive drugs, our understanding of the factors that drive this behaviour is limited.

As mentioned in Chapter 1, some studies suggest that burying behaviour in mice reflects compulsiveness, given the inhibitory effect of selective serotonin reuptake inhibitor (SSRI's) treatments on this behaviour (Egashira et al., 2018), and the fact that this behaviour persists with little change across multiple exposures (Njung'e & Handley, 1991). However, our results do not support compulsion as the driver of the burying behaviour observed in the MB test. If burying is indeed compulsive, the number of burying marbles would remain relatively consistent upon repeated exposure to the stimulus (glass marbles), which is not the case, as seen in Chapter 4. We observed a drastic decrease in the digging response of WT mice upon re-testing (**Figure 3F**), suggesting a certain degree of habituation to the marbles. Overall, our results suggest that burying may be an expression of defensiveness stimulated by novelty.

Like other studies, we also failed to find a correlation between the MB and other anxiety-like tests, such as OF. As observed in Chapter 3, 4 and 5, WT mice in the OF explore more than AS mice but also bury more marbles in the MB tests. If burying behaviour is indeed an indication of anxiety, it would be expected for "stressed" mice to explore less in a bright and open arena and bury more of the perceived aversive stimulus. However, our results failed to show a negative correlation between both behaviours.

So how do we interpret the MB phenotype in AS mice? The results presented in Chapter 3, where postnatal reactivation of *Ube3a* in AS mice successfully rescues the locomotor impairments in the RR but not the MB deficit, makes it unlikely that locomotor impairments interfere with the marble-burying behaviour. This is in accordance with other experiments that also failed in relating a decrease in marble burying with locomotor impairments (Jimenez-Gomez et al., 2011). In addition, our data suggests that the act of burying marbles is an active and directed behaviour rather than a secondary measurement of the digging behaviour, as suggested by Gyertyán (Gyertyán, 1995).

Further experiments have demonstrated that digging is a natural behaviour part of the normal behavioural routine of rodents (Wolmarans et al., 2016). Therefore, the impaired burying

behaviour of AS mice, as well as the nesting deficit, indicate that the lack of expression of the *Ube3a* gene disrupts hard-wired innate behaviours, crucial for survival in mice.
Altogether, the deficits observed in AS mice in the MB, NB and even FST, allow us to conclude that our behavioural battery is a sensitive method for detecting subtle behavioural dysfunction in innate behaviours, a class of tests that rely on the animal's performance without training (Remedios et al., 2017).
The work by Deacon and colleagues gives us an indication that many "species-typical behaviours", including MB and NB, are hippocampal dependent (Deacon & Rawlins, 2005), a brain area markedly affected in AS (Kaphzan et al., 2011)(Hallengren & Vaden, 2014)(Sun et al., 2015)(Mardirossian, Rampon, Salvert, Fort, & Sarda, 2009)(Dindot et al., 2008)(Miura et al., 2002). Both burrowing test/marble burying and nest building assays also reflect the integrity of cortical regions (Deacon, 2006b)(Deacon et al., 2002)(Deacon & Rawlins, 2005)(Teissier et al., 2020).

6.7.4 **THE MEANING OF A BEHAVIOUR: FST**

In previous studies conducted on rats, it was observed that forced swim test caused an increase in Fos-like immunoreactivity in various regions of the brain, including the medial prefrontal cortex, nucleus accumbens, locus coeruleus, raphe nuclei, striatum, hypothalamic nucleus, periaqueductal grey, amygdala, habenula, paraventricular nucleus of hypothalamus, and bed nucleus of stria terminalis (Duncan, Knapp, Johnson, & Breese, 1996)(Romero-grimaldi & Moreno-lo, 2008)(Choi et al., 2013).
These studies suggested that the FST behaviour is associated with the activation of different brain regions, but all agree that the medial prefrontal cortex plays a crucial role.

In a study conducted by Siderov et al. on AS mice, it was found that these mice exhibit exaggerated operant extinction behaviour in operant tests, a task that is known to be mediated by the prefrontal cortex. This study demonstrated that enhanced extinction behaviour in AS mice is associated with enhanced excitability of infralimbic (IL) neurons in the medial prefrontal cortex (Sidorov et al., 2018).
Interestingly, a meta-analysis study showed that inhibition of prelimbic or infralimbic mPFC causes a significant drop in immobility time in the FST, indicating that prelimbic or infralimbic cortices are more relevant to the control of immobility in the FST than other subregions (Domingues, Melleu, & Lino de Oliveira, 2021).
This makes us speculate that perhaps the increased floating time phenotype of AS mice in the FST could also be a reflection of enhanced excitability of infralimbic (IL) neurons in the medial prefrontal cortex. If proven to be the case, this would improve the FST's overall face validity

for AS. However, it is worth noting that the FST is a complex behaviour that can be influenced by many factors, including stress response, age, and endocrine manipulations (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013). In conjunction with the data provided by the study from Rotaru et. al. (D. C. Rotaru et al., 2018), it could be possible that the FST phenotype in AS mice may be influenced by factors other than mPFC dysfunction. To clarify this uncertainty, further studies will be necessary to fully understand the neural circuits underlying the FST phenotype in AS mice and how these circuits are impacted by *Ube3a* expression.

6.7.5 **THE MEANING OF A BEHAVIOUR: FINAL CONSIDERATIONS**

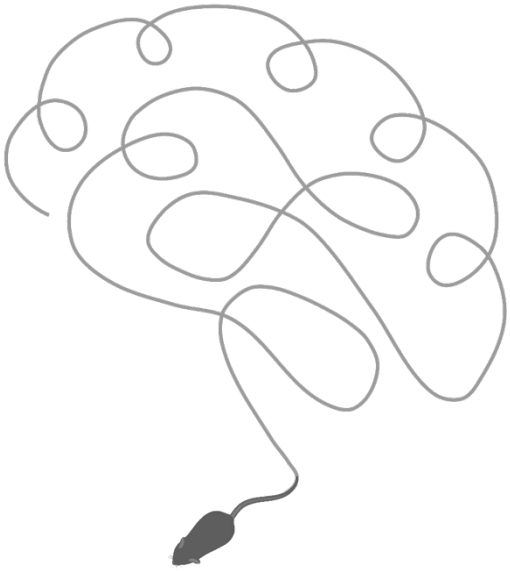
Although we can speculate about the abnormal behavioural phenotypes observed in AS mice, it remains uncertain whether these phenotypes have clinical relevance, given that mice lack cognitive processing analogous to humans. This poses a limitation in our studies. Nevertheless, we know that a phenotype is not a random event, but rather results from the interplay between genetic and environmental factors.

One advantage of studying AS is that its aetiology is relatively straightforward compared to other disorders, such as autism, which are characterized by complex genetic and environmental interactions (Amaral, 2011). AS, on the other hand, is a monogenic disorder caused by a disruption of the maternal *UBE3A* gene. In this thesis, we conducted experiments using genetically identical mice that differed only in their expression of the *Ube3a* gene, and standardized lab conditions ensured that any differences in observed behaviours were due to the genetic alteration or treatment. Because of this, we have a high degree of certainty that the behaviours described in our studies can be used to characterize new models of AS and assess the effects of possible treatments in this syndrome. Therefore, despite our current lack of understanding of the cellular populations responsible for the observed behavioural phenotypes, we demonstrated that the behavioural battery we describe is robust and consistent across multiple AS mouse strains. The reliability of this battery has already been tested in laboratories around the world (Moreira-de-Sá et al., 2020)(Shi et al., 2022)(M. C. Judson et al., 2021)(Milazzo et al., 2021).

The behavioural battery presented in this thesis can increase the accuracy and reproducibility of future studies and accelerate the development of effective treatments for AS. Our studies also revealed that some behavioural tests may be more sensitive and specific to AS phenotypes than others and which tests are stable upon re-testing. With the knowledge we provide, researchers can more accurately evaluate the effects of potential treatments for AS and optimize clinical trial design. Overall, the results from Chapter 4 of this thesis are of significant value as they accelerate the development of effective treatments for AS.

CHAPTER 7

FUTURE DIRECTIONS & CLOSING REMARKS



FUTURE DIRECTIONS & CLOSING REMARKS

In this thesis, our main focus was to study the impact of *Ube3a* reinstatement in Angelman syndrome (AS) mouse models, particularly concerning the timing and extent of its effects. To achieve this, we employed a tamoxifen-inducible Cre recombinase system to control the temporal and spatial expression of *Ube3a* in the AS mouse model. By doing so, we were able to investigate the effects of UBE3A reinstatement across different developmental stages.
Our study encompassed the entire prenatal development period as well as specific postnatal periods, including the early postnatal phase when synaptogenesis and circuit formation are particularly active. By evaluating UBE3A reinstatement at different time points, we aimed to gain insights into how its expression influences the development and function of neural circuits, which are crucial for understanding the underlying mechanisms of AS.
Through this comprehensive approach, we aimed to shed light on the potential therapeutic benefits of *Ube3a* reinstatement in AS and provide valuable information on the critical periods during which such interventions may have the most significant impact on ameliorating the AS phenotype. Our findings contribute to the growing understanding of AS pathophysiology and may inform future therapeutic strategies aimed at improving the lives of individuals with AS.

In light of our experiments' scope, which mainly involved *Ube3a* reinstatement or deletion throughout the entire brain, we believe that investigating the effects of targeted *Ube3a* reinstatement in different brain regions would be of significant interest and scientific value.
We propose conducting a comparative analysis to examine the effects of UBE3A reinstatement and deletion specifically in the cortex, striatum, hippocampus, and cerebellum. These brain regions are known to be involved in AS pathogenesis, exhibit distinct developmental trajectories, and play functional roles that are relevant to the behaviours included in our test battery.
By selectively targeting these specific brain areas, we can gain a more comprehensive understanding of how UBE3A expression influences behavioural outcomes in a region-specific manner. This focused investigation will allow us to decipher the intricate interactions between UBE3A expression, neural circuitry, and behaviour in the context of AS.

Given the significant impact of sleep disturbances on the quality of life for individuals with AS and the burden it places on their caregivers, we propose conducting future experiments aimed at reinstating *UBE3A* expression in brain regions known to control circadian behaviour, such as the suprachiasmatic nucleus (SCN). By targeting the SCN, which serves as the master pacemaker of the body's internal clock, regulating the timing of sleep and wakefulness we could simultaneously assess the impact of *Ube3a* reinstatement on the regulation of circadian

behaviour and sleep patterns in AS mice models while investigating the presence or absence of a therapeutic window for intervention for sleep disturbances in AS. Conducting this research has the potential to provide crucial information on the optimal timing for therapeutic interventions aimed at effectively manage sleep-related challenges in AS.

We could even expand our research scope beyond the brain. By employing our established techniques, we can delve into the specific ramifications of *Ube3a* loss and reinstatement within the Enteric nervous system (ENS). Our current understanding of AS reveals a research gap concerning the role of *Ube3a* in the ENS, despite the prevalence of gastrointestinal or stomach issues in affected individuals. To bridge this gap, we propose utilizing the same techniques that we have employed in our studies to investigate the specific role of *Ube3a* in the ENS and its potential contribution to gastrointestinal manifestations. By doing so, we would have the potential to advance our knowledge of AS and improve our ability to develop targeted interventions aimed at ameliorating the gastrointestinal issues enhancing the overall well-being of individuals affected by the syndrome.

To further validate the utility of the behavioural test battery for mouse models of AS, additional follow-up experiments could be performed to assess its sensitivity and specificity in detecting behavioural phenotypes in other AS mouse models and other genetic or pharmacological interventions. This could involve comparing the performance of the behavioural test battery across different AS mouse models that exhibit distinct genetic or pharmacological manipulations, such as *Ube3a* deletion, *Ube3a* mutation, or pharmacological treatments targeting specific molecular pathways.

One limitation of our behavioural battery for mouse models of AS is the lack of a specific test to assess cognitive and memory functions in the mice. Cognitive deficits are a common feature of AS, and the underlying neural mechanisms are poorly understood. Therefore, we recommend developing a reliable and sensitive cognitive test for AS mouse models to investigate the nature of cognitive impairments in these mice and to evaluate potential therapeutic interventions. Although we have run cognitive tests such as the Morris water maze and Fear Conditioning in our mice, we were not successful in finding a phenotype in the AS mice. We also attempted other cognitive tests, such as the Barnes maze, Novel Object Recognition Test, and Y-maze, but none of these tests yielded significant results in the AS mice (data not shown). Therefore, we suggest exploring alternative cognitive tests or adapting existing tests to better suit the cognitive profile of AS mice.

To explore the potential translational relevance of *UBE3A* reinstatement and delayed *UBE3A* loss for developing effective therapies for AS, further follow-up experiments could involve testing the effects of *UBE3A* reinstatement or delayed *UBE3A* loss in animal models that more closely resemble human physiology and behaviour. This is important because, like our studies showed, no single behavioural test can fully capture the complex nature of AS in mice. While the use of non-human primates may not be feasible due to ethical or practical considerations, alternative animal models that share more similarities with human brain structure and function could be considered. For example, advanced rodent models with more complex cognitive abilities or other mammalian species that exhibit relevant behavioural and neurodevelopmental characteristics could be evaluated.
It is important to note that while animal models can provide valuable preclinical data, ultimately conducting experiments in AS patients would offer the most direct and informative insights. Clinical trials and studies involving human patients are critical to fully understand the therapeutic potential of *UBE3A* interventions and their applicability to human AS cases.

Our studies have indicated that the critical period for intervention in AS for most behaviours closes between birth and postnatal week 3, encompassing a relatively broad timeframe. To refine our understanding and precisely determine when the critical window for rescuing AS-related phenotypes closes, conducting targeted experiments with a narrower focus would be of great value. In particular, investigating the effects of *Ube3a* reinstatement at specific time points, such as 1 week and 2 weeks after birth, could yield more precise information about the temporal dynamics of *Ube3a* expression and its impact on AS phenotypes. By focusing on these specific time points, we can potentially pinpoint the optimal window for therapeutic interventions, thus enhancing the efficacy of treatments.
However, a limitation in conducting experiments during this early postnatal period is the challenge of effectively delivering tamoxifen to 1-week-old mice. The young age and small size of the mice make precise drug delivery more challenging. Additionally, ethical considerations may limit the feasibility of performing experiments at such an early developmental stage. Additionally, ethical considerations, as determined by the committee, may restrict the feasibility of performing experiments at such an early developmental stage. One potential solution to overcome this limitation could be the development of novel tamoxifen delivery methods, such as using nanoparticle-based drug carriers or viral vectors to specifically target *Ube3a* expression in the brain at the desired time points.

Finally, the findings from the studies conducted as part of this thesis shed light on the presence of critical periods for *UBE3A* expression in the treatment of certain AS symptoms. These critical periods indicate that reinstating *UBE3A* after the closure of this sensitive timeframe

does not yield any therapeutic effects. Unfortunately, this observation dashes the hopes for an improvement therapy targeting older AS patients.

The closure of critical periods is achieved through molecular brakes that constrain plasticity and allow for permanent structural consolidation, thereby ending a critical period. As extensively revised by Carulli et al., the formation of perineuronal net (PNN) is the mechanism that coincides precisely with closure of CPs in the cerebral cortex, hippocampus, hypothalamus and amygdala (Carulli & Verhaagen, 2021). PNNs are a specialized, condensed form of extracellular matrix (ECM), composed largely of hyaluronan and chondroitin sulfate proteoglycans (CS-GAG) which, gradually and tightly, enwrap the soma and dendrites of PV+ cells as they mature, restricting plasticity at the end of the CPs (Hockfield, Kalb, Zaremba, & Fryer, 1990) (Kwok, Dick, Wang, & Fawcett, 2011).

However, it has been demonstrated in previous studies that certain experimental manipulations, such as injection of the enzyme chondroitinase ABC (ChABC) in the adult visual cortex, successfully digested CS-GAG and resulted in the reactivation of ocular dominance plasticity after closer of the CP.(Pizzorusso et al., 2002a)(Pizzorusso et al., 2002b)(Willis, Pratt, & Morris, 2022).

The restoration of critical period-like plasticity upon the digestion of the main component of PNNs was also observed in other brain areas, like in the hippocampus (Carstens, Phillips, Pozzo-Miller, Weinberg, & Dudek, 2016) and amygdala (Gogolla, Caroni, Lüthi, & Herry, 2009).

A promising avenue for further investigation involves the re-opening of critical periods in adult AS mice, followed by reinstatement of *Ube3a* expression, with the aim of assessing its potential to rescue AS-like phenotypes that we have demonstrated to be already closed during that specific developmental stage.

In closing, this thesis has laid the groundwork for further exploration of AS and has provided a foundation for the development of targeted therapies. The discoveries made thus far underscore the importance of a comprehensive approach, integrating genetic and behavioural factors in our quest to unravel the complexities of AS. By working collaboratively and building upon the findings presented here, we can continue to make significant strides towards improving the lives of individuals affected by Angelman syndrome.

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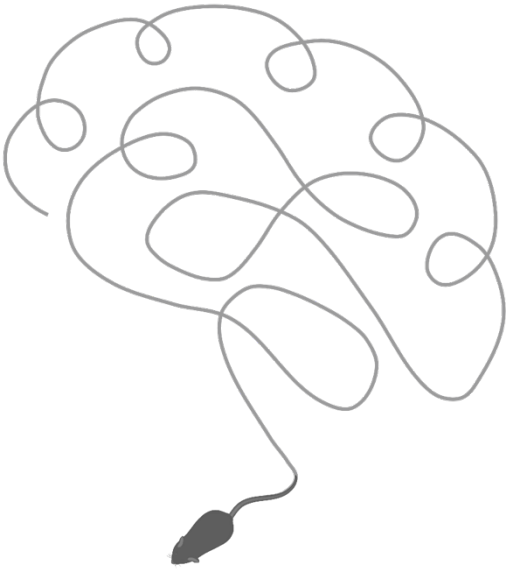

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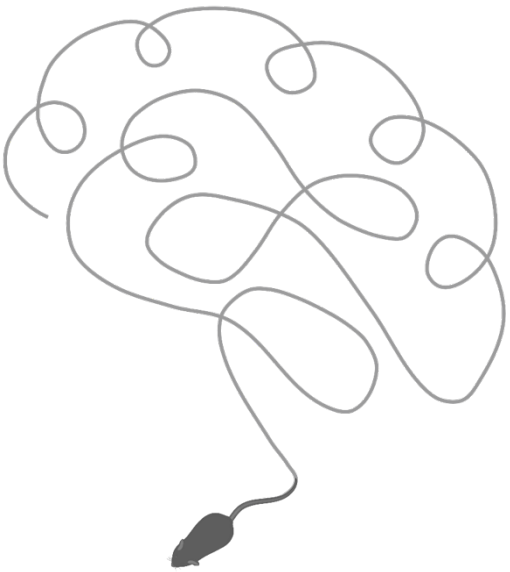

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***

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"*The reward for a job well done is the opportunity to do more.*"
- Jonas Salk

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