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REVIEW

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Angelman Syndrome: From Mouse Models to Therapy

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Abstract—The *UBE3A* gene is part of the chromosome 15q11-q13 region that is frequently deleted or duplicated, leading to several neurodevelopmental disorders (NDD). Angelman syndrome (AS) is caused by the absence of functional maternally derived UBE3A protein, while the paternal *UBE3A* gene is present but silenced specifically in neurons. Patients with AS present with severe neurodevelopmental delay, with pronounced motor deficits, absence of speech, intellectual disability, epilepsy, and sleep problems. The pathophysiology of AS is still unclear and a treatment is lacking. Animal models of AS recapitulate the genotypic and phenotypic features observed in AS patients, and have been invaluable for understanding the disease process as well as identifying apropriate drug targets. Using these AS mouse models we have learned that loss of UBE3A probably affects many areas of the brain, leading to increased neuronal excitability and a loss of synaptic spines, along with changes in a number of distinct behaviours. Inducible AS mouse models have helped to identify the critical treatment windows for the behavioral and physiological phenotypes. Additionally, AS mouse models indicate an important role for the predominantly nuclear UBE3A isoform in generating the characteristic AS pathology. Last, but not least, the AS mice have been crucial in guiding *Ube3a* gene reactivation treatments, which present a very promising therapy to treat AS.

Key words: angelman syndrome, mouse model, UBE3A, neurodevelopment, autism, critical period.

INTRODUCTION

Human neurodevelopment is a relatively long process resulting from complex interactions between genes and environment, with a crucial impact on the formation of differentiated neurons, synapses and ultimately functional neuronal networks (Silbereis et al., 2015). Dysfunction of the ubiquitin ligase gene *UBE3A* which is specifically imprinted in the brain (Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997; Hsiao et al., 2019) leads to two severe human neurodevelopmental disorders (NDDs): Angelman syndrome (AS) caused by the deletion or dysfunction of the maternal *UBE3A* allele and the 15q11.2-q13.3 duplication (Dup15q) syndrome, resulting from the duplication of the allele (Glessner et al., 2009; Urraca et al., 2013) and reviewed in (Elgersma, 2015). It remains largely unclear how UBE3A contributes to the pathophysiology of these two different NDDs, but the levels of UBE3A protein are strongly correlated with the phenotypes of these two different disorders (Elgersma, 2015). Thus loss of UBE3A protein in the brain leads to severe neurological and cognitive deficits while increased levels of UBE3A lead to autism spectrum disorders (ASD) phenotypes or increased risk for schizophrenia (Urraca et al., 2013; Elgersma, 2015). In this review, we specifically focus on AS, and review how AS mouse models have contributed to what we know about UBE3A today, and guide the development of therapies.

AS AND THE IMPORTANCE OF UBE3A FOR NEURODEVELOPMENT

AS is a severe debilitating NDD with an estimated incidence of 1 in 20,000 (Mertz et al., 2013), caused by the absence of functional maternally derived UBE3A protein. The developmental delay emerges around 6 months of age and becomes gradually more apparent after 12 months (Williams et al., 2010). The developmental milestones are not only delayed, but plateau at a developmental level of 24–30 months (Williams et al., 2006). Children with AS have strong deficits of fine and gross motor skills, absence of speech, intellectual disability and abnormal demeanour, occasionally resembling ASD (Williams et al., 2006). Additionally, 80% of the patients have

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Abbreviations: AIS, axon initial segment; AS, angelman syndrome; ASD, autism spectrum disorders; ASOs, anti-sense oligonucleotides; EEG, electroencephalographic; LTP, long term potentiation; NDD, neurodevelopmental disorders; PWS, Prader Willi syndrome; USV, ultrasonic vocalization.

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epilepsy and sleep problems (Williams et al., 2006; Bindels-de Heus et al., 2019). Currently, only symptomatic treatment is available, which is predominantly aimed at reducing seizures and improving sleep (Tan and Bird, 2015).

Affected locus in humans

The *UBE3A* gene encodes the prototype of a subfamily of C-terminal HECT (Homologous to the E6-AP Carboxy Terminus) E3 ligases (Scheffner et al., 1993). Ubiquitin ligases are essential enzymes in the ubiquitin–proteasome pathway, involved in a number of processes including protein degradation, intracellular trafficking, DNA repair and replication (Hamilton et al., 2013). The identification of UBE3A function in 1993 as a ubiquitin ligase, originated from its role in human papilloma virus mediated cervical cancer (Huibregtse et al., 1993a,b; Scheffner et al., 1993; Talis et al., 1998). It would take another 4 years before the *UBE3A* gene was linked to AS (Kishino et al., 1997; Matsuura et al., 1997; Sutcliffe et al., 1997), a syndrome first described by Harry Angelman in 1965 (Angelman, 1965).

The *UBE3A* gene is located on chromosome 15q11.2 in humans (Matsuura et al., 1997; Sutcliffe et al., 1997), which is part of the 15q11-q13 region that is frequently deleted or duplicated. Deletion of this region [del(15)(q11-q13)] results in two distinct disorders, AS or Prader Willi syndrome (PWS), that are cytologically indistinguishable from each other but give rise to distinct clinical disorders (Ledbetter et al., 1981; Kaplan, 1987; Magenis et al., 1987). The fact that some patients afflicted with PWS carry 2 copies of maternal chromosome 15 whereas a subset of individuals with AS harbour 2 paternal copies of chromosome 15 led to the recognition of the parent-of-origin effect in PWS and AS (Nicholls et al., 1989; Malcolm et al., 1991). The subsequent identification of various missense mutations and small deletions, that directly impacted the *UBE3A* gene in a number of AS patients, led to the identification of the gene responsible for AS (Kishino et al., 1997; Matsuura et al., 1997). Interestingly, during evolution the *UBE3A* gene was already present before the development of the nervous system (Rapkins et al., 2006), but its imprinting was established after the diversification of marsupials and placental mammals via multiple chromosomal rearrangements that formed the domain controlling maternal expression of *UBE3A* from non-imprinted regions (Rapkins et al., 2006). The importance of *UBE3A* imprinting coinciding with higher mammalian cognition remains unclear, but it may have been important for its role in neuronal function. Understanding how UBE3A regulates brain development may uncover the specific pathways affected by UBE3A-associated disorders (Zhang et al., 2014).

The imprinting of *UBE3A* is highly unusual for two reasons; firstly, it is specifically imprinted in most neurons of the brain (Albrecht et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997; Runte et al., 2001; Varon et al., 2004; Jones et al., 2016), and secondly it actually falls just outside the methylated region that is responsible for the parent-of–origin imprinting. As shown in Fig. 1, a number of genes around the Prader Willi imprinting center (PWS-IC) are transcribed solely from the paternal locus, which includes the large *UBE3A*-ATS (SNHG14) transcript. Initiation of *UBE3A*-ATS transcription starts at the first exon of SNRPN which overlaps with the PWS-IC, a stretch of CpG islands methylated early during female gametogenesis as a consequence of AS imprinting center (AS-IC) transcriptional activity, inhibiting SNRPN transcription (Smith et al., 2011). The paternal PWS-IC counterpart is left unmodified and allows transcription initiation forming the *UBE3A*-ATS transcript that stretches beyond SNRPN to include the SNORD116 cluster of RNAs until the IPW where transcription ceases. Specifically in mature neurons of the brain, a shift in chromatin structure occurs whereby the *UBE3A*-ATS transcript is elongated beyond the IPW to now include the SNORD115 RNA cluster (Hsiao et al., 2019) after which the *UBE3A*-ATS transcriptional complex is believed to clash with that originating from the *UBE3A* promoter, leading to the cessation of both transcripts around exon 4/5 of *UBE3A* (Meng et al., 2012, 2013) (Fig. 1). This phenomenon, referred to as the collision model, is believed to form the basis of the paternal *UBE3A* transcriptional silencing encountered in mature neurons. Experimental evidence for this model was provided by engineering a mouse model in which transcription of the *Ube3a*-ATS was forced to stop before it reached the *Ube3a* gene, which resulted in bi-allelic *Ube3a* expression (Meng et al., 2013).

THE AS ANIMAL MODEL

To identify targeted treatments and ensure the successful translation of these therapies to clinical trials, mouse models are needed that have high construct (similarity at genotypic level) and face validity (similarity at phenotypic level), as well as robust behavioural phenotypes (Katz et al., 2012; Sonzogni et al., 2018).

Construct validity

There is a high degree of genetic similarity shared between the locus affected in human individuals with AS (15q11-q13) and the syntenic region on mouse chromosome 7 (Albrecht et al., 1997) (Fig. 1) with the exception of the orientation of the region flanked on human chromosome 15 by BP2 and BP3, which in mice is inverted. Both mouse and human loci contain the same genes and undergo parent-of-origin specific imprinting, including the *Ube3a*-ATS mediated silencing of the paternal *Ube3a* allele (Nicholls and Knepper, 2001; Lalande and Calciano, 2007). For these reasons, the construct validity of mouse models for AS is potentially very high.

Mutation spectra in AS individuals. Due to the monoallelic nature of *UBE3A* transcription in neurons, any maternal mutation causing a non-functional UBE3A protein will result in a neuronal UBE3A knockout and hence in AS. Although all AS patients have a loss of functional UBE3A protein, the genetic lesions can be variable. The majority of AS individuals (≥75%) carry a large deletion within the maternally inherited chromosome 15 that encompasses the *UBE3A* gene

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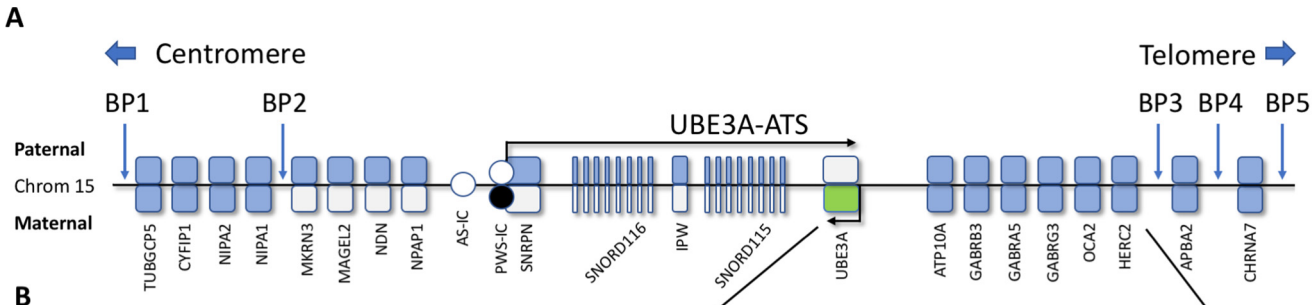
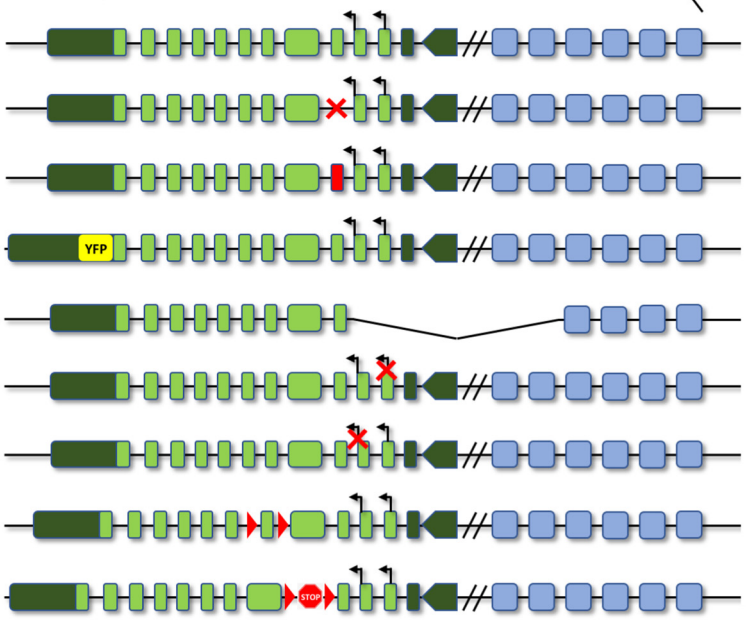


B

Mouse line	MGI ID/Lab	Description (referred to as:)
		Wild-type
Ube3a^{tm1Alb}	94337 Beaudet[1]	Deletion exon 5#
Ube3a^{tm2Yelg}	5995931 Elgersma[2]	PTC in exon 5# (PTC *Ube3a*)
Ube3a^{tm2Alb}	1919907 Beaudet[3]	C-terminal YFP (*Ube3a-YFP*)
Del(7Gabrb3-Ube3a)^{1Yhj}	4830894 Jiang[4]	Large deletion
Ube3a^{em2Yelg}	6000966 Elgersma[5]	Loss of 1st ATG (Iso2 KO)
Ube3a^{em1Yelg}	6000964 Elgersma[5]	Loss of 2nd ATG (Iso3 KO)
Ube3a$^{tm1.1Bdph}$	5994032 Philpot[6]	Floxed exon 7# (Floxed *Ube3a*)
Ube3a^{tm1Yelg}	5987009 Elgersma[7]	Floxed stop cassette in intron 5# (Lox-STOP)



Exon/intron numbering relative to mouse *Ube3a* isoform 2

Fig. 1. Mouse models of AS. A schematic representation of human chromosome 15q11.2-q13 region. **(A)** Expressed (blue and green) and silenced (grey) genes on both the maternal and paternal chromosomes are indicated. **(B)** Frequently used AS mouse-models showing the mutations applied. Note that the direction of transcription of *Ube3a* is towards the centromere. Crosses on exons indicate their deletion and crosses on arrows indicate the loss of an initiating ATG codon. Red hexagons: transcriptional stop. Red exon: contains a stop codon. Red triangles: loxP site. YFP: in frame fusion of YFP at 3′ end of *Ube3a*. BP: breakpoint. PWS-IC: Prader Willi imprinting center. AS-IC: The filled circle represents the methylated AS-IC allele. Angelman syndrome imprinting center. Cited references: 1) Jiang et al. (1998); 2) Wang et al. (2017); 3) Dindot et al. (2008); 4) Jiang et al. (2010); 5) Avagliano Trezza et al. (2019); 6) Judson et al. (2016); 7) Silva-Santos et al. (2015).

and surrounding sequences containing additional genes including GABRB3, GABRA5, GABRG3, encoding gamma-aminobutyric acid type A ($GABA_A$) receptor subunits (Gentile et al., 2010; Buiting et al., 2016) (Fig. 1A). The relatively high frequency of deletions in this area is due to the presence of 2 centromeric (BP1 and BP2) and 4 telomeric (BP3, BP4, BP4a and BP5) breakpoints flanking the 15q11-13q locus which are intrachromosomal low-copy repeats (LCR) that rearrange and result in large deletions/duplications ranging from 5 to 10 Mb in size. The majority of deletions occur between BP1 and BP3 (40% of deletions) and between BP2 and BP3 (60% of deletions) (Amos-Landgraf et al., 1999; Christian et al., 1999; Sahoo et al., 2007). The remaining mutations leading to AS include imprinting defects affecting the AS-IC (3%), paternal uniparental disomy of chromosome 15 (1–2%) and mutations specifically affecting the *UBE3A* gene (5–10%) (Buiting et al., 2016).

There are a number of AS mouse models available to researchers in the field (reviewed in (Mabb et al., 2011; Jana, 2012)) and updated in Fig. 1B). Arguably the first AS mouse model consisted of a partial paternal duplication of chromosome 7 encompassing the *Ube3a* locus, and exhibited characteristic features of AS, such as abnormal EEG (Cattanach et al., 1992, 1997). However, besides being difficult to obtain, the deletion in these mice also included genes that are unaffected in human patients (Cattanach et al., 1992). By far, the most commonly used mouse model to date in AS research, is the one generated by the Beaudet lab in 1998 (*Ube3a*^{tm1Alb}; (Jiang et al., 1998). In this mouse model, exon 5 (numbering based on the long *Ube3a* mouse *isoform 2)* was deleted resulting in an out of frame mutation (Jiang et al., 1998). This mouse model was subsequently used extensively by other labs to investigate changes in behaviour (Jiang et al., 1998; Miura et al., 2002; Allensworth et al., 2011;

Huang et al., 2013; Born et al., 2017) and neuronal function (Kaphzan et al., 2011; Egawa et al., 2012; Wallace et al., 2012) and to test treatment strategies (van Woerden et al., 2007; Egawa et al., 2012; Kaphzan et al., 2013; Sonzogni et al., 2018). The mouse, designed on the premise that loss of UBE3A is the common denominator in all AS patients, has proven to have an excellent construct validity in terms of brain specific imprinting, showing a clear parent-of-origin effect and a lack of UBE3A protein in neurons derived from maternally inherited mutant alleles (Jiang et al., 1998; Judson et al., 2014). These aspects are mirrored in additional mouse models that also directly target the *Ube3a* gene including the deletion of the C-terminal tail (Miura et al., 2002), insertion of a premature stop (PTC) (Wang et al., 2017) and two conditional AS models either with a floxed exon 7 (Judson et al., 2016) or a conditional transcriptional stop cassette in intron 5 (Floxed-STOP) (Silva-Santos et al., 2015) (Fig. 1B).

As stated earlier, only about 5–10% of individuals with AS harbour mutations in *UBE3A* itself. The largest portion of affected individuals carry a large deletion which encompasses a number of flanking genes, leading to a more severe phenotype in individuals with AS (Sahoo et al., 2007; Gentile et al., 2010; Bindels-de Heus et al., 2019). For example, new evidence suggests that contribution of non-*UBE3A* neuronal pathophysiology involving the *GABRB3-GABRA5-GABRG3* gene cluster causes abnormal theta and beta EEG oscillations that may underlie the more severe clinical phenotype in patients with AS (Frohlich et al., 2019). Loss of the $GABA_A$ receptor cluster is partially modelled by a mouse model with a 1.6 Mb deletion encompassing the *Ube3a* gene and the upstream *Atp10a* and *Gabrb3 genes* (Fig. 1B**)**, but does not include the genes flanked by BP1 and BP2 (Jiang et al., 2010). The effect of hemizygosity of the BP1/BP2 flanked genes NIPA-1, NIPA-2, CYF1P1, and GCP5 in Class 1 (between BP1 and BP2) vs Class 2 (between BP2 and BP3) deletions, however, remains unresolved with some studies claiming no difference between the two classes of deletions in terms of symptom severity (Mertz et al., 2014) and others finding a difference (Sahoo et al., 2006, 2007; Valente et al., 2013). Interestingly, a potentially useful deletion mouse model for AS did exist, encompassing all genes between BP1 and BP3 (see Fig. 1A) but unfortunately no behavioural data is available (Gabriel et al., 1999). Finally, imprinting defects have recently been modelled in a mouse with a transcriptional stop cassette inserted upstream of the PWS-IC, resulting in a AS-like imprinting defect when the stop cassette is maternally inherited (Lewis et al., 2019).

Face validity

The face validity of a mouse model is determined by how closely it recapitulates the patient clinical features. The commonly used AS mouse model developed by Beaudet's lab (*Ube3a* exon 5 deletion; Fig. 1), captures many neurological key features of the disorder (*e.g.* epilepsy, motor deficits, abnormal EEG), as well as some of the behavioural abnormalities (*e.g.* abnormal sleep patterns, increased anxiety, repetitive behaviour (Jiang et al., 1998; Miura et al., 2002; Allensworth et al., 2011; Huang et al., 2013; Born et al., 2017; Sonzogni et al., 2018). Below we briefly reviewed the face validity (or lack of it), by the different behavioral domains (Fig. 2).

Cognitive dysfunction. Cognitive function is severely affected in individuals with AS, with psychometric testing suggesting the upper developmental age is between 24 and 30-months (Andersen et al., 2001; Peters et al., 2004).

Deficits in cognitive function of AS mouse models (mostly *Ube3a*^{tm1Alb}), have been shown several times using fear conditioning and water maze learning paradigms (Jiang et al., 1998; van Woerden et al., 2007; Huang et al., 2013). But surprisingly, the learning deficits observed on these tasks tend to be mild, have not yielded consistent results across labs, and should therefore be interpreted with care (Jiang et al., 1998; Born et al., 2017) as discussed by (Sonzogni et al., 2018)). Interestingly, a recent study showed that behavioural extension is changed in the AS model mice (Sidorov et al., 2018). This specific operant behavioural paradigm is an active learning process (De Carvalho Myskiw et al., 2015) known to engage prefrontal circuits such as the infralimbic (IL) medial prefrontal cortex (mPFC) in rodents (Peters et al., 2009). The prefrontal cortex is involved in cognitive function (Miller and Cohen, 2001), and dysfunction in prefrontal circuits likely contributes to cognitive impairments in NDDs such as AS (Yuan and Raz, 2014). In future studies, this new behavioural phenotype may be a more suitable test for assessing the cognitive impairments in AS mice. More so, the behavioural tests that involve the higher-order circuits involved in cognition may become especially important in the preclinical assessment of treatment efficacy, because such circuits may have protracted critical periods for intervention (Rotaru et al., 2018).

Motor deficits

Patients with AS have severe motor dysfunction that first manifests around 6 months of age as hyperkinetic movements of the trunk and limbs, jitteriness or trembling (Fryburg et al., 1991; Williams et al., 2006). Older children have orthopaedic and movement difficulties, gait disturbance, coordination and lack of complex motor skills development. Overall, there is a severe delay in motor milestones such as sitting, which usually occurs after 12 months, and walking, which is often delayed until age 3–5 years (Zori et al., 1992; Buntinx et al., 1995). Eventually walking is either stiff or extremely shaky and jerky and about 10% of the children never achieve walking (Clayton-Smith and Laan, 2003; Beckung et al., 2004; Bindels-de Heus et al., 2019). Voluntary movements are often uncoordinated and children are unable to reach for objects or feed themselves (Clayton-Smith, 1993; Beckung et al., 2004; Grieco et al., 2018).

Several behavioural tasks have been used to assess motor dysfunction in AS mouse models, including the hind-paw footprint analysis, bar crossing ability, wire hanging, paw position in the tail suspension test (Jiang et al., 1998; Heck et al., 2008), and performance on an

Neurological functions		 Phenotype	Test
Cognitive	Severe delay	Not present/ mild	Fear conditioning Water maze
Motor	Severe	Impaired	Rotarod Open field
Language/ Vocalization	Absent	Increased USVs	Ultrasonic vocalization
Behavioral	ASD and Anxiety	Impaired	Nest building, Marble burying Forced swim test Open field
Seizures	Severe	Present	Acute audiogenic or kindling with -flurothyl
EEG	Specific EEG: Increased delta activity Persistent theta activity Spikes and sharp waves	Increased delta power	Extracellular field recordings: EEG, ECoG, LFP
Sleep disturbances	Severe	Mildly impaired	Day/Night cycle EEG

Fig. 2. Face validity of the AS mouse model. Summary of the major neurological deficits described in patients with AS (left) versus similar phenotypes observed in AS mice after specific behavioral tasks (right). The strong phenotypes observed in AS mice are made bold.

accelerating rotating rod (rotarod) (Jiang et al., 1998; Silva-Santos et al., 2015; Born et al., 2017; Sonzogni et al., 2018; Avagliano Trezza et al., 2019). The latter task is the most commonly used assay and yields a robust phenotype which is consistent across labs, and mouse models (Jiang et al., 1998; Huang et al., 2013; Silva-Santos et al., 2015; Born et al., 2017; Sonzogni et al., 2018).

Absence of speech. Most individuals with AS lack speech. Only few individuals can use several words but they are rarely able to make phrases (Trillingsgaard and Østergaard, 2004). Ultimately, communication is achieved by using gestures (Clayton-Smith, 1993), or more recently by communication devices. This distinct feature of AS is not easily recapitulated in the mouse model. Although it is common for vertebrates to use

vocalization for signalling and social communication, the complexity of such behaviours varies extensively among species (Petkov and Jarvis, 2012). Most animals communicate via innate vocalization, and only primates and a few bird species through imitation, which is the main way humans develop language skills (Konopka and Roberts, 2016). Mice are vocal non-learners but they also have rudimentary cortical-striatal circuits similar to those that control production of learned vocalizations in humans and songbirds, and these circuits activate when they vocalize (Arriaga et al., 2012). Ultrasonic vocalization (USV) was investigated in AS mice with a deletion which encompassed the *Ube3a, Atp10a* and *Gabrb3* genes (Jiang et al., 2010). Notably, USV calls were increased in these AS mice. Nevertheless, it should be mentioned that the investigators used newborn pups and their USVs may reflect an innate behavior at this age. Hence, the data may reflect a dysfunction of an innate behavior rather than provide insight into the vocal learning and speech acquisition deficits observed in individuals with AS.

Behavioural dysfunction. There is variability in adaptive behaviour in patients with AS and a substantial subset of children with AS qualify for a comorbid diagnosis of autism, independent of the severity of their cognitive and adaptive behaviour functioning (Steffenburg et al., 1996; Trillingsgaard and Østergaard, 2004; Peters et al., 2012). Children with AS appear hyperactive, with short attention span (Walz, 2007). With age, anxiety becomes significantly stronger manifesting as increased fits and self-harm, cyclic vomiting, tics, or tremors (Thibert et al., 2013; Giroud et al., 2015; Larson et al., 2015; Prasad et al., 2018). To model at least part of these behavioural dysfunctions, a series of behavioural tests are now used to test AS mice (Sonzogni et al., 2018).

Open field assays (Prut and Belzung, 2003; Seibenhener and Wooten, 2015) and the elevated plus maze tests (Pellow et al., 1985; Walf and Frye, 2007) have been extensively used to test the levels of anxiety in rodents. These tests are rapid assessments of well-defined anxiety-mediated fear or flight responses to specific stimuli (Prut and Belzung, 2003; Seibenhener and Wooten, 2015). Rodents for example, show strong aversion when placed in open, bright, and novel environments (Choleris et al., 2001). AS mice show stronger anxiety than their wild type controls when placed in the open field maze (Huang et al., 2013; Silva-Santos et al., 2015; Born et al., 2017; Sonzogni et al., 2018). Although this phenotype has been replicated across different laboratories and different AS mouse lines, this test was shown to have less statistical power compared to other tests that look at behavioural dysfunction in AS mice (Sonzogni et al., 2018).

The marble burying paradigm assesses rodent behaviour related to natural digging and burrowing behaviour (Gyertyán, 1995; Thomas et al., 2009). Digging and building burrows serves different purposes in the wild, including food storage, safety, nesting, thermoregulation (Bouchard and Lynch, 1989). Often, this task shows repetitive digging and burrowing (Sherwin et al., 2004; Thomas et al., 2009) which may have similarity to the repetitive behaviour that forms the core phenotype of ASD. AS mouse models consistently show changes in this behaviour (Meng et al., 2013; Silva-Santos et al., 2015; Born et al., 2017; Sonzogni et al., 2018). However, whereas many mouse models for ASD show increased burying behaviour, AS mice actually show reduced burying behaviour.

Nest building is another animal behaviour serving diverse functions, such as conservation of heat but also for reproduction and shelter (Hansell, 2005; Deacon, 2006; Jirkof, 2014; Baden, 2019). AS mice show a reduced capacity for nest building (Silva-Santos et al., 2015; Sonzogni et al., 2018), but the precise translational value of this observation is unclear as it is unknown which brain areas are underlying this deficit.

It is notable that impaired rotarod performance, reduced open field activity, reduced marble burying behaviour and reduced nest building behaviour could theoretically all be a direct result of impaired motor function. However, this appears not to be the case. Restoring *Ube3a* gene expression in 3-week old AS mice fully restores rotarod impairments but does not rescue any of the other tasks including reduced open field activity, reduced marble burying behaviour and reduced nest building behaviour (Silva-Santos et al., 2015).

Seizure susceptibility. Seizures, including myoclonic, atypical absence, generalized tonic–clonic, and atonic types, usually start between 1 and 3 years (Boyd et al., 1988; Rubin et al., 1997; Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009). Seizures are one of the few AS phenotypes for which there is a treatment, although efficacy of anticonvulsants is sometimes poor (Thibert et al., 2009; Shaaya et al., 2016)

Although spontaneous seizures have not been reported for AS mice, it is important to note that most research on AS models has predominantly made use of animals in a C57BL/6 background, typically a non-seizure permissive mouse strain. Moreover, the use of more seizure permissive strains in combination with chronic EEG monitoring may enable the identification of seizures that are harder to detect by observation only. But even though spontaneous seizures have not been reported, AS mice have a clear increase of seizure susceptibility, either upon audiogenic stimulation or frequent stimulation (kindling) (Jiang et al., 1998; Born et al., 2017). Audiogenic seizures are among the most powerful tests to investigate seizure susceptibility in AS mice, although this phenotype is highly background specific (Jiang et al., 1998; van Woerden et al., 2007; Silva-Santos et al., 2015; Judson et al., 2016; Born et al., 2017; Sonzogni et al., 2018). This phenotype has been demonstrated in four independently derived lines raised in the 129S2 background: the commonly used exon 5 deletion line, the Floxed-STOP line, and the recently generated PTC line (see Fig. 1). Audiogenic seizures can be prevented with anti-epileptic drugs such as valproate, clonazepam, levetiracetam (Silva-Santos et al., 2015;

Sonzogni et al., 2018). Additionally, seizure susceptibility in AS mice induced with the flurothyl $GABA_AR$ antagonist, can be ameliorated with cannabidiol (Judson et al., 2016; Gu et al., 2019a,b).

Electroencephalographic (EEG) abnormalities. Patients with AS have specific EEG abnormalities which often precede clinical features and help support the AS diagnosis in patients without a genetic confirmation (Laan et al., 1997; Valente et al., 2006). The typical EEG pattern includes one or more of the following features: large amplitude of rhythmic delta activity especially in the frontal regions, and persistent rhythmic theta activity and spikes or sharp waves, mixed with 3–4 Hz components of high amplitude, mainly in the occipital regions (Boyd et al., 1988; Laan and Vein, 2005). The delta rhythmicity is the most common EEG abnormality (Vendrame et al., 2012) and it has recently been proposed as a useful biomarker of both patient and mouse models (Sidorov et al., 2017). Moreover, in patients with 15q11-13 deletions including the GABRB3, GABRA5, GABRG3 genes encoding the different subunits of the $GABA_A$ receptor, the EEG disturbances were even stronger (Frohlich et al., 2019).

EEG patterns in mice can be obtained by recording resting-state local field potentials (LFPs) in awake head-fixed mice (Buzsáki et al., 2012). Different AS mouse models including the *Ube3a* exon 5 deletion line and the Floxed-STOP line showed EEG abnormalities that resembled the changes in delta power (Jiang et al., 1998; Judson et al., 2016; Born et al., 2017; Sidorov et al., 2017). These changes in delta power are generalized across the neocortex and has been attributed to the loss of UBE3A specifically from GABAergic interneurons in the brain (Judson et al., 2014; Sidorov et al., 2017).

Sleep disturbances. Patients with AS have sleep disturbances that include: decreased need for sleep, changes in the sleep/wake cycle, early awakening and general hyperactivity (Miura et al., 2002; Bruni et al., 2004; Didden et al., 2004; Walz, 2007; Larson et al., 2015). Moreover, EEG recordings of night time sleep suggest that children with AS have reduced levels of REM sleep and decreased sleep efficiency (Miano et al., 2004) along with increased long-range EEG coherence in the gamma band and fewer and shorter sleep spindles (den Bakker et al., 2018).

Sleep changes can be assessed in mice by investigating their activity in cages equipped with running wheels and by obtaining EEG recordings during the day/night cycle. Two studies evaluated the changes in sleep – wake pattern resulting from loss of UBE3A (Ehlen et al., 2015; Shi et al., 2015) using the *Ube3a* exon 5 deletion line (Jiang et al., 1998). Although both studies point to changes in the sleep–wake architecture in AS mice, Ehlen et al., 2015 showed that loss of UBE3A did not alter circadian rhythmicity, but disrupted sleep homeostasis, while Shi et al., 2015 showed an altered circadian period and phase, which can be further exacerbated by manipulating the environmental light/dark conditions. It is thus important to continue investigating how UBE3A affects the sleep patterns in AS mice.

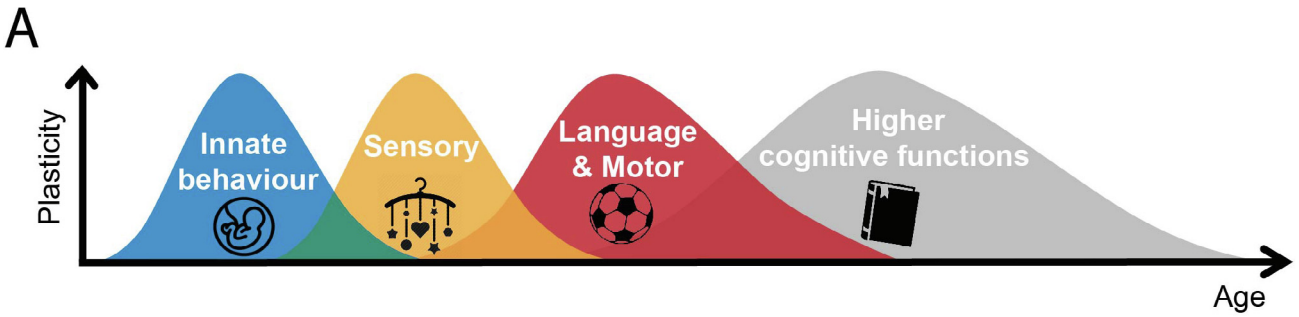
A standardized test battery to reveal AS mouse phenotypes

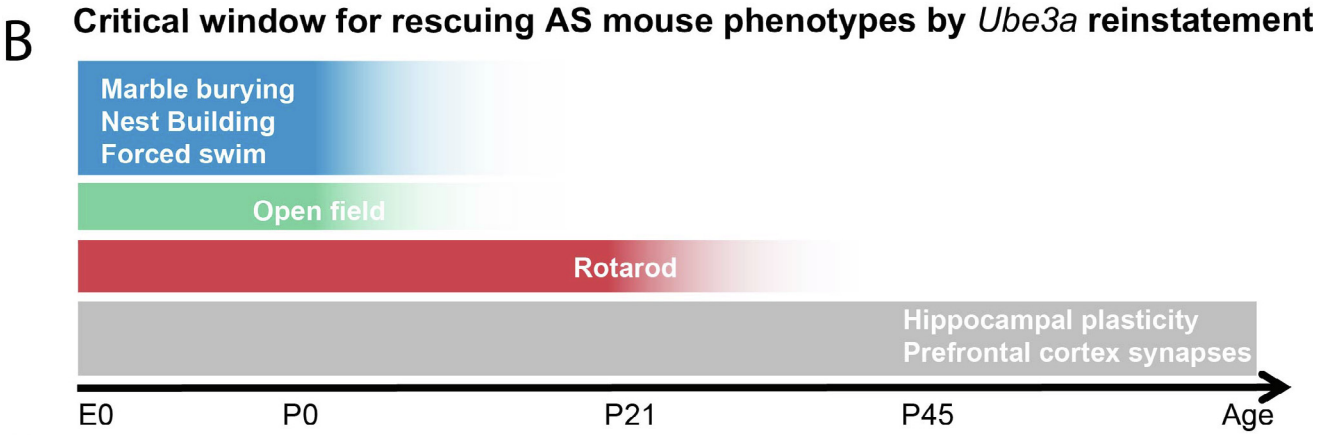
Robust behavioural phenotypes are crucial to identify novel treatments, because they offer sufficient power to detect the effect of the treatment, and minimize type I errors (false effectiveness of a drug). Recently the Elgersma lab described a standardized behavioural test battery which included the rotarod test, marble burying, nest building, open field, and forced swim test, all highly useful for preclinical drug testing in AS (Sonzogni et al., 2018). This study included a meta-analysis that integrated eight independent experiments performed by five different experimenters, using 111 AS and 120 WT littermate mice in the F1 hybrid 129S2-C57BL/6J background Fig. 3C. This standardized behavioural test battery has now been validated in six independently derived *Ube3a* lines: the exon 5 deletion line, the *PTC* line, the conditional *Floxed-STOP* line, the floxed exon 7 line, and the isoform specific *Ube3a* Iso2 KO and Iso3 KO lines (Silva-Santos et al., 2015; Sonzogni et al., 2018; Avagliano Trezza et al., 2019) (Fig. 1).

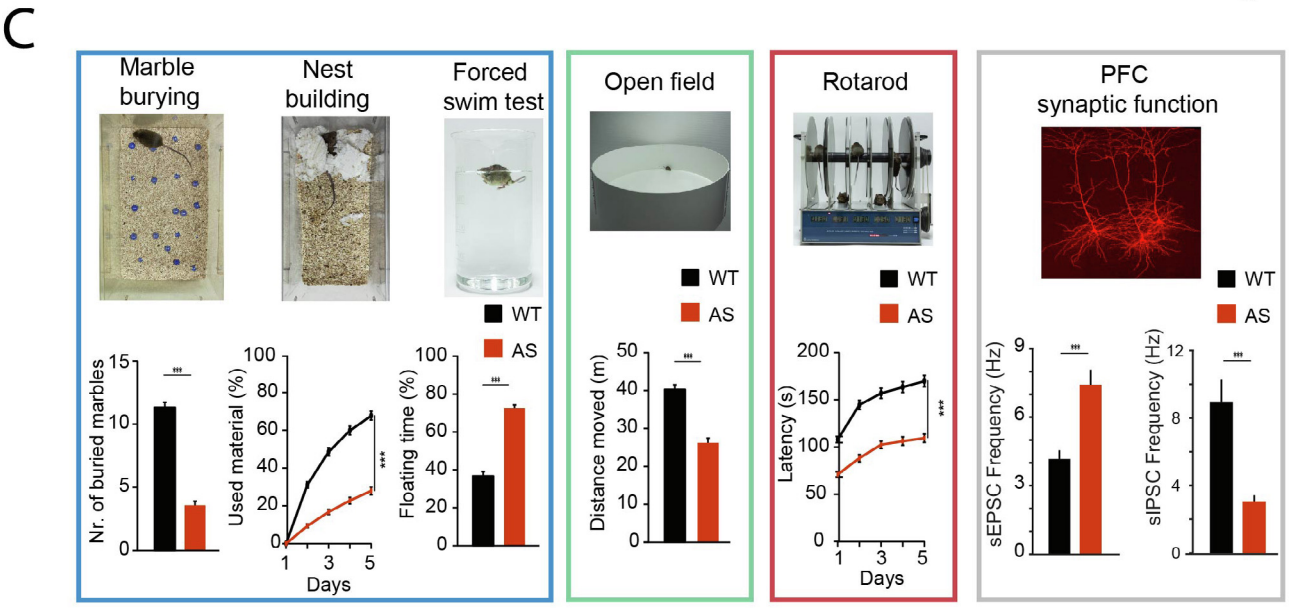
WHAT HAVE AS MOUSE MODELS TAUGHT US ABOUT UBE3A FUNCTION?

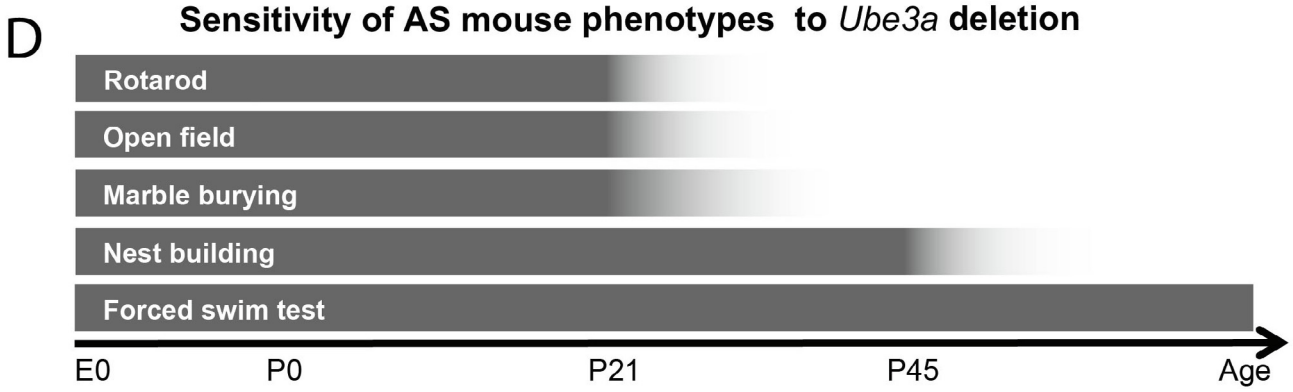
Role of UBE3A in brain development and in the mature brain (critical period)

In general, NDDs are caused by disrupted brain maturation which results in dysfunction of motor, social, cognitive and language function (Thapar et al., 2017). Brain maturation is a relatively long process resulting from complex interactions between genes and environment, with crucial impact on the formation of differentiated neurons, synapses and ultimately mature neuronal networks (West and Greenberg, 2011). Full brain maturation extends long after birth, during so called critical windows when sensory experiences contribute significantly to the fine-tuning of both local and long-range neuronal networks. It is thought that sensory input during critical windows leads to specific gene expression patterns, responsible for transforming sensory experience into long-lasting changes at the level of synapses (Zhou et al., 2008; Colonnese et al., 2010; Kuhlman et al., 2013). Notably, during critical periods, neuronal circuits are flexible and thus able to be easily shaped by sensory input (Innocenti and Price, 2005). Significant impairment often observed in NDDs, is the result of delayed or faster closing of critical periods as well as improper translation of the sensory input into long-lasting changes at synaptic level leading to the formation of dysfunctional neuronal networks (Niwa et al., 2010; Di Martino et al., 2011; Forcelli et al., 2012; Biane et al., 2015; Peng et al., 2016). Importantly, if the brain is not properly shaped during these critical periods, the ability of the neuronal circuits to be modified during adulthood, is severely reduced (Fig. 3A) (Hensch, 2004; Takesian and Hensch, 2013).


A
Plasticity
Innate behaviour
Sensory
Language & Motor
Higher cognitive functions
Age


B
Critical window for rescuing AS mouse phenotypes by Ube3a reinstatement
Marble burying
Nest Building
Forced swim
Open field
Rotarod
Hippocampal plasticity
Prefrontal cortex synapses
E0
P0
P21
P45
Age


C
Marble burying
Nest building
Forced swim test
Open field
Rotarod
PFC synaptic function
WT
AS
Nr. of buried marbles
Used material (%)
Floating time (%)
Distance moved (m)
Latency (s)
Days
sEPSC Frequency (Hz)
sIPSC Frequency (Hz)


D
Sensitivity of AS mouse phenotypes to Ube3a deletion
Rotarod
Open field
Marble burying
Nest building
Forced swim test
E0
P0
P21
P45
Age

An interesting direction is the use of inducible mouse models for AS, as a tool to investigate the role of UBE3A in brain development and adult life, and to explore the mechanisms underlying the critical periods. The conditional Floxed-STOP mouse model allows reinstatement of *Ube3a* gene expression at any desired time throughout the animal life span, and hence can provide insight into the role of UBE3A for brain development (Silva-Santos et al., 2015). The investigators defined the critical periods during which *Ube3a* gene reactivation can ameliorate AS-like phenotypes. They found that the window for improving motor coordination extends furthest into postnatal development, whereas the autism- and anxiety-related phenotypes appear to be established much earlier and cannot be reversed by adult *Ube3a* gene activation (Fig. 3B) (Silva-Santos et al., 2015). It is important to mention that it is unclear at what time point the critical period for these latter phenotypes is precisely closed. Due to the challenge of tamoxifen administration to newborn pups during the perinatal period, it is quite possible that the failure to adequately rescue the marble burying and nest-building deficits at this age was at least in part, due to relatively inadequate UBE3A reinstatement (Silva-Santos et al., 2015).

To study the role of UBE3A in the brain *after* initial brain development, the conditional floxed exon 7 mouse line has been used to delete *Ube3a* during different stages of life (Sonzogni et al., 2019). When *Ube3a* was deleted in animals at 3 weeks or 12 weeks of age, no deficits were observed in motor coordination (rotarod), exploratory behaviour and anxiety (open field), or repetitive behaviour and anxiety (marble burying) Fig. 3D. Additionally, the predisposition toward epilepsy was absent when the gene was deleted after 3 weeks of age (Sonzogni et al., 2019). The combined results obtained from these inducible (*Ube3a* gene on or off) AS mouse models, indicate that UBE3A predominantly plays an important role in the perinatal period. An important caveat however, is that none of these studies addressed the temporal requirement for restoring learning deficits. As discussed above, these phenotypes were too weak for detailed analysis. But importantly, electrophysiological correction such as hippocampal plasticity and excitability of neurons in the prefrontal cortex could be achieved at any time during and after brain development (Silva-Santos et al., 2015; Rotaru et al., 2018).

Affected brain areas

UBE3A is expressed throughout the brain in both glutamatergic and GABA-ergic neurons (Judson et al., 2014, 2016; Rotaru et al., 2018) and loss of UBE3A leads to synaptic and cellular changes in several brain areas. In the last 10 years, AS mouse models have been used to show that loss of UBE3A affects layer 2/3 neurons of the visual cortex (Wallace et al., 2012; Judson et al., 2016), layer 5 pyramidal neurons and fast spiking interneurons in prefrontal cortex (Rotaru et al., 2018), hippocampal pyramidal neurons (Jiang et al., 1998; Van Woerden et al., 2007; Kaphzan et al., 2011, 2013; Silva-Santos et al., 2015), striatal medium spiny neurons (Riday et al., 2012; Steinkellner et al., 2012; Hayrapetyan et al., 2014), dopaminergic mesoaccumbal terminals (Berrios et al., 2016), and neurons of medial nucleus of the trapezoid body (MNTB) (Wang et al., 2017). In the cerebellum, loss of UBE3A affects cerebellar Golgi cells (Egawa et al., 2012; Bruinsma et al., 2015). Despite the high expression of UBE3A in cerebellar Purkinje cells, their function appears to be largely unaffected by loss of UBE3A (Bruinsma et al., 2015).

Cellular dysfunction (electrophysiological phenotype)

The increased excitability appears to be a common feature across neuronal networks from AS mice (Kaphzan et al., 2011; Wang et al., 2017; Rotaru et al., 2018; Sidorov et al., 2018; Avagliano Trezza et al., 2019). However, the exact underlying mechanisms responsible for the hyperexcitable networks observed in AS mice are unknown, and it is notable that loss of UBE3A has a distinct impact on different types of neurons from different areas of the brain. Since a hyper-excitable circuit is often the result of an increased excitation to inhibition ratio (Isaacson and Scanziani, 2011), we will discuss below the role of UBE3A in inhibitory and excitatory synaptic transmission.

Changes in inhibitory synaptic transmission. Because GABA-ergic interneurons regulate neocortical excitability and seizure susceptibility, it is not surprising that GABA-ergic deficits have been implicated in the pathogenesis of multiple NDDs (Le Magueresse and Monyer, 2013; Wamsley and Fishell, 2017). In line with this, selective

Fig. 3. Knowledge of critical periods is important for gene reactivation-based therapy. **(A)** Critical periods have been well described for human behavior. Innate behaviors are evident at birth such as the suckling reflex. However, it is evident that these innate behaviors are not just restricted to reflexes, but that other behaviors that form the basis of our personality (including social interactions) are also formed in the period between the last trimester of pregnancy and the first year of life. Sensory experiences have a critical period of several years (e.g. ocular dominance). Motor skills can be acquired much longer, and there is no clear limit for higher cognitive learning. (Figure adapted from (Hensch, 2005). **(B)** *Ube3a* gene reinstatement experiments in AS mice showed that marble burying, nest building, forced swim-test and open field deficits cannot be fully rescued upon gene reinstatement after three weeks, indicating that the critical period closes before P21. Motor skill can be rescued up to 6 weeks, and no time limit was observed for rescuing hippocampal plasticity or prefrontal cortex function (figure adapted from (Silva-Santos et al., 2015). **(C)** Robust behavioral phenotypes in AS mice. A behavioral test battery consisting of rotarod, open field, marble burying, forced swim test and nest building has been developed and is now routinely used for screening drugs and addressing mechanistic questions. The above experiments depict wild-type and AS mice (about $n = 100$ animals each) put together from 6 independent experiments of vehicle-treated animals. A power analysis of these experiments indicates that 7–15 mice (depending on the test) are needed to have sufficient power for these tests ($\alpha = 0.05$; $\beta = 0.8$). (Figure adapted from (Sonzogni et al., 2018). **(D)** *Ube3a* gene deletion experiments in AS mice showed that UBE3A is not necessary after P21 for normal marble burying, nest building, open field and rotarod behavior, while the forced swim-test remains sensitive to *Ube3a* gene deletion throughout the entire life.

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deletion of *UBE3A* from GABA-ergic neurons causes AS-like EEG abnormalities and enhances seizure susceptibility (Judson et al., 2016).

Several studies point to a decreased inhibitory transmission in AS mice, which may be the result of different mechanisms. For example, Rotaru et al. (2018) showed a decrease of inhibition in layer 5 of prefrontal cortex (PFC) in AS mice possibly resulting from a decreased activity of fast spiking interneurons, while other studies show decreased inhibition in layer 2/3 pyramidal neurons from visual cortex (Wallace et al., 2012), possibly resulting from an increase of clathrin-coated vesicles in presynaptic boutons. These findings may reflect differences between visual cortex and PFC neurons as well as layer specific differences. Alternatively, the differences may result from using different animal models, as the decrease in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in the *Ube3a* exon 7 deletion mice (Wallace et al., 2012), were not observed when using the Floxed-STOP mouse model (Judson et al., 2016). This effect could possibly be due to minor transcriptional read-through in Floxed-STOP mouse model, resulting in slightly "leaky" *Ube3a* expression. If this is the case, it could indicate that small amounts of UBE3A are sufficient to prevent this deficit.

Interestingly loss of UBE3A leads to decreased excitability of fast spiking interneurons in layer 5 PFC (Rotaru et al., 2018) and resembles the profile of immature fast spiking neurons from PFC (Miyamae et al., 2017), suggesting that these cells may fail to properly mature (Stanurova et al., 2016). Although the precise mechanism has to be demonstrated, Rotaru et al. (2018) proposed a decrease via Nav1.1 subtype sodium channels may lead to lower excitability of fast spiking interneurons in AS mice (Yu et al., 2006; Ogiwara et al., 2007; von Schoubye et al., 2018).

Yet, another mechanistically different change in inhibitory transmission in AS involves the GABA transporter-1 (GAT1) which strongly regulates the level of tonic inhibition (Farrant and Nusser, 2005; Bragina et al., 2008), has been proposed as a potential UBE3A substrate (Egawa et al., 2012). Dysfunction of this transporter has been implied to cause changes in tonic inhibition of cerebellar Golgi cells and in GABA co-release from dopaminergic mesoaccumbal terminals (Egawa et al., 2012; Berrios et al., 2016).

Changes in excitatory synaptic transmission. The changes in excitatory transmission in AS mouse models point to a more complex picture. Layer 5 pyramidal neurons in PFC show an action-potential dependent increase in the excitatory transmission (Rotaru et al., 2018). In line with this, increased excitability of excitatory neurons was observed in both PFC (Sidorov et al., 2018) as well as hippocampus (Kaphzan et al., 2011, 2013). Additionally, excitatory neurons in the hippocampus are more excitable due to a decrease in spike threshold (Kaphzan et al., 2011, 2013). Moreover, *in vivo* recordings from visual cortex pyramidal neurons showed increased firing rates of these cells (Wallace et al., 2017). The increased excitability of pyramidal neurons from layer 5 prefrontal cortex may be the result of changes in Kv1 channels. Kv1 channels are highly expressed at the axon initial segment (AIS) (Inda et al., 2006; Kole et al., 2007; Van Wart et al., 2007), a subregion of the axon shown to be enlarged in AS mouse models and potentially responsible for increased excitability of neurons in the hippocampus (Kaphzan et al., 2011, 2013) and the medial nucleus of the trapezoid body (MNTB) (Wang et al., 2017).

In addition to the changes observed in the electrophysiological properties of excitatory neurons, many studies point to a decreased spine density in the visual cortex, cerebellum, and hippocampus of the deletion exon 5 mouse model (Dindot et al., 2008; Yashiro et al., 2009; Sato and Stryker, 2010; Kim et al., 2016; Sun et al., 2016). A decreased spine density would tend to result in a decreased excitatory transmission, which may be the results of homeostatic compensatory mechanism triggered by the increased AP firing rates (Keck et al., 2017). Hence, it remains a question which are the primary versus the compensatory electrophysiological changes in AS. New experiments need to focus on identifying the time points during development when each deficit appears, followed by deficit specific treatment. Such approaches will disentangle the primary and secondary (homeostatic) deficits.

UBE3A targets that could be responsible for the neuronal deficits

The UBE3A protein functions as an E3 ubiquitin ligase by attaching ubiquitin moieties to its protein targets, labelling them for destruction, altered localisation or impacting their function. Although many targets have been put forward, there are a couple of targets of specific interest due to their critical role in neuronal function. For instance, direct interactions have been reported between UBE3A the SK2 channel (a regulator of excitability; Sun et al., 2015), the GABA (GAT1) transporter (a regulator of inhibitory transmission; Egawa et al., 2012), the RhoA guanine nucleotide exchange factor Ephexin5 (responsible for regulating excitatory synapse formation; Margolis et al., 2010) and ARC (a regulator of excitatory transmission; Greer et al., 2010; Smith et al., 2011, Pastuzyn and Shepherd, 2017). However, the finding that ARC is a substrate has more recently been retracted (Mandel-Brehm et al., 2015), and it has been suggested that ARC levels may be regulated at the transcriptional level instead (Kuhnle et al., 2013). Loss of UBE3A also (indirectly) affects CAMK2 activity, an important regulator of neuronal plasticity (Weeber et al., 2003; Van Woerden et al., 2007).

For many of these possible targets is it is unclear whether they are *bona fide* UBE3A substrates. This can only be assessed with well-controlled ubiquitination assays. Since loss of UBE3A has a strong impact on the neurons, it often remains unclear whether changes in protein levels of a presumed target, are truly the result of decreased ubiquitination by UBE3A. It is also noteworthy that UBE3A interacts with the proteasome and may regulate proteostasis in a much broader way, thereby affecting many critical proteins without actually being targets (Martínez-Noël et al., 2012; Lee et al.,

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2014; Tomaić and Banks, 2015; Avagliano Trezza et al., 2019). Hence, proteins with a high turn-over such as for instance proteins of the β-catenin/ Wnt signalling pathway, an important pathway in neurodevelopment, may be affected indirectly by changes in UBE3A activity (Yi et al., 2017; Kühnle et al., 2018; Lopez et al., 2019).

The role of UBE3A isoforms

Searches for protein interactors of UBE3A identified protein interactors/targets of UBE3A that are located in nucleus, cytoplasm or synapse (reviewed in (Lopez et al., 2019)). In the mouse brain, two UBE3A protein isoforms are expressed as a result of alternative splicing; the cytoplasmic (long) isoform (Iso2) and the nuclear (short) isoform (Iso3) which differ only by a 21 amino acid stretch at the amino terminus of UBE3A (Miao et al., 2013; Avagliano Trezza et al., 2019). Although it has been reported that the non-coding (truncated) *Ube3a* transcript (Iso1) plays a role in dendrite growth (Valluy et al., 2015) the relevance for AS was questioned by a recent study (Avagliano Trezza et al., 2019), and the transcript has recently been retracted from the NCBI database.

There is broad consensus on the predominantly nuclear localization of UBE3A in murine derived neurons (Dindot et al., 2008; Burette et al., 2017). Quantification of UBE3A bands on Western blots has determined the ratio of the mouse cytoplasmic UBE3A Iso2 to the mouse nuclear Iso3 protein to be 20%:80% hinting at a potentially strong nuclear role for UBE3A (Avagliano Trezza et al., 2019). This notion was strengthened by data from the same study in which behavioural experiments using isoform-specific *Ube3a* mouse models clearly showed that mice lacking the nuclear isoform were showing the AS mouse phenotypes, while those lacking the cytoplasmic isoform were indistinguishable from WT littermates. UBE3A also displays a predominantly nuclear localization in human neurons as well (Burette et al., 2017; Avagliano Trezza et al., 2019). Unlike its murine counterpart, human UBE3A consists of 3 protein isoforms differing only at their N-terminal end (Yamamoto et al., 1997). Interestingly a mutation that specifically abrogates the expression of the predominantly nuclear (short) human UBE3A isoform 1 (homologous to mouse Iso3) has recently been identified in AS patients (Sadhwani et al., 2018). Together with the finding that some AS associated missense mutations in *UBE3A* interfere with nuclear targeting (Avagliano Trezza et al., 2019), these findings further strengthen the point that UBE3A acts predominantly in the nucleus. However, it is important to note that despite the fact that most UBE3A protein is found in the nucleus, both mouse and human cells also express cytoplasmic forms of UBE3A, indicating that there likely is a cytoplasmic function for UBE3A as well.

Although the findings described above seem to point in the direction of a primarily nuclear role for UBE3A, the role of UBE3A in the nucleus is unknown. UBE3A has been shown to bind to and act as a coactivator to a number of steroid hormone receptors affecting the transcriptional activity of the nuclear receptor targets, although this has been found to be independent of ubiquitin ligase activity (Nawaz et al., 1999), and the relevance to AS is unclear.

Using the AS mouse model to identify a therapy for AS

Currently, only symptomatic treatments are available for AS, which aim at reducing seizures, improving sleep or improve behavioural aspects (Tan and Bird, 2015). Preclinical studies using AS mouse models are not only crucial in the identification of drug targets but also provide important information about the therapeutic dose, optimal age of treatment, and the best outcome measures to be used in a clinical trial. In particular, the motor deficits, seizure susceptibility, sleep deficits EEG anomalies, and some of the behavioural deficits provide good translational value.

Treatment strategies tested in AS mouse models are often aimed at targeting the identified pathophysiological mechanisms (van Woerden et al., 2007; Egawa et al., 2012; Kaphzan et al., 2013; Sun et al., 2015). More recently, a very promising therapy for AS has been described, which involves restoring UBE3A levels. This was achieved by reactivating the intact, but silenced paternal copy of the *Ube3a* gene (Huang et al., 2012; Meng et al., 2013). But regardless of the therapeutic choice, it is essential to determine the critical time window during which appropriate intervention can overcome the neurodevelopmental deficits associated with AS (Silva-Santos et al., 2015).

Pathophysiological and molecular mechanisms as targets for treatment. The observed decrease in hippocampal long term potentiation (LTP) in AS mice (Jiang et al., 1998) prompted further research into the role of calcium/calmodulin-dependent protein kinase II (CaMK2) (Weeber et al., 2003). Thus, one of the first molecular dysfunctions identified in AS mice was the decreased CAMK2 activity via an increased inhibitory phosphorylation at the CAMK2 Thr305 and Thr306 sites (Weeber et al., 2003). Genetically reducing CAMK2 inhibitory phosphorylation was sufficient to rescue both the LTP deficits and the (motor) learning deficits and seizures (van Woerden et al., 2007), but unfortunately, drugs that can mimic this effect have not yet been identified.

Analysis of the electrophysiological properties of hippocampal neurons in AS mice revealed that CA1 pyramidal neurons are more excitable due to altered passive and active intrinsic membrane properties (Kaphzan et al., 2011). These changes were correlated with the increased expression of the α1 subunit of Na/K ATPase (α1-NaKA), as well as increased AIS length (Kaphzan et al., 2011). When α1-NaKA was reduced in AS mice the increased excitability and AIS length were rescued along with a rescue of the impaired LTP and hippocampus-dependent memory deficits (Kaphzan et al., 2013).

Another electrophysiological change observed in hippocampal neurons of AS mice is an increased in the levels of small-conductance calcium-activated potassium channels (SK channels) (Sun et al., 2015). When the currents through these channel were blocked by treatment

with apamine, the deficits in LTP were rescued in AS mice (Sun et al., 2015).

Recently, two unsuccessful clinical trials aiming to treat the motor deficits (Levodopa trial register NCT01281475) and cognitive impairments (Minocycline trial register NCT01531582 and NCT02056665), have been performed. The Levodopa trail is based on the observation that levodopa treatment results in reduced inhibitory phosphorylation of CAMK2 Thr305/6 (Brown et al., 2005), a site shown to be hyper-phosphorylated in AS mice (Weeber et al., 2003; van Woerden et al., 2007). Moreover, it has been suggested that decreased CAMK2 activity leads to decreased striatal dopamine transporter function in AS mice (Steinkellner et al., 2012), a brain area also involved in motor function. Indeed, Levodopa (Tan and Bird, 2015) and Minocycline (Grieco et al., 2014) improved motor function in AS mice. However, more recently these drugs were retested in a study that was better powered and included a number of additional behavioural tests. No significant effects of Levodopa or Minocycline on any of the tests were observed (Sonzogni et al., 2018). These findings emphasize the need to rigorously test potential treatments in AS mouse models using robust assays and sufficiently powered cohorts, before the drugs can be tested in the clinic.

The pronounced motor dysfunction associated with the loss of UBE3A, has been an important driver to investigate cerebellar function, a brain area important for normal motor function (Middleton, 2000). Two studies showed decreased tonic inhibition on cerebellar granule neurons (Egawa et al., 2012; Bruinsma et al., 2015). Pharmacological treatment of AS mice with an extrasynaptic GABAA receptor–selective agonist, THIP/Gaboxadol, which has been previously shown to increase the levels of tonic inhibition (Brown et al., 2002), resulted in a partial rescue of the motor dysfunction (Egawa et al., 2012). Possibly, this partial rescue is due to the finding that gross motor dysfunction of AS mice does not seem to originate from the cerebellum (Egawa et al., 2012; Bruinsma et al., 2015). Gaboxadol is currently being tested in individuals with AS (NCT0299630, NCT04106557).

Gene reactivation. Since the absence of functional UBE3A is the common denominator in all AS cases, reinstating UBE3A protein expression in AS neurons is an attractive treatment strategy to pursue.

One approach to bring back UBE3A protein is through ectopically expressed UBE3A. This has been achieved by the introduction of the dominant short form of *Ube3a* cDNA (mouse Iso 3) through AAV viral mediated gene therapy in AS mice. Although the mouse experiments were statistically underpowered, the authors described a partial rescue of synaptic plasticity (LTP) and water maze phenotypes, and a full rescue of the fear conditioning deficit (Daily et al., 2011). No rescue was observed on the rotarod motor coordination task, which is likely caused by the fact that the AAV injections were limited to hippocampus. Although potentially very promising, the AAV viral approach does bring along some obstacles. The level of UBE3A protein is tightly regulated in brain and too much UBE3A may result in an ASD phenotype (Elgersma, 2015) although little is known about the effect of increased post-natal levels of UBE3A protein. The viral approach, besides it being irreversible, lacks the ability to control the number of viral particles entering each cell, and as a consequence, there is little control over protein levels in individual cells. Also, there will be the need to take into consideration that in human neurons, three UBE3A protein isoforms are expressed and their individual impact on the normal functioning of neurons has not yet been elucidated.

As mentioned earlier, the paternal *UBE3A* allele is silenced by the *UBE3A*-ATS RNA transcript and the reactivation of the silent paternal *UBE3A* gene can be achieved by targeting the *UBE3A*-ATS transcript. A major advantage of targeting the *UBE3A*-ATS transcript to reactivate the dormant paternal *UBE3A* allele, although necessitating repetitive life-long treatment, is that it will not lead to an excessive amount of UBE3A in neurons. Two research groups initiated this line of research and have used mouse models to determine the efficacy of Topoisomerase I inhibitors (Huang et al., 2012; Lee et al., 2018) and anti-sense oligonucleotides (ASOs) to reactivate the paternal *Ube3a* allele (Meng et al., 2015). Topoisomerase I inhibitors were identified through a high throughput small molecule screen carried out using primary neurons derived from mice expressing paternal *Ube3a*-YFP (Huang et al., 2012). Huang and colleagues were able to show that treating neurons with Topoisomerase I inhibitors such as Topotecan and Indotecan resulted in the reduction of *Ube3a-ATS* transcription and a concomitant increase of paternally expressed UBE3A protein (Huang et al., 2012; Lee et al., 2018).

In parallel, the Beaudet lab in collaboration with Ionis Pharmaceuticals, Inc., showed that ASOs can target the *Ube3a-ATS* and activate paternal *Ube3a* transcription (Meng et al., 2015). ASOs are chemically modified oligonucleotides; a chimeric molecule consisting of a central stretch of DNA flanked by ribonucleotides that are able to hybridise with and breakdown a nuclear target RNA in an RNAse H dependant manner (Crooke et al., 2018). By administering ASOs via a single intracerebroventricular (ICV) injection into adult mice, Meng and colleagues targeted the neuronal *Ube3a-ATS* RNA and reactivated the paternal *Ube3a* allele for up to 4 months, rescuing the contextual fear conditioning phenotype in their cohort of adult AS mice (Meng et al., 2015). However, as discussed above, this phenotype is not very robust in AS mice. Moreover, the investigators were not able to rescue other AS related behavioural phenotypes such as the open field, marble burying and accelerating rotarod tests. The experiments with the inducible Flox-STOP mouse model, in which the *Ube3a* gene was switched on at specific times in development, suggests that the failure to observe a behavioural rescue by ASO induced *UBE3A* re-expression, is likely due to the fact that the treated mice were adults (Silva-Santos et al., 2015). This last finding emphasises the importance of animal models in biomedical research, as the critical window for treatment of AS could not have been probed using cell lines.

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Alternatives to the viral, Topoisomerase I inhibitor or ASO-mediated reinstatement of UBE3A have been proposed in literature, such as the zinc-finger, TALEN or CRISPR based artificial transcriptional modulators, but feasibility of such approaches have yet to be demonstrated (Bailus et al., 2016).

AS mouse models: future directions

There are a few major areas in which mouse models may shed further light on AS pathophysiology:

1) Loss of UBE3A affects a multitude of pathways (reviewed in Lopez et al 2019B). The role of the cytoplasmic and in particular nuclear UBE3A in the pathophysiology of AS is still unclear will be a focus for more research. In addition, the role of UBE3A in regulating proteasome activity should be investigated.
2) An important limitation of the *Ube3a* re-activation approach, is the limited therapeutic window in which such a therapy appears to be effective. What dictates this limited critical period for behavioural rescue? Can it be extended by pharmacological means? And why can epilepsy in adult mice efficiently be treated with anti-epileptic drugs but is *Ube3a* gene reinstatement not effective in adult animals? (Silva-Santos et al., 2015). Further studies using the inducible AS mouse model should provide insight into these mechanisms.
3) Although UBE3A is associated with several phenotypes, we do not have a good understanding of which brain areas underlie these deficits. *Ube3a* conditional mice can help us to identify the underlying brain areas. This will also help us identify the cellular (electrophysiological) correlates for these behavioural deficits.
4) Although it is clear that complete loss of (maternal) *UBE3A* expression results in AS, how much UBE3A protein is actually needed to sustain normal development and brain function. And to what extent is bi-allelic *UBE3A* expression during pre-natal development needed? And what are the roles of each of the 3 isoforms in this process? These are important questions for gene reinstatement strategies.
5) The inclusion of large-deletion AS mouse models, such as those described by Gabriel et al. (1999) and Jiang et al. (2010), to help determine the role of all genes that are part of the large deletion in the majority of AS patients.

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