UBE3A reinstatement as a disease-modifying therapy for Angelman syndrome

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ANGELMAN SYNDROME: FROM THREE INDIVIDUALS TO A WELL-CHARACTERIZED SYNDROME

In 1965, the English paediatrician Harry Angelman reported three patients with severe neurodevelopmental delay and overlapping features, now well known as Angelman syndrome.1 The estimated birth incidence of Angelman syndrome is approximately 1 in 20 000 and it is characterized by intellectual disability, impaired motor coordination, seizures, characteristic EEG abnormalities, sleep impairments, increased anxiety, lack of speech, and high comorbidity with autism spectrum disorder.2 The first clinical manifestations usually present during the first year of life, when parents notice the lack of psychomotor activity and seizures.3,5 Currently, only symptomatic treatments are available for Angelman syndrome, which aim to reduce seizures, improve sleep, or improve behavioural aspects.6

Angelman syndrome is caused by the loss of UBE3A protein.7 The UBE3A gene lies in the imprinted Angelman syndrome/Prader–Willi syndrome 15q11.2-q13 locus, such that in neurons only the maternally inherited UBE3A gene is expressed. Four different genetic causes can lead to loss of functional UBE3A protein in neurons.8 The most common cause (approximately 70%) represents a de novo deletion of maternal chromosomal region 15q11.2-q13 encompassing the UBE3A gene. The second frequent cause is a de novo or inherited mutation in the maternal UBE3A gene itself, leading to a loss of functional UBE3A protein. A less frequent cause is paternal uniparental disomy of chromosome 15, which results in decreased UBE3A protein levels owing to two imprinted chromosomes. Similarly, imprinting defects, due to mutations affecting the Prader–Willi syndrome

MOUSE MODELS PROVIDE INSIGHT INTO UBE3A FUNCTION

The identification of the UBE3A gene as the causal gene for Angelman syndrome7 allowed for the
generation of mouse models of Angelman syndrome to study its function.14 The *UBE3A* gene encodes the E3 ubiquitin ligase *UBE3A* (previously identified as E6-associated protein [E6-AP]15) which is an enzyme that is responsible for linking ubiquitin molecules to their target proteins. This can either result in the degradation of these target proteins (poly-ubiquitination) or change the localization or function of target proteins (mono-ubiquitination).

In humans, the *UBE3A* gene encodes for three isoforms that are generated by alternative splicing, such that they all display unique amino (N) termini.16,17 The two most abundant human *UBE3A* isoforms, 1 and 3, make up more than 95% of human *UBE3A* protein and are conserved in nearly all placental animals.17 The human *UBE3A* isoform 2 evolved during early primate evolution and is not found in other mammals.17 Although the significance and function of the different *UBE3A* isoforms has yet to be revealed, it is clear that the unique N terminus of the isoforms dictates the localization of *UBE3A*.17,18 The predominant expression of the nuclear isoforms in both mice and humans explains the highly enriched *UBE3A* staining observed in these neurons.16–20 Notably, the mouse *UBE3A* isoform 2 (homologous to human *UBE3A* isoform 3) that is cytosolic in mice (and given the sequence conservation, probably in most other mammals as well) acquired a mutation during late primate evolution. Hence, in humans and Old World monkeys, this isoform is primarily targeted to the nucleus.17 The finding that most *UBE3A* protein is nuclear could indicate that the critical targets of *UBE3A* might be nuclear as well. In support of that, loss of the cytosolic *UBE3A* isoform does not result in a discernible phenotype in mice. In contrast, loss of the major nuclear isoform results in typical Angelman syndrome phenotypes in mice and humans, although in humans this is milder than in typical cases of Angelman syndrome.12,18 Although several nuclear targets have been identified21 and *UBE3A* function has directly been linked to transcription regulation,22–25 it is not clear which nuclear targets are really critical for Angelman syndrome pathophysiology. This greatly hinders the search for *UBE3A*-target-based treatments.

Apart from the nuclear targets that have been identified, several cytosolic targets have been reported. However, similar to the nuclear targets, it is unclear what their relevance is to Angelman syndrome pathophysiology.14,21,26 It should be further noted that direct ubiquitination by *UBE3A* has not been demonstrated for most of the presumed *UBE3A* targets. Instead, increased levels of target protein in *Ube3a* mice are often used as an indication that the breakdown of a protein is *UBE3A* dependent. But if nuclear *UBE3A* plays a direct or indirect role in gene expression, increased protein levels could also be the result of transcriptional changes. This cautionary note is well illustrated by the observation that the most cited target of *UBE3A*, the synaptic protein ARC,27 is no longer considered to be a direct substrate of *UBE3A*.28,29 Instead, ARC is a target of the ubiquitin ligase TRIAD3A/RNF21630 and *UBE3A* probably regulates ARC at a transcriptional level.29

Several research laboratories have used mouse models of Angelman syndrome (or sometimes induced pluripotent stem cells) to identify novel therapeutics to treat Angelman syndrome. Such therapeutics can be classified into two major categories: (1) targeted treatments aimed at correcting pathophysiological deficits associated with Angelman syndrome.14,15 The *UBE3A* gene encodes the E3 ubiquitin ligase *UBE3A* (previously identified as E6-associated protein [E6-AP]) which is an enzyme that is responsible for linking ubiquitin molecules to their target proteins. This can either result in the degradation of these target proteins (poly-ubiquitination) or change the localization or function of target proteins (mono-ubiquitination).

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syndrome; and (2) treatments aimed at restoring the loss of UBE3A expression in the brain.

**TARGETED TREATMENTS FOR PATHOPHYSIOLOGICAL DEFICITS ASSOCIATED WITH ANGELMAN SYNDROME**

It has been consistently shown that mouse models of Angelman syndrome show marked changes in synaptic plasticity and excitatory/inhibitory balance. Therefore, correcting these changes could provide a great target for treatment. However, over recent years, the view has emerged that these neuronal changes cannot be attributed to just a single mechanism. Several mechanisms have been identified that could underly the changes in neuronal excitability: for instance, changes in the alpha1 subunit of Na/K ATPase, in small-conductance calcium-activated potassium channels (SK channels) in calcium and voltage-dependent big potassium (BK) channels and in GABA (γ-aminobutyric acid) transporter (GAT1) levels. Moreover, multiple changes have been observed in signalling cascades that are indirectly critical to neuronal function, such as CAMK2, Erbin-1-ErbB4, neuregulin-ErbB4, TrkB-PSD-95, ERK, mTORC, and dopamine signalling pathways. These widespread changes are consistent with the suggestion that UBE3A probably plays an important global regulatory role, such as transcriptional regulation and/or by regulating (nuclear) proteasome activity to which it is strongly attached.

In the face of these widespread changes, it is rather surprising that by specifically targeting a single mechanism, an electrophysiological or even a behavioural rescue could be observed in most of the aforementioned studies. It is unclear how these highly specific treatments, which reversed the behavioural phenotypes in mouse models of Angelman syndrome, can be reconciled with the notion that so many mechanisms are affected. We believe that the observed rescue may in part be due to using underpowered studies in combination with behavioural readouts that are only weakly affected in mice with Angelman syndrome. The development of a standardized behavioural test battery of robust phenotypes, and the concomitant power analysis for each test, may allow better drug selection to then be moved to clinical trials. For example, minocycline treatment was successful in a low-powered study that focused on a limited number of readouts. However, when tested in the standardized behavioural test battery with a sufficiently high number of mice with Angelman syndrome, there was no improvement with minocycline on any of the readouts, nor in clinical trials of patients with Angelman syndrome. Similarly, levodopa treatment did not show an improvement in the Angelman syndrome mouse behavioural test battery nor in a clinical trial.

Given that so many molecular mechanisms seem to be affected, a treatment that acts at a more global level may ultimately be more successful in a clinical trial. Several such treatments were successful in Ube3a mice. For some of these treatments, the precise mechanism targeted in Angelman syndrome is unclear, and it is conceivable that they act more generally as cognitive enhancers (e.g., treatment with reelin, ampakines, a neurogenesis stimulator, or IGF-2 treatment). Other drugs target the excitatory/inhibitory imbalance by the GABA modulators, such as ganaxolone and gabaxadol. The latter drug was able to partly correct motor impairments and anxiety phenotypes in mice with Angelman syndrome. But recent data of a randomized, double-blind, placebo-controlled, phase 3 study (Neptune trial, NCT04106557), which enrolled 97 patients treated with gabaxadol (OV101) or placebo, failed to show a change in the primary endpoint (overall score on the Clinical Global Impression-Improvement-Angelman syndrome scale). In addition, secondary outcome measures were not significantly changed. These disappointing results indicate that even identifying more broadly acting treatments is still very difficult.

As a cautionary note of the translational failures, it should be pointed out that all drug testing in mice to date has been performed on mouse models of Angelman syndrome that specifically lack Ube3a, whereas most trials have predominantly included patients with 15q11-13Del Angelman syndrome lacking the entire (maternally inherited) 15q11-13 gene cluster. The deletion of the additional genes in this locus may make it more difficult to obtain a clinical effect of the tested therapies. Hence, some of the translational failures might be caused by using an inappropriate mouse model. Developing additional mouse models of Angelman syndrome that lack the equivalent genes of the 15q11-13 gene cluster, or using 15q11-13Del Angelman syndrome patient-derived induced pluripotent stem cells, might circumvent such issues.

**TREATMENTS AIMED AT RESTORING UBE3A EXPRESSION IN THE BRAIN**

Undoubtedly, the most promising therapeutic approach for treating Angelman syndrome aims at restoring UBE3A expression. Such a therapy would potentially be disease-modifying and get as close to a cure as possible. There are two ways to achieve this: either by gene therapy that introduces UBE3A protein into the brain through viral vectors, or through activation of the imprinted (silenced) paternal UBE3A allele.

Viral-mediated delivery of UBE3A in the brain was shown to be partly effective in mice with Angelman syndrome. However, a major translational challenge will be to create safe viral vectors with a good biodistribution that allow UBE3A expression throughout the human brain. Moreover, getting the correct amount of UBE3A per cell is very important, as too much could potentially be detrimental and may result in autism. Last but not least, such a viral vector would preferably express both dominant UBE3A isoforms (or possibly all three isoforms), to ensure that all UBE3A functions are restored.
To circumvent these issues, a very elegant approach to re-express neuronal UBE3A is by activating the dormant UBE3A gene on the paternally inherited chromosome (commonly referred to as ‘unsilencing’). In both humans and mice, the paternally inherited UBE3A allele is silenced owing to the paternal expression of the long UBE3A-ATS transcript (>460 kilobases in humans, approximately 1000 kilobases in mice) that runs antisense to the UBE3A locus.58 Elegant experiments in mice have demonstrated that this interferes with the expression of the paternally inherited Ube3a allele59,60 (Fig. 2). Interfering with the synthesis of the UBE3A-ATS by topoisomerase inhibitors61 or by Cas9-mediated targeting62 induces paternal UBE3A gene expression, without the risk of inducing too much UBE3A expression.

Alternatively, the UBE3A-ATS can be targeted by antisense oligonucleotide (ASO) treatment. With this approach, binding of the ASO to the UBE3A-ATS leads to RNase-H-mediated cleavage of the ASO-RNA heteroduplex, thereby unsilencing the paternal UBE3A gene63 (Fig. 2). ASO treatment would solve most of the limitations associated with viral injection, especially with respect to controlling the level and the type (isoform) of UBE3A expression as well as its biodistribution. ASOs are readily absorbed by neurons, and given the great success of ASO treatment for spinal muscular atrophy (a motor neuron disorder), such a therapy holds great promise for Angelman syndrome and many other (genetic) neurodevelopmental disorders.

Despite its great therapeutic promise, ASO treatment of mice with Angelman syndrome resulted in a rather disappointing lack of improvement of behavioural phenotypes, despite significant upregulation of UBE3A protein levels in most areas of the brain.63 These disappointing results may have resulted from treating the mice when they were adult. Subsequent studies showed that the critical period for the full recovery of well-established Angelman syndrome mouse phenotypes by Ube3a gene reinstatement lies around birth.64,65 This suggests that UBE3A function is critical for typical brain development, which is further highlighted

![Diagram](image-url)

**Figure 2:** Mechanism of neuronal UBE3A imprinting and antisense oligonucleotide (ASO)-mediated unsilencing of paternal UBE3A gene expression. (a) Overview of the UBE3A locus in neurons of an individual with Angelman syndrome with a mutation in the UBE3A gene (indicated with a star). Note that most patients with Angelman syndrome carry a maternally inherited deletion of the depicted region extending far beyond the Angelman syndrome Imprinting Center (AS-IC) and the UBE3A gene. (b) Overview of paternal UBE3A expression in the Angelman syndrome condition upon ASO treatment (orange). Maternally imprinted genes are depicted in grey, the AS-IC is indicated as an empty purple triangle. The lack of a methylated Prader–Willi syndrome Imprinting Center (PWS-IC; indicated as an empty purple circle) allows for the transcription of the long non-coding SNHG14 gene, also known as UBE3A-ATS, which is responsible for suppressing paternal UBE3A transcription (red rectangle). The administration of ASOs leads to cleavage of the UBE3A-ATS transcript, resulting in the unsilencing of the paternal UBE3A gene (depicted by a green rectangle), allowing restoration of synthesis of the UBE3A protein.
by the observation that deletion of the Ube3a gene in adult mice has little effect.66 How this critical period in mice with Angelman syndrome relates precisely to the optimal treatment of patients with Angelman syndrome is unknown, but it is noteworthy that there is no critical period for restoring hippocampal plasticity.64 Since hippocampal plasticity is often used as a cellular proxy for learning and memory formation, this would suggest that gene reinstatement will improve cognitive function, at least to some extent, at any treatment age.65,66 Another important question is whether UBE3A reinstatement is equally beneficial in patients who carry the 15q11-13 deletion as in those carrying the UBE3A mutation. Owing to the lack of a mouse model of 15q11–13 deletion,14 it is difficult to predict to what extent the effect of losing the additional genes in this locus is masked by the severe consequence of losing UBE3A. However, restoring UBE3A in these patients will probably still significantly improve symptoms.

Since the sequence of the UBE3A-ATS is poorly conserved between mice and humans, the use of patient-derived induced pluripotent stem cells67 has been instrumental in identifying efficient ASOs that can be used in the clinic. Currently, two active clinical trials are taking advantage of the ASO-mediated UBE3A gene reinstatement approach. One trial is sponsored by Hoffmann-La Roche (molecule RO7248824; NCT04428281) and the other is sponsored by GeneTX Biotherapeutics (molecule GTX-102; NCT04259281). A third trial (sponsored by Hoffmann-La Roche) is facilitated by non-homologous isoforms.

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