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Dear CIRM Board Members,

We are honored to hear that our resubmitted TRAN1 application received a score in the fundable range. We propose to perform the necessary preclinical studies to create a first-in-class antisense oligonucleotide (ASO) to treat Timothy syndrome (TS), a disorder caused by a shared pathogenic variant in the gene *CACNA1C* causing cardiac arrhythmia, epilepsy, autism spectrum disorder, and developmental delay/intellectual disability. Given the large number of fundable applications you received this cycle, we wanted to stress the urgency and relevance of our project. In a recent breakthrough paper from our group, the basic science research underlying this TRAN1 proposal was published on the cover of *Nature* on April 24th, 2024. This is a unique moment to make an impact in Timothy syndrome because of the *(1)* high morbidity and mortality associated with Timothy syndrome; *(2)* the lack of attention from pharmaceutical companies for rare disorders despite their potential for significant medical breakthroughs in neuropsychiatry; and *(3)* the significant interest in Timothy syndrome as a proof-of-concept model for the impact of stem cell research in precision medicine.

First, there is a timeliness to our project since children with Timothy syndrome are being diagnosed more readily but also dying young. In the last 18 months, five children with Timothy syndrome have died of unclear, possibly multifactorial causes. Please find included a testimonial from the parent of one of these children. Meanwhile, diagnosis and family engagement are ramping up as more children gain access to genetic testing and then partner with the Timothy Syndrome Alliance (TSA) and the Timothy Syndrome Foundation (TSF), both of whom shared statements of support included within. For example, 38 new families with *CACNA1C*-related disorders have engaged with the TSA in the last year. Moreover, we initiated outreach to other major US pediatric centers to identify additional individuals with Timothy syndrome. In the coming years, we will know of even more individuals, diagnosed even earlier in development, and thus prime candidates for a clinical trial of an ASO geared at early neurologic and psychiatric symptom intervention.

Second, there is currently a lack of (commercial) interest in the pharmaceutical industry to develop treatments for disorders such as Timothy syndrome, even if a potential cure is in sight. Therefore, we are seeking support within academia as there is a moral imperative to act now. This disorder could serve as a "Rosetta Stone" for the field, potentially unlocking broader pharmaceutical interest for neuropsychiatric disorders investigated with human stem cell-derived models. Additionally, Timothy syndrome is an ideal proof-of-concept candidate as one of the only forms of autism spectrum disorders that can be diagnosed in infancy because of the long QT cardiac features. This offers a unique opportunity to intervene early and determine if any complex neuropsychiatric features of this disease can be reversed or prevented. The results of a Timothy syndrome clinical trial will have broad implications for how we approach therapeutics for autism and other neuropsychiatric disorders and could ultimately trigger broader pharmaceutical interest in the future for this and other conditions,

catalyzing a much-needed revolution in psychiatry. Without your support, however, we will not be able to continue research to treat this disorder.

Third, this proposal reflects a foundational shift in how stem cell-based models contribute to identifying disease mechanisms and targeted treatments for neuropsychiatric disorders. The foundational work for this TRAN1 proposal is the culmination of 15 years of research into human cellular models for Timothy syndrome. Timothy syndrome neurons were among the initial disease models created from human induced pluripotent stem (iPS) cells, which we build here at Stanford. Over the years, through a combination of 2D cultures, and then 3D organoids and assembloids that we pioneered at Stanford, we have gained a deep enough understanding of the biology of this complex condition to enable us to design a therapeutic strategy. This ASO intervention, as detailed in our *Nature* publication, was developed exclusively with human stem cell-based models and importantly tested *in vivo*, using an innovative engraftment model, by verifying the effects on patient-derived cells after their integration into rat circuits. This not only showcases the translational potential of assembloids and organoids but also highlights the necessity for a multi-level approach in assessing phenotypes and therapeutic interventions for human neuropsychiatric disorders.

Our recent article on an ASO to treat Timothy syndrome had almost 20,000 downloads within the first week of publication, was accompanied by a News & Views in *Nature*, highlighted in over 30 media sources, including *NPR, El Pais* and *The Financial Times*, and was covered by press release from the *National Institutes of Health*. Parents and clinicians reached out to us following publication to help identify additional individuals with Timothy syndrome due to their interest in engaging in treatment trials. These are kindling new discussions about the need to tackle rare disorders as entry points into finding therapeutics more broadly in psychiatry. We included a statement from the *Autism Science Foundation* that highlights the importance of advancing research in neuropsychiatric and developmental disorders through the study of rare conditions.

In summary, this is one of the first neuropsychiatric diseases that has been understood using a human stem cell model, and our work serves as a proof-of-concept for many other conditions. Even though Timothy syndrome is rare, treatment would still be transformative for affected individuals and would demonstrate that early intervention can impact care for other neurodevelopmental disorders. We are relying on CIRM funds to translate our preclinical work and pave the way for FDA approval, benefitting these children and their families as quickly as possible. We believe this will ultimately attract interest from pharmaceutical companies for other conditions and accelerate therapeutics in the neuropsychiatry field.

We have a strong team of experts spanning basic science, translational development, clinical neurogenetics, and patient outreach. With your support, we are poised to take the next steps toward treating children with Timothy syndrome.

Thank you for your consideration,
Sergiu Pasca & the research team

Timothy Syndrome Alliance

To whom it may concern

Timothy Syndrome Alliance (TSA), as the global patient advocacy non-profit charity organisation run by families of and representing individuals impacted by CACNA1C-related disorders (CRD), including Timothy Syndrome and LongQT8, is in support of clinical treatment advancements of antisense oligonucleotide (ASO) research for our currently incurable disorder.

This work from Sergiu P. Pașca's lab elegantly demonstrates a route to a potential cure for Timothy Syndrome Type 1 (TS1), a CRD diagnosis of a genetic variant with mixed neurologic and cardiac symptoms. This work is proof-of-concept. For our TS1 children, babies and their families there is hope.

Whilst there are potential barriers to be overcome before this can be considered a treatment or a cure, we ask that you also consider our growing wider community of CRD individuals where there is now known to be a large spectrum encompassing neurologic-only, cardiac-only, and mixed neurologic and cardiac symptoms. Our CACNA1C community exceeds 160 affected individuals in number and despite being rare grew by 38 individuals with disease-causing genetic variants in CACNA1C in 2023. It is heartbreaking to share that of these 38 individuals it is not only the TS1 baby who has since passed away. There is significant urgency to translate this research and directly impact clinical outcomes and quality of life.

The team at Stanford engages with the TSA's robust CACNA1C patient registry and Scientific Advisory Board, and it is hoped this collective effort can enhance impact. Transformative targeted therapy possibilities would not only have a long-term impact on our affected CACNA1C individuals and their families but potentially on those with other neurodevelopmental disorders and unmet medical needs.

With hope

Sophie Muir, Chair of Trustees and mum to three boys, one identified as having a CACNA1C-related disorder

Dr Jack Underwood, NMHII, Cardiff University TSA Scientific Advisory Board

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Timothy Syndrome Alliance (TSA)

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Registered Charity No. 1185523

TIMOTHY SYNDROME FOUNDATION

Re: Dr. Sergiu Pasca

April 30, 2024

Dear Funding Review Committee,

It is my pleasure to write in support of Dr. Sergiu Pasca as a recipient of the CIRM funding. As President of the Timothy Syndrome Foundation, I am with pleasure anticipating collaborative efforts with Dr. Pasca in securing Timothy syndrome patients for his future ASO studies.

Having discovered, researched and followed Timothy syndrome (TS) children for over 30 years, I am an ardent advocate for the wellbeing of these children; therefore, this proposed ASO work by Dr. Pasca is of exceptional interest to me. Since I first helped define this disorder three decades ago, I have remained closely involved with families, leading to my continued leadership within the TSF. I can therefore represent medical, research, and family advocacy points of view when I share my excitement and enthusiasm for what these results and potential treatment opportunities mean for the TS community. TS is a most devastating disorder, with known abnormal gene function in almost every cell of the body, causing a myriad of life-threatening health concerns (arrhythmias, hypoglycemia, severe immune dysfunction) in addition to the many debilitating developmental neurological concerns (epilepsy, autism, ADD, ADHD, schizophrenia).

Over the last 30 years there have been numerous studies which have greatly increased the understanding of TS cellular dysfunction, along with advancements in life saving cardiac devices. Unfortunately, despite these needed advancements the TS survival rate remains low and at least 30 percent of all known TS children have died. Dr. Pasca's ASO studies have now offered hope for the children with TS, not only for the important improved neurological function, but possibly improving the lifesaving function of all abnormal cellular and tissue dysfunctions for enhanced overall health and survival of children with TS.

Thank you for your continued consideration of Dr. Sergiu Pasca, an exceptional scientist and friend of children with TS.

Most respectfully,

Katherine W. Timothy

President and Co-Founder of Timothy Syndrome Foundation

Statement of support from Courtney Waller, parent of a child with Timothy syndrome who recently died:

Theodora was born in 2013 and diagnosed with Timothy Syndrome type 1 within moments of her arrival. She passed away February 16, 2024. Unfortunately, she did not survive long enough to see any potential treatments.

Through her life, her father and I knew that she most likely would not live long enough to see meaningful research or a lifesaving treatment. Yet, we also did not want her life and subsequent death to be in vain. Eight years ago, myself and another parent launched the Timothy Syndrome Alliance under the SADS foundation. It was an effort to build community, and ultimately spur research into possible treatments for children born with Timothy Syndrome and other associated *CACN1AC* mutations. The ultimate goal was simple, to keep our children from dying; a harsh reality that the majority of our community will face.

This research has brought so much joy and hope to not just the community, but to myself and Theodora's father. While she may not have survived long enough to be helped, other children may benefit from this treatment. The excitement among the community as a whole is overwhelming. While a full FDA approved treatment is still a ways away from reaching us, all steps towards making this a reality makes all the difference to parents like me, and the kids like Theodora, many of whom will not survive long enough to attend the first grade**.**

National Institutes of Health
Turning Discovery Into Health
Wednesday, April 24, 2024

Gene-based therapy restores cellular development and function in brain cells from people with Timothy syndrome

NIH-supported study shows potential treatment pathway for neurodevelopmental disorder.

In a proof-of-concept study, researchers demonstrated the effectiveness of a potential new therapy for Timothy syndrome, an often life-threatening and rare genetic disorder that affects a wide range of bodily systems, leading to severe cardiac, neurological, and psychiatric symptoms as well as physical differences such as webbed fingers and toes. The treatment restored typical cellular function in 3D structures created from cells of people with Timothy syndrome, known as organoids, which can mimic the function of cells in the body. These results could serve as the foundation for new treatment approaches for the disorder. The study, supported by the National Institutes of Health (NIH), appears in the journal *Nature*.

"Not only do these findings offer a potential road map to treat Timothy syndrome, but research into this condition also offers broader insights into other rare genetic conditions and mental disorders," said Joshua A. Gordon, M.D., Ph.D., director of the National Institute of Mental Health, part of NIH.

Sergiu Pasca, M.D., and colleagues at Stanford University, Stanford, California, collected cells from three people with Timothy syndrome and three people without Timothy syndrome and examined a specific region of a gene known as *CACNA1C* that harbors a mutation that causes Timothy syndrome. They tested whether they could use small pieces of genetic material that bind to gene products and promote the production of a protein not carrying the mutation, known as antisense oligonucleotides (ASOs), to restore cellular deficits underlying the syndrome.

In the lab, researchers applied the ASOs to human brain tissue structures grown from human cells, known as organoids, and tissue structures formed through the integration of multiple cell types, known as assembloids. They also analyzed organoids transplanted into the brains of rats. All of the methods were created using cells from people with Timothy syndrome. Applying the ASOs restored normal functioning in the cells, and the therapy's effects were dose-dependent and lasted at least 90 days.

"Our study showed that we can correct cellular deficits associated with Timothy syndrome," said Dr. Pasca. "We are now actively working towards translating these findings into the clinic, bringing hope that one day we may have an effective treatment for this devastating neurodevelopmental disorder.

The genetic mutation that causes Timothy syndrome affects the exon 8A region of the *CACNA1C* gene. The gene contains instructions for controlling calcium channels—pores in the cell critical for cellular communication. The *CACNA1C* gene in humans also contains another region (exon 8) that controls calcium channels but is not impacted in Timothy syndrome type 1. The ASOs tested in this study decreased the use of the mutated exon 8A and increased reliance on the nonaffected exon 8, restoring normal calcium channel functioning.

Stanford publication showcases new intervention for Timothy syndrome, a known cause of autism

(New York: April 24, 2024) The Autism Science Foundation is excited to announce the publication of a groundbreaking new discovery from Dr. Sergiu Pasca's laboratory at Stanford University, showcasing the transformative potential of utilizing human stem cell models, such as neural organoids and assembloids, in elucidating severe neurodevelopmental disorders. Over the course of 15 years, Dr. Pasca and his team have meticulously identified the fundamental molecular and cellular abnormalities underlying Timothy syndrome—a rare genetic disorder characterized by defects in a calcium channel, which significantly increases the risk of autism spectrum disorder, epilepsy, and developmental delay.

These investigations have yielded invaluable biological insights, culminating in the development of a therapeutic approach capable of effectively reversing these abnormalities both in vitro and in vivo, through the development of a gene therapy that more specifically targets the cause of Timothy Syndrome in model systems and cells obtained from patients. While Timothy syndrome remains a rare condition, the lessons learned from studying it have far-reaching implications. By unraveling its intricacies, we gain a deeper understanding of the broader mechanisms underlying psychiatric disorders.

Autism Science Foundation Chief Science Officer, Dr. Alycia Halladay, is the founder and program officer of the Alliance for Genetic Etiologies in Neurodevelopmental Disorders and Autism, also known as AGENDA, which ASF oversees. AGENDA brings together over 40 patient advocacy groups in an effort to improve outcomes for people with monogenic forms of autism by fostering a genetics-first approach to research and by strengthening collaborations among organizations representing genetically-defined disorders associated with autism. "Patients with rare variants associated with autism, including Timothy Syndrome, are already pursuing and utilizing gene therapies," said Dr. Halladay. "This new approach led by Dr. Pasca's lab improves the precision of that therapy as well as expands ways to validate them in each individual. This is an enormous breakthrough for a community that struggles with finding therapies that are effective and helpful.

The Autism Science Foundation champions the exploration of rare disorders, as they serve as gateways to uncovering insights into more prevalent causes of autism spectrum disorder. By doing so, we pave the way for the identification of novel intervention pathways, ultimately advancing our ability to treat these conditions effectively.

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nature

SWITCHING CHANNELS

Organoids and assembloids offer model way to test potential therapy for Timothy syndrome

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Timothy syndrome (TS) is a severe, multisystem disorder characterized by autism, epilepsy, long-QT syndrome and other neuropsychiatric conditions[1]. TS type 1 (TS1) is caused by a gain-of-function variant in the alternatively spliced and developmentally enriched *CACNA1C* exon 8A, as opposed to its counterpart exon 8. We previously uncovered several phenotypes in neurons derived from patients with TS1, including delayed channel inactivation, prolonged depolarization-induced calcium rise, impaired interneuron migration, activity-dependent dendrite retraction and an unanticipated persistent expression of exon 8A[2–6]. We reasoned that switching *CACNA1C* exon utilization from 8A to 8 would represent a potential therapeutic strategy. Here we developed antisense oligonucleotides (ASOs) to effectively decrease the inclusion of exon 8A in human cells both in vitro and, following transplantation, in vivo. We discovered that the ASO-mediated switch from exon 8A to 8 robustly rescued defects in patient-derived cortical organoids and migration in forebrain assembloids. Leveraging a transplantation platform previously developed[7], we found that a single intrathecal ASO administration rescued calcium changes and in vivo dendrite retraction of patient neurons, suggesting that suppression of *CACNA1C* exon 8A expression is a potential treatment for TS1. Broadly, these experiments illustrate how a multilevel, in vivo and in vitro stem cell model-based approach can identify strategies to reverse disease-relevant neural pathophysiology.

Timothy syndrome type 1 (TS1 or TS) is a severe genetic disorder with significant morbidity and mortality[8–11] caused by the heterozygous c.1216G>A pathogenic variant in exon 8A of *CACNA1C*, resulting in a p.G406R missense variant in the α1 subunit of the L-type voltage-gated calcium channel $Ca_V1.2$ (ref. 8). $Ca_V1.2$ is broadly expressed in both the developing and adult nervous system, primarily in neurons but also in some progenitors and glial cells[12,13]. TS1 affects multiple organ systems and is one of the most penetrant genetic aetiologies of autism spectrum disorder and epilepsy[8]. Common variants in *CACNA1C* have also been strongly associated with other neuropsychiatric disorders including schizophrenia, bipolar disorder and attention deficit hyperactivity disorder[9,11], suggesting that $Ca_V1.2$ is a key susceptibility factor for neuropsychiatric conditions.

Studies in human-induced pluripotent stem (hiPS) cell-derived cardiomyocytes and neurons in both two- and three-dimensional systems reported that cells derived from individuals with TS1 showed delayed voltage-dependent channel inactivation and increased depolarization-induced calcium entry[2,4,14], leading to abnormal excitability. Moreover, using human forebrain assembloids (hFA) generated by the integration of human cortical organoids (hCO) and human subpallial organoids (hSO), we previously described defects in cortical interneuron migration: TS1 interneurons undergo more frequent nucleokinetic saltations driven by enhanced GABA sensitivity but saltation length is reduced due to aberrant cytoskeletal function, leading to overall defective migration[3,4].

Surprisingly, TS1-derived neurons have an abnormally high level of the *CACNA1C* splice form containing exon 8A compared with control neurons[4,6]. Moreover, splicing of *CACNA1C* is developmentally regulated in both mouse and human, with a shift in exon utilization from exon 8A to 8 during early development[15]. Inclusion of either of these mutually exclusive spliced exons has been shown to yield channel isoforms with relatively similar electrophysiological features[16,17]. These findings raise the possibility that decreasing inclusion of the 8A isoform of *CACNA1C* may function as a therapeutic strategy for TS1.

In this study we developed an antisense oligonucleotide (ASO)-based intervention to effectively decrease exon 8A inclusion in neural cells derived from three individuals with TS and an isogenic G406R hiPS cell line. ASOs are short oligonucleotides that can bind to target RNAs, activate cytoplasmic degradation of target RNAs or modulate splicing of pre-messenger RNAs inside the nucleus[18,19]. Several ASOs targeting splicing have advanced from the bench to the clinic as therapeutic options, including for spinal muscular atrophy[20–23]

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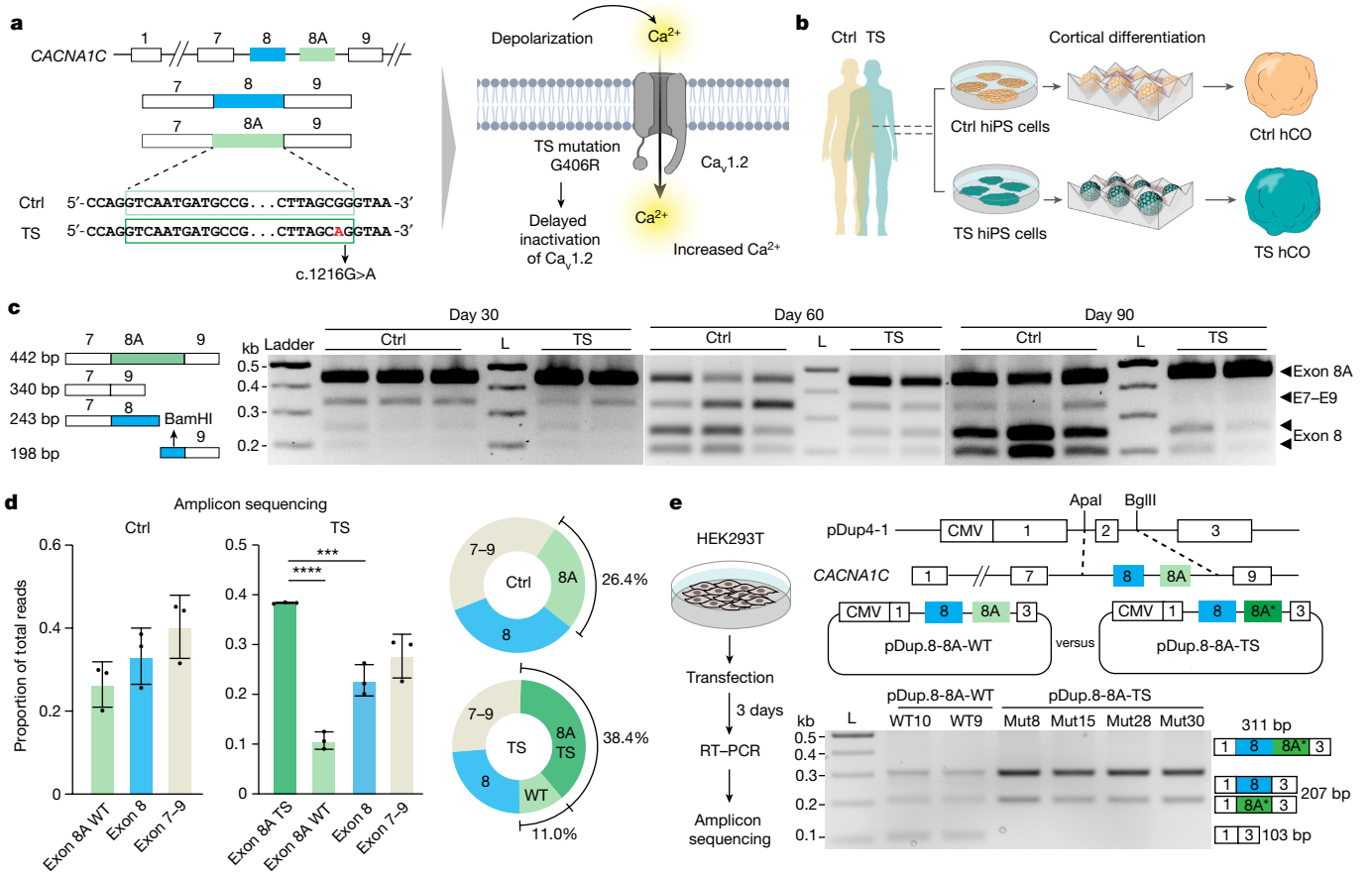

Fig. 1 | The TS G406R variant enhances inclusion of *CACNA1C* exon 8A in human neurons. a, Schematics illustrating the TS pathogenic variant in the alternatively spliced exon 8A (left) and the resulting gain-of-function channel variant (right). The heterozygous G>A variant (black arrow) is located towards the 3′ end of exon 8A. **b**, Generation of hCO from control (Ctrl) and TS hiPS cells. **c**, Schematic of the RFLP assay. Left, PCR products amplified from hCO cDNA; the exon 8-containing amplicon is recognized by restriction enzyme BamHI; exon 8A, 8 and 7–9 amplicons have different molecular weights on agarose gel. Right, RFLP gel image of control and TS hCO at days 30, 60 and 90 of differentiation. Each column represents a hCO derived from different hiPS cell lines. L, ladder. **d**, Next-generation sequencing of amplicons generated from day 60 hCO. Left, PCR products were obtained using a forward primer targeting exon 7 and a reverse primer targeting exon 9; both primers have an Illumina adaptor at their 5′. Right, proportions of exon 8A WT, exon 8A TS, exon 8 and exons 7–9 are shown ($n = 3$ for WT hCO, $n = 3$ for TS hCO). Data presented as mean ± s.d. One-way analysis of variance (ANOVA) with Tukey's post hoc test: for control hCO, $F_{2,6} = 3.246$, $P = 0.1108$; for TS hCO, $F_{3,8} = 50.28$, $P < 0.0001$. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$. **e**, Generation of minigene splicing reporters for exons 8 and 8A of *CACNA1C*. Left, experimental strategy for testing minisplicing reporters in HEK293T cells. Right, a *CACNA1C* DNA fragment (isolated from TS hiPS cells) was inserted into a pDup4-1backbone resulting in two vectors, pDup8-8A-WT and pDup8-8A-TS. bp, base pairs.

and Duchenne muscular dystrophy[24]. Here we first demonstrated that the TS1 p.G406R mutation directly enhanced splicing of the mutated exon 8A. We then performed a screen to identify ASOs that can robustly inhibit splicing of exon 8A, in a time- and dose-dependent manner. Direct application of these ASOs to human cortical neurons in either two- or three-dimensional cultures derived from individuals with TS rescued both delayed channel inactivation and the defect in depolarization-induced calcium elevation. Moreover, these ASOs restored previously identified cortical interneuron migration defects in TS1 forebrain assembloids. Lastly, to verify ASO effectiveness in an in vivo setting, we leveraged a transplantation model that we have recently developed[7]. In this system, human stem cell-derived cortical organoids transplanted (t-hCO) into the somatosensory cortex of newborn athymic rats grow and develop mature cell types that integrate into sensory and motivation-related circuits. We discovered that intrathecal injection of an ASO into rats transplanted with human TS1 cortical organoids resulted in a robust downregulation of exon 8A, accompanied by rescue of both depolarization-induced calcium defects and aberrant activity-dependent dendritic morphology. Taken together, these experiments demonstrate a new genetic rescue strategy for a devastating neurodevelopmental disorder.

Enhanced inclusion of *CACNA1C* exon 8A results in abnormal channel function in human cortical neurons

Exons 8 and 8A are mutually exclusive, 104-nucleotide-long exons of the *CACNA1C* gene (Fig. 1a,b). During cortical differentiation in vitro, in both two-dimensional cultures[2,6] and three-dimensional hCO (Extended Data Fig. 1a), exon 8A is expressed at higher levels in the early stages but this changes in favour of exon 8 over time (Extended Data Fig. 1a; $P < 0.001$). We also verified this using human primary cortical tissues[25] (Extended Data Fig. 2a,b). Interestingly, hCO derived from patients with TS1 expressed considerably higher levels of exon 8A compared with control hCO at days 60–90 of differentiation, which is consistent with our previous observations[4,6]. A restriction fragment-length polymorphism (RFLP) assay that uses the BamHI restriction enzyme to selectively cut exon 8 further confirmed this finding (Fig. 1c), raising the possibility that the G406R mutation may directly interfere with splicing and enhance its own inclusion, which could amplify disease phenotypes by prolonging the expression of mutated $Ca_V1.2$.

To determine whether the TS mutation directly causes increased levels of exon 8A we first analysed its mRNA composition in TS hCO, which contains both wild-type (WT) and p.G406R exon 8A alleles. Given the

heterozygous nature of the mutation in patients with TS1, we reasoned that equal amounts of WT and p.G406R alleles of exon 8A are present in neural cells. However, by sequencing the amplicons spanning exons 7–9 from complementary DNA of both TS and WT hCO, we discovered that the elevated exon 8A expression in TS samples predominantly contained the p.G406R allele (Fig. 1d,e and Extended Data Fig. 1b). We next asked whether this TS-associated enhanced splicing depends on the cellular or genomic context. We generated two minigene splicing reporters in which DNA fragments of around 1 kb spanning exons 8 and 8A (either WT or TS) were inserted into the pDup4-1 reporter backbone (pDup8-8A^{WT} and pDup8-8A^{TS}; Fig. 1e and Extended Data Fig. 1c,d). Transfection and amplification of these two vectors in HEK293T cells showed markedly different splicing outcomes ($P < 0.0001$; Fig. 1e and Extended Data Fig. 1d,e). The WT pDup8-8A^{WT} mostly transcribed exon 8 whereas the mutant pDup8-8A^{TS} preferentially transcribed exon 8A, indicating that the TS mutation is sufficient to shift *CACNA1C* splicing in favour of exon 8A, independently of the cellular context. Previous studies found that the splicing master regulator Ptbp1 modulates exon 8 versus 8A splicing of mouse *Cacna1c*[15]. Both organoids and human primary brain RNA sequencing data show that *PTBP1* expression decreases over time[26] (Extended Data Fig. 2c). To explore the role of PTBP1 in *CACNA1C* splicing, we transfected *CACNA1C* minigene splicing reporters in the presence or absence of human PTBP1 (Extended Data Fig. 2c,d). We found marked changes in splicing patterns following the addition of PTBP1 (Extended Data Fig. 2d), and also increased exon 8A-containing transcripts (Extended Data Fig. 2f). Taken together, these experiments demonstrate that the TS exon 8A *CACNA1C* variant directly and persistently enhances its own abundance, potentially by interfering with splicing machinery, and that splicing regulator PTBP1 affects the selection of exon 8 versus 8A.

Screening of ASOs that can reduce exon 8A in favour of exon 8 *CACNA1C* isoforms in human neural cells

To screen for ASOs that could modify exon 8 splicing, we designed an 'ASO walking' strategy (ref. 21) with 5-nucleotide (nt) spacing covering exon 8A. We used ASOs with a universal 2′-*O*-methoxyethylribose (MOE) modification to avoid potential degradation of *CACNA1C* mRNA[18,19] (Fig. 2a). We differentiated TS hiPS cells into hCO, dissociated them into two-dimensional neural cultures and added 10 μM ASO targeting either exon 8A or a scrambled control ASO (ASO.Scr or A.Scr). Three days later, quantitative PCR with reverse transcription (RT–qPCR) of exons 8 and 8A showed that several ASOs had induced robust downregulation of exon 8A without changing exon 8 expression (Fig. 2b). To validate these results in three-dimensional hCO we selected the top four ASOs (ASO.14, ASO.17, ASO.18 and ASO.20). Exposure to ASOs for 3 days in three-dimensional organoid cultures also yielded selective exon 8A downregulation, as shown by both RT–qPCR analysis (Fig. 2c; $^{*}P < 0.05$, $^{**}P < 0.001$) and RFLP (Fig. 2d). Sequencing these amplicons further confirmed that ASO.14, ASO.17 and ASO.18 targeted and downregulated exon 8A in hCO derived from three patients with TS (Extended Data Fig. 3a). These effects were long-lasting; a single ASO administration effectively suppressed exon 8A up to 90 days post-exposure (Extended Data Fig. 3b,d; $P < 0.05$). Moreover, the switch from 8A to 8 was not associated with changes in the total amount of $Ca_V1.2$ protein, as indicated by immunoblots of hCO (Extended Data Fig. 4a–c).

To study the pharmacodynamics of these ASOs, we treated hCO with different concentrations of ASO.14 ranging from 0.001 to 10 μM at differentiation days 30 and 90. We observed a dose-dependent decrease in exon 8A expression (Fig. 2e; $^{*}P < 0.05$ for day 30, $^{****}P < 0.0001$ for day 90). We then treated hCO with ASO.14 and performed RT–qPCR analysis at 1, 6, 24, 48 and 72 h following exposure. Surprisingly, we found that ASO exposure altered the expression of exon 8A as early as 1 h post exposure in vitro (Extended Data Fig. 3e; $P < 0.001$). The pharmacodynamics of ASO.17 and ASO.18 were similar to that of ASO.14 (Extended Data Fig. 3f,g). Finally, to demonstrate the penetration efficacy of ASOs, we labelled ASO.14 with Cy5 and quantified Cy5$^+$ cells isolated from hCO by flow cytometry. Most cells, including CD90-expressing neurons, were Cy5$^+$ (Fig. 2f). Moreover, 3 days of exposure to Cy5-ASO correlated well with a dose-dependent reduction in Cy5 fluorescence by immunostaining (Extended Data Fig. 5a,b). This indicates that human neurons take up ASO and can, in a dose-dependent manner and within a short period of time, reduce exon 8A expression in this TS model.

Moreover, to identify adverse effects of ASOs in human neural cells we measured their toxicity, immunogenicity and off-target effects[27] (Extended Data Fig. 6). In both TUNEL assay and cleaved caspase 3 (c-Cas3) staining used for estimation of apoptosis we found no differences among ASO.Scr-, ASO.14- and mock-exposed neurons (Extended Data Fig. 6a–d). hTLR9 reporter cells are used to evaluate the immunogenicity of exogenous DNA[28] and we did not detect hTLR9 signalling activation following ASO delivery (Extended Data Fig. 6e). Because the universal MOE modification of our ASOs does not recruit the RNase H1 pathway, this is unlikely to cause off-target gene knockdown. Nonetheless, we performed qPCR analysis for *CACNA1D* encoding $Ca_V1.3$, another L-type calcium channel, and top off-target gene candidates based on sequence homology. We found no significant differences among ASO-treated groups, the ASO.Scr group and the control group (Extended Data Fig. 6f). These preliminary results evaluating ASO adverse effects in vitro are consistent with previous studies on ASO toxicity and off target[27,28].

ASO exposure rescues delayed channel inactivation and interneuron migration defects in TS hCO and hFA

We previously demonstrated that TS cortical neurons show delayed inactivation of barium currents, increased intracellular calcium following depolarization and impaired interneuron migration[3]. To gain further insights into the threshold of TS $Ca_V1.2$ expression necessary to detect a cellular phenotype, we measured depolarization-induced residual Ca^{2+} signal in HEK293T cells expressing 12 variable-ratio combinations of WT and TS $Ca_V1.2$ (Extended Data Fig. 7a–d). We detected a significant difference in residual Ca^{2+} between WT and TS $Ca_V1.2$ (Extended Data Fig. 7c,d) and found that even a small proportion of TS $Ca_V1.2$ is sufficient to perturb the kinetics of channel inactivation (Extended Data Fig. 7c,d). This highlights the effect of TS $Ca_V1.2$ on calcium influx and further indicates ASO therapeutic potential, even at postnatal stages when exon 8A expression is lower than prenatally.

Next we tested whether alteration of exon 8A/8 splicing via ASOs could restore $Ca_V1.2$ channel function. We exposed TS hCO neurons to ASO.14, ASO.17, ASO.18 or ASO.Scr and compared these with control hCO neurons exposed to ASO.Scr in a Fura-2 AM calcium imaging assay (Fig. 3a). As expected, TS neurons showed slower decay kinetics following depolarization compared with control neurons (Fig. 3b,c; $^{****}P < 0.0001$). All three selected ASOs restored residual calcium to control levels, suggesting that ASOs can functionally rescue the TS $Ca_V1.2$ channel (Fig. 3b,c; $P < 0.001$). We then applied ASO.14 and ASO.17 (the latter has effects similar to ASO.18) to both TS and control hCO and performed whole-cell patch-clamping of neurons labelled by SYN1:YFP (Fig. 3a,d). TS neurons showed delayed inactivation of barium currents as measured by percentage channel inactivation following 2 s of current-clamping (Fig. 3e, Extended Data Fig. 8a–d). Similar to the delayed inactivation we observed with calcium imaging, this defect was rescued by both ASO.14 and ASO.17 (Fig. 3f; $^{****}P < 0.0001$). To further explore the functional rescue of ASOs we set up a scalable GCaMP6f imaging readout on dissociated hCO neurons (Extended Data Fig. 7e,f). Following the application of single doses of ASOs at various concentrations for 10 days, we measured GCaMP6f signals before and after acute KCl depolarization. We found that, for all three ASOs tested, both 1 and 10 μM effectively rescued the TS phenotype whereas neither 0.1 nor 0.01 μM for ASO.14 did (Extended Data Fig. 7e–g). This suggests that

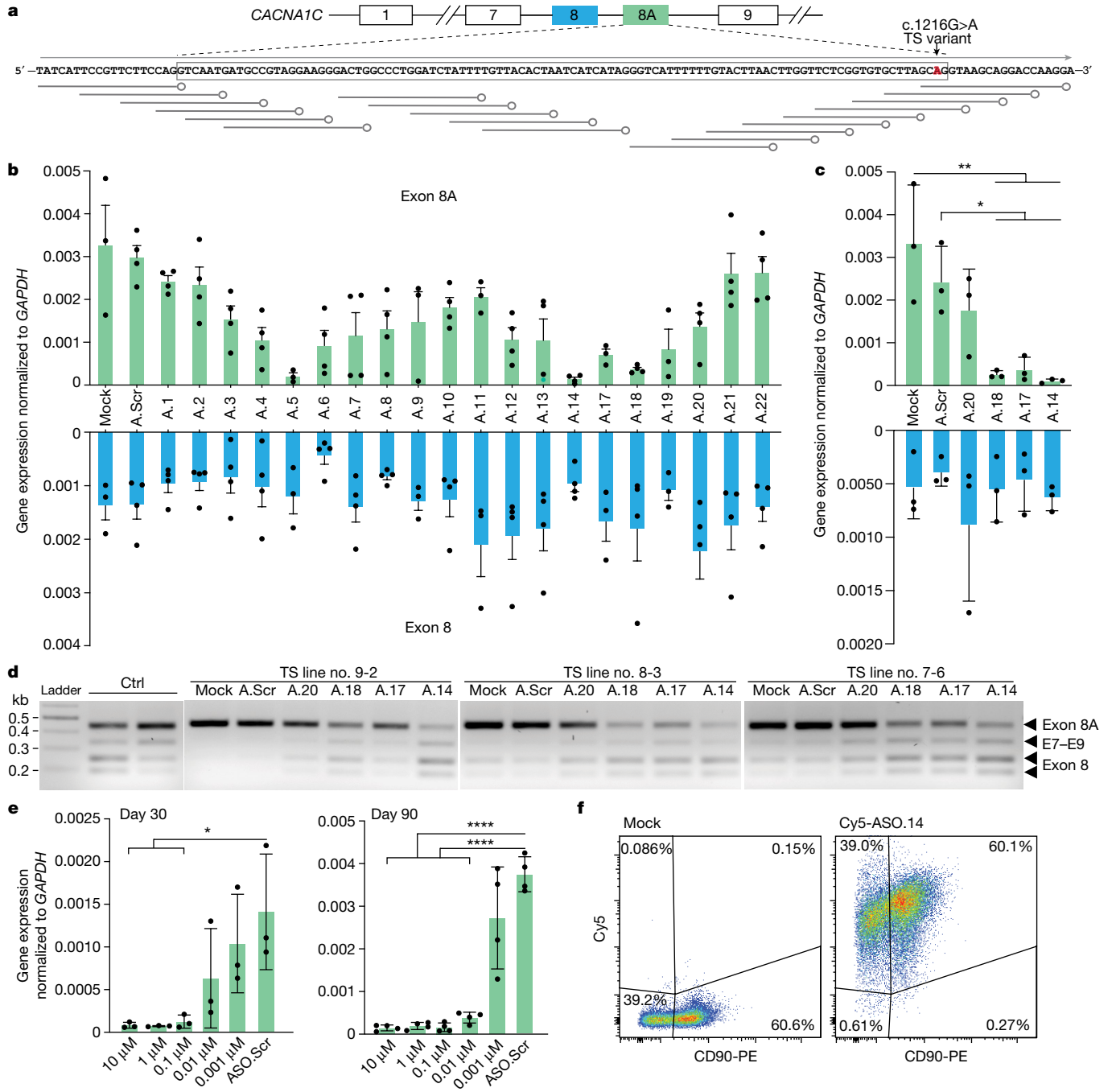

Fig. 2 | Screening of ASOs that can reduce exon 8A in favour of exon 8 *CACNA1C* isoforms in human neural cells. a, ASO design. Arrow denotes the location of the TS variant. **b**, RT–qPCR of exons 8A and 8 in ASO-treated dissociated TS hCO differentiated for 152 days. hCO derived from $n = 2$ TS hiPS cell lines (nos. 9-2 and 8-3) were dissociated and plated. For both TS lines, 10 µM ASO was added to two separated wells resulting in a total of four data points. RNA extraction was carried out 3 days post-exposure. Data are mean + s.e.m. **c**, RT–qPCR analysis of exons 8A and 8 of ASO-treated hCO. Data are mean + s.d. Three TS hiPS cell lines were used ($n = 3$). One-way ANOVA with Tukey's post hoc test: for exon 8A, $F_{5,12} = 8.870$, $P = 0.0010$, $^{*}P < 0.05$, $^{**}P < 0.01$; for exon 8, $F_{5,12} = 0.6689$, $P = 0.6546$. **d**, RFLP analysis from **c**. The size of corresponding amplicons is annotated (black arrowheads). **e**, Serial concentration dilutions of ASO.14 were used to evaluate dose-dependent splicing modulation on *CACNA1C* in hCO. ASO.14 was applied at differentiation day 30 ($n = 3$ individual hCO from three hiPS cell lines, left) and at day 90 ($n = 4$ individual hCO from two hiPS cell lines, right). Data presented as mean ± s.d. One-way ANOVA with Tukey's post hoc test: day 30, $F_{5,12} = 5.131$, $P = 0.0095$; day 90, $F_{5,18} = 36.81$, $P < 0.0001$, $^{****}P < 0.0001$. **f**, Flow cytometry of hCO (day 152) following 2 days of incubation with 1 µM Cy5-ASO.14. hCO were dissociated and stained with neuronal cell surface protein CD90; non-treated hCO were used as control (Supplementary Fig. 1).

there might be a discrepancy between the levels of RNA expression and protein function for TS rescue. Consistent with this, one recent ASO study found that a highly efficient knockdown of UBE3A-ATS was required to elevate the Ube3a protein level, yet UBE3A protein continued to increase with higher ASO concentrations even when mRNA restoration plateaued[29].

We previously discovered that TS interneurons migrate abnormally in hFA[3,4]. To investigate whether ASOs can correct this cellular migratory

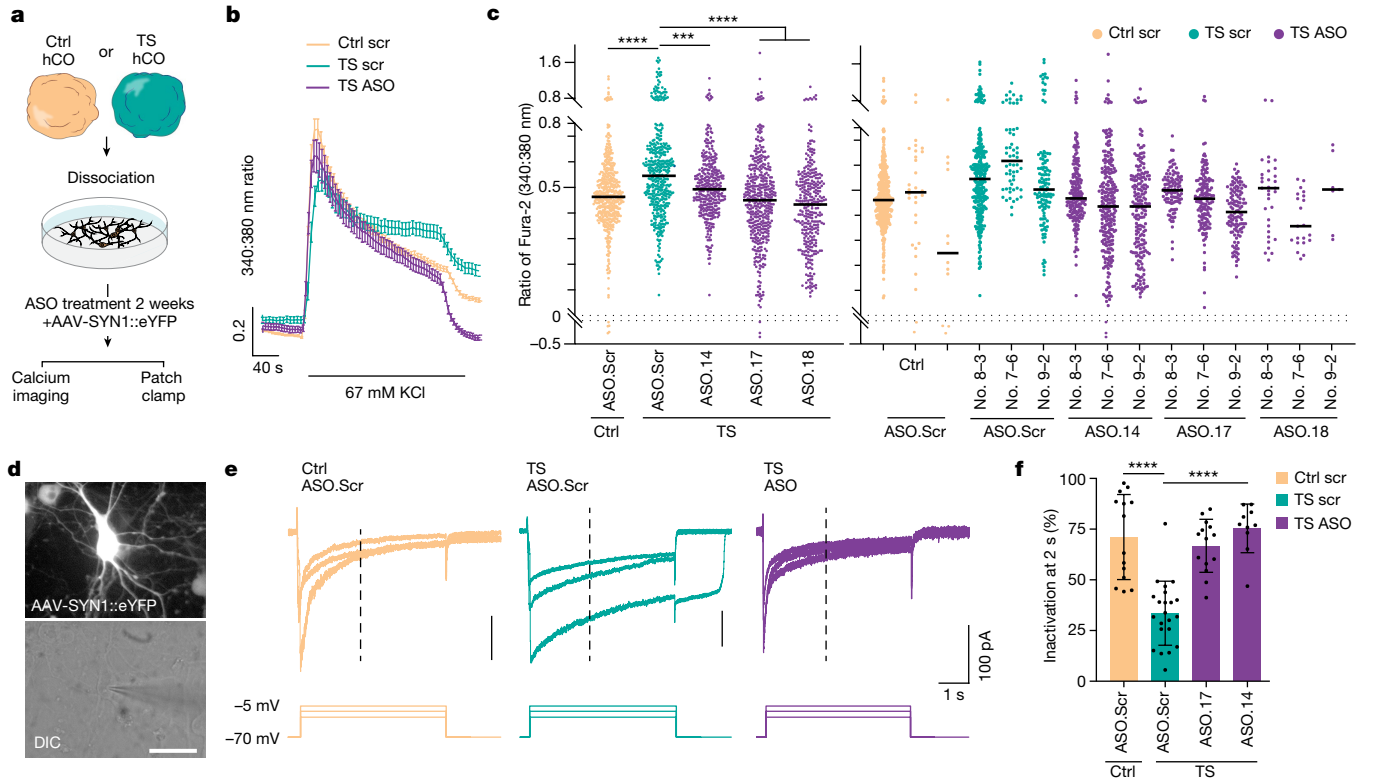

Fig. 3 | ASO exposure rescues delayed channel inactivation in TS cortical neurons. a, Strategy used to evaluate the effect of ASO on human neurons. **b**, Representative traces of depolarization-induced calcium responses measured by Fura-2 imaging (control scramble (Scr), $n = 55$ cells; TS scramble, $n = 31$ cells; TS + ASO.14, $n = 24$ cells). Data presented as mean ± s.e.m. **c**, Residual calcium in ASO-treated neurons (days 100–120 of differentiation). Left, data pooled across hiPS cell lines; right, data separated by cell line. Each dot represents one cell ($n = 2{,}017$ cells); Kruskal–Wallis test, $P < 0.0001$. Control versus TS, $^{****}P < 0.0001$; TS versus ASO.14, $^{***}P < 0.001$; TS versus ASO.17, $^{****}P < 0.0001$; TS versus ASO.18, $^{****}P < 0.0001$. Data presented as mean ± s.e.m. DIC, differential interference contrast. **d**, Representative example of patch-clamp recordings from AAV-SYN1::eYFP-infected hCO neurons. Scale bar, 20 µm **e**, Representative examples of barium currents following 5 s depolarization steps (−70 to −25, −15 and −5 mV, respectively). **f**, Summary graph of barium current inactivation (percentage of inactivated current compared with amplitude of peak current at 2 s) for maximal current. Ctrl Scr, $n = 14$ cells from two lines; TS Scr, $n = 22$ cells from two lines; TS ASO.17, $n = 14$ cells from two lines; TS ASO.14, $n = 10$ cells from one line. Data presented as mean ± s.d. One-way ANOVA with Tukey's post hoc test, $F_{3,56} = 25.34$, $P < 0.0001$, $^{****}P < 0.0001$.

defect in three-dimensional cultures we derived TS and control hCO and hSO, labelled interneurons in hSO with a lineage-specific reporter (LV.Dlxi1/2b::eGFP) and generated hFA, as previously demonstrated[3] (Fig. 4a–d). Three to four weeks post assembly we imaged and quantified saltation frequency and the average saltation length of TS and control interneurons at baseline; we then exposed hFA to ASO.14, ASO.17 or ASO.Scr and performed a further imaging experiment 2 weeks later. At baseline before ASO exposure, as previously described, we found increased saltation frequency (Fig. 4b; $^{*}P < 0.05$, $^{***}P < 0.005$, $^{****}P < 0.0001$) and shortened saltation length in TS interneurons compared with control interneurons (Fig. 4c; $P < 0.05$). Exposure to ASO.14 and ASO.17 reduced the saltation frequency of TS interneurons (Fig. 4b; $P < 0.05$) and increased saltation length (Fig. 4c; $P < 0.05$). In summary, we found that exposure to exon 8A–8-switching ASOs effectively rescued channel function, calcium signalling dynamics and cellular phenotypes in in vitro cultures derived from patients with TS.

ASO delivery in vivo rescues TS-related phenotypes in transplanted human TS cells

Encouraged by these findings and motivated to assess the translational potential of these ASOs in TS, we next validated their effect in an in vivo setting. We have recently developed a strategy for transplantation into the developing cerebral cortex of early postnatal rats that allows hCO to develop mature cell types and integrate both anatomically and functionally into the rodent brain[7]. We now applied this in vivo platform to test the delivery of ASOs in vivo and their ability to rescue genetic and functional defects in cells from patients with TS1 (Fig. 5a,b).

We first tested whether our ASOs would act on rat brain tissue, in particular because rat *Cacna1c* is highly homologous to human *CACNA1C* (Extended Data Fig. 9a). To this end, in the rat cisterna magna we injected 80 µg of ASO.14, an ASO that robustly suppresses exon 8A expression in vitro. Five days later we discovered that ASO.14 had reduced *Cacna1c* exon 8A expression in the cortex, cerebellum and spinal cord (Extended Data Fig. 9b,c).

We next transplanted hCO from three individuals with TS and monitored t-CO and graft position by magnetic resonance imaging (MRI) and immunostaining (Fig. 5b,c). We then injected 300 µg of ASO.14 into rat cisterna magna. Seven to 14 days later we extracted the hCO graft and found that *CACNA1C* exon 8A in TS t-hCO had reduced the level of expression ($P < 0.0001$; Fig. 5d and Extended Data Fig. 10a). This was accompanied by a reduction in the expression of rat *Cacna1c* exon 8a in both cortex and cerebellum (Fig. 5d; $P < 0.05$). Similar to the in vitro ASO experiments, overall $Ca_V1.2$ levels were not affected (Extended Data Fig. 10b). This experiment indicates that ASOs can be delivered intrathecally and can effectively modulate splicing in human transplanted cells. Lastly, we attempted to verify the effects of ASO administration on cellular dysfunction resulting from the TS1 mutation. To do so we extracted t-hCO, sliced the tissue and performed ex vivo calcium imaging using the calcium indicator Calbryte 520 AM (Fig. 5e). We found that ASO.14 normalized the increase in post-depolarization residual calcium found in cortical TS neurons (Fig. 5f,g). Finally, TS is

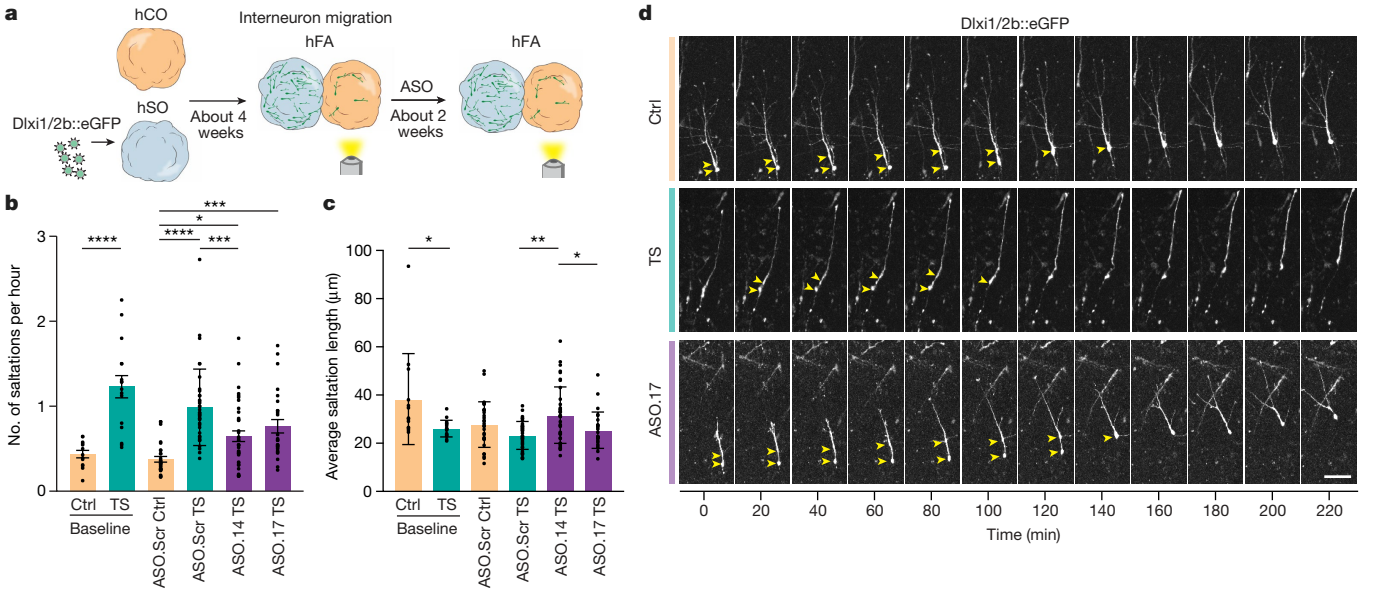

Fig. 4 | ASO exposure rescues delayed migration defects in TS hFA. **a**, Strategy used to test the effect of ASO on interneuron migration using hFA. Preceding fusion of hSO and hCO, hSO were infected with cortical interneuron reporter Lenti-Dlxi1/2b::eGFP around day 40. Imaging was performed at 4 weeks following assembly and again at 2 weeks post ASO incubation. **b**, Saltation frequency of Dlxi1/2b::eGFP$^+$ migrating cortical interneurons in hFA. Pre ASO exposure, $n = 13$ Ctrl cells and $n = 16$ TS cells; post ASO exposure, $n = 30$ Ctrl ASO.Scr, $n = 37$ TS ASO.Scr, $n = 38$ TS ASO.14 and $n = 26$ TS ASO.17 cells. Data presented as mean ± s.d. One-way ANOVA with Tukey's post hoc test for post-ASO exposure groups, $F_{3,125} = 14.03$, $P < 0.0001$, ****$P < 0.0001$, ***$P = 0.0009$, *$P = 0.0177$. Two-tailed unpaired *t*-test with Welch's correction was used to compare baseline control and TS, ****$P < 0.0001$. **c**, Saltation length of Dlxi1/2b::eGFP$^+$ migrating cortical interneurons in hFA. Data presented as mean ± s.d. One-way ANOVA with Tukey's post hoc test for post-ASO exposure groups, $F_{3,125} = 5.648$, $P = 0.0012$, **$P = 0.0007$, *$P = 0.0376$. Two-tailed unpaired *t*-test with Welch's correction was used to compare baseline control and TS, *$P = 0.0386$. **d**, Representative images of saltatory movement (yellow arrowheads) of Dlxi1/2b::eGFP$^+$ migrating cortical interneurons; scale bar, 50 µm.

associated with activity-dependent dendrite morphology defects[5] and this can be detected in patient-derived cortical neurons following transplantation in vivo[7]. To test whether ASOs could rescue this morphological phenotype, we traced neurons using Golgi staining in t-hCO at 14 days post ASO injection. We found that ASO.14 corrected the dendritic morphology of TS neurons in vivo (Fig. 5h,i and Extended Data Fig. 10c–g).

These experiments indicate that ASOs can modulate splicing of human *CACNA1C* both in vitro and in vivo and thereby rescue both molecular and cellular phenotypes of TS1.

Discussion

Developing therapies for neuropsychiatric disorders remains a substantial challenge due to the inaccessibility of human brain tissue. This holds true especially for disorders that emerge during fetal development, such as TS. Despite an understanding of the genetic cause and of some of the molecular mechanisms of TS, we still do not have a promising therapeutic avenue. L-type calcium channel blockers do not restore many of the cellular phenotypes in TS, and roscovitine has extensive off-target effects[2,14]. Some, but not all, of the defects identified with human cellular models have been recapitulated in a mouse[30] expressing the channel with the TS type 2 variant (the p.G406R variant is in exon 8 that also carries a stop codon in exon 8A), suggesting that species-specific differences in gene regulation can change the cellular phenotypes associated with a disease.

Here we developed a potential therapeutic strategy for a severe neurodevelopmental disorder caused by a single nucleotide variant in an alternatively spliced exon. To do this we first investigated splicing profiles in human neurons and found that the persistent elevation of exon 8A in TS is biased towards the TS gain-of-function variant, which probably amplifies defects downstream of this dysfunctional calcium channel. We subsequently screened and identified ASOs that can effectively modulate splicing in TS to reduce exon 8A without changing the overall level of $Ca_V1.2$ protein. We demonstrate, in human neurons derived from three patients with TS1 in human organoid and assembloid models that these ASOs can, in a dose- and time-dependent manner, modulate exon 8A and rescue ion flux kinetics, calcium dynamics and associated cellular movement defects. Lastly, we show that ASOs can be delivered in vivo using a organoid transplantation platform that we previously developed and, importantly, that they can rescue splicing and intracellular calcium flux defects in human neurons integrated into the rat cerebral cortex.

There are a number of limitations to our study. First, our current ASOs do not distinguish WT exon 8A and TS exon 8A. Further refinement, including testing ASOs of varying length, chemical modifications and targeting upstream and downstream of the TS variant, may be needed to increase specificity. Longer and earlier exposure to ASOs may also be needed to fully restore migration defects. The p.G406R mutation in TS1 modelled here is in exon 8A of *CACNA1C*. It would be clinically relevant to investigate whether TS2, caused by the same amino acid mutation but in exon 8, also shows abnormal splicing of exon 8/8A and whether ASOs can correct splicing defects and rescue channel function. Because patients with TS have cardiac arrythmias, it would be useful to test the ability of these ASOs to rescue defects in cardiac organoids. Finally, our assessment of ASO toxicity was performed in vitro. Recent studies have shown that ASOs containing a gapmer design can show dose-dependent acute neurotoxicity in the central nervous system[31,32]; therefore, in vivo short- and long-term pharmacology will be necessary to evaluate the relative toxicity of the full MOE-modified, splicing-modulating candidates that we identified. Notably, for some ASOs we observed a strong dose–response with full splice modulation at 10 nM. Futures studies should explore the contribution to this effect by ASO sequence and chemical modification, endogenous pre-mRNA levels, the cell type context as well as the potential off-mechanism effects. Moreover, evaluation of efficacy

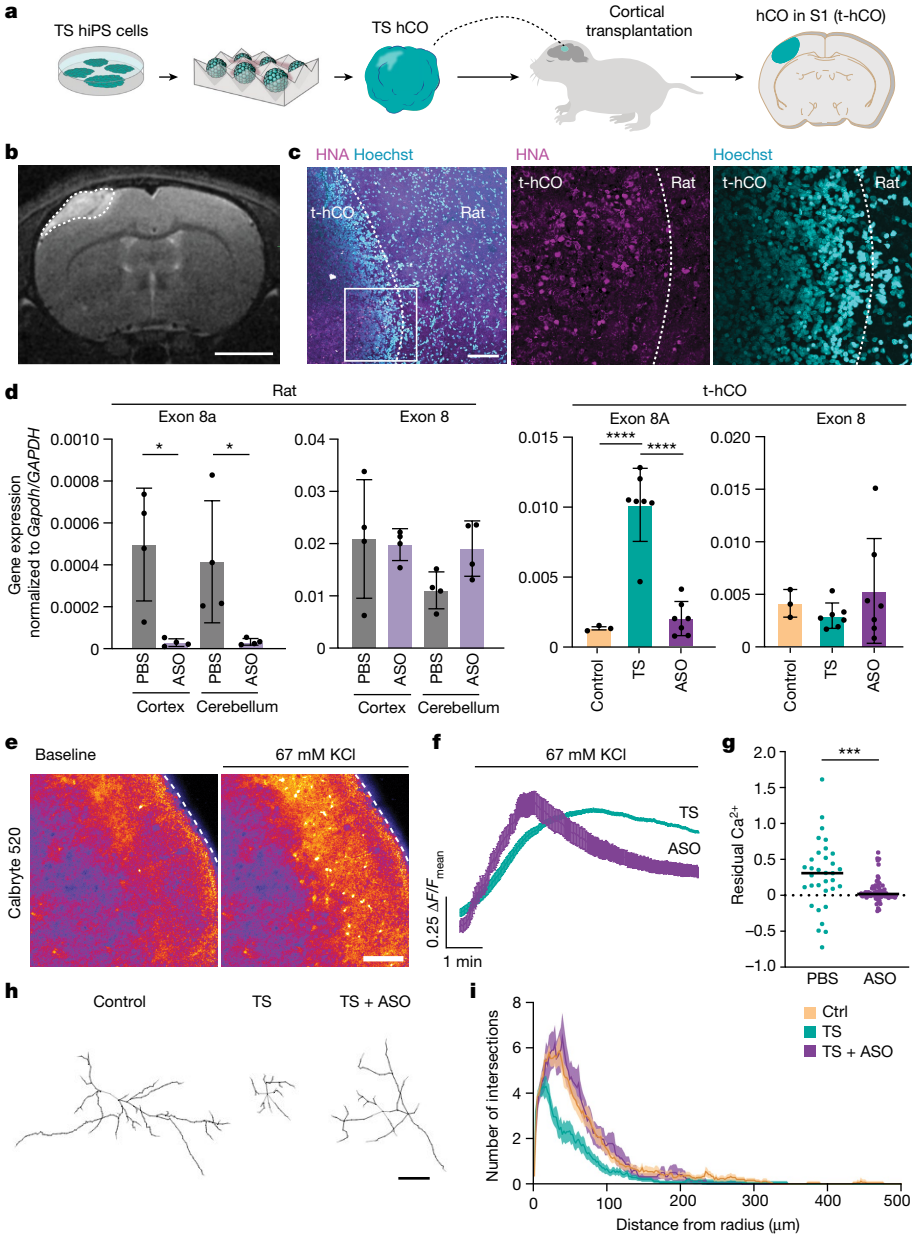

Fig. 5 | ASO delivery in vivo rescues TS-related phenotypes in transplanted human TS cells. a, Schematic illustrating transplantation of hCO (t-hCO) into rat somatosensory cortex. **b**, Representative MRI showing t-hCO (scale bar, 4 mm). **c**, Immunostaining in t-hCO for the human-specific marker HNA (scale bar, 2 mm). **d**, RT–qPCR analysis of t-hCO (days 162–258) and rat neural tissue following ASO injection. Data presented as mean ± s.d. Left, exons 8A and 8 of rat *Cacna1c* in cerebral cortex and cerebellum ($n = 4$ animals per group); two-sided unpaired student's *t*-tests were used to compare ASO versus PBS in cortex ($P = 0.0129$) and ASO versus PBS in cerebellum ($P = 0.0382$). Right, exons 8A and 8 of human *CACNA1C* (Ctrl, $n = 4$; TS, $n = 7$; TS + ASO, $n = 7$; t-hCO. each point represents either qPCR or average qPCR value from t-hCO from the same animal. The same t-hCO samples were also used for the RFLP assay shown in Extended Data Fig. 10a). One-way ANOVA with Tukey's post hoc test: for exon 8A, $F_{2,14} = 40.40$, $P < 0.0001$, $^{****}P < 0.0001$; for exon 8, $F_{2,14} = 0.8211$, $P = 0.4601$. **e**, Calbryte 520-based calcium imaging of t-hCO. Slices of t-hCO were incubated with the dye for 1 h and then imaged on a confocal microscope before and after stimulation by 67 mM KCl; scale bar, 100 µm. **f**, Representative traces of responses to Calbryte 520 imaging. **g**, Residual calcium in Calbryte 250-based imaging of t-hCO (PBS treated, $n = 33$; ASO.14, $n = 77$; Mann–Whitney test, two-tailed, $^{***}P = 0.0002$). **h**, Representative images of cell morphology tracing with Golgi staining; scale bar, 50 µm. **i**, Sholl analysis of Ctrl, TS and TS + ASO neurons in t-hCO ($n = 24$ Ctrl t-hCO neurons, $n = 24$ TS t-CO neurons, $n = 11$ TS + ASO t-hCO neurons). Data presented as mean ± s.e.m.

in vivo will benefit from using nontargeting control of ASO rather than simply PBS. Transplantation of hCO allows unpreceded maturation and circuit integration of human neurons into animals. Of note, this therapeutic approach is unlikely to influence cell specification defects that may take place prenatally, but rather will correct channel dysfunction and associated defects postnatally. Future directions include understanding how the TS variant affects circuit development in vivo, how splicing of exons 8/8A is regulated across brain regions in postnatal primate brain and the functional consequences of this switch. This may give insights into whether there is an optimal developmental window for ASO treatment to rescue these cellular phenotypes.

Our proof-of-concept study, which includes a combination of in vitro and in vivo studies with human patient-derived, three-dimensional, multicellular models, illustrates how this platform could be used to study other neuropsychiatric diseases, and to evaluate

therapeutic efficiency and safety, including but not limited to ASOs, viral vectors and small molecules. This will be particularly relevant when animal models are not available or do not fully recapitulate human pathophysiology.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-024-07310-6.

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