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EXECUTIVE SUMMARY

The CDKL5 Forum is the flagship conference for therapeutic development in CDKL5 deficiency disorder (CDD), a devastating rare disorder with neurodevelopmental delay and early-onset, intractable epilepsy. First held in 2015, the Forum assembles clinicians, scientists, and industry leaders from around the world to share the latest findings in research ranging from basic science to gene therapy and clinical trials, all focused on developing treatments and eventual cures for CDD. The Forum is organized by the Loulou Foundation, a private non-profit UK foundation dedicated to advancing research to better understand CDKL5 and develop therapeutics for CDD.

The third annual CDKL5 Forum was hosted for the first time in the United States on November 29-30th, 2017 in Cambridge, MA. Research advances were highlighted through investigator presentations and posters. An industry panel discussion and breakout discussions stimulated brainstorming about the future directions of research and therapeutic approaches. The Loulou Foundation will use conclusions from this meeting to guide funding decisions for the upcoming year.

The Forum featured two keynote presentations with three distinguished speakers: Feng Zhang, David Liu, and James Wilson.

During Day 1, Zhang, Professor at MIT and the Broad Institute, gave an overview of the revolutionary CRISPR gene editing systems and discussed their various applications. He talked about the RNA editing technique REPAIR, which ultimately converts an adenosine to a guanosine. Doing so could modify an RNA sequence of interest and alter protein expression, potentially providing therapeutic benefit.

Liu, Chemical Biology Professor at Harvard University and the Broad Institute, elaborated on two DNA base editing applications of CRISPR: BE3, which effectively creates a cytidine to thymidine conversion; and Adenine Base Editor (ABE), which ultimately converts adenosine to guanosine. Base editing allows the programmable, direct, and permanent conversion of one base pair to another without double-stranded DNA breaks, which decreases the off-target effects.

On Day 2, Wilson, Professor in the Perelman School of Medicine at the University of Pennsylvania, discussed a brief history of AAV-mediated gene therapies and provided insights into their application to the central nervous system (CNS). Adeno-associated virus (AAV) was initially chosen as a vector due to
its efficacy, safety, and ability to confer stable gene expression. Notably, it was used to create Luxturna, the first FDA-approved gene therapy in the United States to target an inherited form of blindness.

However, the CNS is particularly hard to treat due to low blood-brain barrier penetration. One type of AAV, AAV9, naturally targets the CNS and partially permeates the blood-brain barrier. Intracisternal injection gave the most efficient transduction and gene expression in spinal cord motor neurons. This led to the development of AVXS-101, a drug to treat the motor neuron disease Spinal Muscular Atrophy (SMA) Type I.

AAV9-based gene therapies for a type of lysosomal storage diseases called mucopolysaccharidoses (MPS) have shown more global CNS targeting. Wilson said that AAV-mediated treatment for CNS disorders will emerge soon from the successes seen in motor neuron and storage diseases. For CDKL5, basic science questions need to be answered before gene therapy will be successful.

The main Forum program was divided into 5 sections and breakout discussions, covering topics from basic biology to clinical trials and novel therapeutics.

Session 1, ‘CDKL5 Function,’ was moderated by Dario Alessi and discussed the identification and validation of CDKL5 substrates, biological impacts of CDKL5 substrate phosphorylation, and utility of state-of-the-art phospho-specific monoclonal antibodies against CDKL5 substrates as biomarkers to monitor biology.

The CDKL5 kinase is involved in regulating other biological systems and most known pathogenic mutations occur in the kinase domain. Identifying proteins phosphorylated by CDKL5 could clarify CDKL5’s role in signaling pathways and guide treatment development.

Ivan Muñoz identified and validated two CDKL5 substrates: microtubule-associated protein 1S (MAP1S), and centrosomal protein 131 (CEP131). Pathogenic CDKL5 kinase domain mutations did not produce any phosphorylated MAP1S or CEP131 at the identified sites. Therefore, these phosphoproteins could be used as biomarkers for CDKL5 kinase activity, and can be used to validate novel therapeutics that rescue CDKL5 substrate phosphorylation.

Using Kevan Shokat's specific kinase labeling method, Sila Ultanir also identified three CDKL5 substrates. The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

Vera Kalscheuer identified netrin G1 ligand (NGL-1) as a phosphorylation substrate of CDKL5. She went further and identified CDKL5-NGL-1 complex interactors. The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

CDKL5’s presynaptic role in neurotransmitter release was studied via synaptic vesicle recycling. Michael Cousin demonstrated altered clathrin-mediated endocytosis after high frequency stimulation in CDKL5 knockout mouse neurons. Although the CDKL5 knockout neurons were found to have more synaptic terminals performing bulk endocytosis, preliminary data suggested there is no change in the number of bulk endosomes between the knockout and wild type mouse neurons. Questions remain regarding the consequences on neuron function and regulation of endocytic imbalances.

In summary, having a robust CDKL5 phenotype knockout mouse model was identified as the limiting factor for validating relevant therapeutic approaches. There is also currently no good substrate for monitoring the restoration of CDKL5 kinase activity, which would be the ideal therapeutic strategy.

Moving forward, being able to genetically mimic phosphorylated peptides or amino acids would be useful to study and manipulate phosphorylation and assess the impact of any particular phosphorylation target on CDKL5-associated function. Understanding how a phosphorylation event is clinically relevant is crucial and better antibodies against CDKL5 isoforms are needed to specifically study and elucidate the function of each isoform.
Session 2, ‘Model Systems and Phenotyping,’ highlighted the data on and limitations of the various animal models of CDD currently available, including mouse, human iPSC, and organoid models. Peter Kind, who moderated this session, noted that his lab was developing a rat model.

Rodney Samaco reported on the five currently available animal models of CDD. He highlighted the need for natural history studies in mice, the lack of behavioral phenotype data overall, and the utility of data collection standardization moving forward. The emphasis for this type of foundational work was highlighted as an important consideration for the intent of preclinical testing of novel interventions, which he proposed would safeguard against challenges historically reported in other fields of neurodevelopmental and neurological disorders. He showed that exon 6 deletion mice (Zhou lab model) had impaired cued and conditional fear memory, increased pain sensitivity, and some impaired sociability phenotypes. The mouse model from his lab (Cdkl5 c.1412delA, D471fs) appeared to have no overt CDD phenotype and had no visible spontaneous seizures at approximately 5 weeks of age. The mice had reduced contextual fear memory, indicating hippocampal- and amygdala-dependent memory impairment, and hypersensitivity to heat pain, similar to the phenotypes of the exon 6 deletion knockout mouse.

Using the visual system to study early brain development and its impact in CDD is relevant for two reasons: visual processing can be easily studied in both mice and humans, and patients with CDD show visual processing impairment and abnormal eye tracking, said Michela Fagiolini. By combining visual evoked potentials (VEP), resting state functional MRI (rs-fMRI) and diffusion tensor imaging (DTI), Fagiolini and collaborators have begun identifying selective functional and structural impairments in CDD mouse models that can also be evaluated in patients.

Alysson Muotri discussed using organoids to model CDD. Both patient-derived iPSC neurons and CDKL5 knockout iPSC organoids were studied. Initially, CDD patient-derived neurons showed longer processes both in vitro and in vivo in chimeric mice brains, showed reduced synaptogenesis, and had a 50% reduction in calcium transient potential frequency.

Organoids can be studied for over a year and mature over time, allowing for longitudinal developmental analysis. Mutant CDKL5 brain organoids were slightly smaller in diameter and showed an initial increase in electrical activity that plateaued, leading to lower activity later. Mutant CDKL5 neurons also showed global gene expression downregulation; reduced synaptic and excitatory/inhibitory proteins; reduced spine motility and neuronal functionality; and altered mTOR pathway signaling.

In summary, knowing the natural history of CDD in mice will assist in measuring treatment outcomes. Creating more models and standardizing data collection will assist in comparing and confirming results between models. VEP represents a promising clinical endpoint to measure cognitive impairment in patients and mice. Further characterization of brain organoids and using caution when interpreting their data will be key moving forward.

Session 3, ‘Clinical Trials and Clinical Endpoints,’ discussed the current status of CDD-specific and related epileptic treatments, development of a CDD clinical severity scale, and progress on the first CDD clinical trials. The moderator, Orrin Devinsky, remarked that “the pace of progress that the Loulou Foundation has created is really unparalleled in my 30 years in medicine.”

Helen Cross highlighted the history of rare epilepsy treatments and what can be applied to CDD. The mechanisms of anti-epileptic drugs are not always well-defined and surgery that removes portions of the brain remains the main targeted therapy for epilepsies. However, for epilepsy disorders related to gene mutations, like CDD, determining how to directly address the cause would be the ideal treatment. Cross highlighted results from a few clinical trials using repurposed medications to control epilepsy in other related epileptic disorders but cautions that the studies have limitations. She also states that standard randomized controlled trial methodologies are often impractical for rare epilepsies.

The first CDD clinical severity scale was proposed by Tim Benke, who asked for feedback and collaboration. He discussed the four CDKL5 Centers of Excellence, highlighted some challenges of expanding clinical sites, and summarized how a “typical” CDD patient presents, which guided the clinical severity scale
creation. The proposed scale drew from current Rett and epilepsy scales and included multiple criteria that should not saturate in severity.

**Lorianne Masuoka** reported on the findings from and status of the first CDD clinical trial performed by Marinus Pharmaceuticals. Ganaxolone, a neurosteroid synthetic analog, has been previously used in pediatric populations with West and Fragile X syndromes. It has been shown to have anti-seizure, antidepressant, and anti-anxiety activities and to be well-tolerated in both children and adults. At the time of this conference, the Phase 2 trial was ongoing. In the 7 enrolled patients, a 43% reduction in seizure frequency and 78% increase in seizure-free days were seen. Four patients were enrolled into a one-year extension and 2 discontinued due to the lack of efficacy. It could be taken with other anti-epileptic drugs and was well-tolerated, as no serious adverse effects were reported.

Another Phase 2 clinical trial by PTC Therapeutics for epilepsy control in nonsense mutation CDD and Dravet patients was described by **Orrin Devinsky**. Ataluren, a ribosome-binding molecule that allows selective readthrough of premature mRNA stop codons, has been shown to penetrate the blood-brain barrier in mice and be relatively well-tolerated in previous clinical trials. Data to assess its safety and efficacy were beginning to be collected from eight CDD patients at the time of the conference, with top-line results expected to be available before fall 2018.

Devinsky also discussed the use of cannabidiol (CBD) to control seizures in Dravet syndrome and its application to CDD. In collaboration with **Helen Cross**, a Phase 3 clinical trial studying CBD (drug name: epidiolex) in Dravet syndrome patients showed a 26% reduction in convulsive seizures. Although CBD was relatively well-tolerated overall, some serious adverse events occurred more frequently in the treated group. Previous open-label studies with CDD patients showed more than a 50% reduction in convulsive seizures over 48 weeks, but the placebo effect could not be quantified.

In summary, seizure control should not be the only clinical endpoint measured. Establishing a natural history study with a global registry, tracking patients, improving clinical trial methodologies, and standardizing outcomes will all be important moving forward. The CDD clinical severity scale still needs validation in a patient cohort. Phase 2 clinical trial data suggested that ganaxolone is a safe and well-tolerated treatment for CDD and the Phase 3 trial was being developed. The Phase 2 clinical trial assessing ataluren for epilepsy control is underway, as described above. Epidiolex (CBD) has obtained positive results in two Phase 3 clinical trials for Lennox-Gastaut syndrome and one Phase 3 clinical trial for Dravet syndrome. There is some data on effectiveness in CDD, but some adverse effects have been reported.
Session 4, ‘Novel Therapies,’ discussed a variety of therapies either currently being explored for CDD or available for other related diseases. Yael Weiss, who moderated this session, summarized the “translation toolbox” into four therapy categories: up/downstream regulation, replacement therapies, restoring function, and knockdown.

Jeannie Lee first discussed her work in X reactivation to treat Rett syndrome, which could potentially be applicable to other X-linked neurodevelopmental disorders, like CDD. Heterozygous patients have “mosaic” gene expression due to X-inactivation, which randomly silences one of the two X chromosomes in each of their cells. Reactivation of those silent chromosomes could increase normal protein expression, potentially rescuing the disease phenotype.

For Rett syndrome, the combination of a DNA methylation inhibitor (5-Aza-dC) and two Aurora kinase inhibitors caused up to an 800-fold reactivation of MECP2 in a human cell line. Robust X reactivation was also only seen when a combination of inhibitors was used, indicating synergy in X-inactivation. Her lab also created a female Rett mouse model with a severe phenotype mimicking male Rett mouse models. Partial X reactivation in the brain did not seem to affect the mice or their lifespan.

Amicus Therapeutics is developing a protein replacement therapy for CDD, drawing from their knowledge of drug development for lysosomal storage disorders. Hung Do gave an overview of some of the challenges faced during drug development, such as drug dosing, scaling up during manufacturing, and designing and conducting clinical trials. Especially in rare diseases, smaller patient populations lead to smaller clinical trials that may not provide adequate statistical power. Learning from past solutions to these challenges should assist in therapeutic development for CDD.

Frances Jensen examined the interplay between epilepsies and neurodevelopmental disorders. The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

Exploring how to deliver gene therapies to the brain, Jan Nolta highlighted the utility of mesenchymal stem cells (MSCs). MSCs can be readily engineered with viral vectors, remain in the brain for 1-2 months, have a strong safety profile, and have facilitated cargo delivery to neurons through nanotubules or exosomes. Direct injection into the brain or intrathecal injection are required as MSCs do not cross the blood-brain barrier.

In summary, the knowledge from the X-inactivation Rett studies could be applied to CDKL5 studies. Lee is making a female CDKL5 knockout mouse model and plans to study the reactivation compounds identified for MECP2 with CDKL5 as the target. For clinical trials, adequate natural history studies and functional endpoints to demonstrate clear benefit of a drug are needed, especially for rare diseases. An imbalance in excitatory and inhibitory neurotransmitters is thought to contribute to epilepsies and is relevant in CDKL5 deficient mice. Cell and gene therapies are stepping into the spotlight, making them attractive potential therapeutic strategies.

Session 5, ‘The Industry Panel,’ was a panel discussion moderated by Majid Jafar that provided an overview of rare disease research in the biotechnology and pharmaceutical industry. Each panelist also shared how and why they transitioned from clinical to industry careers and how CDD has touched their lives.

When asked what the driving force behind the current industry interest in rare diseases was, Jeremy Levin reflected on where the immune-oncology field was about 10 years ago. With little interest from large companies, a few smaller companies were pushing the science forward, eventually leading to breakthroughs that catalyzed the currently flourishing industry. He said that similar breakthroughs in scientific understanding and technology could bring a similar change for rare neurological diseases.

Philip Reilly highlighted the impact of patient groups in pushing rare diseases forward. From the pharmaceutical company perspective, Omar Khwaja said that Roche is committed to rare diseases due to a higher confidence in the disease target, an expanded therapeutic toolbox, and the changing shape
of the pharmaceutical industry. Andrew Plump added that Takeda puts the patient at the center of everything they do, which is why they partner with smaller companies to drive rare disease research forward.

To help accelerate rare disorder treatment development, collaboration, partnership, and the power behind the patient groups are key, Khwaja said. Putting patient needs first is also extremely powerful for capturing capital and attention for the disease. When a venture capital firm is looking to invest in a rare disease company, Reilly said that they focus on developing biological processes that could help a wide range of disorders. Levin added that biotechnology companies facilitate drug discovery because there is a smaller distance between them and the patient, so their constant focus is on how to benefit the patient. Plump commented that some pharmaceutical companies are turning away from neurological diseases because of the large intrinsic risks and safety databases required for clinical trials. Rare diseases tend to be easier to work with, but only if the molecular biology is deeply understood in relation to the patient.

**IN CONCLUSION**

A review of the progress reported at the 2017 Forum on the basic and clinical research in CDD illustrates that while much has advanced since the last CDKL5 Forum, providing hope and excitement to the community, there are many important challenges remaining. On the topic of CDKL5 function, proteins which are downstream kinase targets for the kinase have been identified, but it remains to be seen how, or if, these proteins are involved in the pathology of the disorder, or how they can be used as clinical biomarkers for restored CDKL5 function. The rapid developments in the field of genome editing have the potential to transform our approach to genetic disorders, but technical hurdles on the proper balance between efficacy and off-target activities of these novel modalities still need to be resolved. Finally, for therapeutic approaches using viral delivery, the effective delivery of the gene therapy remains to be addressed.

In clinical studies, important milestones are planned for the coming year, including a phase 3 trial for Marinus Therapeutics’ ganaxolone, representing the first phase 3 clinical trial with CDD as the targeted indication, as well as investigator-initiated trials that are ongoing or planned for several other new therapeutics. These are likely to include seizure reduction among their primary clinical endpoints; however, more work is needed to define and validate clinical outcome measures for complex symptomatic domains beyond seizure counts, such as cognitive, motor, and visual function. Research continues on these outcome measures, as well as on establishment and validation of clinical biomarkers and non-invasive functional measures such as EEG and MRI, as reported by several investigators at the Forum.

The partnership between scientists, clinicians, companies, and patient groups creates the unique atmosphere of the CDKL5 Forum. The Loulou Foundation set the ambitious goals of having treatments by 2020 and cures by 2025. With the rapid progress made over the past few years, those goals are getting steadily more achievable. Thank you to everyone working to turn these goals into realities.
INTRODUCTION

On November 29-30th, 2017, the Loulou Foundation hosted the third annual CDKL5 Forum in collaboration with the Orphan Disease Center at the University of Pennsylvania at the Royal Sonesta Boston Hotel in Cambridge, MA.

The meeting hosted over 180 attendees from over 40 academic institutions, 35 pharmaceutical and biotech companies, and patient advocacy groups from six countries. Presentations and posters from investigators provided research updates and breakout discussions facilitated brainstorming about current and future therapeutic approaches for CDKL5 deficiency disorder (CDD).

Majid and Lynn Jafar, co-founders of the Loulou Foundation, welcomed the attendees: “This promises to be the largest and most engaging meeting we have ever had. [...] By accepting our invitation and being here, you’ve shown your commitment and are automatically a member of the CDKL5 Forum.”

Majid highlighted the CDKL5 Forum online portal (www.cdkl5forum.org), which he encouraged researchers to use regularly to “keep the conversation going for the other 363 days of the year.” Personal usernames should have been found on the back of each name tag and the initial password is “hopelovecure”. A new CDKL5 Forum app was also introduced this year with meeting program information.

As in previous years, the conclusions from this meeting will guide the Loulou Foundation’s funding decisions for the upcoming year. The Foundation awarded 10 new CDKL5 Program of Excellence pilot grants since last year. So far, the Foundation has funded 25 projects in 34 labs at 26 institutions and several ongoing corporate programs.

CDD is a rare X-linked genetic disease affecting over 1,600 children worldwide, about 80% of whom are girls. Patients carry a mutation in the cyclin-dependent kinase-like 5 (CDKL5) protein, which is essential for normal brain development. CDD was initially thought to be an early-onset form of Rett syndrome due to similar symptoms, including seizures, severe cognitive deficits, visual impairment, and sleep disturbances. However, the definitive association of CDKL5 gene loss-of-function mutations with this disorder has permitted CDD to be recognized as a distinct clinical entity and has allowed for a more precise description of its specific clinical manifestation.
KEYNOTE ADDRESSES

On the first day, James Wilson hosted the keynote session on genome editing with lectures from Feng Zhang and David Liu. Wilson gave the keynote address on the second day about gene therapy.

EXPANDING THE GENOME EDITING TOOLBOX
FENG ZHANG, Broad Institute/MIT

Rapid advances in DNA sequencing have paved the way for identifying disease-causing mutations. Precisely altering those mutations back to the wild type gene via genome editing presents a potentially revolutionary way to treat these diseases. The latest genome editing technology has leveraged the microbial immune systems known as CRISPR-Cas (clustered regularly-interspaced short palindromic repeats (CRISPR)-CRISPR associated). The first CRISPR-Cas system harnessed for genome editing uses the Cas9 nuclease, which specifically cuts DNA at a sequence complementary to a small RNA molecule (called a guide RNA) complexed with Cas9. Upon sequence recognition via RNA-DNA base pairing, Cas9 will be activated and create a double-stranded DNA break. This break will trigger one of two DNA repair processes, which could allow efficient and precise gene changes to be made. Cas9 has also been engineered to create a so-called dead variant (dCas9) that no longer functions as a nuclease; it is a specific DNA binder to which other enzymes can be attached. A dCas9-enzyme complex could be directed by guide RNAs to a specific location on the chromosome to turn expression of the adjacent gene on or off or to precisely change (or even add) a DNA base.

There are two major classes of CRISPR systems based on the proteins needed for functionality: class I, which uses a multi-protein complex, and class II, which uses a single protein. There are three main class II systems: two DNA-targeting systems (Cas9 and Cas12/Cpf1) and one RNA-targeting system (Cas13), which was recently discovered. This novel system was characterized by transferring it into E. coli to study and verify its RNA cleavage function. These experiments showed that, analogous to Cas9, which requires a protospacer-adjacent motif (PAM) for targeting, Cas13 requires an activating motif (in this case, C, A, or U) flanking the complementary RNA sequence.

The CRISPR-Cas13 system was harnessed to create a diagnostic platform that can detect the presence of bacteria, viruses, or specific genetic mutations. Another application based on Cas13 is a technique called REPAIR (RNA editing for programmable A to I replacement). By converting the base adenosine (A) to inosine (I), which is read by the ribosome as a guanosine (G), REPAIR has the potential to correct pathogenic G to A changes; for example, some patients with Rett syndrome harbor G to A changes in the MECP2 gene. Adenosine deaminase (ADAR) was attached to dead Cas13 (dCas13), which can specifically bind the mRNA sequence of interest. To specify the target A, the guide RNA encodes a C at the complementary position to create a mismatch, which makes a bubble around the target A, allowing ADAR to access that base. The first generation of this tool exhibited many off-target mutations, but by modifying ADAR residues involved in non-specific RNA interactions, the specificity was increased to the point that there were almost no off-target mutations.

When asked about his opinion on how to detect and assess the off-target mutations, Zhang discussed two areas of progress: detection methodology, such as cell-free genomic DNA treatment, to determine
all possible off-target locations; and engineering Cas9 to be more specific. “A system is only as specific as we are able to evaluate. [...] and continued testing in animal models as well as clinical trials will be required to understand the full safety profile,” Zhang said.

Future directions include increasing specificity and efficacy, expanding base conversion activity, establishing delivery systems for in vivo applications, and utilizing animal models. Regarding his expectations for the pace of CRISPR-based therapeutics, Zhang commented “We can’t rush into it, but we do want to move it forward as quickly as possible in a thoughtful and careful way so that we can begin helping people.”

**BASE EDITING: CHEMISTRY ON THE GENOME OF LIVING CELLS**

**DAVID LIU, Broad Institute/Harvard University**

About two-thirds of all known human genetic variations associated with disease are point mutations. As Liu highlights, “the irony though is that standard genome editing methods actually have difficulty correcting point mutations.” Base editing tends to be inefficient because the double-stranded DNA breaks generated get fixed via one of two pathways: end-joining, the most common method that results in either insertions or deletions (indels) at the break site; or homology-directed repair (HDR), the less common method that uses a donor DNA template to fill in the complementary bases in the double stranded break. Unfortunately, HDR is less efficient than end-joining and requires cellular machinery that is poorly expressed in non-dividing cells. It would be ideal to chemically convert one base to another without making a double-stranded DNA break.

The initial scheme involved tethering a DNA-modifying catalyst to a programmable DNA-binding protein. However, DNA modification typically occurred on a large span of nucleotides rather than a few nucleotides, causing the cell to recognize the newly converted base as a mismatch and attempt to correct it back to the original base. Liu’s group tethered dCas9 (a selective DNA binder), a cytidine deaminase (an enzyme that catalyzes a C to U base conversion), and a uracil glycosylase inhibitor (a molecule that prevents the excision of U from DNA), effectively creating a C to T mutation. By taking advantage of the single-stranded DNA bubble created by dCas9, a single-stranded DNA-specific cytidine deaminase was used to minimize off-target conversion. This system, named BE3, can efficiently and selectively convert a C-G base pair to a T-A base pair. This could be used to create stop codons from the ubiquitous Arg, Gln, and Trp codons, creating early stop codons and efficiently introducing a nonsense mutation. The cleanliness of the BE3 base editing system is attractive compared to the mixture of products produced from repair of a double-stranded DNA break.

Subsequent work has focused on:
- Improving purity and editing efficiency
- Maximizing genome-targeting breadth
- Reducing off-target base editing
- Establishing post-mitotic somatic cell base editing in vivo
- Developing editors that convert other bases

A novel base editor, named Adenine Base Editor (ABE), performs an A to G base edit, converting an A-T base pair to a G-C base pair. As Liu remarks, this edit is a major source of de novo mutation because “cytidine spontaneously deaminates to uridine about 300 times a day in every one of your cells.” About 50% of all pathogenic point mutations are caused by G-C to A-T base pairs and would be corrected by such an ABE.
If a deoxyadenosine deaminase is fused to the base editor scaffold, then an A could be selectively converted to an I, which would be read as a G. Unfortunately, there is no known adenosine deaminase for DNA, so the Liu group decided to evolve a novel enzyme. They chose *E. coli* TadA as the beginning enzyme, generated a library of mutant enzymes attached to dCas9, and screened the mutant enzyme-dCas9 complexes in *E. coli* against a mutant antibiotic resistance gene, which needed to have an A-T base pair converted to a G-C base pair to become functional. The evolved enzymes would be tested in human cells for activity. After multiple rounds of screening, the best enzyme (ABE7.10) averaged over 50% editing of sites with various sequences. Upon editing a wide range of sites in the human genome, the average editing efficiency was 53%. ABE-mediated base editing is remarkably clean because there is no natural DNA adenosine deaminase, so enzymes that remove I from DNA are not very abundant or active. For comparison, Cas9-mediated A-T to G-C mutation gives 4.2% conversion with 10.6% indels, whereas ABE-mediated mutation gives 55-68% conversion with <0.1% indels. Therefore, the selectivity of ABE conversion is over 1,000 times greater than Cas9 conversion. As for applications, ABE editing in the promoter region of the fetal hemoglobin gene leads to constitutive expression, which could compensate for lack of hemoglobin in disorders such as beta-thalassemias. ABE was also used to correct a G-C to A-T mutation in *HFE* in hereditary hemochromatosis.

In summary, base editing allows the programmable, direct, and permanent conversion of one base pair to another without double-stranded DNA breaks, donor templates, or homology-directed repair. Efficient base editing in human cells can be achieved by manipulating base excision and repair processes. Delivery methods can affect specificity, with transient RNA and protein delivery giving increased specificity. With the recent developments of ABE, all four transition mutations (C to T, T to C, A to G, and G to A) are possible, representing about two-thirds of all pathogenic point mutants. Liu emphasized that, although base editing is not ready for clinical use quite yet, development of the chemical machinery to selectively and efficiently perform base editing is an important start.

**AAV-MEDIATED GENE THERAPY IN THE CENTRAL NERVOUS SYSTEM**

**JAMES WILSON, University of Pennsylvania**

“To summarize where we are with respect to the technology of gene transfer, I think it’s ready for primetime for diseases that have a neurologic basis,” Wilson remarks.

It is difficult to deliver genes as therapeutics to the correct cells, get the genes into the nucleus, and have the genes be stable after delivery. Initially, viruses were chosen as shuttle vehicles for delivery, specifically adeno-associated virus (AAV). There are 6 known serotypes, with AAV2 being the initially chosen as a vector due to its efficacy, safety, and ability to confer stable gene expression. In theory, stable gene expression would enable a one-time therapy that could provide lasting genetic changes, and thus therapeutic benefits.

One of the first applications of AAV gene delivery was to restore sight to patients with inherited blindness due to retinal cell defects. To overcome the problem of delivery, the viral therapeutic was injected directly under the retina, causing a transient retinal detachment and allowing AAV to penetrate nearby retinal cells.

Jean Bennett began that work and Wilson went on to highlight her career milestones developing curative therapies for congenital blindness. In 1994, Wilson and Bennett demonstrated that mouse retinal cells could be engineered by gene delivery. AAV was used to engineer monkey retina using a fluorescent reporter in 1999, followed by a proof-of-concept canine model in 2001. Showing safety and efficacy in a large animal model was crucial to de-risking gene therapy. In 2008, results from a Phase 1 clinical trial demonstrated partial regain of sight in Leber Congenital Amaurosis Type 2 patients. The drug, Luxturna, was licensed to Spark Therapeutics for efficacy studies and is currently being reviewed by the FDA for approval.

(On Dec 19th, the FDA approved Luxturna, making it the first gene therapy approved in the United States to target an inherited disease.)
Although AAV2 worked efficiently when directly injected at the target sight, the issue of broader delivery was still a challenge. Work done from 2002-2004 with GlaxoSmithKline led to the development of novel AAV platforms by studying other AAV serotypes. Taking advantage of the normal target tissue of each serotype can allow development of specific vectors. Specifically, AAV9 and AAVrh10 were found to target the central nervous system (CNS) and AAV8 targets the liver.

The liver was chosen as the first whole organ target due to its unique pore-containing capillaries, into which the viral vectors could directly penetrate. Hemophilia, an X-linked bleeding disorder, was chosen because patients lack expression of certain clotting factors produced in the liver. A prophylactic protein replacement therapy is currently available which infuses a recombinant version of the clotting factors into the blood. However, clotting factor levels fluctuate as they are broken down, requiring administration three times per week. This leads to a high financial burden, costing a patient approximately $300,000 per year.

By using the AAV8 viral vector to deliver the clotting factor gene to the liver, a one-time dose should provide sustained therapeutic levels of protein in the patient. This would drastically lessen the financial burden and make therapy less time consuming. In 2005, AAV8-mediated delivery of the gene encoding Factor IX was tested in a canine model of Hemophilia B, showing sustained therapeutic levels of Factor IX throughout the lifetime.

The AAV8 vector was provided to labs at the University College London and St. Jude Children’s Research Hospital for clinical trial development. Phase 1 trial results showed a one-time injection of AAV8-Factor IX gave sustained Factor IX levels in Hemophilia B patients. Currently, there are about 8 companies developing gene therapy for hemophilia.

Unlike the liver, the CNS has two main drug delivery problems: getting drugs across the impenetrable blood-brain barrier into the brain and scaling the drug dosage appropriately based on animal models due to the vast heterogeneity of brain sizes between mice and humans. CNS delivery could be achieved directly via two intrathecal approaches: lumbar puncture or intracisternal injection. Ultimately, an intravenous (IV) infusion would be ideal, but blood-brain barrier penetration would be required.

AAV9 can partially permeate the blood-brain barrier via binding to the terminal galactose of a unique receptor on the blood side. Wilson speculated that the AAV9-receptor complex undergoes transcytosis to the brain side of the barrier. AAV9 delivery to various brain subsections and organs were evaluated in mature macaques through either IV injection, cisterna magna injection, or lumbar puncture. At best, IV injection gave uptake in 10% of brain and spinal cord tissue with expression likely being lower. A 10-fold lower dose via cisterna magna injection gave 10 times more uptake than seen with IV injection. Unfortunately, lumbar puncture administration was not efficient, showing uptake in up to 1% of brain and 10% of spinal cord tissue. Overall, both cisterna magna injection and lumbar puncture gave lower uptake in the heart, lung, and liver, indicating lower off-target effects.

Immunizing the host against AAV does not affect CNS gene transfer via intrathecal injection, but it completely blocks peripheral gene transfer. Intracisternal injection showed efficient transduction and gene expression in spinal cord motor neurons, the hippocampus, and the cerebellum. The cerebral cortex showed heterogeneity, with some highly expressed regions and other regions with very little gene expression.

Spinal cord motor neuron transduction following the various administration routes was examined between infant and adult macaques. In adults, intracisternal injection achieved up to 60% transduction while lumbar puncture only achieved less than 20% transduction. Remarkably, transduction levels after all 3 routes of administration were between 60-80% in infants. The doses were extremely high; in fact, enough vector for IV administration in adults could not be made and such doses might cause severe toxicity via inflammatory response, indicating the lack of clinical practicality.

Because AAV9 was so efficient at targeting motor neurons, treatment for the motor neuron disease Spinal Muscular Atrophy (SMA) Type I was developed. SMA Type I patients carry mutations in the
SMN1 gene and do not produce functional SMN1, leading to muscle degeneration. Delivering SMN1 via modified AAV9 into the spinal cord of infant macaques gave over 90% motor neuron transduction. However, when higher doses were used, severe systemic inflammation was seen in 1 out of 3 macaques. In collaboration with a group at Nationwide Children’s Hospital, IV injection of AAV9-SMN1 in a mouse model showed complete survival in mutant mice who otherwise would have died prematurely. In a Phase 1 clinical trial with SMA Type I patients, a low dose (3 patients) or high dose (12 patients) of AAV9-SMN1, called AVXS-101, was delivered as soon as they were diagnosed (all before or around six months old). Remarkably, all patients survived past the anticipated 12-15 months without treatment, despite the over 75% mortality rate typically expected. Only one patient in the low dose cohort experienced a pulmonary event that required intervention. The CHOP INTEND score was used to assess motor function, which revealed no improvement in the low dose cohort and improvement in all but one patient in the high dose cohort. Earlier in November, a follow up report showed that most patients hit their motor milestones, with a majority able to talk, roll over, and control their head (*NEJM*, 377:1713, 2017). An intrathecally-delivered antisense oligonucleotide product from Ionis Pharmaceutical and Biogen, called Spinraza, was also brought forward recently, providing two options for a previously devastating disease. Wilson added that, although both show very impressive clinical data, the high transduction efficacy of a viral vector in motor neurons is beneficial.

“This is the kind of result, when I thought about gene therapy back as a graduate student, that I had hoped gene therapy would produce: a truly transformative, disease-altering therapy in a group of patients that really had nothing else available to them,” Wilson shared.

Although targeting motor neurons was simple, there are only a limited number of diseases originating in the motor neurons. To pursue global CNS disease treatment, a group of lysosomal storage diseases, called mucopolysaccharidoses (MPS) was studied. The non-CNS symptoms are currently treated with enzyme replacement therapy, but the only treatment for CNS symptoms is a bone marrow transplant, which had limited success. To affect the majority of neurons in the brain, the bystander effect was utilized, where a few cells would be corrected and promote cross correction of neighboring cells. In a canine model of MPS VII, intracisternal administration showed gene transfer in a majority of cells in the frontal cortex, cerebellum, and spinal cord as well as decreased ganglioside storage comparable to wild type. A recent review highlighted active clinical trials using AAV9 gene therapy in neurological disorders (*Science*, 358:582, 2017).

A variant of AAV9, called AAV PHP.B, was identified as a potent vector for widespread gene transfer to the adult mouse brain administered via IV infusion (*Nat Biotechnol*, 34:204, 2016). AAV PHP.B was 50-times more potent than AAV9 transfer in C57BL/6 mice. Wilson’s group replicated those results in C57BL/6J mice but failed to see any transfer in BALB/cJ mice and saw some transfer in CB6F1/J mice. Therefore, the increased transduction of AAV PHP.B is unique to C57BL/6J mice. Whole genome sequencing analysis is being conducted to determine the basis of this difference. Accordingly, neither high nor low doses of AAV PHP.B in macaques showed increased transduction compared to AAV9.

Applying lessons from previous gene therapies towards developing a CDKL5 gene therapy poses the following questions:
• What are the target cells and how many need to be corrected to be therapeutic?
• What level of transgene expression is important?
• What is the therapeutic gene?
• Are there animal models and what type of safety data are available?
• What clinical endpoints are available to measure efficacy?

There are a handful of basic science questions that need to be answered before gene therapy would be successful in CDD patients.

Wilson discussed some unpublished results regarding AAV-mediated gene therapy for CDD. The full results summary will be disclosed in an amended report upon publication.

In summary, the first wave of AAV-mediated treatment for CNS disorders will emerge soon from the successes seen in motor neuron and lysosomal storage diseases. For CDKL5 specifically, the above basic science questions need to be answered to have better success with gene therapy; defining the target cells will define the delivery challenges. Preliminary work has shown that non-specific transgene expression is non-toxic, but more work is needed. However, initial pre-clinical gene therapy studies should be pursued simultaneously.

During the question session, Zika virus was proposed as a potential CNS vector. Wilson commented that recombinant viruses typically trigger an immune response and AAV was chosen because it is uniquely anti-inflammatory.

The psychological impacts on patients with CDD who receive gene therapy in the future were also discussed. A question was posed about how reintroducing functional CDKL5 in patients’ brains will affect them, and how we can help them cope with the presumed increased neurological function. Wilson related this to the congenital blindness study, where it was unclear whether the brain could process sight even though the child had never had such sensory input. Adrian Bird’s 2007 Rett syndrome paper was also mentioned because it noted that when the Rett gene was reintroduced too quickly, the mice did poorly and did not tolerate the acute change in functionality (Science, 315:1143, 2007). Neurodevelopmental disorders are network diseases that change over time, so thinking about how to deliver gene changes gradually could be beneficial to promote readjustment.
Moderator: Dario Alessi, University of Dundee
The key points to address regarding CDKL5 function were:
• identification and validation of CDKL5 substrates
• biological impacts of CDKL5 substrate phosphorylation
• utility of state-of-the-art phospho-specific monoclonal antibodies against CDKL5 substrates as biomarkers to monitor biology and as therapeutics
• determination of the most relevant substrates to understanding CDD

IDENTIFICATION AND VALIDATION OF THE FIRST PHYSIOLOGICAL SUBSTRATES OF THE CDKL5 KINASE MUTATED IN CDKL5 DEFICIENCY DISORDER
IVAN MUÑOZ, University of Dundee

Most pathogenic CDKL5 missense mutations reside in the kinase domain, indicating the relevance of kinase functionality in CDD. Determining the function of CDKL5 mutations is critical to understand whether they are loss- or gain-of-function mutations. By identifying proteins that are phosphorylated by CDKL5, we could understand its role in relevant signaling pathways and develop treatments involving those substrates.

Differences in global phosphorylation between CRISPR/Cas9 CDKL5 knockout cells and CDKL5 overexpressing cells were examined. Phosphorylated proteins found exclusively in the presence of CDKL5 were labeled using tandem mass tag (TMT), identified and quantified via HPLC-MS/MS. Many substrates were found to be involved in cytoskeletal formation, microtubule dynamics, and primary cilia regulation, biological processes that play an important, yet poorly understood, role in brain development. Muñoz suggested that CDKL5 may be involved in ciliogenesis, making CDD a possible type of ciliopathy.

Of the substrates identified, Muñoz discussed the validation of two hits: microtubule-associated protein 1S (MAP1S, sequence RPL$^{800}$AR), and centrosomal protein 131 (CEP131, sequence RPG$^{35}$AATTKP). CDKL5-dependent MAP1S and CEP131 phosphorylation was demonstrated in HEK293 cells. Mutant CDKL5 with pathogenic kinase domain point mutations failed to phosphorylate MAP1S or CEP131 at the identified sites, indicating loss-of-function mutations. Screening for small molecules or gene deletions that rescue CDKL5 substrate phosphorylation via phospho-specific antibodies against MAP1S and CEP131 could help identify novel therapeutics.

Future work includes understanding the functional consequences of CDKL5-dependent MAP1S and CEP131 phosphorylation, determining the role of CDKL5 in ciliogenesis, examining the CDKL5 activation and regulation mechanisms, and defining the role of CDKL5 in the nucleus, particularly in DNA repair. Determining the role of MAP1S phosphorylation in neurons using phospho-specific antibodies will be important.
PRESYNAPTIC DYSFUNCTION IN CDKL5 DEFICIENCY DISORDER
MICHAEL COUSIN, University of Edinburgh

Synaptic vesicles are recycled by a number of different endocytosis modes, which are triggered by different patterns of neuronal activity. These endocytosis modes are essential to maintain neurotransmission therefore, the study of synaptic vesicle recycling provides insight into CDKL5’s presynaptic role in neurotransmitter release. Clathrin-mediated endocytosis was studied using synaptophysin-pHluorin (syp-pH), a pH-sensitive GFP fused to the vesicle protein synaptophysin. The fluorescence of syp-pH increases upon vesicle fusion during neurotransmitter release into neutral medium and is non-fluorescent in the acidic vesicle after endocytosis. This assay can measure both the number of vesicles being fused and the speed of a specific endocytosis mode called clathrin-mediated endocytosis. There was no difference in the total number of vesicles either fused or retrieved at either low (10 Hz) or high (40 Hz) stimulation frequencies. However, during high frequency stimulation, the CDKL5 knockout neurons displayed altered clathrin-mediated endocytosis.

During intense activity (i.e. high frequency stimulation), activity-dependent bulk endocytosis (ADBE) is the dominant mode of synaptic vesicle endocytosis. Using a fluorophore-labelled large dextran, ADBE can be studied exclusively because the dextran is too large to fit into the smaller vesicles created using clathrin-mediated endocytosis. CDKL5 knockout neurons were found to have more terminals performing ADBE compared to wild type neurons in both mouse and rat hippocampal cultures. However, preliminary data suggested that there is no change in the number of bulk endosomes formed by ADBE in wild type and CDKL5 knockout neurons.

Cousin discussed some unpublished results from a Huntington’s disease mouse model. The full results summary will be disclosed in an amended report upon publication.

ADBE is hypothesized to limit neurotransmission during high frequency stimulation because bulk endosomes take longer to form functional vesicles compared to clathrin-mediated endocytosis. ADBE
is also regulated via glycogen synthase kinase 3 (GSK3), raising the question of whether GSK3 is hyperfunctioning in CDKL5 knockout neurons.

Questions regarding increased ADBE in CDKL5 knockout models remain:
• What is the mechanism - i.e., is there a decreased threshold for triggering ADBE or is the balance of inhibitory and excitatory neurons different in CDKL5 knockout cells?
• What are the consequences on neuron function - i.e., are short-term plasticity or circuit activity altered?
• How can we correct it - e.g., modulate GSK3 function or find new molecules to study, or regulate ADBE?

IDENTIFICATION OF NOVEL CDKL5 COMPLEX PARTNERS AND KINASE SUBSTRATE CANDIDATES
VERA KALSCHEUER, Max Planck Institute, Berlin

In 2003, Kalscheuer’s group linked CDKL5 gene truncation to a clinical phenotype of infantile spasms and mental retardation by genetic mapping. Also, disruption of the netrin G1 gene was identified in a patient with intellectual disability and other clinical features related to those found in CDD. The protein interactor for netrin G1, netrin G1 ligand (NGL-1), was identified as a phosphorylation substrate of CDKL5. The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

During the discussion after the presentations, the limiting factor and key for validating relevant therapeutic approaches was identified as the lack of a robust CDD phenotype in knockout mouse models. Current models show mild symptoms compared to the human phenotype in the assays tested to date. There is also currently no good substrate for monitoring the restoration of CDKL5 kinase activity, which would be the ideal therapeutic strategy, whether by gene therapy, genome editing, or small molecule approaches. CDKL5 is expressed ubiquitously in the brain, especially in the frontal cortex and hippocampus, with the total levels increasing during development and maintained in adults. CDKL5 is known to be shuttled from the cytoplasm into the nucleus, but its nuclear function is relatively unknown - could it be contributing to the detection and repair of DNA damage?
Moving forward, being able to genetically mimic phosphorylated peptides or amino acids would be useful to study and manipulate phosphorylation and assess the impact of any particular phosphorylation target on CDKL5-associated function. Understanding how a phosphorylation event is clinically relevant is crucial. Examining various cell types containing CDKL5 at different developmental stages could identify where and when CDKL5 is most active. Better antibodies against CDKL5 isoforms are needed to specifically study them and elucidate a function of each. Examining other CDKL proteins may give some insights into their function and specific substrates, how CDKL5 differs functionally from these other CDKL family members, and whether increasing expression of these other CDKL family members might be a viable therapeutic strategy.

To conclude, Alessi posed the following questions that remain to be answered:

• What are the pre- and post-synaptic mechanisms of CDKL5 functions in the neural network?
• How does understanding CDKL5 function converge on therapeutic strategies?
• How is CDKL5 function and regulation different in the subcellular compartments?
• Could we assign functions to different CDKL5 isoforms generated by alternative splicing or promoter usage?
SESSION 2: MODEL SYSTEMS AND PHENOTYPING

Moderator: Peter Kind, University of Edinburgh

Kind opened by saying that “previous sessions have highlighted how we need good preclinical models to translate fundamental research into real clinical effectiveness.” New genetic editing techniques allow for easier creation of novel, non-rodent animal models, paving the way for a rapid period of development for pre-clinical models. This session will first highlight mouse models followed by human iPSC models. Kind also noted that his lab was developing a rat model, which was covered in several posters at the conference.

IMPROVING THE IDENTIFICATION OF REPRODUCIBLE AND RELIABLE PHENOTYPIC ENDPOINTS: EARLY FINDINGS FROM A NATURAL HISTORY STUDY OF A MOUSE MODEL OF CDKL5 DEFICIENCY DISORDER

RODNEY SAMACO, Baylor College of Medicine and the Jan and Dan Duncan Neurological Research Institute (NRI) at Texas Children’s Hospital

Systematic and in-depth characterization of existing Cdkl5 rodent models is key for comparison to human genetic variants. Natural history studies evaluating phenotypes of the model over the animal’s lifespan will gather meaningful information, such as sex-specificity, differences between sex, when the onset of the disease is, and how the disease progresses. Multiple animal models, including other species, are needed because each has their own values and limits. Samaco highlighted the creation of a tissue repository for distribution as part of a larger effort at NRI, which should be available next year. Currently, there are five CDD mouse models:

- Cdkl5\textsuperscript{Δex6} from the Zhou lab (Wang, 2012)
- Cdkl5\textsuperscript{Δex4} from the Gross lab (Amendola, 2014)
- Cdkl5\textsuperscript{Δex2} from the Tanaka lab (Okuda, 2017)
- Cdkl5\textsuperscript{c.175C>T} (R59X) from the Zhou lab (unpublished; results presented by Jensen on 11/30)
- Cdkl5\textsuperscript{c.1412delA} (D471fs) from the Samaco lab (unpublished)

Behavioral phenotypes in mouse models of CDD are sparse. To date, rotarod findings from the exon 6 and 4 deletion models are consistent; learning and memory tests are replicable for the exon 4 deletion model, but the data are from one lab; and the mice’s general health seems mostly normal with some evidence of hindlimb clasping. However, home cage activity findings in the initial reports from the Zhou and Gross labs appeared different between the exon 6 and 4 deletion models and the genetic backgrounds may be different due to different breeding schemes. Standardization without limiting innovation and transparent reporting about how assays are performed will be crucial moving forward.

Identifying at least one phenotypic readout of CDD should be useful for preclinical studies. Samaco highlights the utility of the Biomarkers, Endpoints, and other Tools (BEST) Resource published by the FDA and NIH on how to translate potential biomarkers and outcome measures from animal models to humans.

The following data were collected on the exon 6 deletion mice. The open field activity showed increased spontaneous exploratory activity in male mice at both early and later time points, but female mice only showed increased activity at the later time points. Therefore, “CDD causes increased activity in the
open field,” Samaco commented. Both cued and conditional fear memory in male mice were impaired, in agreement with the original 2012 report (PNAS, 109:21516, 2012). Counterintuitively, the mice had a reduced latency corresponding to increased pain sensitivity. Mice spent equal time in the chamber of the 3-chamber test, indicating impaired sociability, but the mice investigated partner animals more than novel objects (time at the cup was different), indicating normal sociability. Female mice had reduced prepulse inhibition of the acoustic startle response only at the second time point whereas males did not have a detectable phenotype.

D471fs mice do not differ from wild type mice in body weight and display no observable spontaneous seizures. There was no detectable expression of either full length or truncated protein and there seemed to be no off-target mutagenesis from CRISPR/Cas9 editing. Therefore, the D471fs frameshift mutant CDKL5 seems to function like a null mutant. At 4-5 weeks old, both male and female mice have reduced contextual fear memory, which is consistent with previously found phenotypes at a younger age, and are hypersensitive to thermal pain. Samaco said, “overall, the data do confirm that there is impaired hippocampal-dependent memory.”

In summary, natural history studies of animal models will provide insight into the disease biology, model phenotypes, and the model’s pre-clinical utility. “Modeling the analysis of model organisms after human natural history studies will be very important to tease apart the developmental and group-level changes, as well as confirm [these changes] by cross-sectional [analysis],” Samaco concludes.

When asked whether the discrepancy in the mouse and human models highlights something functional about the translation research process, Samaco pointed out the utility of different models, including rat, to be able to identify species-specific versus evolutionarily conserved CDKL5-dependent phenotypes.

CALLOSAL REFINEMENT IN CDKL5 DEFICIENCY DISORDER
MICHELA FAGIOLINI, Boston Children’s Hospital

Early development can be studied using the visual system as an entry point. This is relevant because CDD patients show visual processing impairment, sideways glancing, and abnormal eye tracking. Visual processing can be measured in mouse and human development using the same recording techniques.

Ongoing work shows that exon 6 deletion mice (Zhou lab) exhibit delayed and impaired visual development as well as more callosal inputs, indicating improper refinement. Resting state functional MRI (rs-fMRI) and diffusion tensor imaging (DTI) in the adult knockout mice indicate a significant over-connectivity in the callosum between sensory cortices. Reduced visual evoked potential (VEP) response and acuity was seen in both exon 6 (Zhou lab) and exon 4 (Gross lab) knockout mice, indicating a reproducible endpoint. Both DTI and VEP data are just beginning to be collected in patients.

Fagiolini discussed some unpublished results; the full results summary will be disclosed in an amended report upon publication.
To establish a human-relevant *in vitro* culture system to study the impact of CDKL5 mutation on neuronal function, iPS cell lines were derived from biopsies of CDD patients. In addition, CRISPR-edited iPS cell lines in which wild type CDKL5 was mutated to carry pathological mutations were used to confirm results and provide true isogenic control cell lines. All cell lines had homogenous CDKL5 knockout.

The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

During the ending discussion, Fagiolini commented that although organoids are very powerful, she cautioned against comparing them directly to a post-natal brain. Longitudinal trajectory analysis from the organoids will be useful in studying very early development and guiding other model analyses. Muotri replied that organoids really represent fetal development and changes seen at very early stages, which is hard to predict the translatability to the adult brain. Exercising caution when comparing organoids to mouse models will be important.
SESSION 3: CLINICAL TRIALS AND CLINICAL ENDPOINTS

Moderator: Orrin Devinsky, New York University

“It’s nice for those of us in clinical medicine to be moving from cells and organoids and various animal models to humans. [...] We will see some translational examples of how we try to bring some of the basic science findings into clinical medicine,” Devinsky opened.

OPTIMISING THERAPEUTIC DEVELOPMENT IN RARE EPILEPSIES: LESSONS FOR CDKL5 DEFICIENCY DISORDER
HELEN CROSS, University College London/Great Ormond St Hospital

Looking at the progress of epilepsy treatment over the past 100 years, there are now significantly more anti-epileptic drugs available, yet the outcomes may not be different, especially in early-onset epilepsies. The mechanism of many anti-epileptic drugs was discovered after being used in the clinic. Rather than epilepsy being viewed as a single disease, the epilepsies should be more accurately viewed as a group of diseases themselves. Traditionally, the pattern of seizures and age of onset have been used to categorize which kind of epilepsy a child is experiencing. However, this does not address the epileptic cause, and many diseases with distinct causes will have epilepsy as a symptom.

The International League Against Epilepsy (ILAE) introduced a more detailed classification framework that increasingly recognizes the importance of epilepsy etiology. Many gene mutations have been associated with childhood epilepsy, raising the question of treating the cause rather than the epileptic pattern.

Surgery has been the main targeted therapy by removing portions of the brain believed to cause seizures in carefully selected patients. This has proven successful with one study reporting 72% of patients being seizure-free post-surgery (ILAE Paediatric Epilepsy Surgery Task Force, 2009). However, for diseases with associated genetic mutations, such as CDD, surgery may not be the best treatment option.

“Perhaps we could learn from other diseases as to how the mutations have been targeted with repurposed medications,” Cross states. For example, a gain-of-function mutation in potassium channel subfamily T member 1 (KCNT1) is found in 50% of certain early onset epilepsy patients. The antiarrhythmic drug quinidine reversed gain-of-function in vitro (Annals of Neurology, 75:581, 2014) and various clinical studies indicated favorable response in patients. Another potassium channel mutation (KCNQ2 encephalopathy) has been associated with a severe neonatal onset epilepsy. Retigabine, a potassium channel opener, has been beneficial in a limited patient population with known sensitivity to sodium channel blockers (Epilepsia, 56:685, 2015). However, these were not randomized controlled trials (RCTs), have limited patient populations, do not have a biomarker to show improvement, and do not take disease natural history into consideration.

Standard RCT methodology is often impractical for rare diseases due to small patient populations and the limited outcome of reduced seizures. Considerations moving forward should include tracking patients (creating patient registries, studying disease natural history), improving methodology (within patient comparisons versus n-of-1 and crossover trials, pragmatic RCTs, hybrid design RCTs, adaptive trials), or standardizing outcomes (inclusion criteria, protocols, composite endpoints). In Europe, a European...
Reference Network for rare and complex epilepsies has been created and approved, EpiCARE, providing clinical trial, diagnosis, and treatment information.

“It’s recognized that the endpoint of clinical trials shouldn’t only be seizure control. Seizure control doesn't necessarily lead to improved neurodevelopmental outcomes,” Cross added. In an early onset epilepsy disease, Dravet syndrome, a composite endpoint has been identified including 5 key activities deemed important to both clinicians and caregivers: seizures, cognitive functioning (both expressive and receptive), daily activities, and social functioning. However, to date, the only outcomes used as primary endpoints in clinical trials for Dravet syndrome have been seizure-related.

CDKL5 DEFICIENCY DISORDER: TOWARDS A CLINICAL SEVERITY SCALE
TIM BENKE, University of Colorado, Denver

The International Foundation for CDKL5 Research (IFCR) has three Centers of Excellence:
• the Benke group at the University of Colorado/Children's Hospital Colorado
• the Olson group at Harvard University/Boston Children’s Hospital
• the Parikh group at the Cleveland Clinic.

Benke stated, “our goal for the Centers of Excellence was to provide clinical care tailored to CDKL5 [deficiency disorder]. We wanted to determine based on this what the standards of care were and to provide, in a clinical setting, a research platform to determine clinical profiles as prelude to trials.”

There will be a fourth center at Washington University in St. Louis. He highlighted some challenges of expanding clinical sites: collecting uniform data, lacking a shared data repository, and gathering the funding needed to accomplish such things. Moving forward, two data repositories are currently being built.

A “typical” CDD patient presents with early onset epilepsy, cortical visual impairment, severe global developmental delays, breathing and sleep disturbances, and gastrointestinal issues. However, there is a wide clinical spectrum. For diagnosis, the inclusion criterion was an altered CDKL5 gene confirmed to be pathogenic, such as a non-sense mutation in exons 1-19 or a missense mutation either in the kinase domain or not present in the parents. CDKL5 duplication should be an exclusion criterion.

When designing a clinical severity scale, one must consider the following criteria:
• including issues specific to the disease
• usefulness (reflect severity, be predictive, can be completed in a standard clinical visit)
• be dynamic (variable values do not saturate, include both static and non-static values)
• be validated (ensure the same patient receives the same score from different clinicians)

Learning from the usefulness and drawbacks of current scales will help when developing a CDD scale. The Rett Clinical Severity Score (CSS) and Rett Clinical Global Impression of Severity (CGI-S) rank either 13 or 7 items, respectively, with only one relating to seizures. The FoxG1 Developmental Encephalopathy Inventory is a parental assessment that ranks 12 items but does not assess movement or seizures. The Tuberous Sclerosis Early Childhood Epilepsy Severity Scale (ECHESS) is more detailed, but only includes seizure information.
Benke proposed the first version of the CDKL5 IFCR-COE clinical scale, which scored multiple items within 4 parts:

- **Part 1: Epilepsy** (any seizure, convulsive seizure, number of seizure types, status epilepticus, number of anticonvulsants used, response to treatment, history of epileptic spasms, and longest seizure free period)
- **Part 2: Motor** (gross motor, tone, and fine motor)
- **Part 3: Cognition, Vision, and Speech** (alertness, irritability, vision, speech, non-verbal communication, two object choice, receptive language, and mood)
- **Part 4: Autonomic** (swallowing, gastrointestinal, toileting, historical pain, response to pain during exam, sleep, and daytime sleepiness)

Although this has not been validated in a cohort yet, it has broad features that should not saturate in severity and could be completed during a standard doctor visit. Benke highlighted the need for feedback and a validation trial.

GANAXOLONE, A NOVEL INVESTIGATIONAL TREATMENT FOR CHILDREN WITH CDKL5 DEFICIENCY DISORDER: RESULTS FROM AN ONGOING PHASE 2 CLINICAL STUDY

**LORIANNE MASUOKA**, Marinus Pharmaceuticals

Neurosteroids are endogenous steroid molecules that regulate many functions, such as promoting positive mood, calming anxiety, reducing hyperexcitability, and preventing seizures. Ganaxolone is a synthetic analog of the neurosteroid allopregnanolone. It binds uniquely to both synaptic and extrasynaptic GABA<sub>a</sub> receptors and calms over-excited neurons. Both *in vitro* and *in vivo* data have demonstrated the anti-seizure, anti-depressant, and anti-anxiety activity of ganaxolone. It has been safe, effective, and well-tolerated in over 1,500 children and adults and is available in multiple dosage forms to fit patient needs. Ganaxolone has been previously used in pediatric populations, such as West (*Epilepsy Res*, 42:133, 2000) and Fragile X (*J Neurodev Disord*, 9:26, 2017) syndromes.

Regarding the current clinical trial using ganaxolone for CDD, Masuoka said, “this community of scientists, patient advocates, and the Loulou Foundation is so strong. You're helping us so much that this company of 15 has managed to accomplish an unbelievable amount of work in the last 6 months, including raising the money to be able to do our Phase 3 program.” Currently, Orphan Drug designation has been granted to ganaxolone by the FDA.

The current Phase 2 clinical trial began with a 12-week baseline period to establish a baseline seizure rate, then a 26-week treatment period, followed by a 1-year extension if the treatment was successful. Patients were enrolled based on having a confirmed CDKL5 mutation, stable background treatment, and more than four seizures per 28-day period in the baseline period. Some patients in the trial have been on ganaxolone for over two years, allowing long-term data to be collected. The primary endpoint was the change in seizure frequency per 28 days relative to baseline. Secondary endpoints include the increase in seizure-free days relative to the baseline, safety and tolerability measurements, and Clinical Global Impression of Severity (CGI-S) data collection.

Of the seven patients enrolled, there was a large variability in the number of seizures and concomitant anti-epileptic drugs, although
almost all were on a benzodiazepine. A median seizure reduction of 43% and median increase in seizure-free days of 78% was seen and four of the patients entered the one-year extension. It was generally well-tolerated with no serious adverse effects reported. However, two patients discontinued treatment due to lack of efficacy. In the future, more endpoints that focus on aspects other than seizures will be important.

When asked how to set trial guidelines to obtain reliable and interpretable scientific outcomes yet include the most CDD patients, Masuoka said there are two key points to designing good clinical trials: mimicking real-world settings, such as by relaxing concomitant medication exclusions, and ensuring use of a placebo control even though the disease is life-threatening.

In summary, preliminary data suggest that ganaxolone is a safe and well-tolerated treatment for CDD. Planning for the Phase 3 clinical trial is currently underway. Moving forward, establishing a natural history study within a global registry would benefit future clinical trials. Masuoka concludes, “It is clear that if you have this type of natural history data, the health authorities in this type of rare condition may, under certain circumstances, allow you to use those data as your control group. Once you have those data, then pharmaceutical companies will see that as an incentive to get into CDD research.”

READTHROUGH THERAPY OF CDKL5 NONSENSE MUTATIONS AND CBD FOR CDKL5 DEFICIENCY DISORDER
ORRIN DEVINSKY, New York University

“The pace of progress that the Loulou Foundation has created is really unparalleled in my 30 years in medicine,” Devinsky remarked.

Ataluren is a unique molecule that binds to the ribosome and allows for selective “readthrough” of a premature mRNA stop codon, producing a full-length functional protein similar to the endogenous protein. Importantly, it does not affect normal stop codons and does not modify transcription or mRNA stability. However, it does not work on stop codons due to a frameshift mutation. Its activity has been demonstrated in vitro and in vivo, both in mice and humans. Ataluren was shown to penetrate the blood-brain barrier in mice, which is crucial for use in central nervous system disorders. Previous clinical trials have also shown that ataluren is relatively well-tolerated, with side effects similar to the placebo (Lancet Respir Med, 2:539, 2014; Muscle Nerve, 50:477, 2014).

Together with the manufacturer of ataluren, PTC Therapeutics, a Phase 2 randomized, double-blind, placebo-controlled crossover study of ataluren for epilepsy control in nonsense mutation CDD and Dravet syndrome has begun. “There are probably 500-1,000 monogenic disorders that have nonsense mutations as a contributing factor. […] About 20% of patients with CDD have those,” Devinsky said.

First, there was a 4-week screening period to track seizures, followed by a 12-week period of blinded treatment, a 4-week washout period, and another 12-week period of treatment. An open-label 12-week extension will be offered to patients who had success on the treatment. Eight patients with each syndrome have been enrolled in the trial. The primary study objective is to characterize the safety profile. Secondary objectives include evaluating changes in convulsive and drop seizure frequency and determining the changes in minor seizure types.
Other aspects, such as cognitive, motor, and behavioral function changes and quality of life will also be monitored. The data should be gathered and analyzed over the next six months.

Devinsky also discussed the use of cannabidiol (CBD) in CDD. CBD is the non-psychoactive main ingredient of cannabis known for its anti-seizure properties. However, there are some challenges in studying CBD, such as increased placebo effect rates in children and potential acute and chronic side effects. More studies are needed as there is not enough data to be confident about the safety and efficacy of CBD use in children.

In collaboration with Helen Cross, a Phase 3 clinical trial studying the effects of CBD (drug name: epidiolex) in Dravet syndrome was performed in the United States and Europe involving 120 patients ages 2-18 years old. Overall, CBD was relatively safe and well-tolerated with 84% of adverse events being mild or moderate; however, some serious adverse events occurred and were three times as common in the treated group versus the placebo group. After a 14-week treatment period, a net reduction in number of convulsive seizures of 26% was seen with CBD compared to the placebo group. Two similar studies in Lennox-Gastaut syndrome found similar results (Lancet, 391:1085, 2018; NEJM, 378:1888, 2018).

Previous open-label studies with 16 CDD patients showed a greater than 50% reduction in the number of convulsive seizures after treatment for 48 weeks, which is past the honeymoon period of most drugs (Devinsky, et al., Epilepsy Behav, 2018, in press). The main drawback of these findings is that it was an open-label study, so the placebo effect could not accurately be known.
SESSION 4: NOVEL THERAPIES

Moderator: Yael Weiss, Ultragenyx

The translational toolbox includes many modalities that can target the main causes of disease:

- Up/downstream regulation (small molecules, antibodies, siRNA, miRNA)
- Replacement therapies (enzyme or mRNA replacement, gene therapy, gene editing)
- Restoring function (X-activation, exon skipping, chaperones)
- Knockdown (siRNA, antisense oligonucleotides, gene editing, antibodies)

REACTIVATING X-LINKED GENES TO TREAT X-LINKED NEURODEVELOPMENTAL DISORDERS

JEANNIE LEE, Massachusetts General Hospital

The CDKL5 gene is located on the X chromosome and female CDD patients tend to be heterozygous, containing one wild type and one mutant CDKL5 gene. Patients are “mosaic” with about half of their cells expressing the wild type gene and the other half expressing the mutant gene. This is caused by X-inactivation, which silences one of the X chromosomes at random in each cell. All cells contain the wild type gene, which could be targeted and re-expressed in the cells expressing the mutant gene in a process called X reactivation. Reactivation is therapeutically relevant because there are over 100 disease genes on the X chromosome, including many neurodevelopmental genes. Unfortunately, X reactivation is difficult because there are multiple layers of silencing.

Both unbiased high throughput small molecule screens and rational design approaches have been used to identify molecules that promote X reactivation. Over 365,000 compounds were screen for activity and 3 confirmed hits were found: a DNA methylation inhibitor (5-Aza-dC) and two Aurora A and B kinase inhibitors (VX680 and MLN8237). Although each compound promotes some reactivation, there is almost complete (up to 16 times more) reactivation when 5-Aza-dC is used with an Aurora kinase inhibitor, indicating synergism between DNA methylation and Aurora kinase pathways. A 600- to 800-fold reactivation of MECP2 was also seen in a human cell line. Promisingly, 5-Aza-dC is known to cross the blood-brain barrier, though it is yet unknown whether VX680 and MLN8237 can cross.

In the MECP2 studies, mice were treated with 5-Aza-dC for a short period of time, reducing toxicity. Modifying the epigenetic state allows for changes to be passed on to daughter cells, thus decreasing the time of treatment needed for long-term effects.

X-inactive specific transcript (Xist) is a crucial gene involved in X-inactivation and therefore an ideal target to prevent X-inactivation. Once specific proteins that interact with Xist RNA have been identified, protein inhibitors could be rationally designed that may target X reactivation to specific genes on the X chromosome. An RNA proteomic method, called iDRIP, was developed and identified approximately 100 protein binders, many of which are epigenetic factors with well-characterized small molecule modulators. Robust reactivation was only seen when 2 or more factors were targeted, again indicating synergy in X-inactivation.

Lee talked about using the identified compounds in a Rett syndrome mouse model, which parallels the ongoing work with CDD. The inspiration came from the landmark study done by the Bird lab where the
neurological symptoms of Rett disease were reversed in mice by supplying the MECP2 gene (Science, 315:1143, 2007). To study X-reactivation, a female mouse model with increased disease penetrance needed to be created for both Rett syndrome and CDD.

Lee discussed unpublished results showing that increased disease penetrance resulted in animals that mimic severe Rett Syndrome in male mice. Low-level MECP2 expression in the brain of affected females significantly improved both survival and neuromotor phenotypes. The full results summary will be disclosed in an amended report upon publication.

Moving forward, the knowledge from the Rett studies can be applied to CDKL5 studies. A female CDKL5 knockout mouse model is being created and the reactivation compounds identified for MECP2 will be applied to study CDKL5 upregulation using CDKL5 expression as a biomarker.

(Some of these results were recently published: Carrette, et al. A Mixed Modality Approach towards Xi Reactivation for Rett Syndrome and Other X-Linked Disorders. PNAS, 2018, 115(4) E668-E675. DOI: 10.1073/pnas.1715124115.)

DEVELOPING NEW THERAPEUTICS FOR CDKL5 DEFICIENCY DISORDER: LESSONS LEARNED FROM LYSOSOMAL STORAGE DISORDERS
HUNG DO, Amicus Therapeutics

Amicus Therapeutics is developing a protein replacement therapy using recombinant CDKL5. Previously, Amicus has developed drugs for lysosomal storage disorders, so lessons learned during those drug development processes can be applied to current CDD drug development.

Drug dosing is a challenge for multiple reasons. Optimizing clinical dosage based on preclinical results can be problematic because animal models may not accurately reflect the human disease. Drug concentrations are up to 100-fold lower in the target tissue than in the blood, where readouts are measured. This is especially true for central nervous system targets and gives inaccurate readouts. The drug must also have an effective mechanism for cellular uptake at low concentration and have a good safety profile at higher doses, if high doses are needed for efficacy.

Manufacturing can be difficult on multiple levels. Scale up of large, complex biologics is very challenging due to the time and cost constraints. For proteins, understanding and replicating post-translational modifications at large production scale is key. Controlling protein production in mammalian systems is feasible but modulating carbohydrate processing is very difficult. Variations in protein glycosylation can directly affect drug processing and clearance. Stringent regulatory rules need to be adhered to for ensuring batch-to-batch consistency, maintaining the critical quality attributes of therapeutic protein that enable efficacy, and quantifying drug impurities to ensure patient safety. Quality control encompassing all aspects of manufacturing process must be ensured by the drug developer to meet rigorous guidelines from FDA and EMA.

The final challenge involves designing and conducting clinical trials. Especially in rare diseases, small patient populations only allow
small clinical trials, resulting in increased genetic and phenotypic heterogeneity. Small patient cohorts may not give adequate statistical power to demonstrate clinical efficacy. The lack of appropriate clinical endpoints makes analysis of drug efficacy and drug approval difficult. Biochemical improvements can be used to understand dosing, but functional endpoints are needed to demonstrate a clear benefit. Having inadequate natural disease history also makes it difficult to differentiate between drug-promoted improvement rather than disease course. Demonstrating the ability of the drug to change the disease course is crucial in determining its efficacy.

The knowledge gained in overcoming these known challenges from other therapeutic programs can be used in the development of new therapies for CDD.

**EVIDENCE OF EXCITATORY: INHIBITORY IMBALANCE IN CDKL5 DEFICIENCY DISORDER**

**FRANCES JENSEN,** Perlman School of Medicine, University of Pennsylvania

The presented work is unpublished; therefore, the full summary will be disclosed in an amended report upon publication.

During the panel questions at the end, the correlation of age and development between mice and humans was discussed. It was suggested to view up to a 3-month-old mouse like a newborn, rather than a mature mouse. Following treatment efficacy longer than two months will be crucial to better understand how it will be translated to patients.

**STRATEGIES FOR DELIVERING GENE THERAPY TO THE BRAIN**

**JAN NOLTA,** University of California, Davis

Nolta began by describing the infrastructure at the UC Davis Medical Center, including fee-for-service cores and a good manufacturing practice (GMP) facility, to promote collaboration: “We are very open to collaborating to help leverage the Foundation money to further collaborative grant funding to help move things into clinical trials.”

Nolta also highlighted graduate student Julian Halmai’s poster about using TALEs or CRISPR/Cas9 to reactivate the silenced CDKL5 allele to rescue CDKL5 deficiency phenotypes. A 2- to 3-fold increase in CDKL5 expression was seen using various guide RNAs. Lee noted similar findings in her lab but determined that the upregulated allele was from the active X chromosome, not the inactive allele as hoped. Ongoing allelic analysis is being conducted by Halmai to determine which allele is being upregulated.

Delivering gene editing cargo is always a challenge, but especially so for entry into the brain. One delivery method utilizes mesenchymal stem cell (MSC)-mediated exosome or microparticle
encapsulation of the gene editing cargo. MSCs can be readily engineered using viral vectors, remain in the brain for 1-2 months, do not require immunosuppression, have a strong safety profile, and have demonstrated delivery of cargo to neurons via tunneling nanotubes or exosomes. They specifically migrate throughout the brain and communicate with sick or distressed neurons. MSCs are introduced into the brain via intrathecal injection or direct injection into the brain, as they do not cross the blood-brain barrier. Intranasal delivery is being explored to allow the MSC exosomes to cross the blood-brain barrier.

Kyle Fink's lab at the University of California, Davis used MSCs to express fluorophore-labeled gene editing proteins in target cells for up to 10 days. MSCs were used to deliver fluorophore-labeled TALEs into the mouse brain.

“The time is now, there is a ‘New Era of Living Medicine.’ Cell and gene therapies are now being prescribed to treat disease and injuries,” Nolta concluded. A few drugs are now available, such as Kymriah immunotherapy to treat childhood leukemia and Strimvelis gene therapy to treat severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID). Sangamo Therapeutics recently treated the first patient with gene editing therapy in the liver to treat Hunter syndrome.
Majid Jafar opened the panel discussion by quoting the late Henri Termeer, a world-renowned biotechnology entrepreneur and member of the Board of Fellows at Harvard Medical School: “When you’re working to rescue the life of a child, the system readjusts itself to help you.” Each panelist discussed their career paths – all transitioning from clinical to industry careers – and connections with CDD.

Andrew Plump is “one of a few American internists who ultimately went on to become a clinical geneticist.” After getting excited about basic science during his educational training, he transitioned into an industry career at Merck to more closely impact the translation of basic science advances to patients. He then transitioned to Sanofi and is now at Takeda, where he is leading their effort to discover drugs for rare diseases. Plump also recounted the first time he met Jafar, where he saw a picture of Jafar’s daughter, Alia, and realized that she looked like one of his best friend’s daughters who has CDD. “It’s just an incredible story when you consider how rare this condition actually is,” he added.

Jeremy Levin began as a zoologist but decided to become a doctor after realizing his passion for patients and medicine. He came to the US to explore the biotech industry, where he worked for and was inspired by Termeer. After working in large companies, he returned to his patient-focused roots by joining Ovid Therapeutics after meeting its founder, Matthew During. Ovid is a biotech company focused on finding cures for rare neurodevelopmental disorders and epileptic encephalopathies. “It’s absolutely marvelous – we see patients, we see families, and yes, we don’t have a drug yet, but I’m very hopeful. I can’t tell you how gratifying it is to take the risk every day to devote one’s time to doing that.”

Jafar shared the story of when he met Omar Khwaja before Alia’s diagnosis. He highlighted how helpful his advice was and how uplifting it was to see him pursuing rare disease cures at Roche, a large pharmaceutical company. After obtaining his Ph.D. in molecular genetics, Khwaja decided to become a
pediatric neurologist because he thought it was a “segment of children with chronic diseases that was highly underserved.” He decided to go into biopharma at Roche because it offered the best resources for developing and bringing drugs forward. Since he has been at Roche, they have formally entered the pediatric rare genetic disease area.

Both Khwaja and Jafar highlighted Philip Reilly’s book, called Orphan, which is about children with rare diseases and their families. Like Plump, Reilly was trained as an internist who became a clinical geneticist and worked with hopelessly ill, drug-addicted, or undiagnosed patients over his 35-year clinical career. After overseeing 1,200 institutionalized patients with undiagnosed neurological disorders and seeing the conditions they lived in, he said to himself, “there must be something better that I can do for these people.” That led him to Third Rock Ventures, which invests in and starts up rare genetic disease companies. They have funded 45 companies thus far, focusing on areas such as gene therapies for the brain, X chromosome reactivation, and rare epilepsies.

**What is the driving force behind the current interest in rare diseases?**
Levin reflected on the immuno-oncology field in 2005, where only a few companies existed, and most pharmaceutical and venture capitalism companies did not want to get involved. However, a few companies were pushing the science forward, leading to promising clinical results. “That catalyzed an industry – between now and then, the change is fundamental. The catalytic moment was understanding that you could address a problem in a patient in a way that you could measure and understand,” Levin comments. Similar breakthroughs in scientific understanding and technology for orphan neurological disorders could bring about a similar change.

Reilly stressed that the number and power of tools have improved drastically, contributing to more rapid scientific progress. He also pointed out that it is somewhat easier to target a disease caused by one gene. “The success rate, overall, to getting to drug approval is actually higher for single-gene diseases and the time course is a bit shorter. I don't know if that will pan out overall, but it seems that way,” he said. He highlighted the impact of the patient groups in pushing rare diseases forward and the partnership of the regulatory agencies to create the most impactful clinical trials.

Speaking about the motivation for a large pharma company moving into the rare disease space, Khwaja mentioned three reasons driving Roche’s commitment to rare diseases: 1) a changing industry shape with the end of the “blockbuster drug” era, 2) having more confidence in the disease target of a single-gene
disease to decrease the risk of drug development, and 3) an expanded toolbox of therapeutic modalities, and drug delivery.

Plump discussed that Takeda puts the patient at the center of everything they do, which is why they have partnered with smaller companies, like Ovid, to work on rare diseases. He stated that two-thirds to three-quarters of approved medications originate in small biotech companies, not pharma companies. This drives pharma companies to partner with biotech companies to create synergy and share the risks, benefits, and scientific expertise.

How can we help accelerate and de-risk developing treatments for rare disorders?
“IT really starts with collaboration and partnership,” Khwaja began. The power of patient groups is key to putting the disease and science first and doing some of the riskier work to draw interest from biotech companies. Researchers and clinicians should ensure that clinical scales and databases are being built to facilitate drug development and optimize future workflow. Putting the patient needs first is also extremely powerful for capturing capital and attention around a disease area.

When Third Rock is looking to start a rare disease company, Reilly said that it used to be more opportunistic, but it is now focused on developing a “product engine” to discover products possibly applicable to multiple disorders. For example, they focus on larger biological processes that could impact a wide range of disorders. For venture capitalism, “there are two key currencies: one is the great scientists and clinicians who know the space and the other is the patient groups,” Reilly adds.

Addressing why some companies have turned away from neuroscience, Plump said there are “massive intrinsic risks. The very large safety databases that you have to develop in Phase 3 [trials] are prohibitive.” The rare disease space tends to be easier to work with if there is a deep understanding of how molecular changes relate to the disease. New technologies provide treatment tools, making progress easier. He also highlighted the immense opportunities currently available to drive patient-specific drug development.

In terms of facilitating drug discovery efforts of biotech companies, Levin discussed the dichotomy within larger pharma companies between the budget imposed by the marketing department and the scientific or medical unmet need. Comparatively, issues within a small company are different because there is a smaller distance between decisions made and the patient. Levin said that “you have to consistently ask only one question: How will this benefit the patient?” He also highlighted that one family meeting with Termeer is what started Genzyme and impacted Gaucher’s disease. “The power of an organization today is the ability to coalesce information far more fundamental than any company can do by itself. I believe it’s that interchange of information – that exchange of ideas, that exchange of experience – which makes it such an extraordinary lever in driving innovation in the area,” Levin concluded.
BREAKOUT DISCUSSIONS

During the afternoon of Day 1, five discussion sessions were held on topics ranging from animal models to clinical trial design. These allowed smaller groups of scientists, clinicians, and CDD patient advocates to dissect each topic and identify what needs to be defined and the best ways to do so. Daniel Lavery, CSO of the Loulou Foundation and Director of the CDKL5 Program of Excellence in the Orphan Disease Center at the University of Pennsylvania, said these sessions and their summaries “should help set the table for some good discussions later on.”

Breakout A: CDKL5 Models: What is the Relevance of Animal and Cell Models to the Phenotypes of Human Disease?
Moderator: MICHAEL GREEN, UMass Medical School

This very lively discussion dissected the three currently used model systems: mouse, rat, and human. All five mouse models are of null or truncated CDKL5 alleles, warranting the creation of additional missense mutation models that more closely mimic patients. A few issues were that most studies published at the time of this conference presented data on male mice (a few papers have reported information on female mice) and there was some variability between labs, possibly due to differences in the genetic backgrounds or small sample sizes. The mice also do not display the seizure phenotype seen in patients, but there had only been eight papers published reporting behavior in Cdkl5 mice at that time, indicating that it might be too early to find a behavioral phenotype consensus.

The one rat model, containing a null allele with an LEH outbred background, from Peter Kind’s lab is currently being characterized and distributed to other labs.

A limited number of cellular-based human models were assessed. Human iPSC-derived neurons and glia have great potential on a cellular and molecular level but are currently limited to studying early developmental stages. Human iPSC-derived organoids have the potential to carry out complex neural activity, with a three-dimensional organization that in some ways mirrors some aspects of brain structure; however, these organoids are admittedly relatively early in their development as a reliable model of post-embryonic disease states.

The discussion also focused on the status and translational relevance of behaviors measured in preclinical assays, such as rotarod analysis, to patient phenotypes. The usefulness of modalities other than behavior, especially for biomarker studies, and the bi-directionality of translational research was also discussed. Kind noted that an isolated behavioral assay does not tell you much; “it must be viewed in the context of behavioral repertoire and in a species-specific context.”

Challenges with animal models include identifying what makes a good preclinical phenotype for drug studies and understanding the source of phenotype differences reported between labs (potentially due to procedural or environmental variables). As housing conditions are rarely reported, either transparency in reporting conditions or moving towards condition standardization were brought up.

In summary, the best phenotypes for rodent preclinical trials must be identified. Visual evoked potential (VEP) seems to be the best phenotype to date. Developing standardized protocols with some flexibility and having open communication
between researchers about the benefits and limitations of preclinical models are also key. Human iPSC-derived neurons and organoids are exciting tools for drug testing as they become more high-throughput. Human post-mortem brain tissue analysis could provide important disease insights, despite known technical issues. The question of whether it was too early to consider larger animal models, such as non-human primates, was raised.

During questions, Kind highlighted the need to focus on studies of female mice. “There was quite a long discussion about tracking X-inactivation in females, but right now we don’t really have the tools to do that. In terms of interpreting data, that will be a very important aspect to follow up on. It’s very early days and we have a lot to do.” Joe Zhou also noted the importance of assessing the X-inactivation ratio in the brain for use in interpreting female behavior data. “We have made a mouse model that allows us to track this ratio. Again, this is in early stages and hopefully next year we will be able to share with everybody.”

Breakout B: Bioinformatics and Drug Screening/Repurposing
Moderator: ISAAC KOHANE, Harvard Medical School
Rapporteur: MICHAEL JOHNSON, Imperial College London

Two key issues were discussed: which modalities would provide a screenable readout for evaluating drugs, and what the parent perspectives were on the off-license repurposing of FDA-approved drugs. Even though phenotypic and computational drug screens are complimentary approaches, there is no validated signature for drug response. However, there was disagreement on what data are required to develop those drug response signatures. Whether the drug readout should be quantitative, such as ranking drugs by potential efficacy, and if drugs should be filtered by additional criteria, such as blood-brain barrier penetration and safety, were discussed. Parents said they would be open to off-license use of FDA-approved drugs if there was enough evidence for efficacy, low toxicity, and good safety profiles.

Overall, the current research bottleneck is the lack of reliable models for drug screening, indicating the importance of exploring multiple models and developing model-specific efficacy readouts. Ideally, convergence across models would accelerate drug discovery. There was agreement upon needing more data but disagreement about which data to collect to develop reliable readouts. It is important to keep in mind that these issues are not specific to CDD and that we should learn from related diseases.

During questions, mining existing databases to learn about wild type CDKL5 function was discussed. Green stated that neurotypical human brain gene expression datasets have been examined to look at where and when CDKL5 is expressed and study the RNA interactome partners of CDKL5.

Breakout C: Imaging and EEG as Tools for Probing CDKL5 Function and Disease Natural History
Moderator: HELEN CROSS, Great Ormond St Hospital
Rapporteur: MICHELA FAGIOLINI, Boston Children’s Hospital

Three main topics were discussed: evolution of the EEG phenotype with disease progression, applicability of MRI and the tools available, and examining cortical visual impairment as a means of studying neurophysiological behavior. EEG abnormality is not initially seen in mice, is not always seen in CDD, and
is difficult to interpret in infants. Because EEG studies are still in the early stages, much remains unknown. Studying the stages of hypsarrhythmia progression might be important. A natural history study of EEG is needed to determine its relation to disease phenotype.

Initial patient MRIs appear relatively normal and MRIs are difficult to obtain due to requiring patient sedation. Previous MRI studies should be studied for volumetric irregularities. Moving forward, standardization of data collection will be important.

Studying cortical visual impairment offers a non-invasive, low-cost, quantitative, and seemingly highly correlated readout method for mapping disease trajectory. A visual evoked potential (VEP) phenotype was identified in CDD mouse models. In humans, there is anecdotal evidence that cortical visual impairment improves with age but the reason for improvement is unknown. Standardization of stimulus and compiling a natural history are needed.

In conclusion, natural history studies are needed for both EEG and cortical visual impairment. Developing standardized MRI protocols and cortical visual impairment stimuli will facilitate future data collection. Using VEP to determine CDD biomarkers seems to be the most promising, once a standardized protocol is developed for longitudinal evaluation.

Involving patients from Europe and Asia to collect more data and creating a consortium to aid in standardized data collection was also discussed.

Breakout D: Biomarker Discovery and Validation in the Periphery and CNS
Moderator: LAURA MAMOUNAS, NINDS/NIH

Mamounas began by stating that biomarker identification for neurodevelopmental disorders (NDDs) is a priority for the NIH and NINDS. Toward this end, the NINDS convened a workshop in December 2017 to consider biomarker development in a number of rare, genetic forms of NDD, including CDD. During the breakout session, the range of FDA-defined biomarkers was presented (i.e., the FDA BEST guidelines: www.ncbi.nlm.nih.gov/books/NBK326791/), and subsequent discussion centered on determining which biomarkers are most urgent for CDD. For epileptic seizures in CDD, EEG and clinical assessment of seizures are currently the accepted endpoints because they are feasible and well-validated measures. However, there are no equivalent “surrogate” biomarkers for the cognitive and behavioral components of CDD.

The challenges of CDD biomarker development for NDDs, including CDD, were also discussed. Challenges include the lack of detailed natural history, heterogeneity of disease phenotypes even within Mendelian disorders such as CDD, feasibility of testing in children, the connection between biomarkers and clinical outcomes, understanding developmental trajectories in pediatric populations, a limited patient population, the lack of data and data acquisition standards, and the development of translatable biomarkers in models to guide clinical trials.

Overall, biomarkers should be “fit for purpose,” such as protein expression and molecular readouts to measure a therapeutic response to gene therapy or imaging biomarkers to help
stratify subjects for clinical trials. Given the heterogeneity both within and across the different NDDs, functional and cognitive measures will need to be tailored to specific disorders and even to specific populations within an NDD (e.g., those children who develop autism in CDD). Unbiased approaches for biomarker discovery, such as continuous symptom monitoring and telemonitoring, are needed along with quality natural history studies. Rigorous standardized protocols must be developed to obtain reproducibility across studies and neurodevelopmental diseases.

**Breakout E: Improving Clinical Outcome Measures for CDKL5 Deficiency Disorder**

**Moderator: TIM BENKE, University of Colorado-Denver**

The goal of this discussion was to develop a scale to measure clinically meaningful changes in CDD for use in clinical trials, natural history studies, and clinical care. The session focused on identifying useful scales for CDD; summarizing common seizure type definitions; balancing data collection, clinical care, and parents’ needs during clinical visits; collecting objective rather than subjective measurements; identifying both seizure and non-seizure endpoints; receiving feedback from families about what is clinically meaningful (walk, sleep, communication, seizures, etc.); and defining the time, money, and resources needed to design, develop, and validate the scale.

Identifying a relevant time frame as a reference period is difficult. There is a disconnect between clinicians and care-givers as to definitions of seizure types. A potential resource to address that disparity is a video bank of seizure type definitions that provide a unified vocabulary for clinicians and care-givers. Although keeping a seizure diary is ideal, it may not be feasible given the magnitude of daily seizures. A proposed alternative was asking about the frequency of specific seizure types or seizure free days in the past week. Regulators are receptive to non-seizure endpoints, but more data are needed and discussing what data to measure with regulators will be helpful. How to best validate a CDD scale was also discussed.

In summary, identifying the top three readouts to the care-giver (“Care-giver Top 3”) and utilizing them in the scale will be important. An ideal scale should be simple, quick, and executable at most clinical centers. Compiling a scale that can evolve as the disease knowledge evolves will be the most useful.

During questions, the need for standard care practice guidelines for international use was brought up. Benke highlighted that the scale would primarily be useful for clinicians to track disease progress while still incorporating what is important to the care-giver. The data would be collected into a database and stored within the larger orphan disease database. Concerns over the duplication of data, clinician time, and quality of the data collected were also brought up.

The importance of aligning preclinical and clinical readouts was also reiterated. Michael Saxe, Neuroscience Senior Investigator at Novartis, added that the clinical readout of Dravet syndrome is seizure frequency over 28 days, which is not a viable readout for animal models. Instead, mice are either heated until they seize then drugs are evaluated based on their ability to reverse those seizures, or seizure severity is examined after administration of an epileptic drug. Machine learning algorithms will be used to try to identify seizures via either EEG or video signatures. Ideally, videos of animals over days or weeks can be analyzed for spontaneous seizures.
KEY TAKE-AWAYS FROM BREAKOUT DISCUSSIONS

• Natural history studies are needed to guide biomarker discovery and EEG or cortical visual impairment readouts
• Standardization of protocols and data acquisition will be key moving forward in both the pre-clinical and clinical domains
• Developing better preclinical animal models is essential for biomarker discovery and drug screening
• Identifying reliable and translatable readouts in both pre-clinical models and patients is a major research bottleneck
• More data are needed for robust data interpretation, but determining exactly what data to collect was still debated
In this moving presentation, Dr. Frame talked about day-to-day life with her daughter, Kiera Grace, who has CDD. She was honest about the challenges of living with children that have multiple daily seizures, developmental delays, sleep issues, and behavioral problems. However, she also highlighted the good times – the rays of sunshine where her daughter’s and other CDD patient’s personalities can shine through.

“Our children have an inner light that radiates and captures the heart of all of those that they meet. And even on the darkest days of CDKL5 [deficiency disorder], amidst the seizures and the pain, the loneliness and the isolation, their light gives us warmth and comfort and courage and strength to keep fighting for them,” Dr. Frame said.

Dr. Frame gave us a glimpse into the human side of CDD, serving as a reminder of what this research is for and what the ultimate goal is – to improve patients’ lives.
DINNER AND ANNUAL PRIZE AWARDS

After the conclusion of Day 1, a delegate dinner was hosted by the Loulou Foundation at the Museum of Science in Cambridge, MA. Before the meal, Eric Lander, President and Founding Director of the Broad Institute spoke about the history of genetics and how rapidly the field was progressing.

Majid and Lynn Jafar announced the 2017 CDKL5 Forum Awards for Excellence and the inaugural Junior Fellowship Awards:

The Junior Fellowship Awards were presented to five young investigators at the doctoral or post-doctoral level who work in labs focusing on CDD. Awardees were chosen based on their work ethic, research track record, and commitment to CDD. The award includes $10,000 in funding presented to the lab of the awardee. The 2017 winners were:

- Lucas Baltussen – Francis Crick Institute
- Claudia Fuchs – University of Bologna
- Ralph Hector – Glasgow University
- Ivan Muñoz – University of Dundee
- Laura Rusconi – University of Insurbia

The ‘Lab of the Year’ award and $50,000 prize was given to Joe Zhou, University of Pennsylvania. The ‘Company Making a Difference (Pre-clinical)’ award was given to Amicus Therapeutics for their work in advancing enzyme replacement therapy.

The ‘Company Making a Difference (Clinical)’ award was given to Marinus Pharmaceuticals for their work advancing ganaxolone in clinical trials. The award for ‘Outstanding Contribution’ went to Penny, Dustin, and Lily Howard in honor of Harper Howard. A short video about Harper’s story was shown, describing her pioneering use of CBD oil to manage seizures and the selfless decision by the Howard family to donate her brain to CDD research.
CLOSING DISCUSSION

Presentations at the 2017 CDKL5 Forum highlighted the exciting potential of disease-modifying therapeutic approaches, such as gene therapy, genome editing, and X reactivation. As a consequence, the closing discussion session with Forum attendees focused on the immediate challenges that still remain to help advance these therapies to patients and their families. These included the need to address basic questions, such as, which CDKL5 isoform(s) are necessary for reversal of which symptoms of the disorder? This is a key question which will impact gene therapy strategies, such as AAV-mediated gene delivery. Likewise, what are the relevant downstream phosphorylation targets of CDKL5? We saw evidence from several groups pointing to proteins involved in microtubule function (MAP1S and EB2); are these truly disease-relevant targets of CDKL5 kinase activity, or merely convenient biomarkers for this activity? A corollary to this is the need for quantitative and sensitive methods for detecting the CDKL5 protein and the phospho-peptides that it modifies, preferably with a throughput and sensitivity superior to the current Western blotting assays.

Many of the disease-modifying therapeutic approaches will face the same challenge: how to deliver the therapeutic to a sufficient fraction of the target cells in the large and well-protected human brain. While significant progress in targeting motor neurons for SMA using AAV-mediated gene therapy vectors provides hope, other approaches such as intrathecal delivery, and more neurotropic viral vectors or delivery systems need to be investigated.

Another challenge is the need for better validated phenotypes in the different pre-clinical disease models, both human and animal, which are being developed with the goal of demonstrating translational potential. Challenges, such as the lack of a robust seizure phenotype in the animal models and the early stage of human organoid models in their own establishment and validation, need to be addressed to improve the broader utility and value of these platforms. Standardization of testing protocols, particularly in behavioral assays in animal models, is critical, as results from different labs in different models need to be confirmed, or at least harmonized, in order to establish solid, actionable data from these complex (and at times highly variable) assay systems. The potential for functional imaging techniques, such as visual and audio evoked potentials, and the structural imaging technologies, such as DTI, may present an opportunity for direct translation, and even "back-translation" from patients to pre-clinical models.

The announcement of the first advanced clinical trials for CDD therapeutics is an exciting milestone for the entire community. These first trials (Marinus’ ganaxolone trial, and investigator-initiated trials for PTC’s ataluren and GW's epidiolex) will use the reduction in seizure frequency as their primary endpoint, which is an important component of the disorder and still a significant unmet medical need. However, as was often underscored, epilepsy represents only one part of this devastating disease; the bigger challenge will be how industry and the community can tackle neurodevelopmental delay symptoms in a clinically effective way. New outcome measures for CDD neurodevelopmental domains will need to be established and validated before they can become part of a clinical trial for the detection of any significant therapeutic effect on these symptoms. This is a challenge being faced in other neurodevelopmental disorders, and perhaps information from these other disorders can help inform the establishment of cognitive and developmental outcome measures for CDD.

As discussed, many of these topics will drive decisions within the Loulou Foundation on its funding directions in 2018. This will include the priority topics for academic grants within the CDKL5 Program of Excellence pilot grant program, as well as an expanded portfolio of directed research projects within the Foundation.

In conclusion, the 2017 CDKL5 Forum provided a snapshot of the rapid advancement made within the past several years in understanding CDD. At the same time, these advances also illuminate the hopes and the challenges of the future, as we take on the next set of questions in our mission to develop real treatments and, eventually, a cure for this terrible disorder.
The next CDKL5 Forum will be held on October 22-23, 2018, at the Francis Crick Institute in London. The location of the Forum will alternate between Europe and the USA in future years. The upcoming CDKL5 Patient Conference will be held on June 29-30, 2018, at The University of Colorado, Denver, Children’s Hospital, in Aurora, Colorado.