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(71) Applicants: **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; Penn Center for Innovation, 3600 Civic Center Blvd., 9th Floor, Philadelphia, PA 19104 (US). **AMICUS THERAPEUTICS, INC.** [US/US]; 3675 Market Street, Philadelphia, PA 19104 (US).

(72) Inventors: **SCHMID, Ralf**; 415 Strathmore Road, Haverstown, PA 19083 (US). **PERCIVAL, Justin**; 131 Winchester Road, Merion Station, PA 19066 (US). **KATZ, Evan**; 26 Point of Woods Drive, North Brunswick, NJ 08902 (US). **CLARK, Sean**; 74 Manor Drive, Princeton, NJ 08540 (US).

(74) Agent: **KODROFF, Cathy, A.** et al.; Howson & Howson LLP, 325 Sentry Parkway East, Five Sentry East, Suite 160, Blue Bell, PA 19422 (US).

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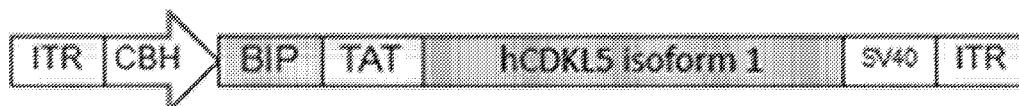
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(54) Title: COMPOSITIONS USEFUL IN TREATMENT OF CDKL5 DEFICIENCY DISORDER (CDD)

FIG 1



(57) Abstract: Provided is a recombinant adeno-associated virus (rAAV) having an AAV capsid and a vector genome which comprises a human CDKL5 (hCDKL5) expression cassette. Also provided are a production system useful for producing the rAAV, a pharmaceutical composition comprising the rAAV, and a method of treating a subject having CDD, or ameliorating symptoms of CDD, or delaying progression of CDD via administering an effective amount of the rAAV to a subject in need thereof.

COMPOSITIONS USEFUL IN TREATMENT OF CDKL5 DEFICIENCY DISORDER (CDD)

5 BACKGROUND OF THE INVENTION

CDKL5 Deficiency Disorder (CDD) is a serious neurodevelopmental disorder affecting young children. The underlying cause is lack of CDKL5 protein expression due to mutations in the X-linked Cyclin-Dependent Kinase-Like 5 gene, CDKL5 (Mendelian Inheritance in Man, MIM: 300203; previously known as STK9), resulting in a range of phenotypes, including EIEE2 (MIM: 300672), a form of early infantile epileptic encephalopathy [Bahi-Buisson, N. et al. Key clinical features to identify girls with CDKL5 mutations. *Brain* 131, 2647-2661, doi:10.1093/brain/awn197 (2008)], and infantile spasms [Fehr, S. et al. *Eur J Hum Genet* 21, 266-273, doi:10.1038/ejhg.2012.156 (2013); Kalscheuer, V. M. et al., *American Journal of Human Genetics*, 72, 1401-1411, doi:10.1086/375538 (2003); Tao, J. et al. *American Journal of Human Genetics* 75, 1149-1154, doi:10.1086/426460 (2004). Weaving, L. S. et al. *American Journal of Human Genetics* 75, 1079-1093, doi:10.1086/426462 (2004)]. In addition to the characteristic early-onset seizures, the phenotype may also include a number of other features, such as stereotypic hand movements, severe psychomotor retardation and general hypotonia. The early postnatal onset of symptoms indicates that CDKL5 plays a crucial role in brain development. CDKL5 is also expressed within the mature adult nervous system. CDKL5 is expressed throughout the cell, including the nucleus and the cytoplasm of the cell soma and dendrites.

CDKL5 gene mutations are the cause of most cases of CDD, a progressive neurologic developmental disorder and one of the most common causes of cognitive disability in females. Males who have the genetic mutation that causes CDD are affected in devastating ways. Most of them die before birth or in early infancy. See, e.g., [ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Rett-Syndrome-Fact-Sheet](https://rarediseases.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Rett-Syndrome-Fact-Sheet) and omim.org/entry/312750.

Various gene therapy vectors designed to treat CDD have been described. However, there remains a need to increase expression of the therapeutic gene in target cells.

SUMMARY OF THE INVENTION

Provided herein is an AAV gene therapy-mediated approach for CDD which provides cross correction in the brain. In certain embodiments, the gene therapy approach provided herein may reverse post-developmental CDD pathology.

- 5 In certain embodiments, a recombinant adeno-associated virus (rAAV) is provided. The rAAV comprises an AAV capsid and a vector genome, wherein the vector genome comprises inverted terminal repeats (ITR) and an expression cassette comprising a nucleic acid sequence comprising coding sequences for BIP-TATk28-hCDKL5 operably linked to regulatory sequences which direct expression thereof, wherein said regulatory sequences
- 10 comprise a chicken β -actin hybrid promoter and a polyA sequence, and said BIP-TATk28-hCDKL5 is a fusion protein comprising the BIP amino acid sequence of SEQ ID NO: 3, the TATk28-amino acid sequence of SEQ ID NO: 7, and the hCDKL5 amino acid sequence of SEQ ID NO: 24. In certain embodiments, the BIP-TATk28-hCDKL5 fusion protein has the amino acid sequence of SEQ ID NO: 11. In certain embodiments, the BIP-TATk28-
- 15 hCDKL5 fusion protein is encoded by SEQ ID NO: 10 or a sequence 95% identical thereto. In certain embodiments, the chicken β -actin hybrid promoter has a sequence of SEQ ID NO: 12. In certain embodiments, the expression cassette further comprises a Kozak sequence. In certain embodiments, the expression cassette comprises the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, and an SV40
- 20 polyA sequence. In certain embodiments, the expression cassette has the nucleic acid sequence of SEQ ID NO: 13 or sequence at least 95% identical thereto. In certain embodiments, the vector genome comprises a AAV-5' ITR (also referenced as 5' ITR), the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, an SV40 polyA sequence, and a AAV-3' ITR (also referenced as 3' ITR).
- 25 In certain embodiments, the vector genome has the nucleic acid sequence of SEQ ID NO: 14 or a sequence at least 95% identical thereto. In certain embodiments, the capsid is an AAVhu68 capsid, an AAVhu95 capsid, or AAVrh91 capsid.

- In certain embodiments, a plasmid useful in producing an rAAV particle which comprises an expression cassette comprising a chicken β -actin hybrid promoter sequence, a
- 30 Kozak sequence, a BIP-TATk28-hCDKL5 coding sequence, and an SV40 polyA sequence is provide. In certain embodiments, the plasmid contains the vector genome which comprises a 5' ITR, the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-

TATk28-hCDKL5 coding sequence, an SV40 polyA sequence, and a 3' ITR. In certain embodiments, a packaging cell is provided which comprises the expression cassette, vector genome or plasmid.

In a further aspect, provided herein is a composition comprising a rAAV or a vector
5 as described herein and an aqueous suspension media.

In another aspect, a method of treating a subject having CDD, or ameliorating symptoms of CDD, or delaying progression of CDD is provided. The method comprises administering an effective amount of a rAAV or a vector as described herein to a subject in need thereof. In certain embodiments, a suspension is formulated for intravenous
10 administration, intrathecal administration, intra-cisterna magna administration or intracerebroventricular administration.

A rAAV is provided or use in treating a patient having a CDKL5-deficiency disorder. Use of a rAAV in preparing a medicament for use in treating a patient having a CDKL5-deficiency disorder is provided.

15 These and other aspects of the invention are apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 is a graphic map of the vector genome in a recombinant AAV particle. The
20 vector genome contains an AAV 5' inverted terminal repeat (ITR) and an AAV 3' ITR at the extreme 5' and 3' end, respectively. The ITRs flank the sequences of the expression cassette packaged into the AAV capsid which have sequences encoding a BIP-TATk28-hCDKL5 fusion protein, in which BIP refers to a signal peptide and TATk28 is a cell penetrating peptide. The expression cassette further comprises regulatory sequences operably linked to
25 the fusion protein coding sequences, including a hybrid chicken beta actin (CBH) promoter, and an SV40 poly A.

FIG 2 provides an illustration of cross-correction, in which a secretable BIP-TATk28-hCDKL5 fusion protein is delivered via an AAV which corrects deficient hCDKL5 in vector-transduced cells and delivers secreted hCDKL5 protein to other brain cells.

30 FIG 3A shows western blot analysis of BIP-TATk28-hCDKL5 protein expression in brain of male mice of ID Nos: 734 and 738 (WT PBS), 753 (CDKL5^{-/-} PBS), 783, 855 and 860 (CDKL5^{-/-} BIP.TATk28.hCDKL5), 772 and 832 (CDKL5^{-/-} CDKL5). Male neonatal

control or CDKL5^{-/-} mice were injected ICV with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5 vector or AAVhu68-CBH-hCDKL5 vector.

FIG 3B shows western blot analysis of BIP-TATk28-hCDKL5 protein expression in brain of male mice of ID Nos: 1157 and 1162 (WT PBS), 1156 and 1160 (CDKL5^{-/-} PBS),
5 980 and 1136 (CDKL5^{-/-} BIP.TATk28.hCDKL5), 1202 and 1206 (CDKL5^{-/-} CDKL5). Male P14 control or CDKL5^{-/-} mice were injected ICV with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5 vector or AAVhu68-CBH-hCDKL5 vector.

FIG 3C shows a graph with densitometric quantitation of CDKL5 expression. BIP-TATk28-hCDKL5 protein expression reached 50 percent of wild type (WT) PBS controls
10 and was higher than untagged CDKL5 expression.

FIG 3D shows BIP-TATk28_hCDKL5co2 and hCDKL5co2 mRNA expression levels plotted as hCDKL5-co2 transcript per 100ng mRNA.

FIG 4 shows representative fluorescent images of sagittal sections of cortex from AAVhu68-CBH-BIP-TATk28-hCDKL5co2 treated CDKL5^{-/-} mice showing the distribution
15 of BIP-TATk28-hCDKL5 protein (yellow) and BIP-TATk28-hCDKL5 mRNA (blue).

FIG 5A shows the number of cross corrected cells positive for Cdkl5 protein in neonatal and CDKL5^{-/-} injected mice that were quantitated using Visiopharm® software.

FIG 5B shows the number of cells positive for Cdkl5 protein (with or without a signal for Cdkl5 mRNA) in the cortex, striatum, thalamus, hippocampus and hypothalamus
20 of P14 injected control and CDKL5^{-/-} injected mice quantitated using Visiopharm® pathology image analysis software.

FIG. 5C further shows the number of cross corrected cells positive for Cdkl5 protein in neonatal and CDKL5^{-/-} injected mice that were quantitated using Visiopharm® software, more specifically in multiple brain regions of P14 BIP-TATk28-hCDKL5 treated CDKL5^{-/-}
25 mice.

FIG 6 shows quantitation of brain BIP-TATk28-CDKL5 (BTC) protein expression one month after intravenous injection of PHP.B-CBH-BIP-TATk28-CDKL5 vector in P18 male CDKL5^{-/-} mice.

FIG 7 shows that intravenous delivery of PHP.B-CBH-BIP-TATk28-CDKL5 into
30 male P18 CDKL5^{-/-} mice reduced hyperactivity (horizontal activity) measured in open field testing.

FIG 8 shows strong expression of BIP-TATk28-CDKL5 (BTC) protein in WT and CDKL5^{-/-} mice at three months after injection in neonatal P0 mice. Figure 8A shows a representative Western blot (upper panel) of BIP-TATk28-CDKL5 protein expression. The lower panel shows the corresponding image of protein loading control using for quantitating BIP-TATk28-CDKL5 protein expression. Figure 8B shows a graph of the densitometric quantitation of BIP-TATk28-CDKL5 protein expression presented as fraction of endogenous wild type CDKL5 expression.

FIG 9 shows that PHP.B-CBH-BIP-TATk28-CDKL5 improves hind limb clasping and nest building but not locomotor behavioral deficits in CDKL5^{-/-} mice. PHP.B-CBH-BIP-TATk28-CDKL5 was injected ICV into neonatal mice and tests of behavior were performed three months later. FIG. 9A shows that treatment had no impact on locomotor activity; however, treatment significantly improved hind limb clasping (FIG 9B) and nest building (FIG 9C).

FIG 10 shows electroencephalogram (EEG) recording approach in 3-4 and 6-7 month old untreated and treated control and female CDKL5^{+/-} mice.

FIG 11 shows the relative power of different brain oscillation frequencies from EEG recordings taken at 12 pm -1 pm during the light cycle in 3-4 month female CDKL5^{+/-} mice and wild type (WT) controls. Figure 11A shows perturbation of the relative power of delta, theta alpha bands in CDKL5^{+/-} mice. Figure 11B shows perturbation of the relative power of the gamma frequency band in CDKL5^{+/-} mice.

FIG 12 shows the relative power of different brain oscillation frequencies recorded by EEG at 12 am -1 am during the night cycle in 3-4 month female CDKL5^{+/-} mice and wild type (WT) controls. Figure 12A shows perturbation of the relative power of delta, theta alpha frequency bands in CDKL5^{+/-} mice. Figure 12B shows perturbation of the relative power of the gamma frequency band in CDKL5^{+/-} mice. FIG 13A and 13B show that BIP-TATk28k-hCDKL5 reduces aberrant delta/theta and gamma power in CDKL5^{+/-} mice during the middle of the light cycle (12pm – 2pm window). FIG 7A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. FIG 7B shows the fraction of total pro-seizure gamma (15-50 Hz) power output.

FIGs 14A and 14B show that BIP-TATk28k-hCDKL5 reduces abnormalities in delta, alpha, and gamma frequency bands in CDKL5^{+/-} mice at the dark to light transition

(6am–7am window). FIG 14A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. The fraction of delta, theta power was decreased in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. FIG 14B shows the fraction of total pro-seizure gamma (15-50 Hz) power output.

FIGs 15A and 15B show that BIP-TATk28k-hCDKL5 reduces abnormalities in theta, alpha, and gamma frequencies in CDKL5^{+/-} mice during the light to dark transition (6pm–7pm window). FIG 15A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. FIG 15B shows the fraction of gamma (15-50 Hz) power output.

FIG 16A shows that BIP-TATk28k-hCDKL5 reduces abnormal gamma frequency power in aged female CDKL5^{+/-} mice during the middle of the light cycle (6am–7am).

FIG 16B shows that BIP-TATk28k-hCDKL5 reduces abnormal gamma frequency power in aged female CDKL5^{+/-} mice during the middle of the light cycle (12pm – 2pm).

FIGs 16C to 16F show that BIP-TATk28k-hCDKL5 reduces abnormalities in gamma frequency power in CDKL5^{+/-} mice at the dark to light transition (6am–7am window). FIG 16C shows a representative 1-hour long electrocardiogram traces from WT PBS treated mice. FIG 16D shows a representative 1-hour long electrocardiogram traces from CDKL5^{+/-} mice PBS treated mice. FIG. 16E shows a representative 1-hour long electrocardiogram traces from WT BIP-TATk28-hCDKL5 mice. FIG. 16F shows a representative 1-hour long electrocardiogram traces from CDKL5^{+/-} BIP-TATk28-hCDKL5 treated mice.

FIG. 17A further shows cortical protein expression (CDKL5) in mice, plotted as percent wild-type-PBS expression in CDKL5^{-/-} with P14 treatment. FIG. 17B further shows cortical mRNA expression, more specifically, BIP-TATk28-CDKL5 (BTC) and hCDKL5 mRNA expression levels plotted as hCDKL5-co2 transcript per 100ng mRNA.

FIGs. 18A to 18F further shows representative fluorescent images (Immunofluorescence and In Situ Hybridization) of cortex from AAVhu68-CBH-BIP-TATk28-hCDKL5co2 treated CDKL5^{-/-} mice showing the distribution of BIP-TATk28-hCDKL5 protein (yellow) and BIP-TATk28-hCDKL5 mRNA (blue). Nuclei (purple) are counterstained with DAPI (4',6-diamidino-2-phenylindole).

FIG. 19A shows a representative microscopy image of CDKL5 expression in hippocampal cross-section tissue of female control CDKL5^{+/-} mice administered with PBS.

FIG. 19B shows a representative microscopy image of CDKL5 expression in hippocampal cross-section tissue of female control CDKL5^{+/-} mice treated with AAV.BIP-TATk28-hCDKL5. FIG. 19C shows hippocampal BIP-TATk28-hCDKL5 expression, plotted as percent wildtype-PBS expression. FIG. 19D shows western blot analysis of BIP-TATk28-hCDKL5 and endogenous CDKL5 protein expression (in comparison with the total protein control) in hippocampal tissue of female CDKL5^{+/-} mice.

FIG. 20 shows biodistribution results for peripheral tissue, brain, dorsal root ganglion (DRG), spinal cord (SC), plotted as GC/diploid cell. The values are summarized in tables 1A, 1B, and 1C below.

FIG. 21 shows RNA biodistribution for select tissues of peripheral organs, brain, DRG and SC. The values are summarized in tables 2A, 2B, and 2C below.

FIG. 22 shows summarized results of the ELISPOT analysis (Tables 3A and 3B) for AAVhu68, plotted as spot forming units (SFU) per 10⁶ (million) cells.

FIG. 23 shows summarized results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5, plotted as SFU per 10⁶ (million) cells. These results indicate some liver transduction with AAVhu68 and hCDKL5 expression following ICM administration.

FIG. 24A shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool A, plotted as SFU per 10⁶ (million) cells. FIG. 24B shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool B, plotted as SFU per 10⁶ (million) cells. FIG. 24C shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool C, plotted as SFU per 10⁶ (million) cells. FIG. 24D shows results of the ELISPOT analysis (Table 3A) for AAVhu68 baseline PBMC (for subjects 171164, 191410, HS1602026 as analyzed with dimethyl sulfoxide (DMSO), Pool A, Pool B, and Pool C), plotted as SFU per 10⁶ (million) cells. FIG. 24E shows results of the ELISPOT analysis (Table 3A) for AAVhu68 D35/36 PBMC (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10⁶ (million) cells. FIG. 24F shows results of the ELISPOT analysis (Table 3A) for AAVhu68 spleen sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10⁶ (million) cells. FIG. 24G shows results of the ELISPOT analysis (Table 3A) for AAVhu68 liver sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10⁶ (million) cells. FIG. 24H shows results of the ELISPOT analysis

(Table 3A) for AAVhu68 lymph node sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells.

FIG. 25A shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool A-1, plotted as SFU per 10^6 (million) cells. FIG. 25B shows results of the
 5 ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool A, plotted as SFU per 10^6 (million) cells. FIG. 25C shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool B, plotted as SFU per 10^6 (million) cells. FIG. 25D shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool C, plotted as SFU per 10^6 (million) cells. FIG. 25E shows results of the ELISPOT analysis (Tables 3A and 3B) for
 10 hCDKL5 PBMC pool D, plotted as SFU per 10^6 (million) cells. FIG. 25F shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 baseline PBMC sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25G shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 D35/D36 PBMC sample (for subjects
 15 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25H shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 spleen sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25I shows results of the ELISPOT
 20 analysis (Tables 3A and 3B) for hCDKL5 liver sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25J shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 lymph node sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides),
 25 plotted as SFU per 10^6 (million) cells. Overall, these results indicate a transgene specific T cell responses seen in all animals.

FIG. 26A shows measured velocity in left medial nerve. FIG. 26B shows measured velocity in right medial nerve. FIG. 26C shows NP amplitude in left medial nerve. FIG. 26D shows NP amplitude in right medial nerve. FIG. 26E shows peak to peak (PP) amplitude in
 30 left medial nerve. FIG. 26F shows PP amplitude in right medial nerve. FIG. 26G shows measured velocity in left sural nerve. FIG. 26H shows measured velocity in right sural nerve. FIG. 26I shows NP amplitude in left sural nerve. FIG. 26J shows NP amplitude in right sural

nerve. FIG. 26K shows PP amplitude in left sural nerve. FIG. 26L shows PP amplitude in right sural nerve.

DETAILED DESCRIPTION OF THE INVENTION

5 Compositions and methods for treating CDD are provided herein. In certain embodiments, the secretable CDKL5 fusion proteins delivered via the AAV provided herein are believed to provide broader brain CDKL5 delivery and expression, contributing to cross-correction (i.e., correction in cells not initially transduced with the rAAV vector).

The various nucleic acid sequences provided herein are useful for packaging BIP-
10 TATk28-hCDKL5 coding sequence into a suitable vector (e.g., a rAAV) or a genetic element useful for manufacture (e.g., a plasmid).

The hCDKL5 sequences herein encode human CDKL5 protein isoform 1. The wild-type hCDKL5 sequences are reproduced in SEQ ID NO: 1. In the fusion protein provided herein, the hCDKL5 sequence is fused at its N-terminus to an exogenous BIP leader
15 sequence (Bip) and a peptide uptake enhancer (TATk28), which is designed to enhance uptake of the peptide by cells following expression of the fusion protein from cells transduced with the expression cassette. The fusion protein contains the human CDKL5 amino acid sequence of SEQ ID NO: 24. In certain embodiments, a sequence having at least 95% to 100% identity to SEQ ID NO: 24 (also amino acid 61 to 1020 of SEQ ID NO: 11)
20 and/or values therebetween, at least 96% identity to 100% identity, at least 97% identity, at least 98% identity, at least 99% identity, 99% to 100% identity to SEQ ID NO: 24 may be used which provides functional hCDKL5 activity.

The hCDKL5 coding sequences provided herein are engineered sequences. SEQ ID NO: 2 provides engineered sequences which are referred to interchangeably as hCDKL5co2
25 or hCDKL5 engineered sequences. Encompassed herein are CDKL5 coding sequences which are 95% to 100% identical to the contiguous sequence of SEQ ID NO: 5 which encode SEQ ID NO: 1 (optionally lacking the initial Met, such as in SEQ ID NO: 24), i.e., at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical to SEQ ID NO: 1, or up to 100% identical, and values therebetween
30 which encode SEQ ID NO: 1 (optionally lacking the initial Met, as in SEQ ID NO: 24).

The CDKL5 fusion protein provided herein further comprises an uptake peptide fused directly or via a short linker (e.g., GGGGS) to the N-terminus of the hCDKL5 protein.

As provided herein, the uptake peptide is TATk28-peptide (also referenced to as the TAT peptide) having the amino acid sequence of SEQ ID NO: 7. In certain embodiments, the TATk28-peptide is encoded by SEQ ID NO: 5 or a sequence at least 99% identical thereto which encodes SEQ ID NO: 7.

5 The CDKL5 fusion protein provided herein further comprises a BIP signal peptide (also referenced to as Bip, or BiP signal peptide) fused to the N-terminus of the TATk28-optional linker-CDKL5 fusion. As provided herein the signal peptide has the sequence of SEQ ID NO: 3. In certain embodiments, the BIP signal peptide is encoded by SEQ ID NO: 4. In certain embodiments, the BIP signal peptide is encoded by a sequence at least 99%
10 identical to SEQ ID NO: 4 which encodes SEQ ID NO: 4.

 In certain embodiments, the BIP-TATk28-hCDKL5 is a fusion protein having the amino acid sequence of SEQ ID NO: 11. Throughout the specification, this is also used interchangeably with BIP-TAT-hCDKL5 or BIP-TATk28-hCDKL5-co2. In certain
15 embodiments, the fusion protein has a sequence at least 99% identical to SEQ ID NO: 11. In certain embodiments, the BIP-TATk28-hCDKL5 is encoded by a nucleic acid sequence of SEQ ID NO: 10 or a sequence at least 95% to 100% identical to SEQ ID NO: 10, and/or values therebetween, at least 96% identity to 100% identity, at least 97% identity, at least 98% identity, at least 99% identity, 99% to 100% identity to SEQ ID NO: 10.

 Without wishing to be bound by theory, in a cross correction, the CDKL5 is made
20 within cell following administration with expression cassette or a vector genome comprising BIP-TATk28-hCDKL5, wherein some expressed hCDKL5 stays within a cell, while some is secreted and taken up by neighboring cells. This approach allows for cross correction (i.e., delivery of hCDKL5 to cells not transduced by the AAV.BIP-TATk28-hCDKL5. For example, an early study shows cross-correction in hippocampus, cortex, striatum, thalamus,
25 and hypothalamus. See, Example 2 below. See also, WO2015/013148A2 and WO2010/129021A1, which are incorporated herein by reference in its entirety. In certain embodiments, the extent of cross correction is examined using visualization of number of

cells positive for Cdkl5 protein (cross corrected and transduced cells) in comparison with or without a signal for Cdkl5 mRNA (transduced cells) in various tissues.

II. Expression Cassette

5 A gene therapy vector is provided herein which comprises an expression cassette comprising a nucleic acid sequence comprising coding sequences for BIP-TATk28-hCDKL5 operably linked to regulatory sequences which direct expression thereof.

As used herein, an “expression cassette” refers to a nucleic acid molecule which comprises a biologically useful nucleic acid sequence (e.g., a gene cDNA encoding a protein
10 (i.e., a CDKL5 coding sequence), enzyme or other useful gene product, mRNA, etc.) and regulatory sequences operably linked thereto which direct or modulate transcription, translation, and/or expression of the nucleic acid sequence and its gene product. As used herein, “operably linked” sequences include both regulatory sequences that are contiguous or non-contiguous with the nucleic acid sequence and regulatory sequences that act in trans or
15 cis nucleic acid sequence. Such regulatory sequences typically include, e.g., one or more of a promoter(s), an enhancer(s), an intron(s), a Kozak sequence, a polyadenylation sequence, and a TATA signal. The expression cassette may contain regulatory sequences upstream (5’ to) of the gene sequence, e.g., one or more of a promoter, an enhancer, an intron, etc., and one or more of an enhancer, or regulatory sequences downstream (3’ to) a gene sequence,
20 e.g., 3’ untranslated region (3’ UTR) comprising a polyadenylation site, among other elements. In certain embodiments, the regulatory sequences are operably linked to the nucleic acid sequence of a gene product, wherein the regulatory sequences are separated from nucleic acid sequence of a gene product by an intervening nucleic acid sequences, i.e., 5’-untranslated regions (5’UTR). In certain embodiments, the expression cassette comprises
25 nucleic acid sequence of one or more of gene products. In some embodiments, the expression cassette can be a monocistronic or a bicistronic expression cassette. In other embodiments, the term “transgene” refers to one or more DNA sequences from an exogenous source which are inserted into a target cell.

Typically, such an expression cassette can be used for generating a viral vector and
30 contains the coding sequence for the gene product described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described

herein. In certain embodiments, a vector genome may contain two or more expression cassettes.

In certain embodiments, an expression cassette comprising the coding sequence for BIP-TATk28-hCDKL5 operably linked to regulatory sequences for directed its expression.

5 In certain embodiments, the regulatory sequences comprise a chicken β -actin hybrid (also referenced to as CBh or CBH) promoter and a polyA sequence. In certain embodiments, the chicken β -actin hybrid promoter has a sequence of SEQ ID NO: 12. In certain embodiments, the expression cassette further comprises a Kozak sequence. In certain embodiments, the expression cassette comprises the chicken β -actin hybrid promoter sequence, a Kozak
10 sequence, the BIP-TATk28-hCDKL5 coding sequence, and an SV40 polyA sequence. In certain embodiments, the SV40 polyA sequence has a sequence of SEQ ID NO: 26. In certain embodiments, the expression cassette has a nucleic acid sequence of SEQ ID NO: 13 or sequence at least 95% identical thereto, or at least 96% identical thereto, or at least 97% identical thereto, or at least 98% identical thereto, or at least 99% identical, or 95% to 100%
15 identical to SEQ ID NO: 13, and/or values therebetween.

Typically, such an expression cassette to be packed into a viral vector contains the coding sequence for the gene product described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein. Thus, in certain embodiments, the vector genome comprises a 5' ITR, the chicken β -actin hybrid
20 promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, an SV40 polyA sequence, and a 3' ITR. In certain embodiments, the vector genome has the nucleic acid sequence of SEQ ID NO: 14 or a sequence at least 95% identical thereto, or at least 96% identical thereto, or at least 97% identical thereto, or at least 98% identical thereto, or at least 99% identical, or 95% to 100% identical to SEQ ID NO: 14, and/or values
25 therebetween.

In certain embodiment, the target cell may be a central nervous system cell. In certain embodiments, the target cell is one or more of an excitatory neuron, an inhibitory neuron, a glial cell, a cortex cell, a frontal cortex cell, a cerebral cortex cell, a spinal cord cell. In certain embodiments, the target cell is a peripheral nervous system (PNS) cell, for
30 example a retina cell. Other cells other than those from nervous system may also be chosen as a target cell, such as a monocyte, a B lymphocyte, a T lymphocyte, a NK cell, a lymph node cell, a tonsil cell, a bone marrow mesenchymal cell, a stem cell, a bone marrow stem

cell, a heart cell, an epithelium cell, a esophagus cell, a stomach cell, a fetal cut cell, a colon cell, a rectum cell, a liver cell, a kidney cell, a lung cell, a salivary gland cell, a thyroid cell, an adrenal cell, a breast cell, a pancreas cell, an islet of Langerhans cell, a gallbladder cell, a prostate cell, a urinary bladder cell, a skin cell, a uterus cell, a cervix cell, a testis cell, or any
5 other cell which expresses a functional CDKL5 protein in a subject without CDD.

In certain embodiments, an additional or alternative promoter sequence may be included as part of the expression control sequences (regulatory sequences), e.g., located between the selected 5' ITR sequence and the coding sequence. Constitutive promoters, regulatable promoters [see, e.g., WO 2011/126808 and WO 2013/04943], tissue specific
10 promoters, or a promoter responsive to physiologic cues may be utilized in the vectors described herein. The promoter(s) can be selected from different sources, e.g., human cytomegalovirus (CMV) immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polymovirus promoter, myelin basic protein (MBP) or glial fibrillary acidic protein (GFAP) promoters, herpes simplex virus (HSV-1) latency associated
15 promoter (LAP), rouse sarcoma virus (RSV) long terminal repeat (LTR) promoter, neuron-specific promoter (NSE), platelet derived growth factor (PDGF) promoter, hSYN, melanin-concentrating hormone (MCH) promoter, CBA, matrix metalloprotein promoter (MPP), and the chicken beta-actin promoter.

In addition to a promoter, a vector may contain one or more other appropriate
20 transcription initiation sequences, transcription termination sequences, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA for example WPRE; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. An
25 example of a suitable enhancer is the CMV enhancer. Other suitable enhancers include those that are appropriate for desired target tissue indications. In one embodiment, the regulatory sequences comprise one or more expression enhancers. In one embodiment, the regulatory sequences contain two or more expression enhancers. These enhancers may be the same or may differ from one another. For example, an enhancer may include a CMV immediate
30 early (CMV IE) enhancer. This enhancer may be present in two copies which are located adjacent to one another. Alternatively, the dual copies of the enhancer may be separated by one or more sequences. In still another embodiment, the expression cassette further contains

an intron, e.g., the chicken beta-actin intron. In certain embodiments, the intron is a chimeric intron (CI) – a hybrid intron consisting of a human beta-globin splice donor and immunoglobulin G (IgG) splice acceptor elements. Other suitable introns include those known in the art, e.g., such as are described in WO 2011/126808. Examples of suitable polyA sequences include, e.g., Rabbit globin poly A, SV40, SV50, bovine growth hormone (bGH), human growth hormone, and synthetic polyAs. Optionally, one or more sequences may be selected to stabilize mRNA. The expression cassettes provided may include one or more expression enhancers such as post-transcriptional regulatory element from hepatitis viruses of woodchuck (WPRE), human (HPRE), ground squirrel (GPRE) or arctic ground squirrel (AGSPRE); or a synthetic post-transcriptional regulatory element. These expression-enhancing elements are particularly advantageous when placed in a 3' UTR and can significantly increase mRNA stability and/or protein yield. In certain embodiments, the expressions cassettes provided include a regulator sequence that is a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) or a variant thereof. Suitable WPRE sequences are provided in the vector genomes described herein and are known in the art (e.g., such as those are described in US Patent Nos. 6,136,597, 6,287,814, and 7,419,829, which are incorporated by reference). In certain embodiments, the WPRE is a variant that has been mutated to eliminate expression of the woodchuck hepatitis B virus X (WHX) protein, including, for example, mutations in the start codon of the WHX gene (See, Zanta-Boussif et al., Gene Ther. 2009 May;16(5):605-19, which is incorporated by reference). In other embodiments, enhancers are selected from a non-viral source. In certain embodiments, no WPRE sequence is present.

III. rAAV

Provided herein is a recombinant adeno-associated virus (rAAV) useful for treating CDD. The rAAV comprises (a) an AAV capsid; and (b) a vector genome packaged in the AAV capsid of (a). Suitably, the AAV capsid selected targets the cells to be treated. In certain embodiments, the capsid is from Clade F.

In certain embodiments, the Clade F AAV capsid is selected from an AAVhu68 capsid [See, e.g., US2020/0056159; PCT/US21/55436; SEQ ID NO: 19 and 21 for nucleic acid sequence; SEQ ID NO: 20 for amino acid sequence] or an AAVhu95 capsid [See, e.g., US Provisional Application No. 63/251,599, filed October 2, 2021; SEQ ID NO: 22 (hu95

nucleic acid sequence) and SEQ ID NO: 23 (hu95 amino acid sequence). In certain embodiments, the AAV capsid is a Clade A capsid, such as AAVrh91 capsid (nucleic acid sequence of SEQ ID NOs: 15 and 17). See, PCT/US20/030266, filed April 29, 2020, now published WO2020/223231, which is incorporated by reference herein and International
5 Application No. PCT/US21/45945, filed August 13, 2021 which are incorporated herein by reference.

In certain embodiments, the AAV capsid for the compositions and methods described herein is chosen based on the target cell. In certain embodiment, the AAV capsid transduces a CNS cell and/or a PNS cell. In certain embodiments, other AAV capsid may be
10 chosen. the AAV capsid is selected from a cy02 capsid, a rh43 capsid, an AAV8 capsid, a rh01 capsid, an AAV9 capsid, an rh8 capsid, a rh10 capsid, a bb01 capsid, a hu37 capsid, a rh02 capsid, a rh20 capsid, a rh39 capsid, a rh64 capsid, an AAV6 capsid, an AAV1 capsid, a hu44 capsid, a hu48 capsid, a cy05 capsid a hu11 capsid, a hu32 capsid, a pi2 capsid, or a variation thereof. In certain embodiments, the AAV capsid is a Clade F capsid, such as
15 AAV9 capsid, AAVhu68 capsid, hu31 capsid, hu32 capsid, or a variation thereof. See, e.g., WO 2005/033321 published April 14, 2015, WO 2018/160582, and US 2015/0079038, each of which is incorporated herein by reference in its entirety. In certain embodiments, the AAV capsid is a non-clade F capsid, for example a Clade A, B, C, D, or E capsid. In certain embodiment, the non-Clade F capsid is an AAV1 or a variation thereof. In certain
20 embodiment, the AAV capsid transduces a target cell other than the nervous system cells. In certain embodiments, the AAV capsid is a Clade A capsid (e.g., AAV1, AAV6, AAVrh91), a Clade B capsid (e.g., AAV 2), a Clade C capsid (e.g., hu53), a Clade D capsid (e.g., AAV7), or a Clade E capsid (e.g., rh10).

As used herein, the term “clade” as it relates to groups of AAV refers to a group of
25 AAV which are phylogenetically related to one another as determined using a Neighbor-Joining algorithm by a bootstrap value of at least 75% (of at least 1000 replicates) and a Poisson correction distance measurement of no more than 0.05, based on alignment of the AAV vp1 amino acid sequence. The Neighbor-Joining algorithm has been described in the literature. See, e.g., M. Nei and S. Kumar, Molecular Evolution and Phylogenetics (Oxford
30 University Press, New York (2000). Computer programs are available that can be used to implement this algorithm. For example, the MEGA v2.1 program implements the modified Nei-Gojobori method. Using these techniques and computer programs, and the sequence of

an AAV vp1 capsid protein, one of skill in the art can readily determine whether a selected AAV is contained in one of the clades identified herein, in another clade, or is outside these clades. See, e.g., G Gao, et al, J Virol, 2004 Jun; 78(10): 6381-6388, which identifies Clades A, B, C, D, E and F, and provides nucleic acid sequences of novel AAV, GenBank

5 Accession Numbers AY530553 to AY530629. See, also, WO 2005/033321.

A rAAV is composed of an AAV capsid and a vector genome. An AAV capsid is an assembly of a heterogeneous population of vp1, a heterogeneous population of vp2, and a heterogeneous population of vp3 proteins. As used herein when used to refer to vp capsid proteins, the term “heterogeneous” or any grammatical variation thereof, refers to a
10 population consisting of elements that are not the same, for example, having vp1, vp2 or vp3 monomers (proteins) with different modified amino acid sequences.

As used herein when used to refer to vp capsid proteins, the term “heterogeneous” or any grammatical variation thereof, refers to a population consisting of elements that are not the same, for example, having vp1, vp2 or vp3 monomers (proteins) with different modified
15 amino acid sequences. The term “heterogeneous population” as used in connection with vp1, vp2 and vp3 proteins (alternatively termed isoforms), refers to differences in the amino acid sequence of the vp1, vp2 and vp3 proteins within a capsid. The AAV capsid contains subpopulations within the vp1 proteins, within the vp2 proteins and within the vp3 proteins which have modifications from the predicted amino acid residues. These subpopulations
20 include, at a minimum, certain deamidated asparagine (N or Asn) residues. For example, certain subpopulations comprise at least one, two, three or four highly deamidated asparagines (N) positions in asparagine - glycine pairs and optionally further comprising other deamidated amino acids, wherein the deamidation results in an amino acid change and other optional modifications.

25 In certain embodiments, AAV capsids are provided which have a heterogeneous population of AAV capsid isoforms (i.e., VP1, VP2, VP3) which contain multiple highly deamidated “NG” positions. In certain embodiments, the highly deamidated positions are in the locations identified below, with reference to the predicted full-length VP1 amino acid sequence. In other embodiments, the capsid gene is modified such that the referenced “NG”
30 is ablated and a mutant “NG” is engineered into another position.

In certain embodiments, the AAV capsid is a AAVhu68 capsid, or an AAVrh91 capsid. In certain embodiments, the AAVhu68 capsid comprises amino acid sequence of

SEQ ID NO: 20. In certain embodiments, the AAVhu68 capsid comprises: (i) AAVhu68 vp1 proteins, AAVhu68 vp2 proteins, and AAVhu68 vp3 proteins produced from a nucleic acid sequence encoding SEQ ID NO: 20; or (ii) heterogeneous populations of AAVhu68 vp1, AAVhu68 vp2 and AAVhu68 vp3 proteins, wherein the subpopulations of the AAVhu68
5 vp1, AAVhu68 vp2 and AAVhu68 vp3 proteins comprise at least 50% to 100% deamidated asparagines (N) in asparagine - glycine pairs at each of positions 57, 329, 452, 512, relative to the amino acids in SEQ ID NO: 20, wherein the deamidated asparagines are deamidated to aspartic acid, isoaspartic acid, an interconverting aspartic acid/isoaspartic acid pair, or combinations thereof, as determined using mass spectrometry. In certain embodiments, the
10 nucleic acid sequence encoding AAVhu68 vp1 protein is SEQ ID NO: 19, or a sequence at least 80% to at least 99% identical to SEQ ID NO: 19 which encodes the amino acid sequence of SEQ ID NO: 20; optionally wherein the nucleic acid sequence is at least 80% to 97% identical to SEQ ID NO: 19. In certain embodiments, the nucleic acid sequence encoding AAVhu68 vp1 protein is SEQ ID NO: 21, or a sequence at least 80% to at least
15 99% identical to SEQ ID NO: 21 which encodes the amino acid sequence of SEQ ID NO: 20; optionally wherein the nucleic acid sequence is at least 80% to 97% identical to SEQ ID NO: 21.

As used herein, the terms “target cell” and “target tissue” can refer to any cell or tissue which is intended to be transduced by the subject AAV vector. The term may refer to
20 any one or more of muscle, liver, lung, airway epithelium, central nervous system, neurons, eye (ocular cells), or heart.

As used herein, a “vector genome” refers to the nucleic acid sequence packaged inside the rAAV capsid which forms a viral particle. Such a nucleic acid sequence contains AAV inverted terminal repeat sequences (ITRs). In the examples herein, a vector genome
25 contains, at a minimum, from 5' to 3', an AAV 5' ITR, coding sequence(s) (i.e., transgene(s)), and an AAV 3' ITR. In certain embodiments, the ITRs are from AAV2, a different source AAV than the capsid, or other than full-length ITRs may be selected. In certain embodiments, the ITRs are from the same AAV source as the AAV which provides the rep function during production or a transcomplementing AAV. Further, other ITRs, e.g.,
30 self-complementary (scAAV) ITRs, may be used. Both single-stranded AAV and self-complementary (sc) AAV are encompassed with the rAAV. Additionally, a shortened version of the 5' ITR, termed Δ ITR, has been described in which the D-sequence and

terminal resolution site (trs) are deleted. In certain embodiments, the vector genome includes a shortened AAV2 ITR of 130 base pairs, wherein the external A elements is deleted.

Without wishing to be bound by theory, it is believed that the shortened ITR reverts back to the wild-type (WT) length of 145 base pairs during vector DNA amplification using the

5 internal (A') element as a template. In other embodiments, full-length AAV 5' and 3' ITRs are used. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed pseudotyped. However, other configurations of these elements may be suitable.

The transgene is a nucleic acid coding sequence, heterologous to the vector
10 sequences, which encodes a polypeptide, protein, functional RNA molecule (e.g., miRNA, miRNA inhibitor) or other gene product, of interest. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a cell of a target tissue. Suitable components of a vector genome are discussed in more detail herein. In one example, a "vector genome"
15 contains, at a minimum, from 5' to 3', a vector-specific sequence, a nucleic acid sequence encoding protein of interest operably linked to regulatory control sequences (which direct their expression in a target cell), where the vector-specific sequence may be a terminal repeat sequence which specifically packages the vector genome into a viral vector capsid or envelope protein. For example, AAV inverted terminal repeats are utilized for packaging into
20 AAV and certain other parvovirus capsids.

In certain embodiments, the vector genome is an expression cassette having inverted terminal repeat (ITR) sequences necessary for packaging the vector genome into the AAV capsid at the extreme 5' and 3' end and containing therebetween a *CDLK5* gene as described herein operably linked to sequences which direct expression thereof.

25 As used herein, the term "host cell" may refer to the packaging cell line in which the rAAV is produced from the plasmid. In the alternative, the term "host cell" may refer to the target cell in which expression of the transgene is desired.

Additionally, provided herein, is an rAAV production system useful for producing a rAAV as described herein. The production system comprises a cell culture comprising (a) a
30 nucleic acid sequence encoding an AAV capsid protein; (b) the vector genome; and (c) sufficient AAV rep functions and helper functions to permit packaging of the vector genome into the AAV capsid. In certain embodiments, the vector genome comprises a AAV 5' ITR,

expression cassette comprising engineered hCDKL5 coding sequence, optionally further comprising BiP and or Tatk28 peptides, operably linked to expression control sequences which control expression thereof, and AAV 3' ITR. In certain embodiments, the vector genome comprises a AAV 5' ITR, expression cassette comprising BiP peptide, Tatk28, and engineered hCDKL5 coding sequence operably linked to expression control sequences which control expression thereof, and AAV 3' ITR. In certain embodiments, the vector genome comprises a AAV 5' ITR, expression cassette comprising nucleic acid sequence of SEQ ID NO: 13, and AAV 3' ITR. In certain embodiments, the vector genome is SEQ ID NO: 14. In certain embodiments, the cell culture is a human embryonic kidney 293 cell culture. In certain embodiments, the AAV rep is from a different AAV. In certain embodiments, wherein the AAV rep is from AAV2. In certain embodiments, the AAV rep coding sequence and cap genes are on the same nucleic acid molecule, wherein there is optionally a spacer between the rep sequence and cap gene.

For use in producing an AAV viral vector (e.g., a recombinant (r) AAV), the vector genomes can be carried on any suitable vector, e.g., a plasmid, which is delivered to a packaging host cell. The plasmids useful in this invention may be engineered such that they are suitable for replication and packaging in vitro in prokaryotic cells, insect cells, mammalian cells, among others. Suitable transfection techniques and packaging host cells are known and/or can be readily designed by one of skill in the art.

In certain embodiments, a plasmid useful in producing an rAAV particle is provided which comprises an expression cassette comprising a chicken β -actin hybrid promoter sequence, a Kozak sequence, a BIP-TATk28-hCDKL5 coding sequence, and an SV40 polyA sequence, said BIP-TATk28-hCDKL5 encoding a protein comprising the BIP amino acid sequence of SEQ ID NO: 3, the TATk28-amino acid sequence of SEQ ID NO: 7, and the hCDKL5 amino acid sequence of SEQ ID NO: 24. In certain embodiments, the expression cassette has the nucleic acid sequence of SEQ ID NO: 13 or sequence at least 95% identical thereto. In certain embodiments, the expression cassette has the nucleic acid sequence of SEQ ID NO: 25 or sequence at least 95% identical thereto.

In certain embodiments, a nucleic acid (e.g., a plasmid) useful in rAAV production comprises a vector genome comprising a 5' ITR, the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, an SV40 polyA

sequence, and a 3' ITR. In certain embodiments, the vector genome has the nucleic acid sequence of SEQ ID NO: 14 or a sequence at least 95% identical thereto.

Methods for generating and isolating AAVs suitable for use as vectors are known in the art. See generally, e.g., Grieger & Samulski, 2005, Adeno-associated virus as a gene
5 therapy vector: Vector development, production and clinical applications, Adv. Biochem. Engin/Biotechnol. 99: 119-145; Buning et al., 2008, Recent developments in adeno-associated virus vector technology, J. Gene Med. 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety. As used herein, a gene therapy vector refers to a rAAV as described herein, which is suitable for use in treating
10 a patient. For packaging a gene into virions, the ITRs are the only AAV components required in cis in the same construct as the nucleic acid molecule containing the gene. The cap and rep genes can be supplied in trans.

In certain embodiments, the manufacturing process for rAAV involves method as described in US Provisional Patent Application No. 63/371,597, filed August 16, 2022, and
15 US Provisional Patent Application No. 63/371,592, filed August 16, 2022, which are incorporated herein by reference in its entirety.

In one embodiment, the selected genetic element may be delivered to an AAV packaging cell by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and
20 protoplast fusion. Stable AAV packaging cells can also be made. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Molecular Cloning: A Laboratory Manual, ed. Green and Sambrook, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

25 The term "AAV intermediate" or "AAV vector intermediate" refers to an assembled rAAV capsid which lacks the desired genomic sequences packaged therein. These may also be termed an "empty" capsid. Such a capsid may contain no detectable genomic sequences of an expression cassette, or only partially packaged genomic sequences which are insufficient to achieve expression of the gene product. These empty capsids are non-
30 functional to transfer the gene of interest to a host cell.

The recombinant adeno-associated virus (AAV) described herein may be generated using techniques which are known. See, e.g., WO 2003/042397; WO 2005/033321, WO

2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional rep gene; an expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into the AAV capsid protein. Methods of generating the capsid, coding sequences therefor, and methods for production of rAAV viral vectors have been described. See, e.g., Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

In one embodiment, a production cell culture useful for producing a recombinant AAV having a capsid selected from AAVhu68, AAVrh91, or hu95 is provided. Such a cell culture contains a nucleic acid which expresses the AAVhu68 capsid protein in the host cell (e.g., SEQ ID NO: 19 or SEQ ID NO: 21; a nucleic acid molecule suitable for packaging into the AAVhu68 capsid, e.g., a vector genome which contains AAV ITRs and a non-AAV nucleic acid sequence encoding a gene operably linked to regulatory sequences which direct expression of the gene in a host cell; and sufficient AAV rep functions and adenovirus helper functions to permit packaging of the vector genome into the recombinant AAVhu68, or AAVrh91 capsid (e.g., SEQ ID NO: 15 or SEQ ID NO: 17), AAVhu95 capsid (e.g., SEQ ID NO: 22). In one embodiment, the cell culture is composed of mammalian cells (e.g., human embryonic kidney 293 cells, among others) or insect cells (e.g., *Spodoptera frugiperda* (Sf9) cells). In certain embodiments, baculovirus provides the helper functions necessary for packaging the vector genome into the recombinant AAVhu68, AAVrh91, or AAVhu95 capsid.

Optionally the rep functions are provided by an AAV other than AAV2, selected to complement the source of the ITRs.

In one embodiment, cells are manufactured in a suitable cell culture (e.g., HEK 293 or Sf9) or suspension. Methods for manufacturing the gene therapy vectors described herein include methods well known in the art such as generation of plasmid DNA used for production of the gene therapy vectors, generation of the vectors, and purification of the vectors. In some embodiments, the gene therapy vector is an AAV vector and the plasmids generated are an AAV cis-plasmid encoding the AAV vector genome and the gene of interest, an AAV trans-plasmid containing AAV rep and cap genes, and an adenovirus helper plasmid. The vector generation process can include method steps such as initiation of cell culture, passage of cells, seeding of cells, transfection of cells with the plasmid DNA, post-

transfection medium exchange to serum free medium, and the harvest of vector-containing cells and culture media. The harvested vector-containing cells and culture media are referred to herein as crude cell harvest. In yet another system, the gene therapy vectors are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, e.g., Zhang et al., 2009, Adenovirus-adenovirus associated virus hybrid for large-scale recombinant adeno-associated virus production, Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following US patents, the contents of each of which is incorporated herein by reference in its entirety: US Patent Nos. 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065.

The crude cell harvest may thereafter be subject to further processing including, concentration of the vector harvest, diafiltration of the vector harvest, microfluidization of the vector harvest, nuclease digestion of the vector harvest, filtration of microfluidized intermediate, crude purification by chromatography, crude purification by ultracentrifugation, buffer exchange by tangential flow filtration, and/or formulation and filtration to prepare bulk vector. An affinity chromatography purification followed anion exchange resin chromatography are used to purify the vector drug product and to remove empty capsids. These methods are described in more detail in WO2017/160360, filed December 9, 2016, entitled "Scalable Purification Method for AAV9", which is incorporated by reference. Purification methods for AAV8, WO2017/100676, filed December 9, 2016, and rh10, WO2017/100704, filed December 9, 2016, entitled "Scalable Purification Method for AAVrh10", also filed December 11, 2015, and for AAV1, WO2017/100674, filed December 9, 2016 for "Scalable Purification Method for AAV1", filed December 11, 2015, are all incorporated by reference herein. Other suitable methods may be selected.

To calculate empty and full particle content, VP3 band volumes for a selected sample (e.g., in examples herein an iodixanol gradient-purified preparation where # of genome copies (GC) = # of particles) are plotted against GC particles loaded. The resulting linear equation ($y = mx + c$) is used to calculate the number of particles in the band volumes of the test article peaks. The number of particles (pt) per 20 μ L loaded is then multiplied by 50 to give particles (pt) /mL. Pt/mL divided by GC/mL gives the ratio of particles to

genome copies (pt/GC). Pt/mL–GC/mL gives empty pt/mL. Empty pt/mL divided by pt/mL and x 100 gives the percentage of empty particles.

Generally, methods for assaying for empty capsids and AAV vector particles with packaged genomes have been known in the art. See, e.g., Grimm et al., *Gene Therapy* (1999) 6:1322-1330; Sommer et al., *Molec. Ther.* (2003) 7:122-128. To test for denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., *J. Virol.* (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the primary antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies, preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE loading buffer containing reducing agent (e.g., DTT), and capsid proteins were resolved on pre-cast gradient polyacrylamide gels (e.g., Novex). Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions or other suitable staining method, i.e., SYPRO ruby or coomassie stains. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further diluted and amplified using primers and a TaqManTM fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the

AAV vector is employed to generate a standard curve in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

5 In one aspect, an optimized q-PCR method is used which utilizes a broad spectrum serine protease, e.g., proteinase K (such as is commercially available from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with
10 proteinase K buffer in an amount equal to the sample size. The proteinase K buffer may be concentrated to 2-fold or higher. Typically, proteinase K treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally conducted at about 55 °C for about 15 minutes, but may be performed at a lower temperature (e.g., about 37 °C to about 50 °C) over a longer time period (e.g., about 20 minutes to about
15 30 minutes), or a higher temperature (e.g., up to about 60 °C) for a shorter time period (e.g., about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (e.g., about 70 to about 90 °C) and the time extended (e.g., about 20 minutes to about 30 minutes). Samples are then diluted (e.g., 1000-fold) and subjected to TaqMan analysis as described in the standard assay.

20 Additionally, or alternatively, droplet digital PCR (ddPCR) may be used. For example, methods for determining single-stranded and self-complementary AAV vector genome titers by ddPCR have been described. See, e.g., M. Lock et al, *Hu Gene Therapy Methods*, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14.

25 In brief, the method for separating rAAVhu68 (or AAVrh91 or AAVhu95) particles having packaged genomic sequences from genome-deficient AAVhu68 (or AAVrh91 or AAVhu95) intermediates involves subjecting a suspension comprising recombinant AAVhu68 (or rh91) viral particles and AAVhu68 (or AAVrh91 or AVhu95) capsid intermediates to fast performance liquid chromatography, wherein the AAVhu68 (or
30 AAVrh91 or AAVhu95) viral particles and AAVhu68 intermediates are bound to a strong anion exchange resin equilibrated at a pH of about 10.2 (or about 9.8 for AAVrh91), and subjected to a salt gradient while monitoring eluate for ultraviolet absorbance at about 260

nanometers (nm) and about 280 nm. Although less optimal for rAAVhu68 and AAVrh91, the pH may be in the range of about 10 to 10.4. In this method, the AAV full capsids are collected from a fraction which is eluted when the ratio of A260/A280 reaches an inflection point. In one example, for the Affinity Chromatography step, the diafiltered product may be applied to an affinity resin (Life Technologies) that efficiently captures the AAV serotype. Under these ionic conditions, a significant percentage of residual cellular DNA and proteins flow through the column, while AAV particles are efficiently captured.

The rAAV.hCDKL5 (e.g., rAAV.BIP-TATk28.hCDKL5) is suspended in a suitable physiologically compatible composition (e.g., a buffered saline). This composition may be frozen for storage, later thawed and optionally diluted with a suitable diluent. Alternatively, the vector may be prepared as a composition which is suitable for delivery to a patient without proceeding through the freezing and thawing steps.

As used herein, the term "NAb titer" a measurement of how much neutralizing antibody (e.g., anti-AAV Nab) is produced which neutralizes the physiologic effect of its targeted epitope (e.g., an AAV). Anti-AAV NAb titers may be measured as described in, e.g., Calcedo, R., et al., Worldwide Epidemiology of Neutralizing Antibodies to Adeno-Associated Viruses. *Journal of Infectious Diseases*, 2009. 199(3): p. 381-390, which is incorporated by reference herein.

The abbreviation "sc" refers to self-complementary. "Self-complementary AAV" refers a construct in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. See, e.g., D M McCarty et al, "Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis", *Gene Therapy*, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, e.g., U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

A "replication-defective virus" or "viral vector" refers to a synthetic or artificial viral particle in which an expression cassette containing a gene of interest is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid

or envelope are replication-deficient; i.e., they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the gene of interest flanked by the signals required for
5 amplification and packaging of the artificial genome), but these genes may be supplied during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

10 In many instances, rAAV particles are referred to as DNase resistant. However, in addition to this endonuclease (DNase), other endo- and exo- nucleases may also be used in the purification steps described herein, to remove contaminating nucleic acids. Such nucleases may be selected to degrade single stranded DNA and/or double-stranded DNA, and RNA. Such steps may contain a single nuclease, or mixtures of nucleases directed to different targets, and may be endonucleases or exonucleases.

15 The term "nuclease-resistant" indicates that the AAV capsid has fully assembled around the expression cassette which is designed to deliver a gene to a host cell and protects these packaged genomic sequences from degradation (digestion) during nuclease incubation steps designed to remove contaminating nucleic acids which may be present from the production process.

20 In certain embodiments, the vector is a viral vector selected from a recombinant parvovirus, a recombinant lentivirus, a recombinant retrovirus, or a recombinant adenovirus; or a non-viral vector selected from naked DNA, naked RNA, an inorganic particle, a lipid particle, a polymer-based vector, or a chitosan-based formulation. The selected vector may be delivered by any suitable method, including transfection, electroporation, liposome
25 delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.

30 A "replication-defective virus" or "viral vector" refers to a synthetic or artificial viral particle in which an expression cassette containing a gene of interest is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid

or envelope are replication-deficient; i.e., they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the transgene of interest flanked by the signals required for
5 amplification and packaging of the artificial genome), but these genes may be supplied during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication. Such replication-defective viruses may be adeno-associated viruses (AAV), adenoviruses, lentiviruses (integrating or non-integrating), or another suitable virus
10 source.

A "nucleic acid", as described herein, can be RNA, DNA, or a modification thereof, and can be single or double stranded, and can be selected, for example, from a group including: nucleic acid encoding a protein of interest, oligonucleotides, nucleic acid analogues, for example peptide-nucleic acid (PNA), pseudocomplementary PNA (pc-PNA),
15 locked nucleic acid (LNA) etc. Such nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins, for example that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example but are not limited to RNAi, shRNAi, siRNA, micro RNAi (mRNAi), antisense oligonucleotides etc.

20 The term "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of nucleic acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired.
25 However, identity among smaller fragments, e.g., of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired.

Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide
30 fragment thereof or the corresponding nucleic acid sequence coding sequences. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids. Generally, when referring to "identity", "homology", or "similarity"

between two different sequences, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments” refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence.

5 Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, e.g., the “Clustal X”, “Clustal Omega” “MAP”, “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter
10 these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, e.g., J. D. Thomson et al, Nucl. Acids. Res., “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

15 Multiple sequence alignment programs are also available for nucleic acid sequences. Examples of such programs include, “Clustal W”, “Clustal Omega”, “CAP Sequence Assembly”, “BLAST”, “MAP”, and “MEME”, which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms
20 known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid
25 sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference.

IV. Compositions

Provided herein is a composition comprising an rAAV or a vector as described herein and an aqueous suspension media. In certain embodiments, the suspension is formulated for intravenous delivery, intrathecal administration, or intracerebroventricular administration.

5 Provided herein are compositions containing at least one rAAV stock and an optional carrier, excipient and/or preservative. As used herein, a “stock” of rAAV refers to a population of rAAV. Despite heterogeneity in their capsid proteins due to deamidation, rAAV in a stock are expected to share an identical vector genome. A stock can include rAAV having capsids with, for example, heterogeneous deamidation patterns characteristic
10 of the selected AAV capsid proteins and a selected production system. The stock may be produced from a single production system or pooled from multiple runs of the production system. A variety of production systems, including but not limited to those described herein, may be selected.

 As used herein, “carrier” includes any and all solvents, dispersion media, vehicles,
15 coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase
20 “pharmaceutically-acceptable” refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host. Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered vector genomes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a
25 nanosphere, or a nanoparticle or the like.

 In one embodiment, a composition includes a final formulation suitable for delivery to a subject, e.g., is an aqueous liquid suspension buffered to a physiologically compatible pH and salt concentration. Optionally, one or more surfactants are present in the formulation. In another embodiment, the composition may be transported as a concentrate
30 which is diluted for administration to a subject. In other embodiments, the composition may be lyophilized and reconstituted at the time of administration.

A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, e.g., such as Pluronic® F68 [BASF], also known as Poloxamer 188, which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy caprylic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. In one embodiment, the surfactant may be present in an amount up to about 0.0005 % to about 0.001% (based on weight ratio, w/w %) of the suspension. In another embodiment, the surfactant may be present in an amount up to about 0.0005 % to about 0.001% (based on volume ratio, v/v %) of the suspension. In yet another embodiment, the surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension, wherein n % indicates n gram per 100 mL of the suspension.

In another embodiment, the composition includes a carrier, diluent, excipient and/or adjuvant. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The buffer/carrier should include a component that prevents the rAAV, from sticking to the infusion tubing but does not interfere with the rAAV binding activity in vivo. A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, e.g., such as Poloxamer 188 (also known under the commercial names Pluronic® F68 [BASF], Lutrol® F68, Synperonic® F68, Kolliphor®

P188) which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-
5 15 Hydroxystearate), LABRASOL (Polyoxy caprylic glyceride), polyoxy -oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10
10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension.

In certain embodiments, the composition containing the rAAV.hCDKL5 is delivered at a pH in the range of 6.8 to 8, or 7.2 to 7.8, or 7.5 to 8. For intrathecal delivery, a pH
15 above 7.5 may be desired, e.g., 7.5 to 8, or 7.8.

In certain embodiments, the formulation may contain a buffered saline aqueous solution not comprising sodium bicarbonate. Such a formulation may contain a buffered saline aqueous solution comprising one or more of sodium phosphate, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and mixtures thereof, in water,
20 such as a Harvard's buffer. The aqueous solution may further contain Kolliphor® P188, a poloxamer which is commercially available from BASF which was formerly sold under the trade name Lutrol® F68. The aqueous solution may have a pH of 7.2.

In another embodiment, the formulation may contain a buffered saline aqueous solution comprising 1 mM Sodium Phosphate (Na_3PO_4), 150 mM sodium chloride (NaCl),
25 3mM potassium chloride (KCl), 1.4 mM calcium chloride (CaCl_2), 0.8 mM magnesium chloride (MgCl_2), and 0.001% poloxamer (e.g., Kolliphor®) 188, pH 7.2. See, e.g., harvardapparatus.com/harvard-apparatus-perfusion-fluid.html. In certain embodiments, Harvard's buffer is preferred due to better pH stability observed with Harvard's buffer.

In certain embodiments, the formulation buffer is artificial CSF with Pluronic F68. In
30 other embodiments, the formulation may contain one or more permeation enhancers. Examples of suitable permeation enhancers may include, e.g., mannitol, sodium

glycocholate, sodium taurocholate, sodium deoxycholate, sodium salicylate, sodium caprylate, sodium caprate, sodium lauryl sulfate, polyoxyethylene-9-laurel ether, or EDTA.

Optionally, the compositions of the invention may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or
5 chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The compositions according to the present invention may comprise a pharmaceutically acceptable carrier, such as defined above. Suitably, the compositions
10 described herein comprise an effective amount of one or more AAV suspended in a pharmaceutically suitable carrier and/or admixed with suitable excipients designed for delivery to the subject via injection, osmotic pump, intrathecal catheter, or for delivery by another device or route. In certain embodiments, the ommya reservoir is used for delivery. In one example, the composition is formulated for intrathecal delivery. In one example, the
15 composition is formulated for intravenous (iv) delivery.

V. Uses

Provided herein is a method of treating CDD, comprising administering an effective amount of an rAAV or a vector as described herein to a subject in need thereof. Optionally,
20 the vectors provided herein encoding BIP-TATk28-hCDKL5 (e.g., rAAV.BIP-TATk28-hCDKL5) may be used in a regimen involving co-administration with the BIP-TATk28-hCDKL5 protein and/or combined with other CDKL5 therapies.

In certain embodiments, an “effective amount” herein is the amount which achieves amelioration of CDD symptoms and/or delayed CDD progression.

25 The vectors are administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired
30 organ (e.g., brain, CSF, the liver (optionally via the hepatic artery), lung, heart, eye, kidney,), oral, inhalation, intranasal, intrathecal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, intraparenchymal, intracerebroventricular,

intrathecal, ICM, lumbar puncture and other parenteral routes of administration. Routes of administration may be combined, if desired.

Dosages of the viral vector (for example, rAAV) depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and can thus vary among patients. For example, a therapeutically effective human dosage of the viral vector is generally in the range of from about 25 to about 1000 microliters to about 100 mL of solution containing concentrations of from about 1×10^9 to 1×10^{16} vector genome copies. In certain embodiments, a volume of about 1 mL to about 15 mL, or about 2.5 mL to about 10 mL, or about 5 mL suspension is delivered. In certain embodiments, a volume of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, or about 15 mL suspension is delivered. In certain embodiments, a dose of about 8.9×10^{12} to 2.7×10^{14} GC total is administered in this volume. In certain embodiments, a dose of about 1.1×10^{10} GC/g brain mass to about 3.3×10^{11} GC/g brain mass is administered in this volume. In certain embodiments, a dose of about 3.0×10^9 , about 4.0×10^9 , about 5.0×10^9 , about 6.0×10^9 , about 7.0×10^9 , about 8.0×10^9 , about 9.0×10^9 , about 1.0×10^{10} , about 1.1×10^{10} , about 1.5×10^{10} , about 2.0×10^{10} , about 2.5×10^{10} , about 3.0×10^{10} , about 3.3×10^{10} , about 3.5×10^{10} , about 4.0×10^{10} , about 4.5×10^{10} , about 5.0×10^{10} , about 5.5×10^{10} , about 6.0×10^{10} , about 6.5×10^{10} , about 7.0×10^{10} , about 7.5×10^{10} , about 8.0×10^{10} , about 8.5×10^{10} , about 9.0×10^{10} , about 9.5×10^{10} , about 1.0×10^{11} , about 1.1×10^{11} , about 1.5×10^{11} , about 2.0×10^{11} , about 2.5×10^{11} , about 3.0×10^{11} , about 3.3×10^{11} , about 3.5×10^{11} , about 4.0×10^{11} , about 4.5×10^{11} , about 5.0×10^{11} , about 5.5×10^{11} , about 6.0×10^{11} , about 6.5×10^{11} , about 7.0×10^{11} , about 7.5×10^{11} , about 8.0×10^{11} , about 8.5×10^{11} , about 9.0×10^{11} GC per gram brain mass is administered in this volume.

The dosage is adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed. The levels of expression of the transgene product can be monitored to determine the frequency of dosage resulting in viral vectors, preferably AAV vectors containing the minigene. Optionally, dosage regimens similar to those described for therapeutic purposes may be utilized for immunization using the compositions of the invention.

The replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^9 GC to

about 1.0×10^{16} GC (to treat an subject) including all integers or fractional amounts within the range, and preferably 1.0×10^{12} GC to 1.0×10^{14} GC for a human patient. In one embodiment, the compositions are formulated to contain at least 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , or 9×10^9 GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , or 9×10^{10} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , or 9×10^{11} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , or 9×10^{12} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , or 9×10^{13} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , or 9×10^{14} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , or 9×10^{15} GC per dose including all integers or fractional amounts within the range.

In one embodiment, for human application the dose can range from 1×10^{10} to about 1×10^{15} GC per kg body weight including all integers or fractional amounts within the range. In one embodiment, the effective amount of the vector is about 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , or 9×10^9 GC per kg body weight including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , or 9×10^{10} GC per kg body weight including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , or 9×10^{11} GC per kg body weight including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , or 9×10^{12} GC per kg body weight including all integers or fractional amounts within the range. In another

embodiment, the effective amount of the vector is about 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , or 9×10^{13} GC per kg body weight including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , or 9×10^{14} GC per kg body weight including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , or 9×10^{15} GC per kg body weight including all integers or fractional amounts within the range.

In one embodiment, for human application the dose can range from 1×10^{10} to about 1×10^{15} GC per gram (g) brain mass including all integers or fractional amounts within the range. In one embodiment, the effective amount of the vector is about 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , or 9×10^9 GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , or 9×10^{10} GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , or 9×10^{11} GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , or 9×10^{12} GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , or 9×10^{13} GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , or 9×10^{14} GC per gram (g) brain mass including all integers or fractional amounts within the range. In another embodiment, the effective amount of the vector is about 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , or 9×10^{15} GC per gram (g) brain mass including all integers or fractional amounts within the range.

These above doses may be administered in a variety of volumes of carrier, excipient or buffer formulation, ranging from about 25 to about 1000 microliters, or higher volumes, including all numbers within the range, depending on the size of the area to be treated, the viral titer used, the route of administration, and the desired effect of the method.

In one embodiment, the volume of carrier, excipient or buffer is at least about 25 μL . In one embodiment, the volume is about 50 μL . In another embodiment, the volume is about 75 μL . In another embodiment, the volume is about 100 μL . In another embodiment, the volume is about 125 μL . In another embodiment, the volume is about 150 μL . In another embodiment, the volume is about 175 μL . In yet another embodiment, the volume is about 200 μL . In another embodiment, the volume is about 225 μL . In yet another embodiment, the volume is about 250 μL . In yet another embodiment, the volume is about 275 μL . In yet another embodiment, the volume is about 300 μL . In yet another embodiment, the volume is about 325 μL . In another embodiment, the volume is about 350 μL . In another embodiment, the volume is about 375 μL . In another embodiment, the volume is about 400 μL . In another embodiment, the volume is about 450 μL . In another embodiment, the volume is about 500 μL . In another embodiment, the volume is about 550 μL . In another embodiment, the volume is about 600 μL . In another embodiment, the volume is about 650 μL . In another embodiment, the volume is about 700 μL . In another embodiment, the volume is between about 700 and 1000 μL .

In certain embodiments, the dose may be in the range of about 1×10^9 GC/g brain mass to about 1×10^{12} GC/g brain mass. In certain embodiments, the dose may be in the range of about 1×10^{10} GC/g brain mass to about 3×10^{11} GC/g brain mass. In certain embodiments, the dose may be in the range of about 1×10^{10} GC/g brain mass to about 2.5×10^{11} GC/g brain mass. In certain embodiments, the dose may be in the range of about 5×10^{10} GC/g brain mass.

In one embodiment, the viral constructs may be delivered in doses of from at least about 1×10^9 GC to about 1×10^{15} , or about 1×10^{11} to 5×10^{13} GC. Suitable volumes for delivery of these doses and concentrations may be determined by one of skill in the art. For example, volumes of about 1 μL to 150 mL may be selected, with the higher volumes being selected for adults. Typically, for newborn infants a suitable volume is about 0.5 mL to about 10 mL, for older infants, about 0.5 mL to about 15 mL may be selected. For toddlers, a volume of about 0.5 mL to about 20 mL may be selected. For children, volumes of up to about 30 mL may be selected. For pre-teens and teens, volumes up to about 50 mL may be selected. In still other embodiments, a patient may receive an intrathecal administration in a volume of about 5 mL to about 15 mL are selected, or about 7.5 mL to about 10 mL. Other suitable volumes and dosages may be determined. The dosage may be

adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed.

The above-described recombinant vectors may be delivered to host cells according to published methods. The rAAV, preferably suspended in a physiologically compatible carrier, may be administered to a human or non-human mammalian patient. In certain embodiments, for administration to a human patient, the rAAV is suitably suspended in an aqueous solution containing saline, a surfactant, and a physiologically compatible salt or mixture of salts. Suitably, the formulation is adjusted to a physiologically acceptable pH, e.g., in the range of pH 6 to 9, or pH 6.5 to 7.5, pH 7.0 to 7.7, or pH 7.2 to 7.8. As the pH of the cerebrospinal fluid is about 7.28 to about 7.32, for intrathecal delivery, a pH within this range may be desired; whereas for intravenous delivery, a pH of about 6.8 to about 7.2 may be desired. However, other pHs within the broadest ranges and these subranges may be selected for other route of delivery.

As used herein, the terms "intrathecal delivery" or "intrathecal administration" refer to a route of administration for drugs via an injection into the spinal canal, more specifically into the subarachnoid space so that it reaches the cerebrospinal fluid (CSF). Intrathecal delivery may include lumbar puncture, intraventricular (including intracerebroventricular (ICV)), suboccipital/intracisternal, and/or C1-2 puncture. For example, material may be introduced for diffusion throughout the subarachnoid space by means of lumbar puncture. In another example, injection may be into the cisterna magna. In certain embodiment, a rAAV, vector, or composition as described herein is administered to a subject in need via the intrathecal administration. In certain embodiments, the intrathecal administration is performed as described in US Patent Publication No. 2018-0339065 A1, published November 29, 2019, which is incorporated herein by reference in its entirety.

As used herein, the terms "intracisternal delivery" or "intracisternal administration" refer to a route of administration for drugs directly into the cerebrospinal fluid of the cisterna magna cerebellomedullaris, more specifically via a suboccipital puncture or by direct injection into the cisterna magna or via permanently positioned tube.

In certain embodiments, treatment of the composition described herein has minimal to mild asymptomatic degeneration of DRG sensory neurons in animals and/or in human

patients, well-tolerated with respect to sensory nerve toxicity and subclinical sensory neuron lesions.

VI. Apparatus and Method For Delivery of a Pharmaceutical Composition into 5 Cerebrospinal Fluid

In one aspect, the vectors provided herein may be administered intrathecally via the method and/or the device provided in this section and described in WO 2018/160582, which is incorporated by reference herein. Alternatively, other devices and methods may be selected. In certain embodiments, the method comprises the steps of CT-guided sub-
10 occipital injection via spinal needle into the cisterna magna of a patient. As used herein, the term Computed Tomography (CT) refers to radiography in which a three-dimensional image of a body structure is constructed by computer from a series of plane cross-sectional images made along an axis. In certain embodiments, vectors and/or compositions thereof as described herein are administered via computed tomography- (CT-) guided sub-occipital
15 injection into the cisterna magna (intra-cisterna magna [ICM]). In certain embodiments, the Ommaya Reservoir is used for delivery of a pharmaceutical composition. In certain embodiments, the apparatus is described in US Patent Publication No. 2018-0339065 A1, published November 29, 2019, which is incorporated herein by reference in its entirety.

20 VII. CDD

“Patient” or “subject”, as used herein interchangeably, means a male or female mammalian animal, including a human, a veterinary or farm animal, a domestic animal or pet, and animals normally used for clinical research. In one embodiment, the subject of these methods and compositions is a human patient. In one embodiment, the subject of these
25 methods and compositions is a male or female human. In certain embodiment, the subject of these methods and compositions is diagnosed with CDD and/or with symptoms of CDD.

The methods and compositions may be used for treatment of any of the stages of CDD. In certain embodiments, the patient is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 month(s) old, or about 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12,
30 13, 14, 15, 16, 17, 18 year(s) old. In certain embodiments, the patient is a toddler, e.g., 18 months to 3 years of age. In certain embodiments, the patient is from 3 years to 6 years of

age, from 3 years to 12 years of age, from 3 years to 18 years of age, from 3 years to 30 years of age. In certain embodiments, patients are older than 18 years of age.

Symptoms in CDD include seizures that usually begin within the first 3 months of life, and can appear as early as the first week after birth. The types of seizures change with age, and may follow a predictable pattern. The most common types are generalized tonic-clonic seizures, which involve a loss of consciousness, muscle rigidity, and convulsions; tonic seizures, which are characterized by abnormal muscle contractions; and epileptic spasms, which involve short episodes of muscle jerks. Seizures occur daily in most people with CDKL5 deficiency disorder, although they can have periods when they are seizure-free. Seizures in CDKL5 deficiency disorder are typically resistant to treatment.

Development is impaired in children with CDKL5 deficiency disorder. Most have severe intellectual disability and little or no speech. The development of gross motor skills, such as sitting, standing, and walking, is delayed or not achieved. About one-third of affected individuals are able to walk independently. Fine motor skills, such as picking up small objects with the fingers, are also impaired; about half of affected individuals have purposeful use of their hands. Most people with this condition have vision problems (cortical visual impairment).

Other common features of CDKL5 deficiency disorder include repetitive hand movements (stereotypies), such as clapping, hand licking, and hand sucking; teeth grinding (bruxism); disrupted sleep; feeding difficulties; and gastrointestinal problems including constipation and backflow of acidic stomach contents into the esophagus (gastroesophageal reflux). Some affected individuals have episodes of irregular breathing. Distinctive facial features in some people with CDKL5 deficiency disorder include a high and broad forehead, large and deep-set eyes, a well-defined space between the nose and upper lip (philtrum), full lips, widely spaced teeth, and a high roof of the mouth (palate). Other physical differences can also occur, such as an unusually small head size (microcephaly), side-to-side curvature of the spine (scoliosis), and tapered fingers.

As described above, the terms “increase” “decrease” “reduce” “ameliorate” “improve” “delay” or any grammatical variation thereof, or any similar terms indicating a change, means a variation of about 5 fold, about 2 fold, about 1 fold, about 90%, about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20%, about 10%, about 5

% compared to the corresponding reference (e.g., untreated control or a subject in normal condition without CDD), unless otherwise specified.

In certain embodiments, the patient receives medications controlling some signs and symptoms associated with the CDD, such as seizures, muscle stiffness, or problems with
5 breathing, sleep, the gastrointestinal tract or the heart. In certain embodiments, the seizures are tonic-clonic seizures, which involve a loss of consciousness, muscle rigidity, and convulsions or tonic seizures, which are characterized by abnormal muscle contractions. In other embodiments, symptoms include epileptic spasms, which involve short episodes of muscle jerks. Other symptoms include repetitive hand movements, such as clapping, hand
10 licking, and hand sucking; teeth grinding; disrupted sleep; feeding difficulties; gastrointestinal problems including constipation and backflow of acidic stomach contents into the esophagus (gastroesophageal reflux); and/or irregular breathing.

In certain embodiments, a diuretic agent may be used in co-therapy in a subject in need thereof. Diuretic agent used may be acetazolamine (Diamox) or other suitable diuretics.
15 In some embodiments, the diuretic agent is administered at the time of gene therapy administration. In some embodiments, the diuretic agent is administered prior to gene therapy administration. In some, embodiments the diuretic agent is administered where the volume of injection is 3 mL.

In certain embodiments, co-therapies may be utilized, which comprise co-
20 administration of Cdk15-isoform 1, isoform 2, isoform 3, and/or isoform 4- expressing vectors, or various two- or three-way combinations thereof. Optionally, co-therapy may further comprise administration of another active agent. In certain embodiments, co-therapy may comprise enzyme replacement therapy.

Optionally, an immunosuppressive co-therapy may be used in a subject in need.
25 Immunosuppressants for such co-therapy include, but are not limited to, a glucocorticoid, steroids, antimetabolites, T-cell inhibitors, a macrolide (e.g., a rapamycin or rapalog), and cytostatic agents including an alkylating agent, an anti-metabolite, a cytotoxic antibiotic, an antibody, or an agent active on immunophilin. The immune suppressant may include a nitrogen mustard, nitrosourea, platinum compound, methotrexate, azathioprine,
30 mercaptopurine, fluorouracil, dactinomycin, an anthracycline, mitomycin C, bleomycin, mithramycin, IL-2 receptor- (CD25-) or CD3-directed antibodies, anti-IL-2 antibodies, ciclosporin, tacrolimus, sirolimus, IFN- β , IFN- γ , an opioid, or TNF- α (tumor necrosis factor-

alpha) binding agent. In certain embodiments, the immunosuppressive therapy may be started 0, 1, 2, 3, 4, 5, 6, 7, or more days prior to or after the gene therapy administration. Such immunosuppressive therapy may involve administration of one, two or more drugs (e.g., glucocorticoids, prednelisone, micophenolate mofetil (MMF) and/or sirolimus (i.e., rapamycin)). Such immunosuppressive drugs may be administered to a subject in need once, twice or for more times at the same dose or an adjusted dose. Such therapy may involve co-administration of two or more drugs, the (e.g., prednelisone, micophenolate mofetil (MMF) and/or sirolimus (i.e., rapamycin)) on the same day. One or more of these drugs may be continued after gene therapy administration, at the same dose or an adjusted dose. Such therapy may be for about 1 week (7 days), about 60 days, or longer, as needed. In certain embodiments, a tacrolimus-free regimen is selected.

The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using "comprising" language, under other circumstances, a related embodiment is also intended to be interpreted and described using "consisting of" or "consisting essentially of" language.

The term "expression" is used herein in its broadest meaning and comprises the production of RNA or of RNA and protein. With respect to RNA, the term "expression" or "translation" relates in particular to the production of peptides or proteins. Expression may be transient or may be stable.

It is to be noted that the term "a" or "an", refers to one or more, for example, "an enhancer", is understood to represent one or more enhancer(s). As such, the terms "a" (or "an"), "one or more," and "at least one" is used interchangeably herein.

Throughout the specification, exponents are referred to using the term "e" followed by a numerical value (n). It will be understood with this refers to " $\times 10^n$ ". For example, "3e9" is the same as 3×10^9 and "1e13" is the same as 1×10^{13} .

As described above, the term "about" when used to modify a numerical value means a variation of $\pm 10\%$, ($\pm 10\%$, e.g., ± 1 , ± 2 , ± 3 , ± 4 , ± 5 , ± 6 , ± 7 , ± 8 , ± 9 , ± 10 , or values therebetween) from the reference given, unless otherwise specified.

Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

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VIII. EXAMPLES

The following examples are illustrative only and are not intended to limit the present invention.

Several models for CDD have been developed and may selected for use in evaluating therapeutic effect. These following models are null for CDKL5 expression: g., a Cdkl5-ko mouse having a deletion in exon 6 (Δ exon 6), Wang et al, Proceedings of the National Academy of Sciences Dec 2012, 109 (52) 21516-21521; DOI: 10.1073/pnas.1216988110; a Cdkl5-ko mouse having a deletion in exon 4 (Δ exon 4) (see, Amendola et al. (2014) Mapping Pathological Phenotypes in a Mouse Model of CDKL5 Disorder. PLoS ONE 9(5): e91613. doi.org/10.1371/journal.pone.0091613 (2014); a Cdkl5 (R59X knock-in) model (available from Jackson Laboratory; see, Tang et al (2019), Tang S, et al. Altered NMDAR signaling underlies autistic-like features in mouse models of CDKL5 deficiency disorder. Nat Commun. 2019;10(1):2655. Published 2019 Jun 14. doi:10.1038/s41467-019-10689-w, and a Cdkl5 (D471fs) model (Rodney Samaco, Baylor College of Medicine, Houston, TX).

We have developed an AAV gene therapy-mediated cross correction approach for CDD to broaden CDKL5 expression in the brain. In these studies, we have examined AAV gene therapy-mediated cross correction using different Clade F capsids, ages of injection, routes of administration and observed robust expression and mitigation of behavioral deficits.

25

Example 1. Production of rAAV comprising BIP-TATk28-hCDKL5

In the studies herein, a fusion protein comprising an exogenous leader and cell penetrating peptide fused to engineered sequences encoding human CDKLK5 isoform 1 were generated and comparative studies were performed with the corresponding construct without the exogenous BIP-TAT sequences, containing the native leader sequence. The rAAV are generated using triple transfection techniques, utilizing (1) a cis plasmid encoding AAV2 rep proteins and the AAVhu68 VP1 cap gene, (2) a cis plasmid comprising

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adenovirus helper genes not provided by the packaging cell line which expresses adenovirus E1a, and (3) a trans plasmid containing the vector genome for packaging in the AAV capsid. See, e.g., US 2020/0056159. The trans plasmid is designed to contain either the vector genome of BIP-TAT28k28-CDKL5 as illustrated in FIG 1 or the comparative vector genome containing the sequences encoding hCDKL5 with the native hCDKL5 leader peptide. FIG. 1 shows a graphic map of the vector genome in a recombinant AAV particle. The vector genome contains an AAV 5' inverted terminal repeat (ITR) and an AAV 3' ITR at the extreme 5' and 3' end, respectively. The ITRs flank the sequences of the expression cassette packaged into the AAV capsid which have sequences encoding a BIP-TATk28-hCDKL5 fusion protein, in which BIP refers to a signal peptide and TATk28 (also referenced to as TAT) is a cell penetrating peptide. The expression cassette further comprises regulatory sequences operably linked to the fusion protein coding sequences, including a hybrid chicken beta actin (CBH) promoter, and an SV40 poly A.

15 Example 2. Gene Therapy-Mediated Cross Correction with BIP-TATk28-CDKL5

In this study, we used AAVhu68 vector to deliver an engineered CDKL5 transgene. FIG 2 provides an illustration of cross-correction, in which a secretable BIP-TAT-hCDKL5 fusion protein is delivered via an AAV which corrects deficient hCDKL5 in vector-transduced cells and delivers a secreted hCDKL5 protein to other brain cells. We observed that AAVhu68.BiP. TATk28.CDKL5 achieved broader brain CDKL5 delivery and expression for CDKL5-deficiency disorder (CDD). In gene therapy mediated cross correction, we use an AAVhu68 vector to deliver a carefully engineered CDKL5 transgene designed for optimal expression, secretion and uptake so that a larger number of cells express CDKL5 protein, while using a 800-bp CBh (truncated chicken beta-actin hybrid) promoter which provides strong, long-term, and ubiquitous CNS expression.

Briefly, in this study, mice were administered with AAVhu68.CBH.BIP-TAT28k-hCDKL5co2.SV40 or AAVhu68.CBH.hCDKL5co2.SV40 (control) at a dose of 1×10^{11} (1e11) GC/mouse via intraventricular (ICV) injection, at age P0 or P14. In life analysis was collected for 1 month, following which mice were necropsied and tissues were collected. Dual immunofluorescence (IF) and in-situ hybridization (ISH) were performed for expression and quantification studies.

First, we examined CDKL5 expression using western blot analysis. FIG. 3A shows western blot analysis of BIP-TATk28-hCDKL5 protein expression in brain of male mice of ID Nos: 734 and 738 (WT PBS), 753 (CDKL5^{-/-} PBS), 783, 855 and 860 (CDKL5^{-/-} BIP.TATk28.hCDKL5), 772 and 832 (CDKL5^{-/-} CDKL5). FIG. 3B shows western blot analysis of BIP-TATk28-hCDKL5 protein expression in brain of male mice of ID Nos: 1157 and 1162 (WT PBS), 1156 and 1160 (CDKL5^{-/-} PBS), 980 and 1136 (CDKL5^{-/-} BIP.TATk28.hCDKL5), 1202 and 1206 (CDKL5^{-/-} CDKL5). Male neonatal control or CDKL5^{-/-} mice were injected ICV with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5 vector or AAVhu68-CBH-hCDKL5 vector. Vector dose was 1×10^{11} (1e11) genome copies (GC) per animal. After 1 month, in life brains were harvested and subject to Western blotting analysis to measure tagged and untagged CDKL5 protein expression. FIG. 3A also shows a loading control below a CDKL5 immunoblot. FIG. 3C shows a graph with densitometric quantitation of CDKL5 expression. BIP-TATk28-hCDKL5 protein expression reached 50 percent of wild type (WT) PBS controls and was higher than untagged CDKL5 expression. FIG. 3D shows BIP-TATk28-CDKL5co2 and hCDKL5co2 mRNA expression levels plotted as hCDKL5-co2 transcript per 100ng mRNA. FIG. 17A further shows cortical protein expression (CDKL5) in mice, plotted as percent wild-type-PBS expression in CDKL5^{-/-} with P14 treatment. FIG. 17B further shows cortical mRNA expression, more specifically, BIP-TATk28-CDKL5 (BTC) and hCDKL5 mRNA expression levels plotted as hCDKL5-co2 transcript per 100ng mRNA. These results show that BIP-TATk28-hCDKL5 (BTC) protein expression with P14 treatment is higher than untagged hCDKL5 in CDKL5^{-/-} mice. Overall, P14 intracerebroventricular (ICV) delivery with hu68-CBH-BIP-TATk28-hCDKL5 (1e11 GC/mouse (1×10^{11})) results in 27% of WT-PBS cortical expression with untagged hCDKL5 at 2.3%. Initial P0 ICV study results in ~50% of WT-PBS cortical expression (untagged hCDKL5 at ~25%). Expression of mRNA in the cortex was similar for BIP-TATk28-hCDKL5 and untagged hCDKL5.

Next, male neonatal control or CDKL5^{-/-} mice were injected ICV with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5 vector or AAVhu68-CBH-hCDKL5 vector. Vector dose was 1e11 (1×10^{11}) genome copies per animal. After 1 month in life brains were harvested and subject to dual immunofluorescence and in situ hybridization analysis. FIG. 4 shows a representative fluorescent images of sagittal sections of cortex from AAVhu68-CBH-BIP-TATk28-hCDKL5co2 treated CDKL5^{-/-} mice showing the distribution of BIP-

TATk28-hCDKL5 protein (yellow) and BIP-TATk28-hCDKL5 mRNA (blue). Nuclei (purple) are counterstained with DAPI (4',6-diamidino-2-phenylindole). FIG. 18 further shows representative fluorescent images (Immunofluorescence and In Situ Hybridization) of cortex from AAVhu68-CBH-BIP-TATk28-hCDKL5co2 treated CDKL5^{-/-} mice showing the distribution of BIP-TATk28-hCDKL5 protein (yellow) and BIP-TATk28-hCDKL5 mRNA (blue). Nuclei (purple) are counterstained with DAPI (4',6-diamidino-2-phenylindole). Arrows highlight cross corrected cells which are positive for CDKL5 protein only. CDKL5 protein was identified by immunofluorescence while BIP-TATk28-hCDKL5 mRNA was identified by in situ hybridization using a custom RNAscope probe. These results show the increased CDKL5 expression and cross-correction in the cortex of CDKL5^{-/-} mice treated at P14 with BIP-TATk28-hCDKL5.

Next, we examined cross correction in the cortex of BIP-TATk28-hCDKL5 treated P0 CDKL5^{-/-} mice. In a cross correction studies, the CDKL5 is made within the cell following expression from the rAAV-CBH-BIP-TATk28-hCDKL5 vector, wherein a subset of the expressed BIP-TATk28-hCDKL5 is secreted and taken up by neighboring cells. This approach allows for cross correction for many surrounding cells in addition to the cells into which hCDKL5 gene was transduced. The extent of cross correction was examined using visualization of number of cells positive for Cdkl5 protein (cross corrected and transduced cells) in comparison with or without a signal for Cdkl5 mRNA (transduced cells) in the cortex, striatum, thalamus, hippocampus and hypothalamus. FIG. 5A shows the number of cross corrected cells positive for Cdkl5 protein that were quantitated using Visiopharm® software. FIG. 5C further shows the number of cross corrected cells positive for Cdkl5 protein in neonatal and CDKL5^{-/-} injected mice that were quantitated using Visiopharm® software, more specifically in multiple brain regions of P14 BIP-TATk28-hCDKL5 treated CDKL5^{-/-} mice.

Mean fraction of cross-corrected cell for BIP-TATk28-CDKL5 protein (P0 injection)

- 4.78% in Cortex
- 3.2% in Striatum
- 1.69% in Thalamus
- 4.03% in Hippocampus

Untagged hCDKL5 control showed no significant cross correction as expected.

Next, we examined cross correction in the cortex of BIP-TATk28-hCDKL5 treated P14 CDKL5^{-/-} mice. FIG. 5B further displays the number of cells positive for Cdkl5 protein (with or without a signal for Cdkl5 mRNA) in the cortex, striatum, thalamus, hippocampus and hypothalamus quantitated using Visiopharm® pathology image analysis software. The number of cross corrected cells positive for Cdkl5 protein that were quantitated using Visiopharm® software. The mean fraction of cross corrected cells in the cortex was 2.46 %. The mean fraction of cross corrected cells in the striatum was 2.71 %. The mean fraction of cross corrected cells in the thalamus was 1.37 %. The mean fraction of cross corrected cells in the hippocampus was 4.8 %. The mean fraction of cross corrected cells in the hypothalamus was 0.24 %.

We examined the expression of BIP-TATk28-hCDKL5 with treatment in aged (6-7 month) seizure-prone female CDKL5^{+/-} mice in hippocampus, and observed that BIP-TATk28-hCDKL5 expression is primarily hippocampal. FIG. 19A shows a representative microscopy image of CDKL5 expression in hippocampal cross-section tissue of female control CDKL5^{+/-} mice administered with PBS. FIG. 19B shows a representative microscopy image of CDKL5 expression in hippocampal cross-section tissue of female control CDKL5^{+/-} mice treated with AAV.BIP-TATk28-hCDKL5. FIG. 19C shows hippocampal BIP-TATk28-hCDKL5 expression, plotted as percent wildtype-PBS expression. FIG. 19D shows western blot analysis of BIP-TATk28-hCDKL5 and endogenous CDKL5 protein expression (in comparison with the total protein control) in hippocampal tissue of female CDKL5^{+/-} mice.

We also tested the ability of intravenously and intracerebroventricular injected PHP.B-CBH-BIP-TATk28-CDKL5 vector to reduce behavioral deficits in CDKL5^{-/-} mice, specifically increased cage locomotor activity. We packaged BIP-TATk28-CDKL5 into the AAV9 variant PHP.B because it can cross the blood brain barrier when injected intravenously and effectively transduces cells of the brain in C57BL/6J mice. In mice, brain CDKL5 protein levels rise around two weeks of age; therefore, to mimic this natural increase we retroorbitally injected P18 CDKL5^{-/-} mice with PHP.B-CBH-BIP-TATk28-CDKL5 at a dose of 1e11 (1x10¹¹) GC/animal. When mice reached 3 months of age we tested the impact of treatment on cage locomotor activity using an open field testing system (FIG 7) and harvested brains for determination of BIP-TATk28-CDKL5 protein expression by Western immunoblot (FIG 6).

BIP-TATk28-CDKL5 protein was strongly expressed in CDKL5^{-/-} mice with little expression in treated control mice (FIG 6). As expected, PBS treated WT and CDKL5^{-/-} controls showed no BIP-TATk28-CDKL5 protein expression. Open field testing showed a stereotypic increase in horizontal (locomotor) activity in CDKL5^{-/-} mice compared with controls (FIG 7). Intravenously injected PHP.B-CBH-BIP-TATk28-CDKL5 significantly reduced hyperactivity in CDKL5^{-/-} mice (FIG 7).

Next we compared the reversal of hyperactivity by intracerebroventricular injection of PHP.B-CBH-BIP-TATK28-CDKL5 into CDKL5^{-/-} neonatal mice at a dose of 1e11 GC/animal. In contrast to intravenous delivered vector, ICV delivered PHP.B-CBH-BIP-TATK28-CDKL5 did not affect increased locomotor activity in CDKL5^{-/-} mice suggesting a role for route of administration in determining therapeutic efficacy (FIG 9A). We then tested if ICV injection of PHP.B-CBH-BIP-TATK28-CDKL5 vector could reduce other abnormal behaviors in CDKL5^{-/-} mice including excessive hind limb claspings (FIG 9B) and poor nest building (FIG 9C). Treatment significantly reduced hind limb claspings in CDKL5^{-/-} mice (FIG 9B). PHP.B-CBH-BIP-TATK28-CDKL5 vector treatment also significantly improved nest building behavior in CDKL5^{-/-} mice (FIG 9C). Taken, together these data indicate that PHP.B-CBH-BIP-TATK28-CDKL5 vectors were effective in reducing behavioral deficits in CDKL5^{-/-} mice.

EEG is a potential clinical biomarker in CDD and a method to visualize and/or quantify electrical activity of the brain (i.e., potentially translational functional readout in CDD mice). EEG is used clinically and findings may be more easily extrapolated from mice to humans than behavioral readouts. Therefore, we also evaluated the utility of electroencephalography (EEG) to evaluate vector therapeutic efficacy in CDKL5 mutant mice. FIG.10 shows the recording approach in 3-4 month old or 6-7 month old untreated and treated control and female CDKL5^{+/-} mice. Briefly, intracranial EEG recordings from the cortex were performed over a 2 to 3 day period and data from same time periods combined and averaged. As indicated in FIG. 10, analysis windows were middle of the day, middle of the night, light to dark transition (active period), dark to light transition (sleep period). Most EEG abnormalities were seen during the transition to inactivity and sleep when the vivarium lights go on at 6 am.

EEG analysis revealed significant perturbation of neural oscillations in young three to four month old female CDKL5^{+/-} mice compared to wild type controls. FIG 11A shows

that from 12 pm to 1 pm during the light cycle, the relative power of delta frequencies (0.5-4 Hz) was significantly reduced in young female CDKL5^{+/-} mice compared to wild type (WT) controls. CDKL5^{+/-} mice also showed both significant reductions and increases in theta (4-8 Hz) frequency power and an increase in alpha (8-13 Hz) frequency power. Beta frequency power (13-24 Hz) was unaffected. FIG 11B shows that from 12 pm to 1 pm during the light cycle, the relative power of gamma frequencies (25-50 Hz) was modestly but significantly increased in young female CDKL5^{+/-} mice compared with wild type (WT) controls.

FIG 12A shows that from 12 am to 1 am during the dark cycle, delta frequency (0.5-4 Hz) power was significantly reduced in young female CDKL5^{+/-} mice compared to wild type (WT) controls. CDKL5^{+/-} mice also showed both significant reductions and increases in theta (4-8 Hz) frequency power and an increase in alpha (8-13 Hz) frequency power. Beta frequency power (13-24 Hz) was unaffected. FIG 12B shows that from 12 am to 1am during the dark cycle, the relative power of gamma frequencies (25-50 Hz) was modestly but significantly increased in young female CDKL5^{+/-} mice compared with wild type (WT) controls. Taken together, the findings of FIG 11 and 12 show that the absence of normal CDKL5 expression disrupts delta, theta, alpha and gamma oscillations in the young adult murine brain during the light and dark cycles.

Next, we examined if BIP-TAT-CDKL5 reduce existing neurophysiological EEG abnormalities in aged epileptic spasm-prone female CDKL5^{+/-} mice. In this study, CDKL5^{+/-} female mice and controls were aged to 6-7 months of age, where they are known to exhibit epileptic spasms (Mulcahey et al., Exp Neurol. 2020 Oct;332:113388. doi: 10.1016/j.expneurol.2020.113388. Epub 2020 Jun 22. PMID: 32585155). Next, bilateral ICV injections were performed at a dose of 1×10^{11} (1e11) GC/mouse with hu68-CBH-BIP-TATk28-CDKL5. At one week post-injection, intracranial EEG implant (head cap) was mounted (Pinnacle 3 channel-video tethered electroencephalography (EEG) system). At weeks two and four post-injection we recorded activity of the left and right frontal cortices over 48 hours. EEG activity was analyzed between 6 am and 7 am when mice are transitioning to low activity and sleep states. Additionally, we performed behavioral testing (open field, hind limb clasping, nest building). Next, mice were necropsied, and tissues were collected and/or stored for further analysis.

FIG. 13A and 13B show that BIP-TATk28k-hCDKL5 reduces aberrant delta/theta and gamma power in CDKL5^{+/-} mice during the middle of the light cycle (12pm – 2pm

window). FIG. 13A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. The fraction of delta, theta power was decreased in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. The fraction of alpha power in CDKL5^{+/-} mice was increased relative to wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly increased delta and theta power towards control levels. Beta power was similar between all groups. FIG. 13B shows the fraction of total pro-seizure gamma (15-50 Hz) power output. Gamma power was significantly elevated in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly decreased gamma power in CDKL5^{+/-} mice but not WT control mice.

FIG. 14A and 14B show that BIP-TATk28k-hCDKL5 reduces abnormalities in delta and gamma frequency bands in CDKL5^{+/-} mice at the dark to light transition (6am–7am window). EEG activity was analyzed between 6 am and 7 am when mice are transitioning to low activity and sleep states. FIG. 14A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. The fraction of delta, theta power was decreased in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. The fraction of alpha power in CDKL5^{+/-} mice was increased relative to wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly increased delta and alpha power. Beta power was similar between all groups. FIG. 14B shows the fraction of total pro-seizure gamma (15-50 Hz) power output. Heightened gamma frequency band activity occurs in Cdk15-deficient patients (Ren et al., 2015) and in Cdk15 deficient mice (Mulcahey et al., 2020). Gamma power was significantly elevated in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly decreased gamma power in CDKL5^{+/-} mice but not WT control mice.

FIG. 15A and 15B shows that BIP-TATk28k-hCDKL5 reduces abnormalities in theta, alpha, and gamma frequencies in CDKL5^{+/-} mice during the light to dark transition (6pm–7pm window). FIG. 15A shows the fraction of total power output at delta (0-4 Hz), theta (4-8 Hz), alpha (8-13 Hz) and beta (13-24 Hz) frequency bands. The fraction of delta, low theta power was decreased in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. The fraction of alpha power and high theta power in CDKL5^{+/-} mice were increased relative to wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly improved theta and alpha power. Beta power was similar between all groups.

FIG. 15B shows the fraction of gamma (15-50 Hz) power output. Gamma power was significantly elevated in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls. BIP-TATk28-hCDKL5 vector treatment significantly decreased gamma power, particularly in the 31-38 Hz range, in CDKL5^{+/-} mice but not WT control mice. These results show that treatment with BIP-TATk28-hCDKL5 in 6-7 month old CDKL5^{+/-} mice shows significantly changed relative power in delta, alpha, and gamma frequencies compared to CDKL5^{+/-} PBS treated mice.

FIG. 16A shows that BIP-TATk28k-hCDKL5 reduces abnormal gamma frequency power in aged female CDKL5^{+/-} mice during the dark to light cycle transition (6am-7am).

FIG. 16B shows that BIP-TATk28k-hCDKL5 reduces abnormal gamma frequency power in aged female CDKL5^{+/-} mice during the middle of the light cycle (12pm – 2pm). As described above, briefly, we aged CDKL5^{+/-} female mice and controls to 6-7 months of age when they are known to exhibit epileptic spasms. CDKL5^{+/-} female mice and controls were subject to intracerebroventricular injection with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5co2 vector at a dose of 1×10^{11} (1e11) GC/mouse. One week after injection, an intracranial electroencephalogram (EEG) implant was cemented onto the head, and at one month after injection the electrical activity of the left and right cortices was recorded over a 48-hour period. EEG activity was analyzed between 12 pm and 2pm in the middle of the light cycle where mice are less active and sleep frequently, and between 6am and 7am. FIG. 16B shows that summed delta (0-4 Hz) power is significantly decreased in PBS treated CDKL5^{+/-} mice compared with PBS treated wild type controls (12pm – 2pm). The fraction of summed gamma power in CDKL5^{+/-} mice was significantly increased relative to wild type PBS controls. BIP-TATk28-hCDKL5 vector treatment significantly decreased pro-seizure gamma power.

FIG. 16C shows that BIP-TATk28k-hCDKL5 reduces abnormalities in gamma frequency power in CDKL5^{+/-} mice at the dark to light transition (6am–7am window). FIG. 16C shows representative 1-hour long electrocardiogram traces from PBS and BIP-TATk28-hCDKL5 vector treated control and CDKL5^{+/-} mice. PBS treated CDKL5^{+/-} mice show substantial increase (hotter colors) in gamma (25 -50 Hz) frequency power compared with PBS treated controls. BIP-TATk28-hCDKL5 vector reduced gamma frequency power in CDKL5^{+/-}, but not WT control mice. These results show that treatment with BIP-TATk28-hCDKL5 in adult CDKL5^{+/-} mice affects EEG spectral power.

Overall, treatment with BIP-TATk28-hCDKL5 demonstrates cross-correction and reduces neurophysiological dysfunction in aged CDKL5^{+/-} mice. AAV delivered BIP-TATk28-hCDKL5 achieved higher and broader expression than untagged hCDKL5 and between 0.24 – 4.8% cross correction in various brain regions of CDKL5^{-/-} mice. EEG activity in CDKL5^{+/-} mice shows abnormal shifts in delta, theta, alpha, and gamma frequency power. ICV treatment in aged (>6 months) seizure-prone CDKL5^{+/-} mice shows a positive effect on abnormal EEG activity during the transition from active to inactive/sleep (6-7 am) and other times of day analyzed. Expression of BIP-TATk28-hCDKL5 is strongest in the hippocampus with partial expression in the cortex, striatum, and thalamus close to the injection site.

More specifically, overall the results show that BIP-TATk28-CDKL5 mitigates electrical abnormalities in the brains of aged CDKL5^{+/-} mice. We observed that aged heterozygous CDKL5 females showed a decrease in delta and gamma frequency band power. Additionally, we observed that at 1 month after ICV injection, BIP-TATk28-hCDKL5 ameliorated established EEG abnormalities in aged and seizure prone CDKL5^{+/-} female mice. As observed during light to dark transition 6am-7am (activity to inactivity/sleep), BIP-TATk28-hCDKL5 improved the power of a subset of delta frequencies, and also decreased seizure-associated increase in gamma power. As observed during middle of the light cycle 12pm-2pm (inactivity/sleep), BIP-TATk28-hCDKL5 improved the power of a subset of delta and theta frequencies, and also decreased pro-seizure increase in gamma power. It is important to note that gamma power increased over time with age in CDKL5^{+/-} acting as a potential biomarker of disease severity and progression. Furthermore, we observed that overexpression of BIP-TATk28-hCDKL5 isoform 1 on wild type background did not significantly impact summed frequency band power, and did not significantly impact behavior (open field (OF), nesting behavior (NB), home caged (HC)) either.

Recent genetic studies in male CDKL5^{-/-} mice by Terzic et al., (2021) J. Clin. Invest., suggest that CDKL5 plays important roles in maintaining adult brain structure and function and that restoration of Cdkl5 expression in adult brains is sufficient to mitigate CDD pathology. Importantly, from a therapeutic point of view, gene therapy-mediated cross correction with BIP-TATk28-CDKL5 was able to mitigate established neurophysiological pathology in adult epileptic spasm prone female CDKL5^{+/-} mice. Our initial studies of Cdkl5 expression suggest that we do not need high Cdkl5 for efficacy. Taken together, these

findings provide compelling evidence that restoration of Cdkl5 function after early brain development can mitigate CDD pathology. Additionally, these results show that gene therapy mediated cross correction can reduce established CDD pathology in adult mice the CDD treatment time window is broader than previously thought.

5

Example 3. Methods

Western Blotting

Brains were harvested and separated into halves or dissected into specific regions then homogenized in RIPA buffer. Equal amounts of protein were loaded onto SDS-PAGE stain free ready gels (Bio-Rad Laboratories) and transferred to PVDF membranes. Proteins were detected using West Femto Maximum Sensitivity Substrate (Thermo Scientific) and imaged on a ChemiDoc imager (Bio-Rad Laboratories) and quantitated using ImageLab software (Bio-Rad Laboratories).

15 Cross correction analysis

Brains were formalin fixed and paraffin embedded and cut into sagittal sections. To simultaneously detect Cdkl5 protein and mRNA, we used a RNA protein codetection kit from Advanced Cell Diagnostics according to the manufacturer's instructions. We used commercially available sheep anti-CDKL5 antibody (S957D, MRC Protein Phosphorylation and Ubiquitylation Unit (PPU) Reagents and Services) and RNAscope probe to BIP-TATk28-hCDKL5 (Advanced Cell Diagnostics). Dual labeled sections were scanned and the numbers of cells in each section positive for Cdkl5 mRNA and/or protein were quantitated using Visiopharm® software.

25 Behavioral assays

To test locomotor activity we used an open field testing system (Med Associates, Inc., Fairfax, VT). Mice were singly housed in a new cage with bedding and the cage then placed in the open field system within an array of infrared cross beams. Mice were allowed to freely roam their cage for 30 minutes during which the number of horizontal beam breaks (a measure of locomotor activity) or total ambulatory distance, was automatically recorded then analyzed. To test nest building ability, singly housed mice were transferred a new cage

with a pre-weighed square nestlet. After 24 h, mice were returned to their home cage and the quality of the nest was scored on a scale of 1 to 5 according to Deacon R.M. Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. Nat. Protoc. 2006;1(1):122–12 Nature Prot. To test the impact of BIP-TATk28-CDKL5 treatment on hind limb clasping, the mouse was lifted up into the air by grasping the base of its tail. Hind limb positioning was then observed and assigned a score between 0 (normal) to 3 (fully abnormal) according to the method of Guyenet et al., J Vis. Exp. 2010 May 21;(39):1787. doi: 10.3791/1787. PMID: 20495529; PMCID: PMC3121238.

10 EEG recordings

Electroencephalogram (EEG) recordings of brain electrical activity were performed using a 3 channel-video tethered electroencephalography system (Pinnacle Technology Inc). Trace analyses were performed using Pinnacle™ software and Labchart (AD Instruments). An intracranial EEG implant was cemented onto the head of untreated 3-4 month old or 6-7 month-old control and Cdk15^{+/-} mice treated with PBS or AAVhu68-CBH-BIP-TATk28-hCDKL5. In the aged mouse study, the implant was cemented onto the heads of treated mice one month after injection. The implant containing two recording electrodes and the electrical activity of the left and right cortices was recorded over a 48-hour period. EEG activity was analyzed during different time periods during the dark and light cycle including: the dark to light transition where mice transition from active to less active and sleep states (6am-7am), the middle of the light cycle (12 pm and 2pm), the beginning of the dark cycle (6pm -7 pm) and the middle of the dark cycle (12 am to 2 am) where mice are more active and sleep less.

Example 4. Evaluation of the pharmacology and toxicity of an AAVhu68.CBH.BIP-TATk28-CDKL5-1.SV40 vector delivered into the cisterna magna of rhesus macaques

In this study, we tested the safety and toxicity of rAAVhu68.CBH.BIP-TATk28-CDKL5-1.SV40 in non-human primates. Briefly, rAAVhu68.CBH.BIP-TATk28-CDKL5-1.SV40 was administered intracisterna magna (ICM) injection at a dose of 3×10^{13} (3e13) GC/animal (n=3). Study duration was 35 days. The readouts of the study included: BW, Clinpathology with coag., NAb/Bab, peripheral blood mononuclear cells (PBMCs),

cerebrospinal fluid (CSF) & serum biomarkers (DRG), Neuro-exam, NCV; histopathology: DRG toxicity, H&E, CDKL5 ISH, CDKL5 IHC; biochemical: western blot; bio-distribution; neurofilament light chain (Nfl). Sample collected for analysis included baseline, day 0 (D0), D7, D15, D28, D35/36. Furthermore, necropsies were completed on D35/36.

5 Throughout the study, subject 191410 showed mild lymphocytic pleocytosis of D15 (animal stable). On D22 showed normal CSF values, which indicated a resolved mild pleocytosis from D14, and showed signs of doing well cage-side. No D22 clinical chemical analysis was performed due to low serum volume. On D28, CSF sample indicated signs of mild lymphocytic pleocytosis (similar to D15), with normal cbc/chem analysis. On D36,
10 CSF sample indicated to be normal, subdural hematoma was visible at the spinal cord and dorsal surface of cerebellum.

 Throughout the study, subject 171164 showed mild lymphocytic pleocytosis on D7 on CSF readout, but observed to be doing well on cage-side observation, and appeared to be clinically normal. No notable observations were observed at D15. On D22, moderate
15 mononuclear/lymphocytic pleocytosis was indicated in CSF results. On D28, moderate lymphocytic pleocytosis was indicated on CSF analysis (216 WBC/ μ L, 98% lymphocytes, approximately doubled since D22), while CBC/chem was normal with clinically normal observation. On D36, reddening along margins of lungs was observed, with a mild lymphocytic pleocytosis, values improved from D28 (216 to 37 WBC).

20 Throughout the study, subject HS1602026 showed mild neutrophilic leukocytosis (14.1k WBC, 10k neutrophils) on bloodwork D7. Similar pattern was observed at baseline. There were no signs of systemic infection, with cage-side observation and CSF were observed to be normal as well. On D15, mild lymphocytic pleocytosis was indicated in collected sample (no blood work abnormalities, animal stable). On D22, mild mononuclear
25 pleocytosis was indicated on CSF, slightly higher than D15 results (4 WBC on 5/25 to 20 WBC on 6/1). On D28, subject HS1602026 had low glucose (39mg/dl) on the chem panel with history of hypoglycemia on some previous blood collections (CW suspect due to fasting), but subject recovered normally and did not display any clinical signs of hypoglycemia. On D35, there were no gross lesions, but observed mild lymphocytic
30 pleocytosis.

FIG. 20 shows biodistribution results for peripheral tissue, brain, dorsal root ganglion (DRG), spinal cord (SC), plotted as GC/diploid cell. The values are summarized in tables 1A, 1B, and 1C below.

Table 1A.

Animal ID	NAb to hu68	Peripheral organs		
		Liver	Heart	Kidney
171164	20	0.0004	0.0007	0.0076
191410	< 5	6.4012	0.1188	0.0918
HS1602026	160	0.0732	0.0004	0.0027

5

Table 1B.

Animal ID	DRG				SC			
	DRG-C	DRG-T	DRG-L	DRG-S	SC-C1-2	SC-C	SC-T	SC-L
171164	1.9633	0.4485	0.3858	0.3279	0.1505	0.1616	0.2238	0.0923
191410	3.0297	1.7444	1.1603	0.9731	1.1320	0.2669	0.1316	0.1113
HS1602026	1.1841	1.7828	0.4516	2.1628	0.1119	0.0139	0.2677	0.3970

Table 1C.

Animal ID	Brain								
	Frontal Cortex	Parietal Cortex	Temporal Cortex	Occipital Cortex	Caudate nucleus	Thalamus	Hippo campus	Cerebellum	Medulla
171164	0.5125	0.0534	0.2381	0.1417	0.0041	0.0046	0.0438	0.0238	0.4272
191410	1.1788	0.0879	0.1529	0.9063	0.0177	0.0965	0.6367	0.0448	0.1008
HS1602026	0.2900	0.0367	0.0964	0.0801	0.0113	0.0036	0.1200	0.0922	0.0469

FIG. 21 shows RNA biodistribution for select tissues of peripheral organs, brain, DRG and SC. The values are summarized in tables 2A, 2B, and 2C below.

Table 2A.

		NAb to hu68	Peripheral organs	
	Animal ID		Liver	Heart
RNA	171164	20	not detectable	not detectable
	191410	< 5	1761.7095	339.1970
	HS1602026	160		
DNA	171164	20	0.0004	0.0007
	191410	< 5	6.4012	0.1188
	HS1602026	160	0.0732	0.0004

5

Table 2B.

		NAb to hu68	DRG	SC
	Animal ID		DRG-C	SC-T
RNA	171164	20	711.4605	451.9906
	191410	< 5	227.0279	531.9238
	HS1602026	160	63.4719	358.4638
DNA	171164	20	1.9633	0.2238
	191410	< 5	3.0297	0.1316
	HS1602026	160	1.1841	0.2677

Table 2C.

			Brain
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	Animal ID	NAb to hu68	Frontal Cortex	Occipital Cortex	Hippocampus	Medulla
RNA	171164	20	178.0911	100.6302	below detection	1502.0304
	191410	< 5	127.4421	111.5017	below detection	178.8797
	HS1602026	160	62.0617	below detection	58.6366	77.4974
DNA	171164	20	0.5125	0.1417	0.0438	0.4272
	191410	< 5	1.1788	0.9063	0.6367	0.1008
	HS1602026	160	0.2900	0.0801	0.1200	0.0469

Tables 3A (AAVhu68) and 3B (hCDKL5) below show a summary of the enzyme-linked immunospot (ELISpot) assay analysis from collected samples as indicated. For the ELISPOT peptide library, hCDKL5 pool A-1 includes the Bip signal, TATk28 signal and peptide linker, while pool A, B, C, and D are for the remainder of the hCDKL5 sequence and excludes the BIP signal, TATK28 signal, and peptide linker.

Table 3A.

			AAVhu68			Positive Controls	
Sample ID	PBMC	DMSO no Stim.	A	B	C	CD3	PMA/ION
HS1602026	Baseline	10	23	35	18	1	108
HS1602026	Day 35	23	50	150	90	1	135
HS1602026	Day 35 spleen	50	100	123	155	3	172
HS1602026	Day 35 liver	385	530	473	275	19	350
HS1602026	Day 35 lymph node	0*	0*	0*	X	0	31

171164	Baseline	18	45	38	35	4	197
171164	Day 36	25	63	73	53	2	144
171164	Day 36 spleen	80	138	108	118	6	237
171164	Day 36 liver	105	180	213	225	2	354
171164	Day 36 lymph node	3	3	3	0	1	114
191410	Baseline	15	10	25	40	1	233
191410	Day 36	23	13	15	28	1	202
191410	Day 36 spleen	75	73	65	93	2	280
191410	Day 36 liver	78	113	203	165	24	456
191410	Day 36 lymph node	25	35	30	30	1	180

Table 3B.

Sample ID	PBMC	hCDKL5				
		A-1	A	B	C	D
HS1602026	Baseline	15	15	8	25	25
HS1602026	Day 35	83	20	58	68	25
HS1602026	Day 35 spleen	130	50	55	75	88
HS1602026	Day 35 liver	530	435	448	933	288
HS1602026	Day 35 lymph node	X	X	X	X	X
171164	Baseline	50	45	28	15	45
171164	Day 36	53	20	15	18	40
171164	Day 36 spleen	135	60	85	78	103
171164	Day 36 liver	848*	243	163	203	98

171164	Day 36 lymph node	3	3	8	8	0
191410	Baseline	18	20	13	13	15
191410	Day 36	115	20	18	30	13
191410	Day 36 spleen	163	75	50	63	63
191410	Day 36 liver	100	88	55	75	95
191410	Day 36 lymph node	28	60	38	23	25

As indicated in the tables 3A and 3B above, values less than 200 SFU per million are considered low response, values 200-500 SFU are considered moderate response, and values above 500 SFU per million are considered high response. In the samples tested, hCDKL5 pool A-1 comprised peptides 1-11 to include BiP signal, Tatk28 signal and peptide linker. In hCDKL5 pool A comprised peptides 12-50 to exclude BiP signal, Tatk28 signal and peptide linker. For HS1602026 Day 35 lymph node sample, values were inconclusive as low number of viable cells were recovered. For HS1602026 Day 35 liver sample (DMSO data point 385) high non-specific background was observed. For HS1602026 Day 35 sample low positive response against AAVhu68 pools B and C, and against hCDKL5 pool A-1 was observed. For HS1602026 Day 35 spleen sample, a low positive response against AAVhu68 pool C was observed. For 171164 Day 36 spleen sample, high non-specific background (DMSO) was observed. For 171164 Day 36 liver sample, high non-specific background (DMSO) was observed, and high positive response against hCDKL5 pool A-1 was observed despite high background. For 191410 Day 36 sample, low positive response against hCDKL5 pool A-1 was observed. For 191410 Day 36 spleen, high non-specific background (DMSO) was observed. For 191410 Day 36 liver sample, high non-specific background (DMSO) was observed.

FIG. 22 shows summarized results of the ELISPOT analysis (Tables 3A and 3B) for AAVhu68, plotted as SFU per 10^6 (million) cells. FIG. 23 shows summarized results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5, plotted as SFU per 10^6 (million) cells. These results indicate some liver transduction with AAVhu68 and hCDKL5 expression following ICM administration.

FIG. 24A shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool A, plotted as SFU per 10^6 (million) cells. FIG. 24B shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool B, plotted as SFU per 10^6 (million) cells. FIG. 24C shows results of the ELISPOT analysis (Table 3A) for AAVhu68 pool C, plotted as SFU per 10^6 (million) cells. FIG. 24D shows results of the ELISPOT analysis (Table 3A) for AAVhu68 baseline PBMC (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells. FIG. 24E shows results of the ELISPOT analysis (Table 3A) for AAVhu68 D35/36 PBMC (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells. FIG. 24F shows results of the ELISPOT analysis (Table 3A) for AAVhu68 spleen sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells. FIG. 24G shows results of the ELISPOT analysis (Table 3A) for AAVhu68 liver sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells. FIG. 24H shows results of the ELISPOT analysis (Table 3A) for AAVhu68 lymph node sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A, Pool B, and Pool C), plotted as SFU per 10^6 (million) cells.

FIG. 25A shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool A-1, plotted as SFU per 10^6 (million) cells. FIG. 25B shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool A, plotted as SFU per 10^6 (million) cells. FIG. 25C shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool B, plotted as SFU per 10^6 (million) cells. FIG. 25D shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool C, plotted as SFU per 10^6 (million) cells. FIG. 25E shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 PBMC pool D, plotted as SFU per 10^6 (million) cells. FIG. 25F shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 baseline PBMC sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25G shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 D35/D36 PBMC sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25H shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 spleen sample (for subjects 171164,

191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25I shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 liver sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. FIG. 25J shows results of the ELISPOT analysis (Tables 3A and 3B) for hCDKL5 lymph node sample (for subjects 171164, 191410, HS1602026 as analyzed with DMSO, Pool A-1, Pool A, Pool B, Pool C, Pool D peptides), plotted as SFU per 10^6 (million) cells. Overall, these results indicate a transgene specific T cell responses seen in all animals. And which are only against peptide pool A-1 that contain the TAT tag.

The collected samples were further analyzed for peripheral nerve function (NCV results), which showed overall no signs of toxicity.

FIG. 26A shows measured velocity in left medial nerve. FIG. 26B shows measured velocity in right medial nerve. FIG. 26C shows NP amplitude in left medial nerve. FIG. 26D shows NP amplitude in right medial nerve. FIG. 26E shows PP amplitude in left medial nerve. FIG. 26F shows PP amplitude in right medial nerve. FIG. 26G shows measured velocity in left sural nerve. FIG. 26H shows measured velocity in right sural nerve. FIG. 26I shows NP amplitude in left sural nerve. FIG. 26J shows NP amplitude in right sural nerve. FIG. 26K shows PP amplitude in left sural nerve. FIG. 26L shows PP amplitude in right sural nerve. Variability, low values with sural nerve may be due to size/age of animals (age ~6-8 years, weights between 3-10 kg). Velocity is an indication of how myelinated the neurons are, i.e., if slowed velocity then demyelinating lesion. Amplitude is an indication of how strong the signal from the nerve is, i.e., if lower amplitude then less neurons contributing to the signal propagation of the nerve, indication of axonopathy.

Additionally, expression of hCDKL5 was confirmed by IHC (immunohistochemistry) and ISH (in situ hybridization) microscopy (representative images are not shown). Tables 4A and 4B shows a summary of quantification of expression of hCDKL5 as analyzed by IHC microscopy of spinal cord tissue samples (SC-C: cervical; SC-T: thoracic; SC-L: lumbar).

Table 4A.

		SC
--	--	----

Animal ID	NAb to hu68	SC-C1-2	SC-C	SC-T	SC-L
171164	20	0.1505	0.1616	0.2238	0.0923
191410	< 5	1.1320	0.2669	0.1316	0.1113
HS1602026	160	0.1119	0.0139	0.2677	0.3970

Table 4B.

		NAb to hu68	SC
	Animal ID		SC-T
RNA	171164	20	451.9906
	191410	< 5	531.9238
	HS1602026	160	358.4638

Tables 5A and 5B shows a summary of quantification of expression of hCDKL5 as
 5 analyzed by ISH microscopy of DRG tissue samples (DRG-C: cervical; DRG-T: thoracic; DRG-L: lumbar; DRG-S: sacral).

Table 5A.

		DRG			
Animal ID	NAb to hu68	DRG-C	DRG-T	DRG-L	DRG-S
171164	20	1.9633	0.4485	0.3858	0.3279
191410	<5	3.0297	1.7444	1.1603	0.9731
HS1602026	160	1.1841	1.7828	0.4516	2.1628

Table 5B.

			DRG
	Animal ID	NAb to hu68	DRG-C
RNA	171164	20	711.4605
	191410	<5	227.0279
	HS1602026	160	63.4719

All documents cited in this specification are incorporated herein by reference, as is US Provisional Patent Application No. 63/274,351, filed November 1, 2021, US Provisional Patent Application No. 63/288,911, filed December 13, 2021, and US Provisional Patent Application No. 63/341,693, filed May 13, 2022, are incorporated herein by reference. The

5 electronic sequence listing filed herewith named “UPN-22-9932PCT_SequenceListing_20221101.xml” with size of 87,313 bytes, created on date of November 1, 2022, and the contents of the electronic sequence listing (e.g., the sequences and text therein) are incorporated herein by reference in entirety. While the invention has been described with reference to particular embodiments, it will be appreciated that

10 modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

CLAIMS:

1. A recombinant adeno-associated virus (rAAV) useful for treating CDKL5 deficiency disorder (CDD), wherein the rAAV comprises an AAV capsid and a vector genome, wherein the vector genome comprises inverted terminal repeats (ITR) and an expression cassette comprising a nucleic acid sequence comprising coding sequences for BIP-TATk28-hCDKL5 operably linked to regulatory sequences which direct expression thereof, wherein said regulatory sequences comprise a chicken β -actin hybrid promoter and a polyA sequence, and said BIP-TATk28-hCDKL5 is a fusion protein comprising the BIP amino acid sequence of SEQ ID NO: 3, the TATk28-amino acid sequence of SEQ ID NO: 7, and the hCDKL5 amino acid sequence of SEQ ID NO: 24.
2. The rAAV according to claim 1, wherein the BIP-TATk28-hCDKL5 fusion protein has the amino acid sequence of SEQ ID NO: 11.
3. The rAAV according to claim 1 or 2, wherein the BIP-TATk28-hCDKL5 fusion protein is encoded by SEQ ID NO: 10 or a sequence 95% identical thereto.
4. The rAAV according to any one of claims 1 to 3, wherein the chicken β -actin hybrid promoter has a sequence of SEQ ID NO: 12.
5. The rAAV according to any one of claims 1 to 3, wherein the expression cassette further comprises a Kozak sequence.
6. The rAAV according to any one of claims 1 to 5, wherein the expression cassette comprises the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, and an SV40 polyA sequence,
7. The rAAV according to any one of claims 1 to 6, wherein the expression cassette has the nucleic acid sequence of SEQ ID NO: 13 or sequence at least 95% identical thereto.

8. The rAAV according to claim 7, wherein the expression cassette has the nucleic acid sequence of SEQ ID NO: 13.
9. The rAAV according to any one of claims 1 to 8, wherein the vector genome comprises a 5' ITR, the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, an SV40 polyA sequence, and a 3' ITR.
10. The rAAV according to any one of claims 1 to 9, wherein the vector genome has the nucleic acid sequence of SEQ ID NO: 14 or a sequence at least 95% identical thereto.
11. The rAAV according to claim 10, wherein the vector genome has the nucleic acid sequence of SEQ ID NO: 14.
12. The rAAV according to any one of claims 1 to 11, wherein the capsid is an AAVhu68 capsid, an AAVhu95 capsid, or AAVrh91 capsid.
13. A composition comprising a stock of rAAV according to any one of claims 1 to 12 and one or more excipients, one or more preservatives, and/or one or more carriers.
14. The composition according to claim 13, wherein the suspension is formulated for intravenous administration, intrathecal administration, intra-cisterna magna administration or intracerebroventricular administration.
15. A rAAV according to any one of claims 1 to 12 or a composition according to claim 14 for use in treating a patient having a CDKL5-deficiency disorder.
16. Use of a rAAV according to any one of claims 1 to 12 or a composition according to claim 13 or 14 in preparing a medicament for use in treating a patient having a CDKL5-deficiency disorder.
17. A plasmid useful in producing an rAAV particle which comprises an expression cassette comprising a chicken β -actin hybrid promoter sequence, a Kozak

sequence, a BIP-TATk28-hCDKL5 coding sequence, and an SV40 polyA sequence, said BIP-TATk28-hCDKL5 encoding a protein comprising the BIP amino acid sequence of SEQ ID NO: 3, the TATk28-amino acid sequence of SEQ ID NO: 7, and the hCDKL5 amino acid sequence of SEQ ID NO: 24.

18. The plasmid according to claim 17, wherein the expression cassette has the nucleic acid sequence of SEQ ID NO: 13 or sequence at least 95% identical thereto.

19. The plasmid according to claim 17 or 18, wherein the expression cassette has the nucleic acid sequence of SEQ ID NO: 13.

20. The plasmid according to any one of claims 17 to 19, wherein the vector genome comprises a 5' ITR, the chicken β -actin hybrid promoter sequence, a Kozak sequence, the BIP-TATk28-hCDKL5 coding sequence, an SV40 polyA sequence, and a 3' ITR.

21. The plasmid according to any one of claims 17 to 20, wherein the vector genome has the nucleic acid sequence of SEQ ID NO: 14 or a sequence at least 95% identical thereto.

22. The plasmid according to any one of claims 17 to 21, wherein the vector genome has the nucleic acid sequence of SEQ ID NO: 14.

23. A packaging cell comprising a plasmid according to any one of claims 17 to 22.

FIG 1

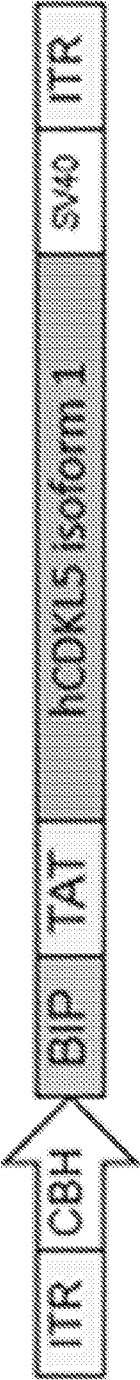


FIG 2

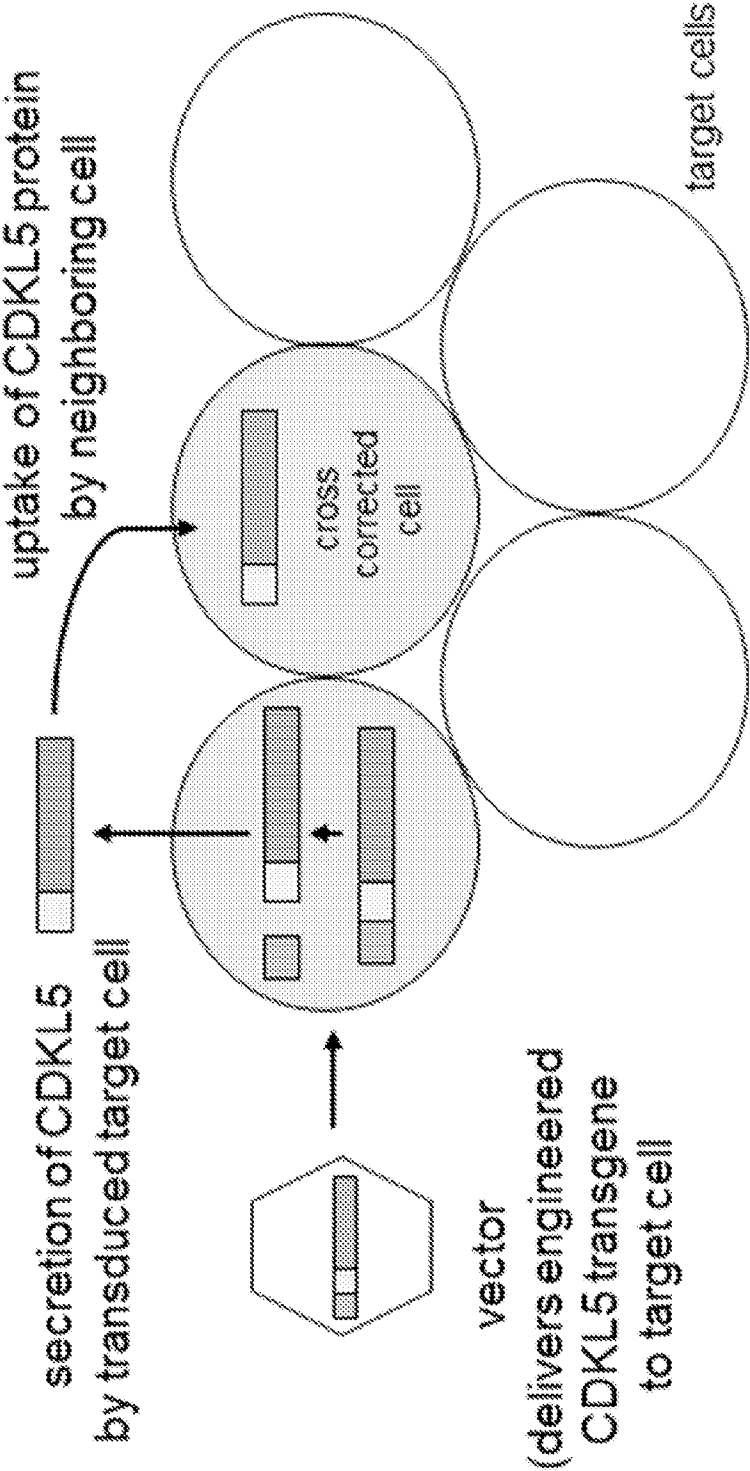


FIG 3B

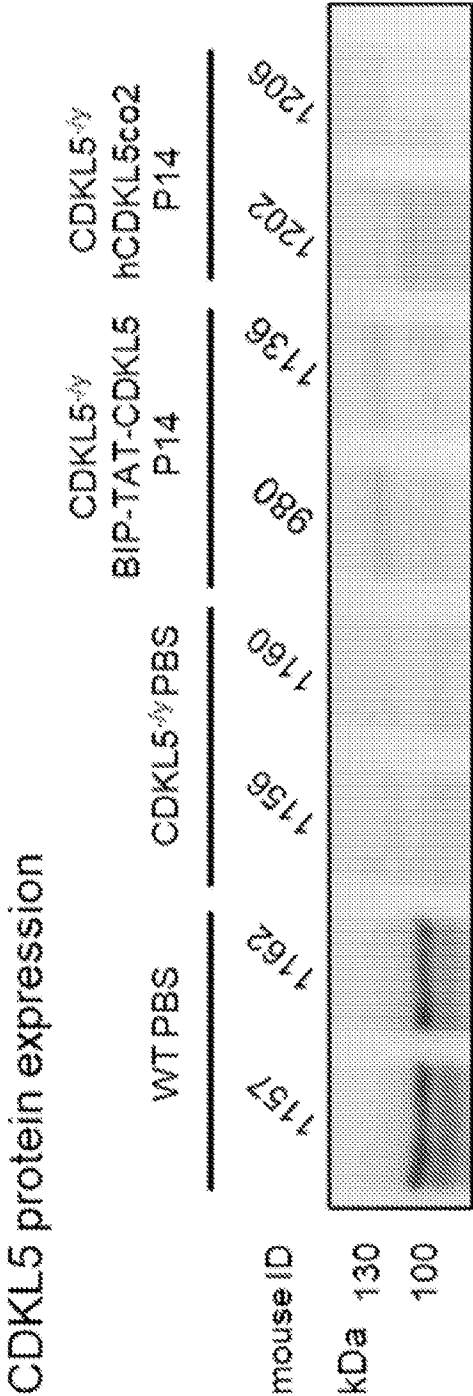


FIG 3C

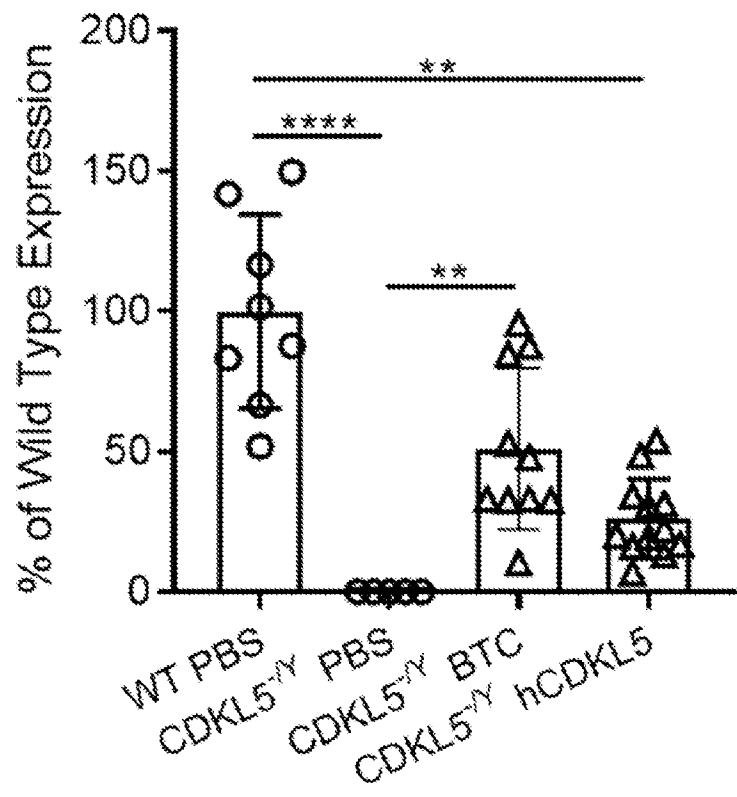


FIG 3D

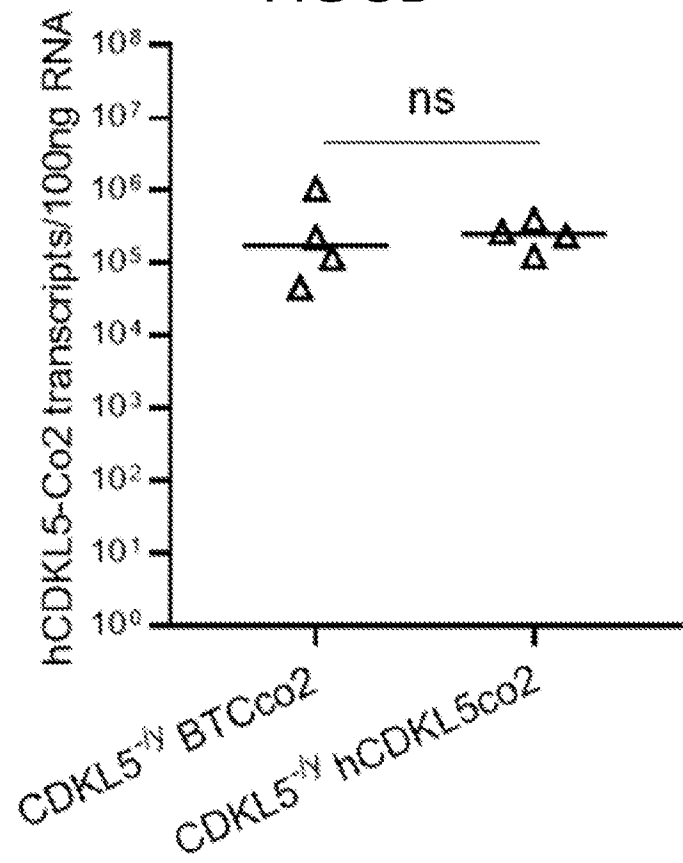


FIG 4

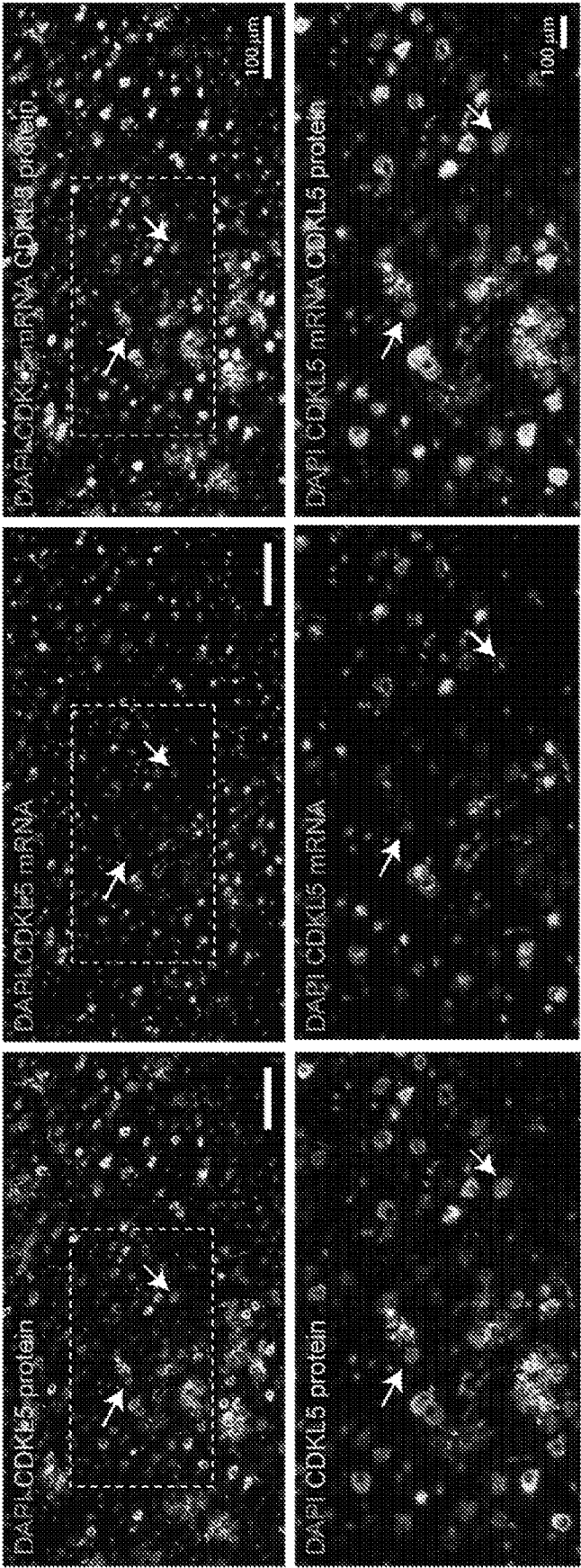


FIG 5A

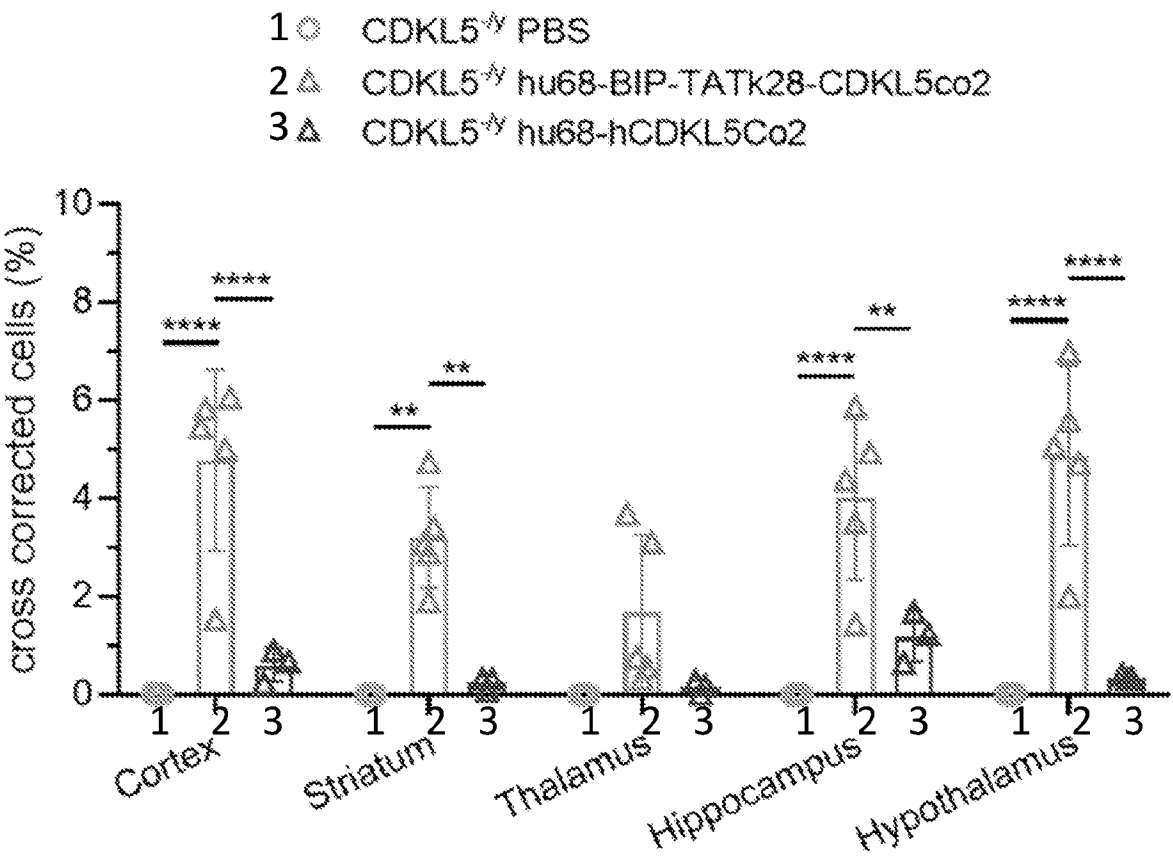


FIG 5B

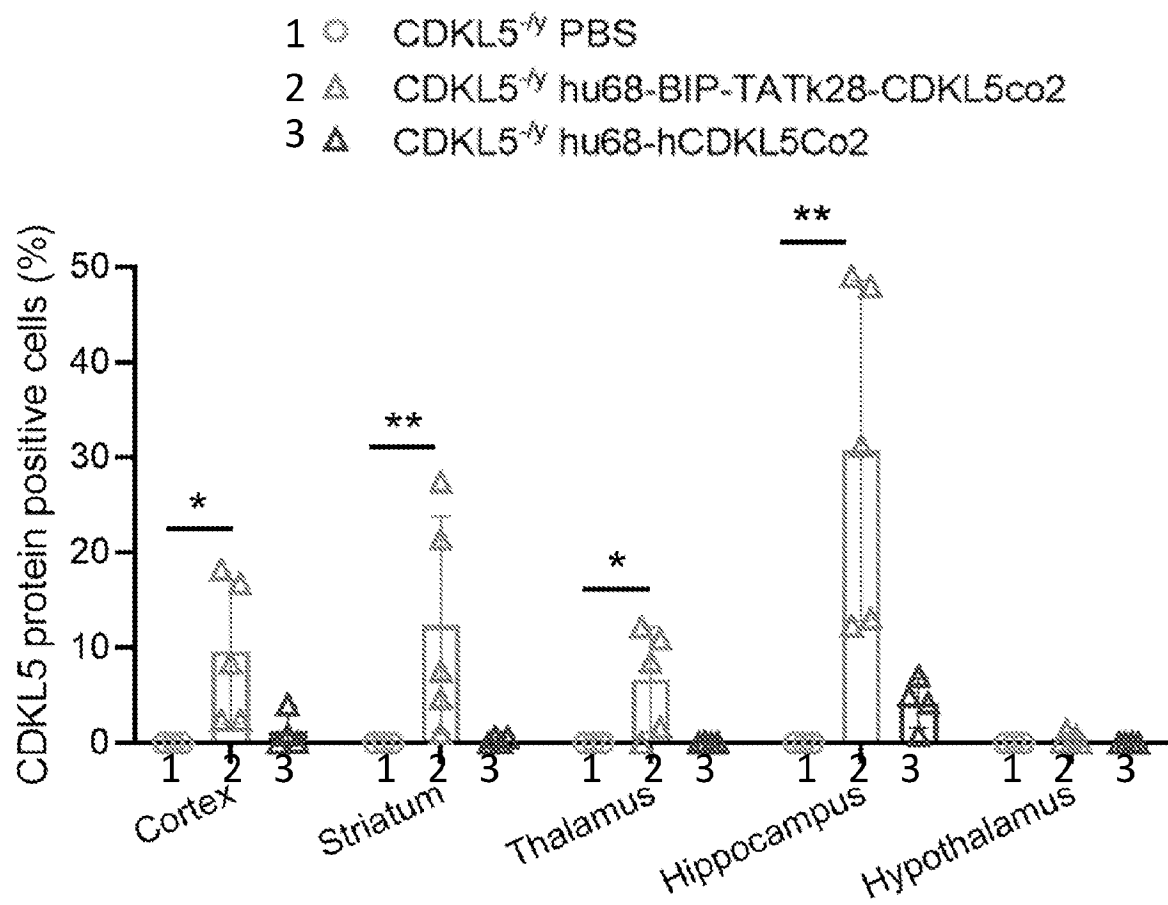


FIG 5C

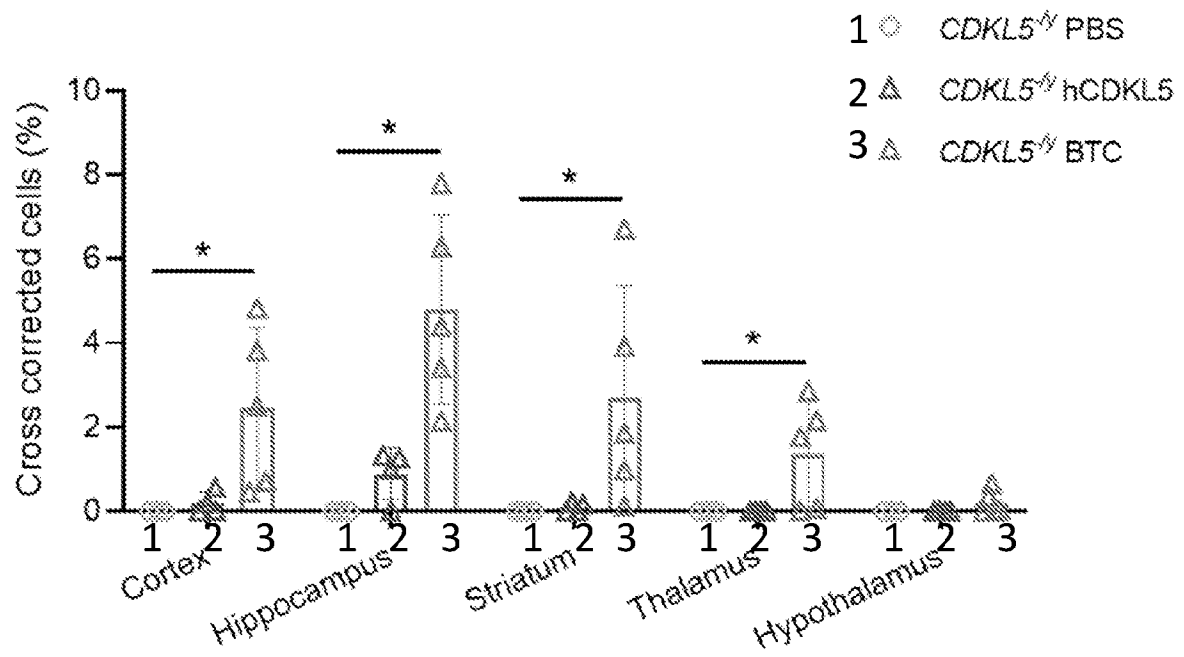


FIG 6

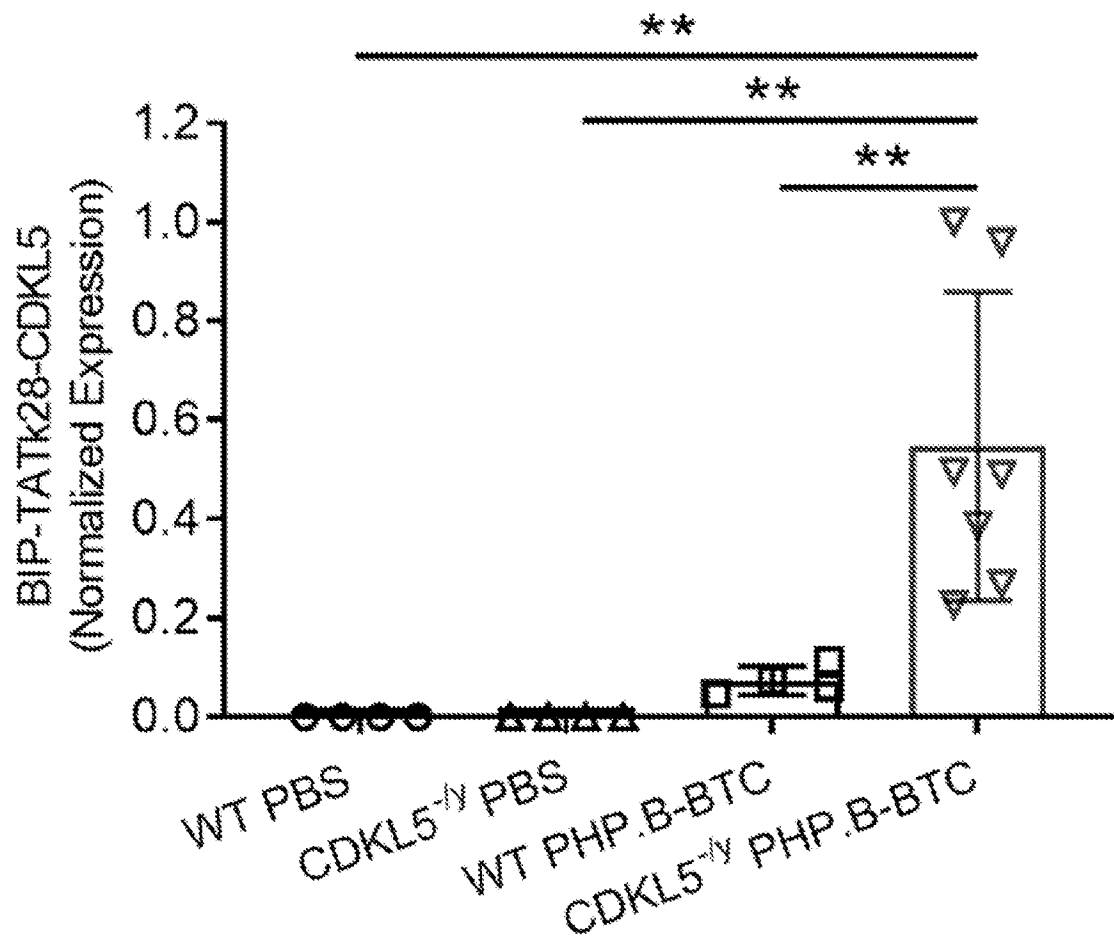


FIG 7

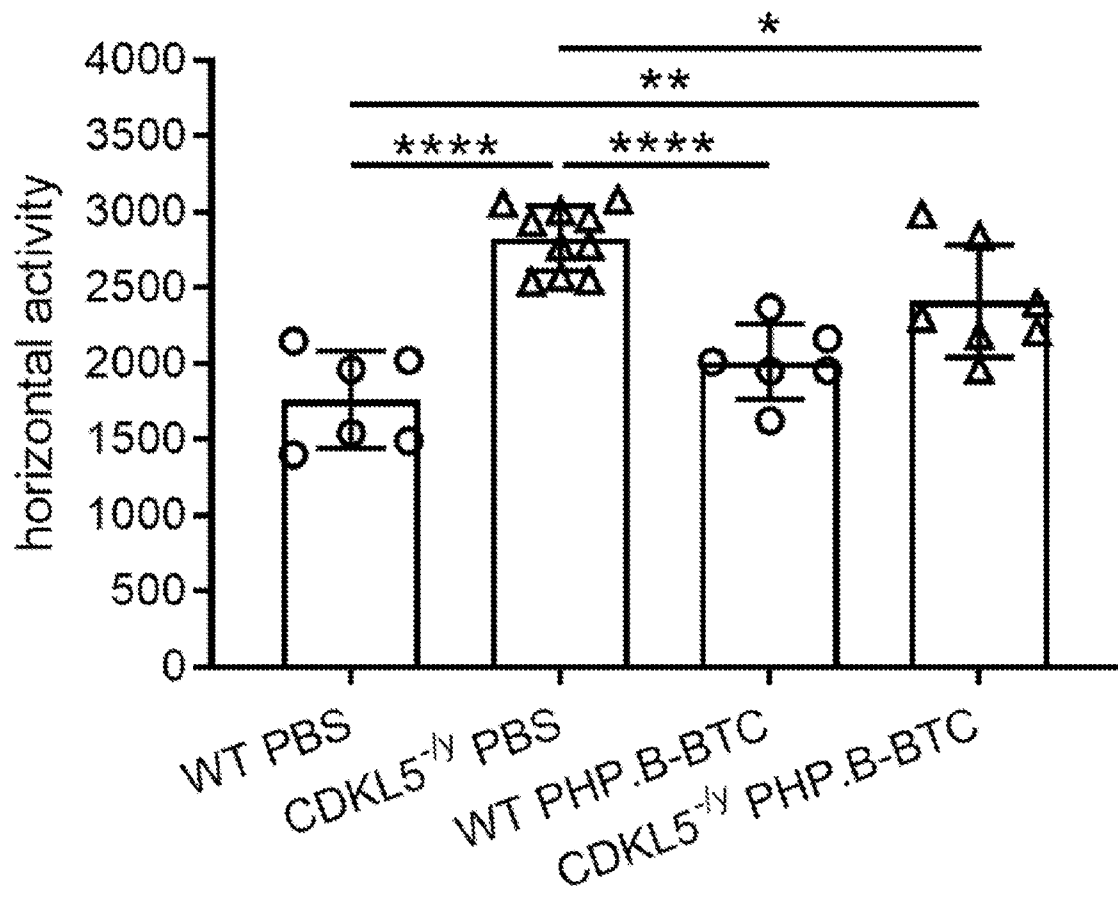


FIG 8A

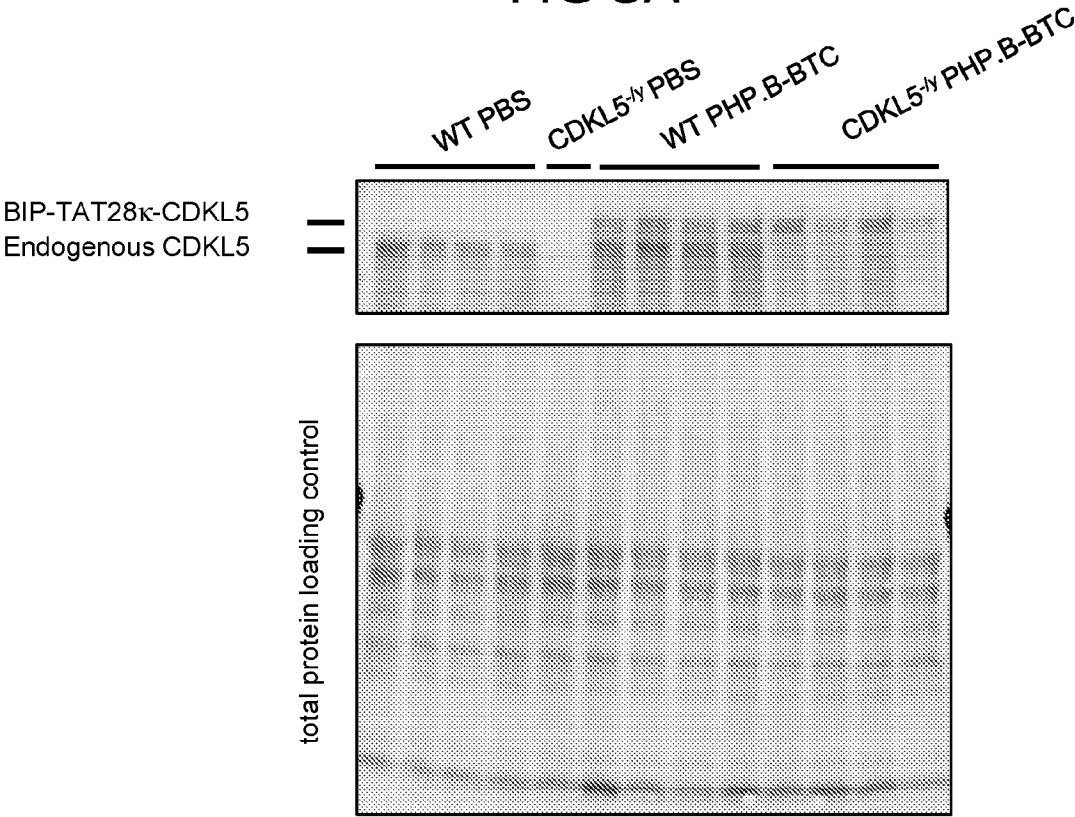


FIG 8B

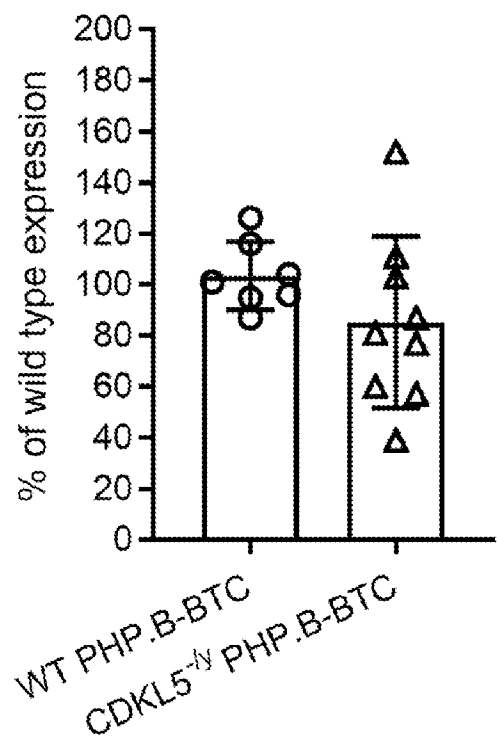


FIG 9A

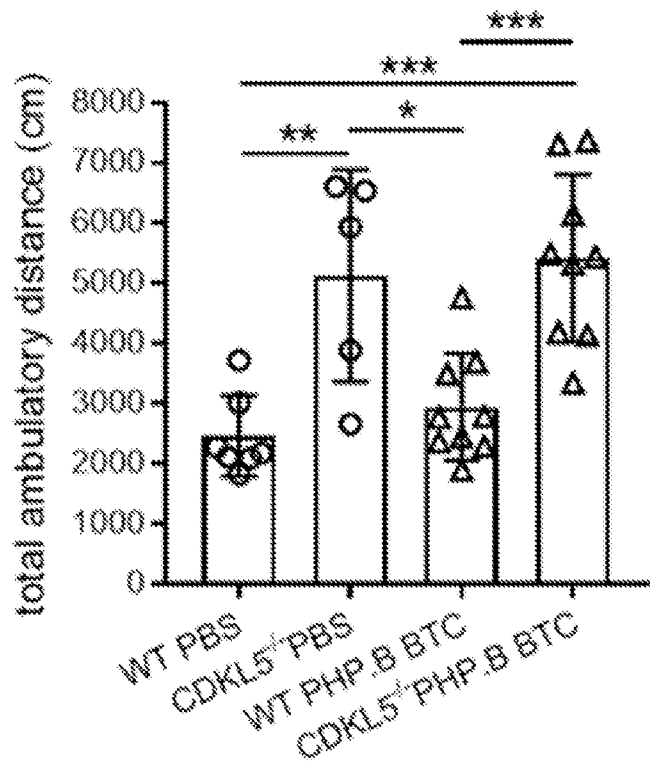


FIG 9B

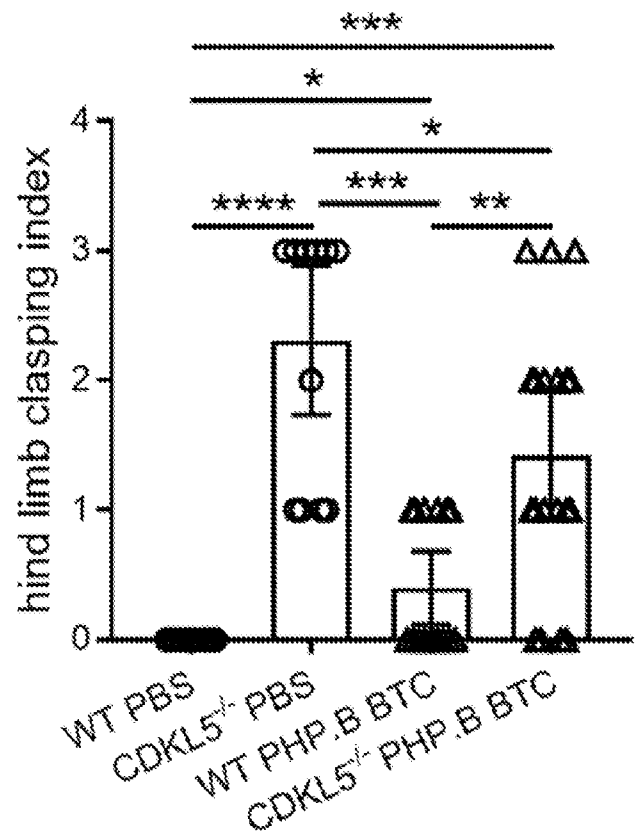


FIG 9C

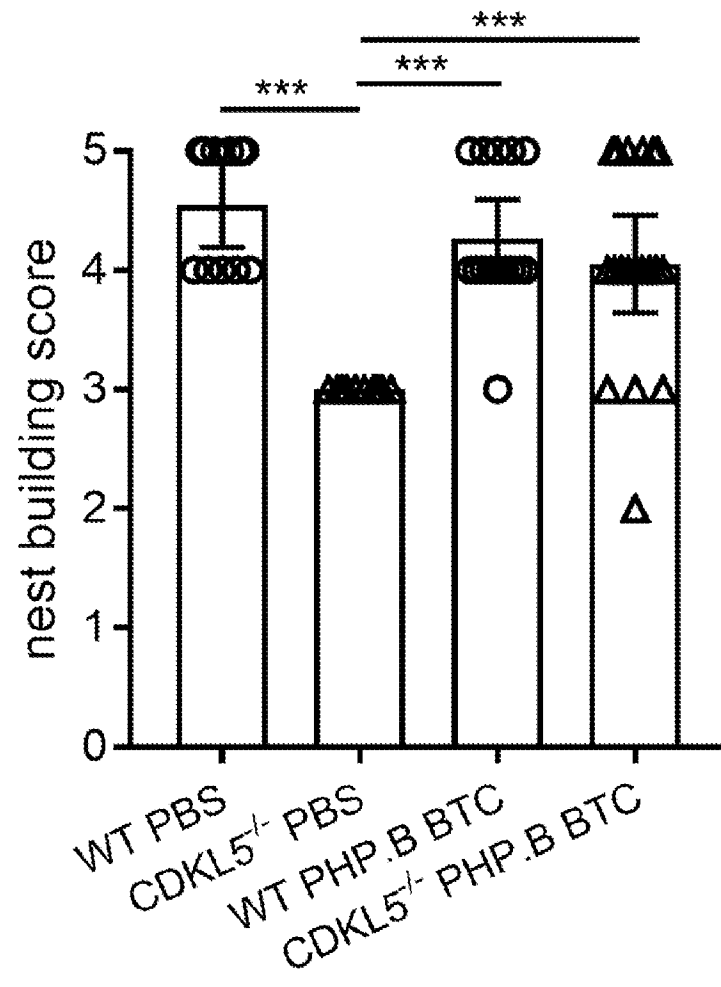


FIG 10

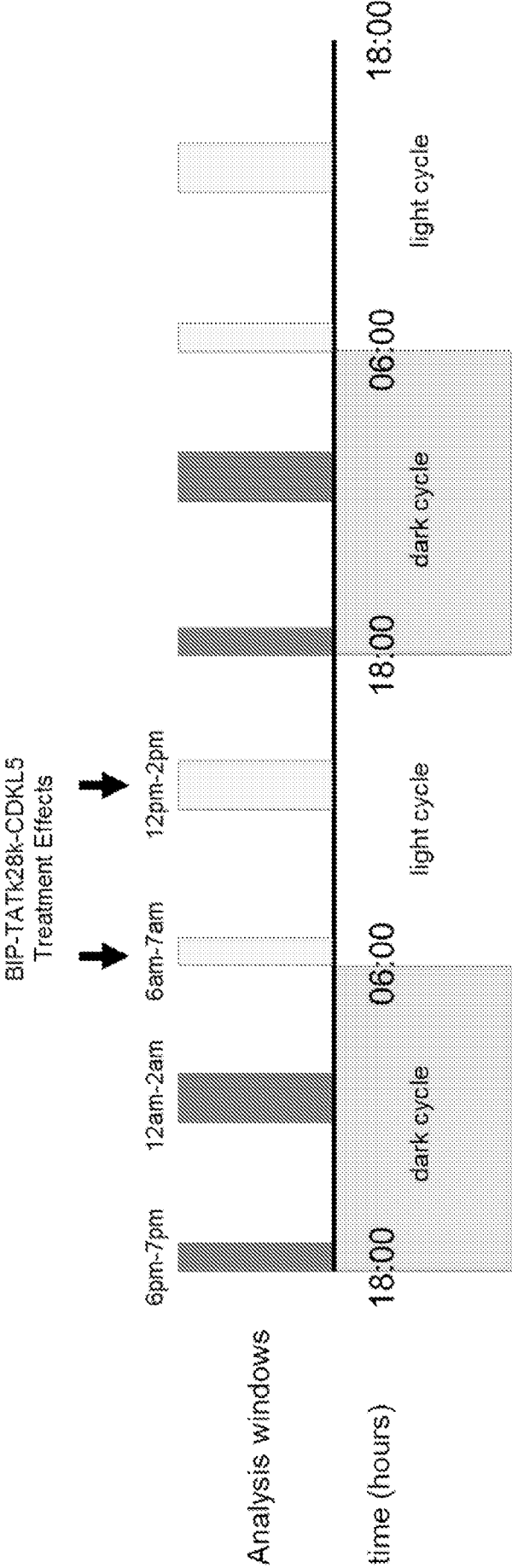


FIG 11A

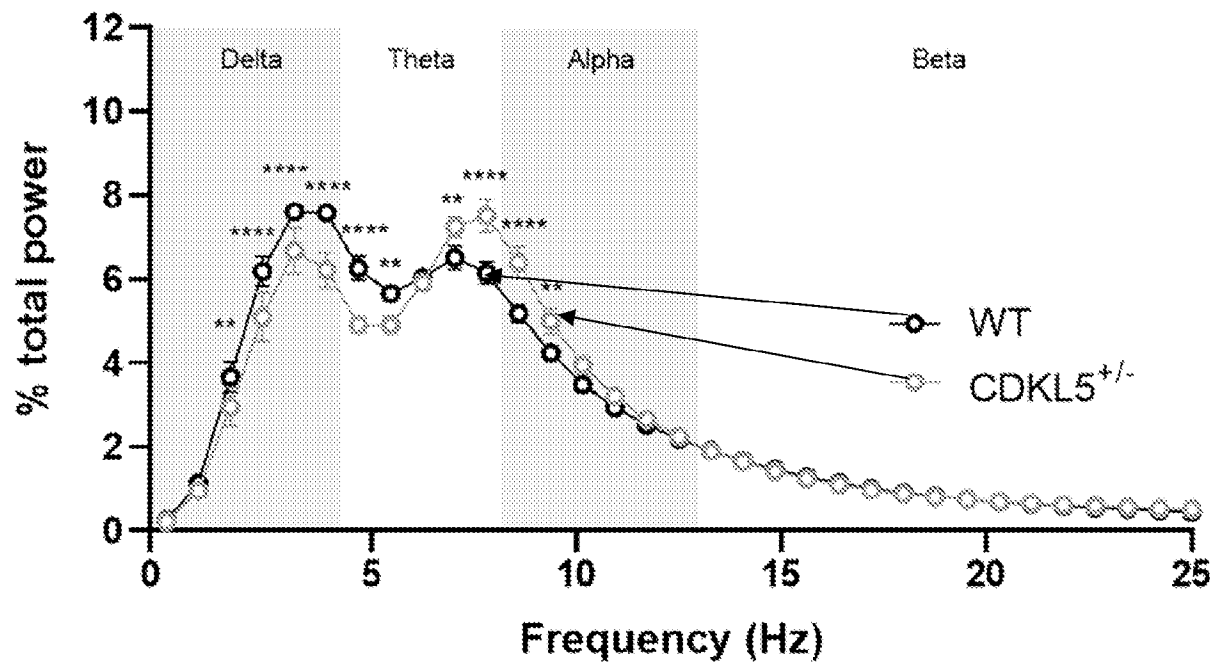


FIG 11B

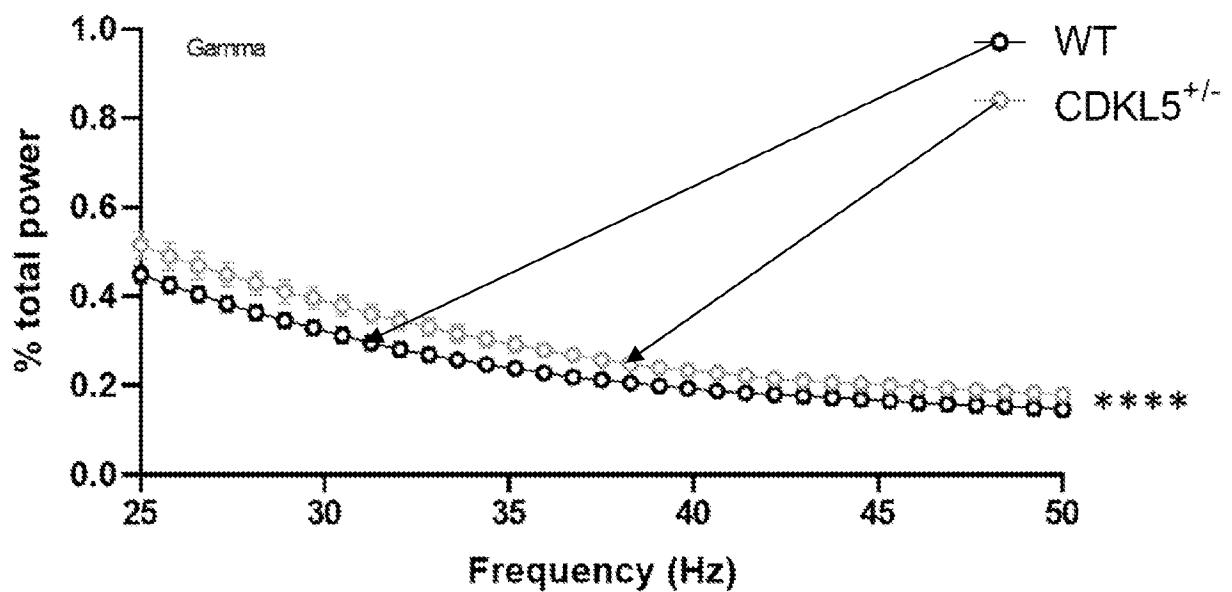


FIG 12A

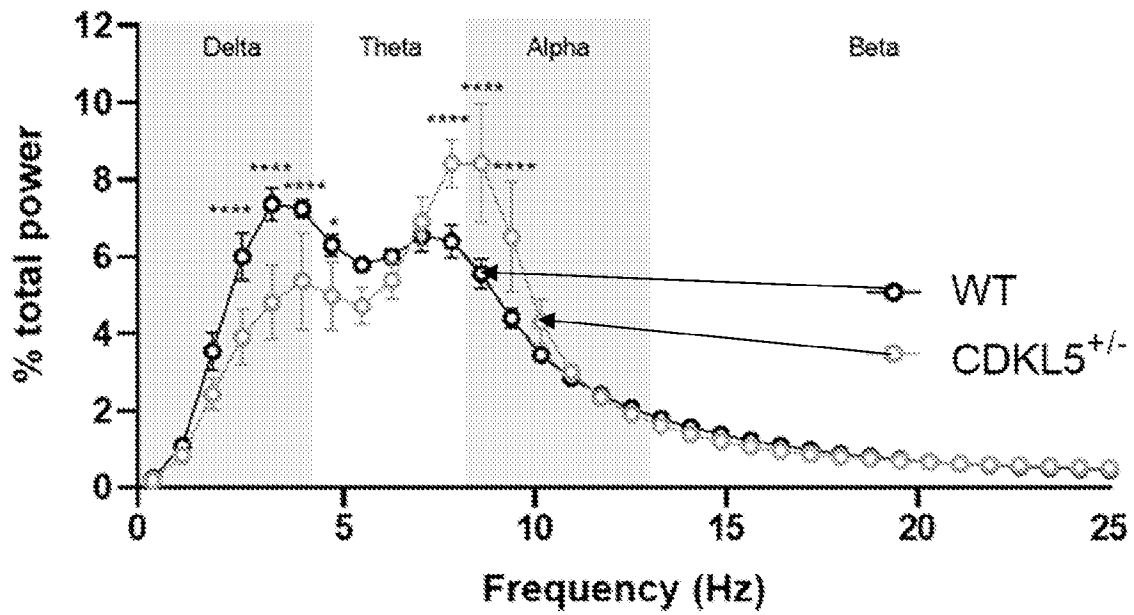


FIG 12B

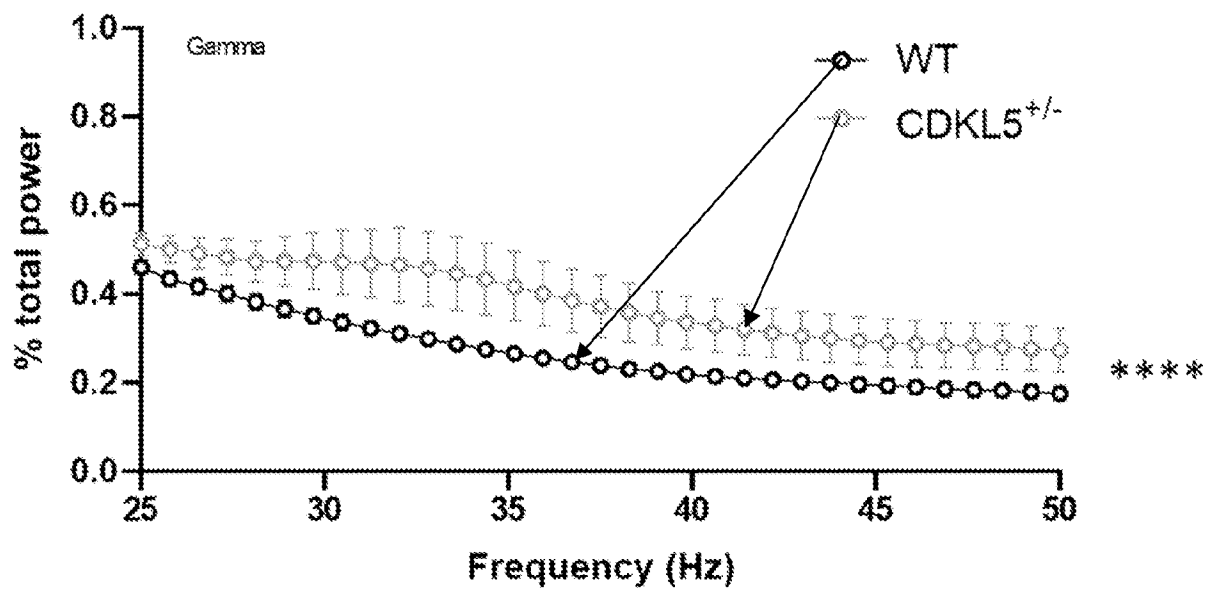


FIG 13A

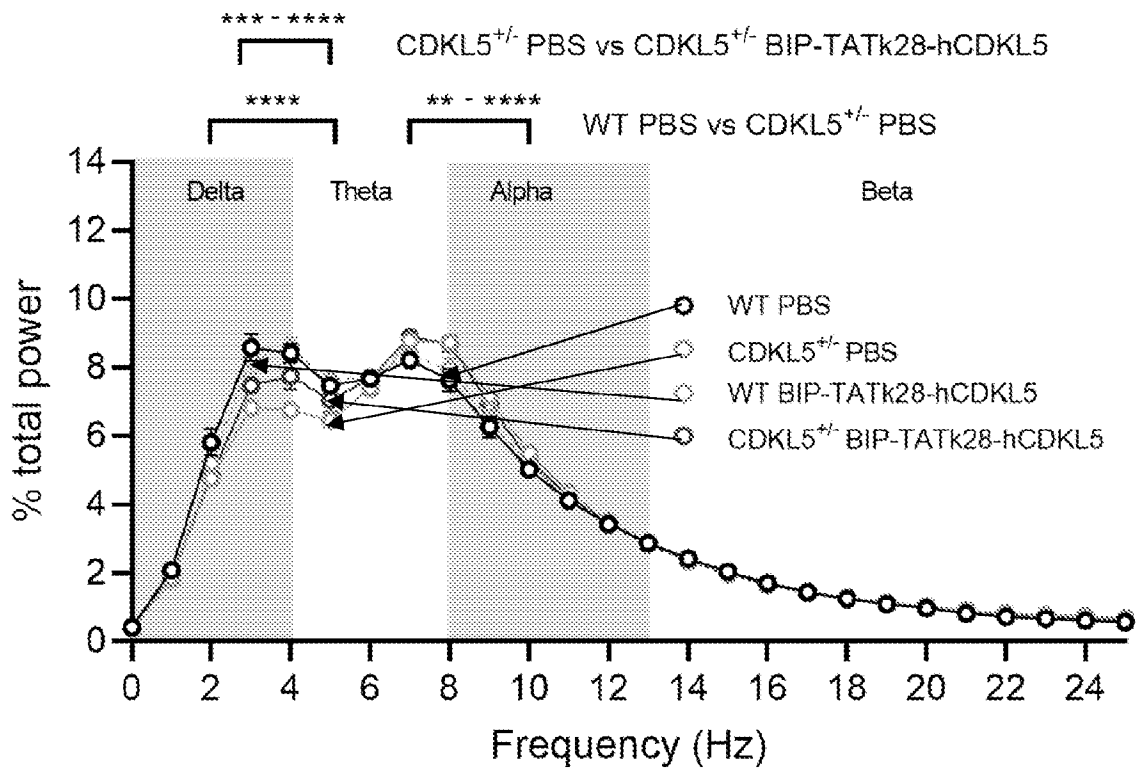


FIG 13B

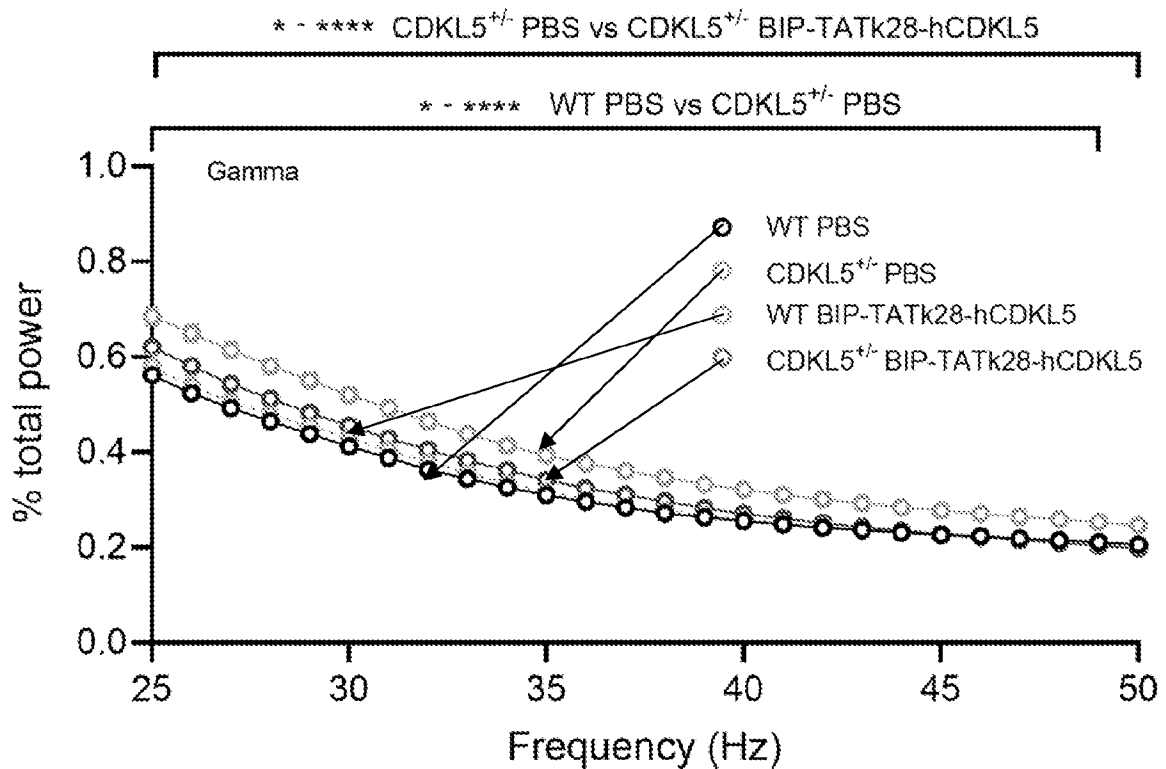


FIG 14A

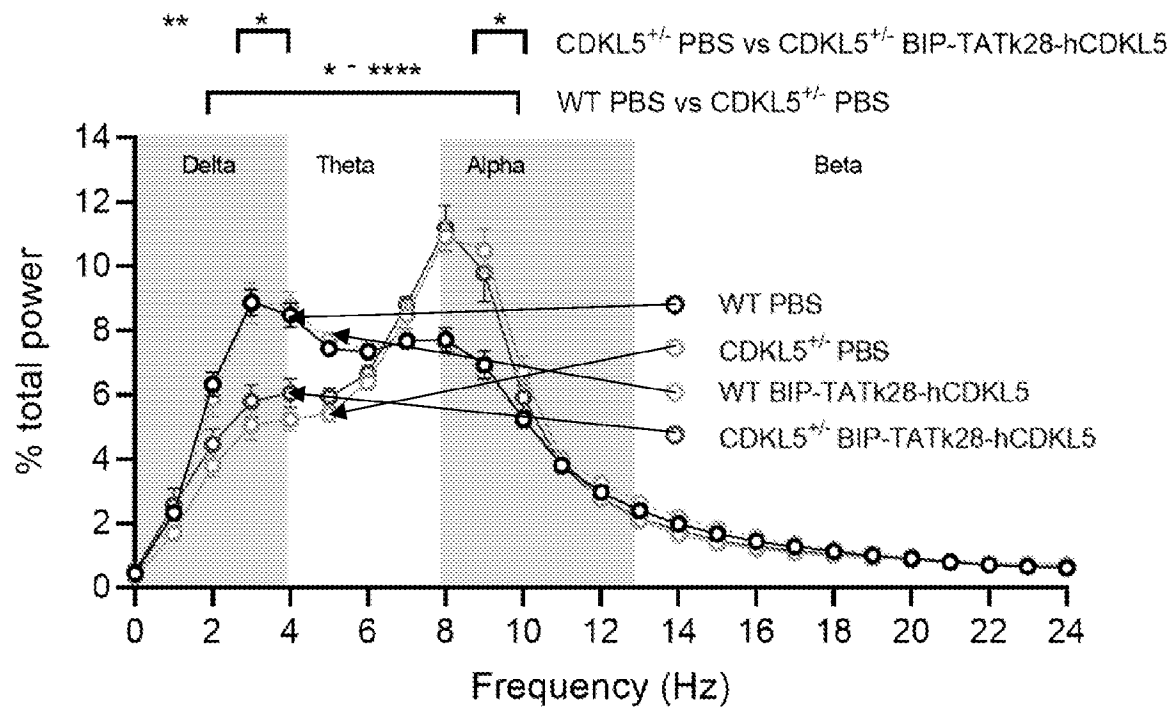


FIG 14B

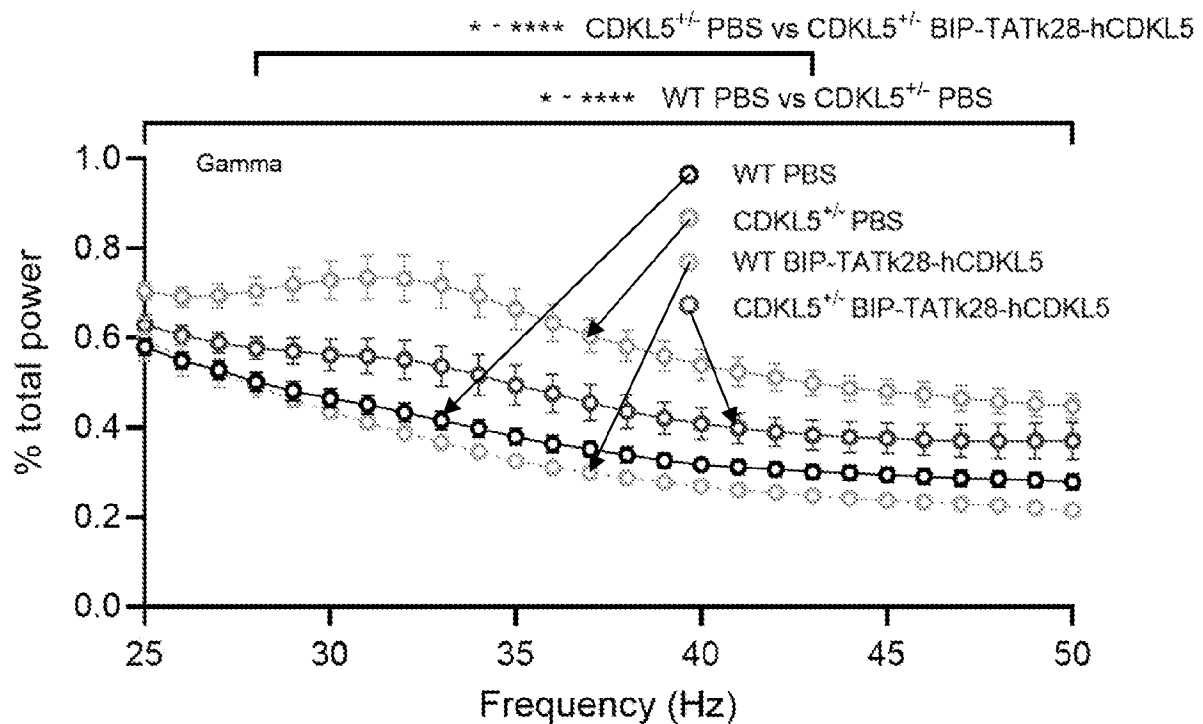


FIG 15A

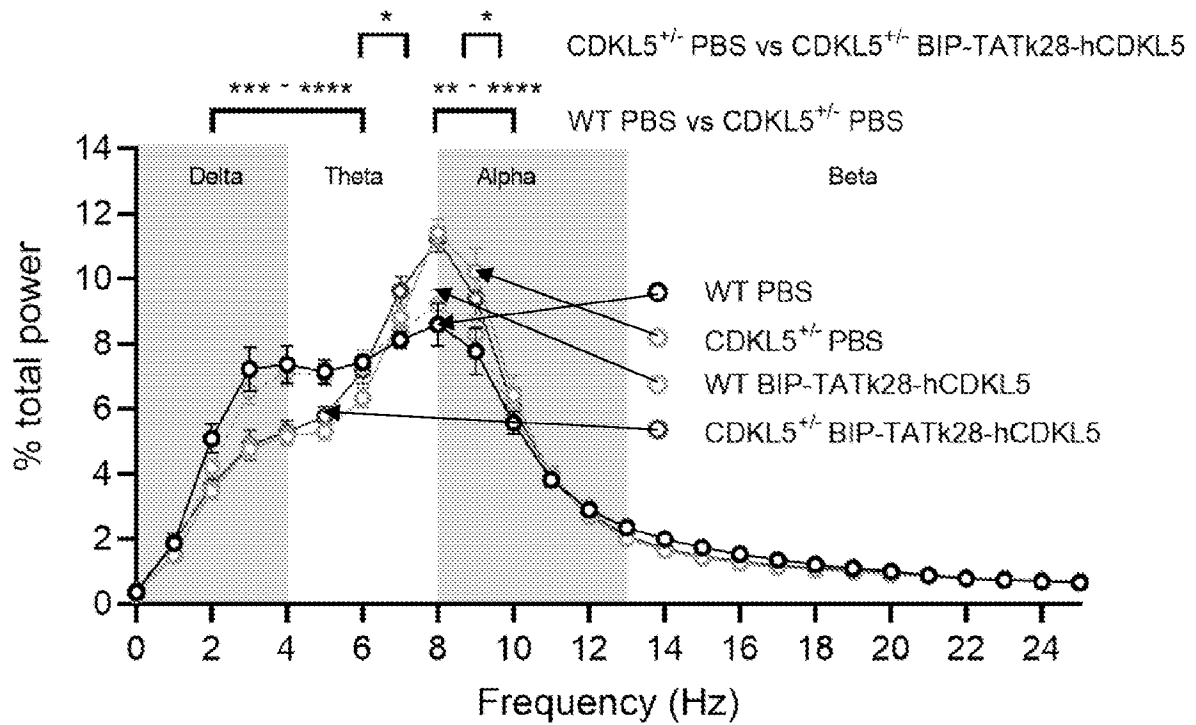


FIG 15B

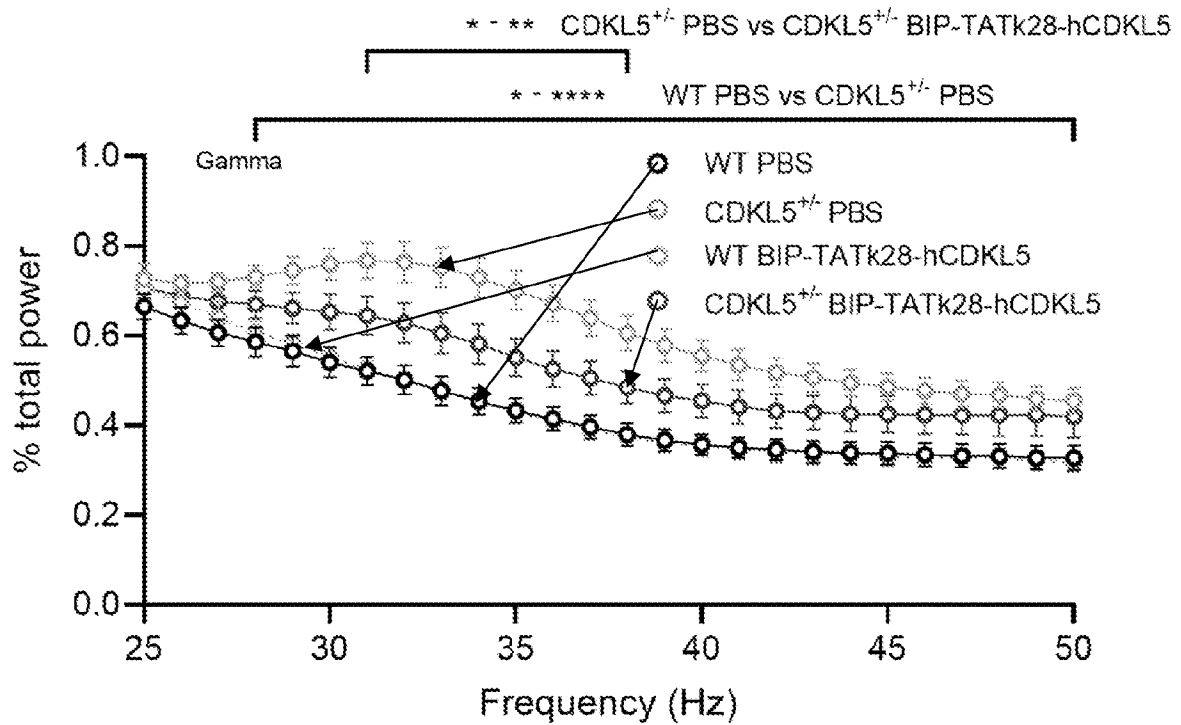


FIG 16A

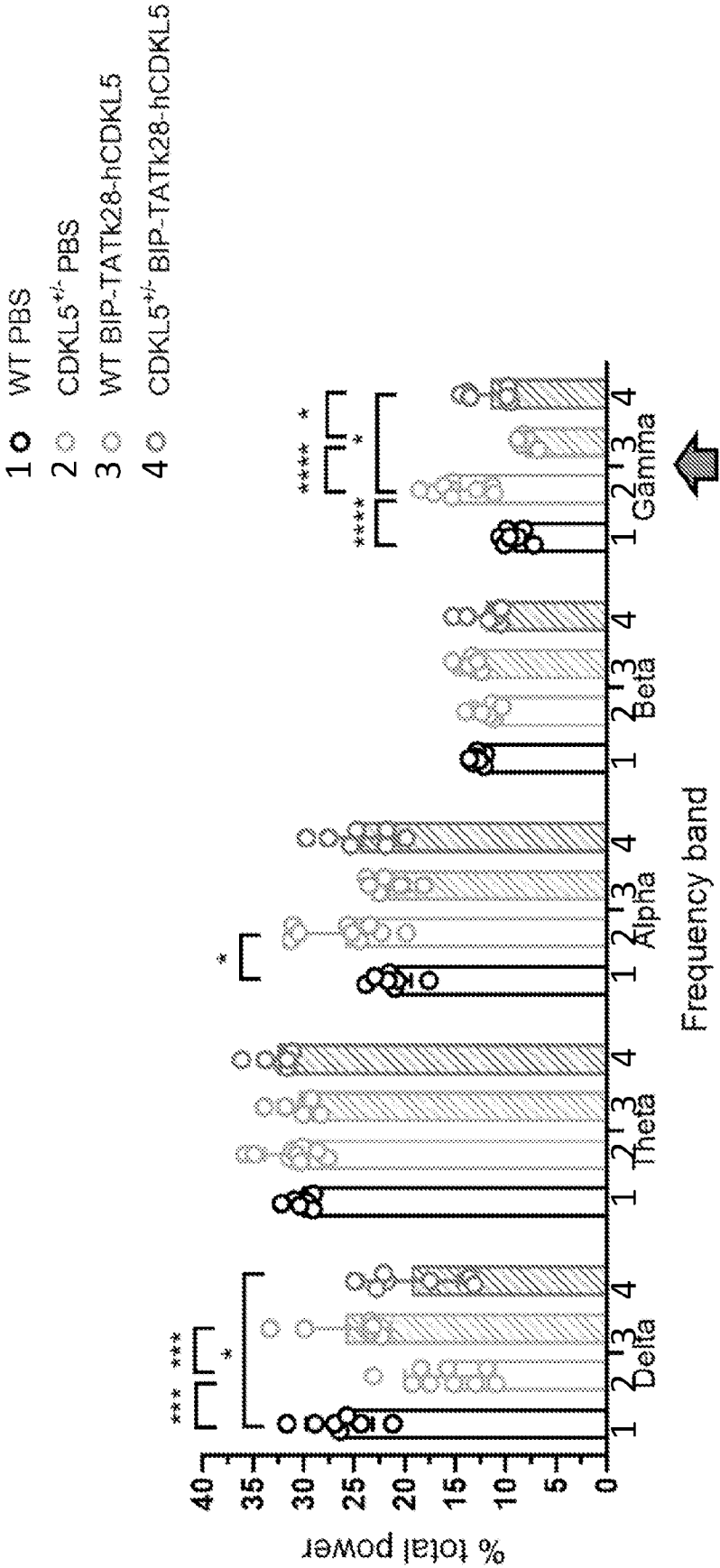


FIG 16B

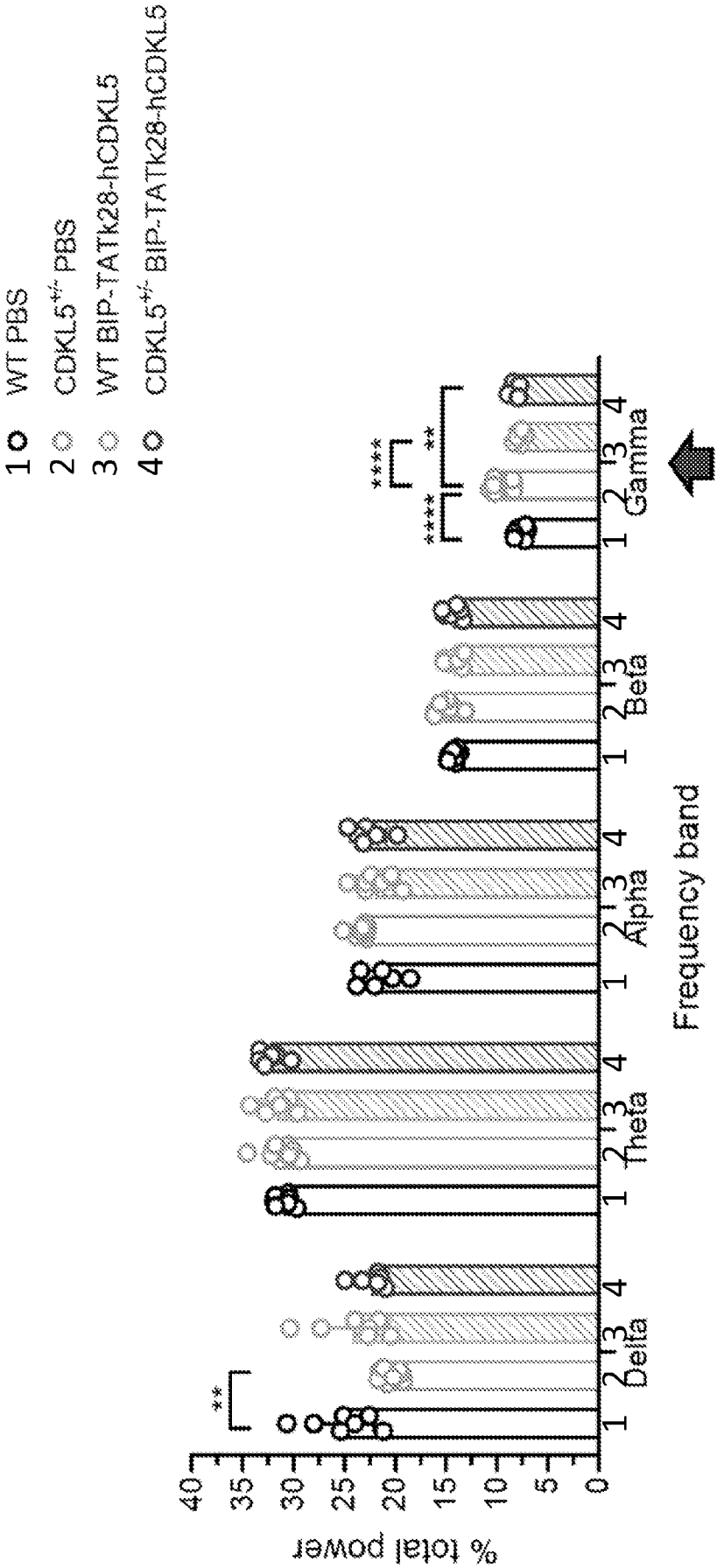


FIG 16C

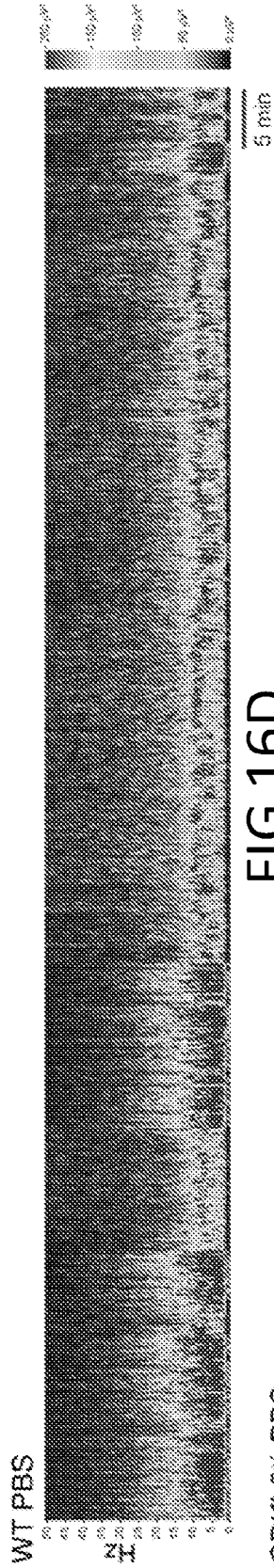


FIG 16D

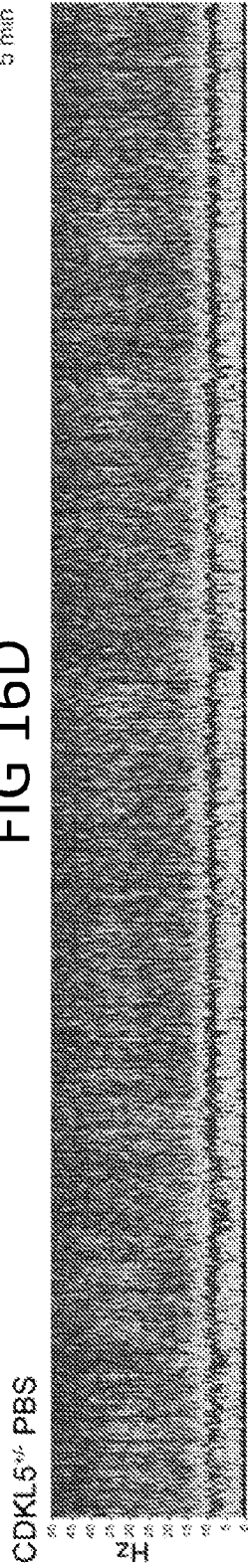


FIG 16E

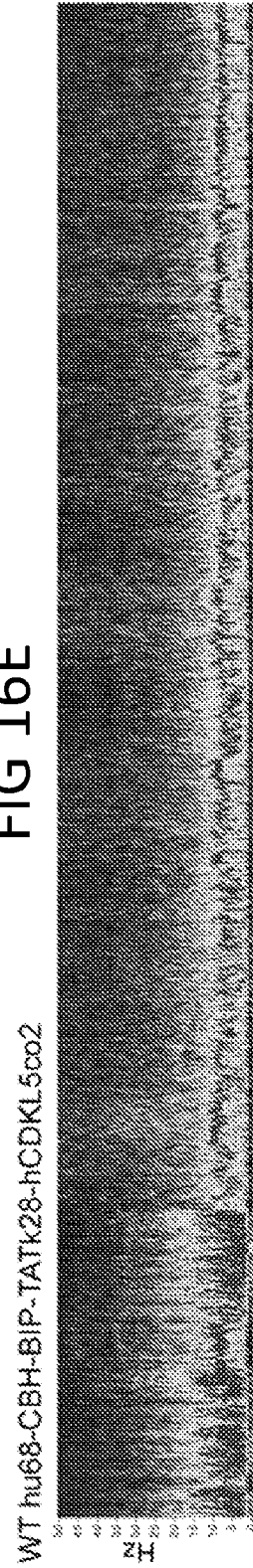


FIG 16F

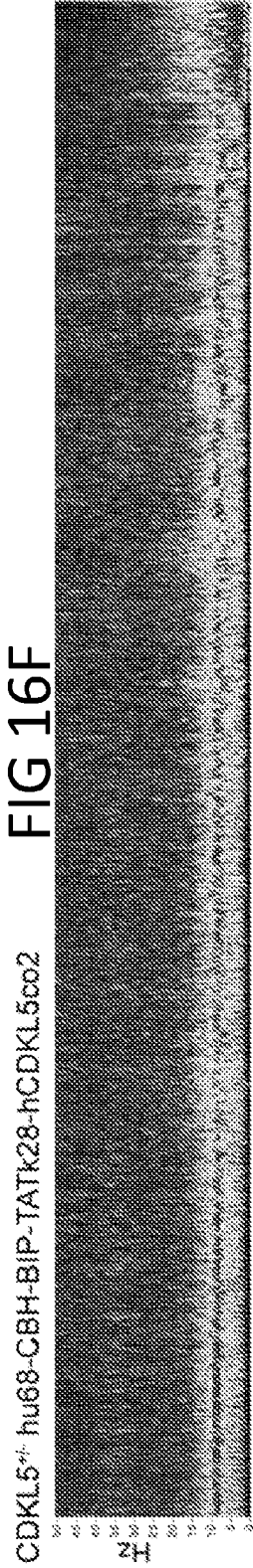


FIG 17A

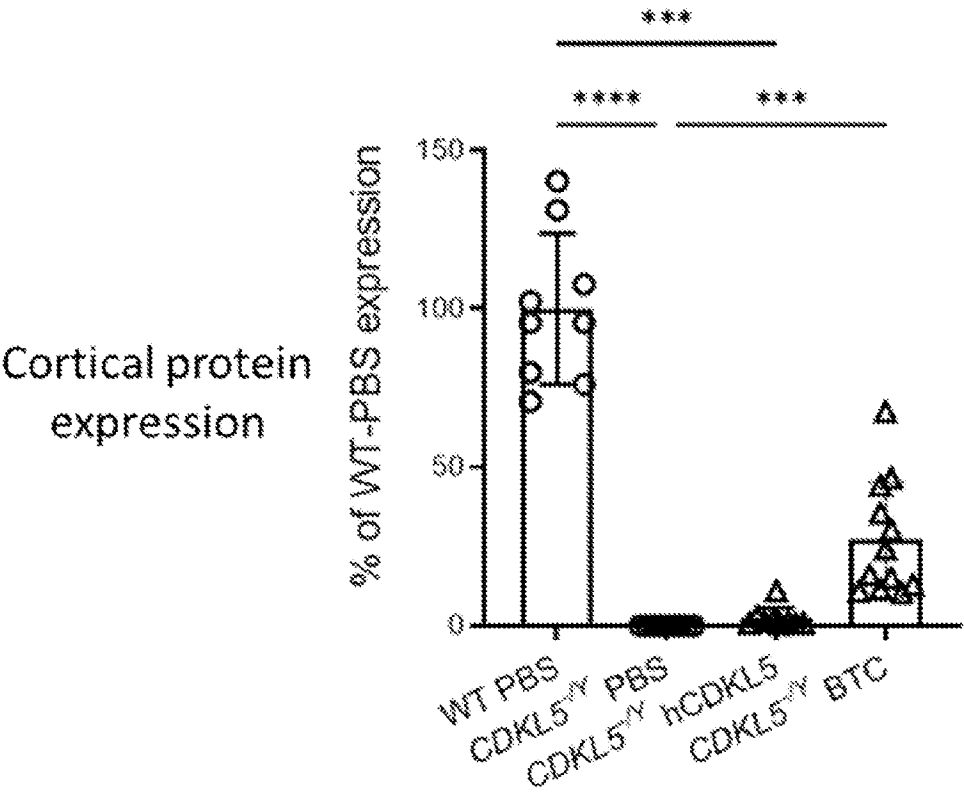
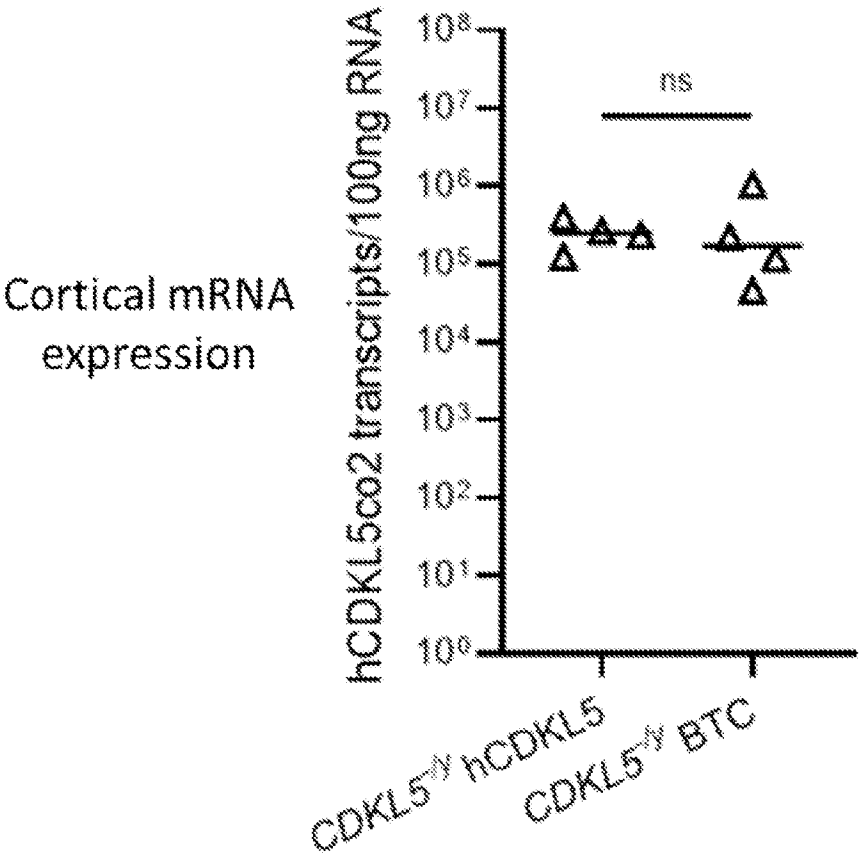


FIG 17B



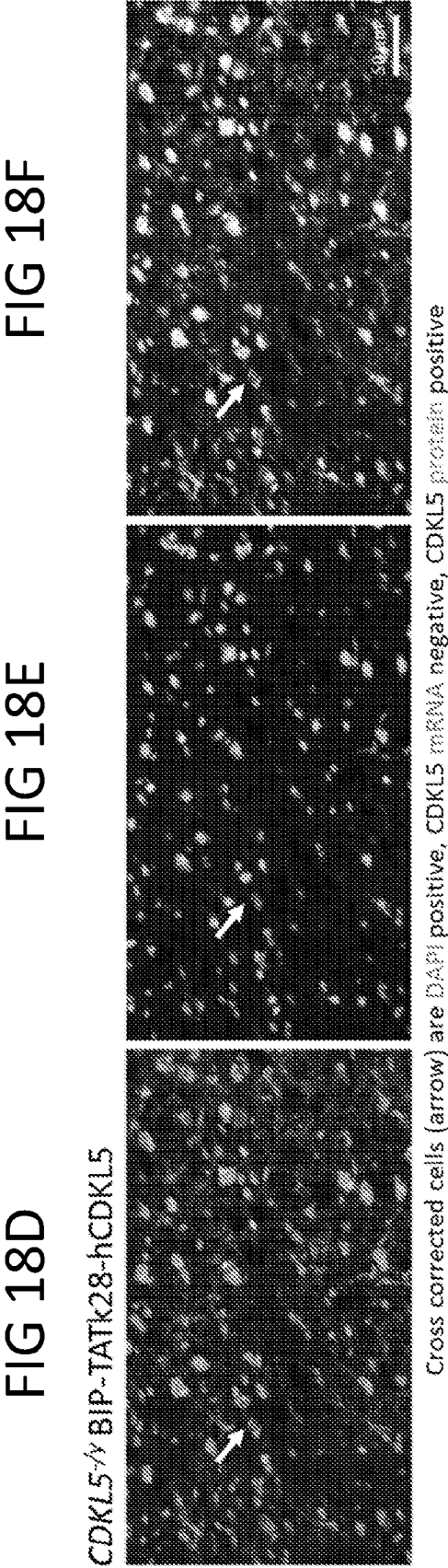
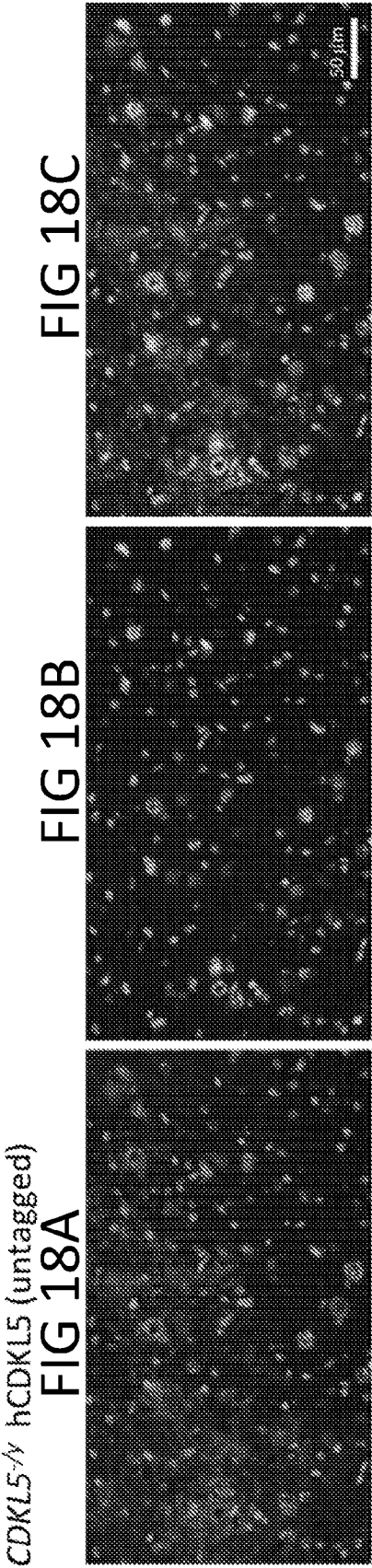


FIG 19A

CDKL5 / NeuN / DAPI

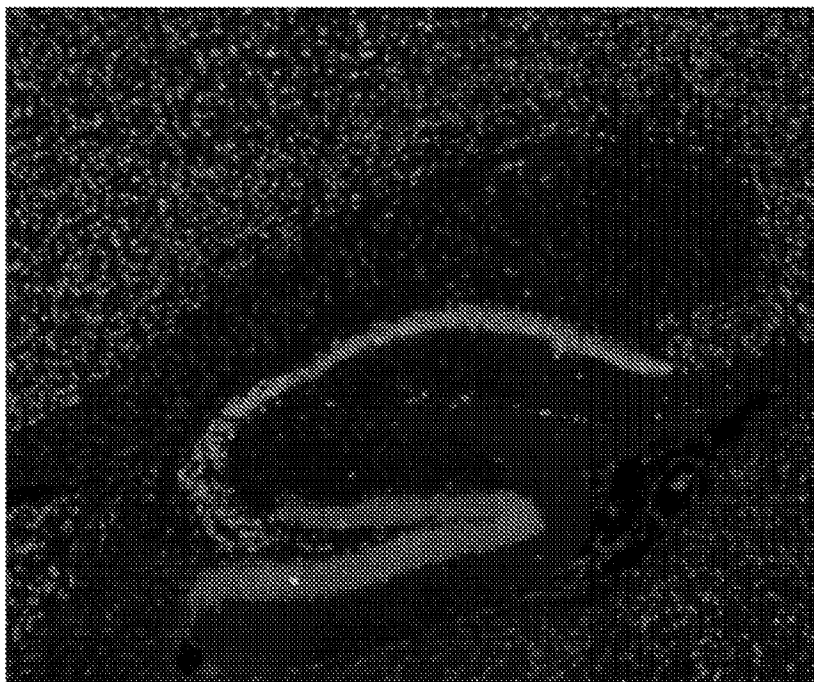


FIG 19B

CDKL5 / NeuN / DAPI



FIG 19C

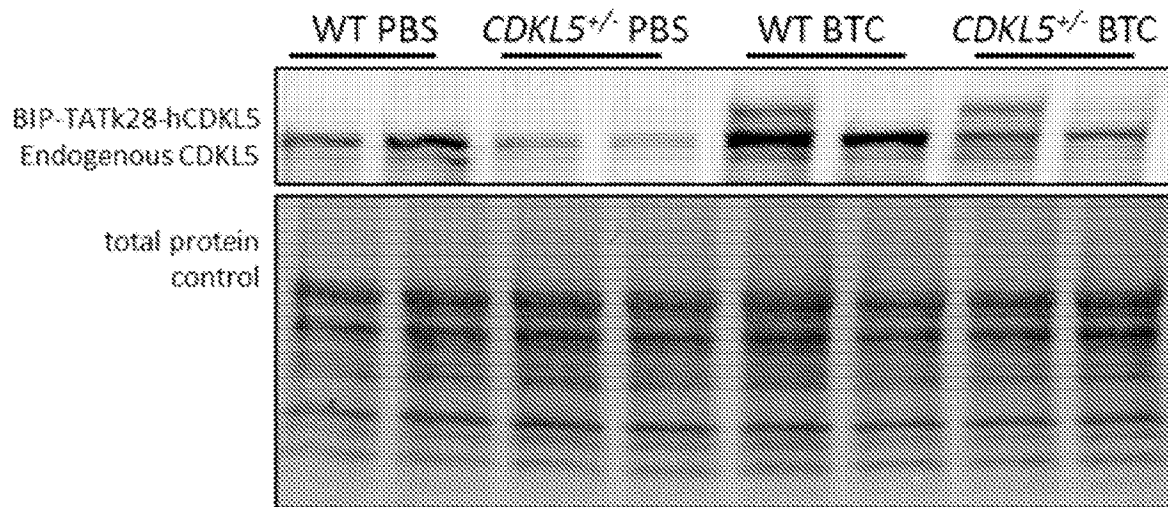


FIG 19D

Hippocampal BIP-TATk28-hCDKL5 expression

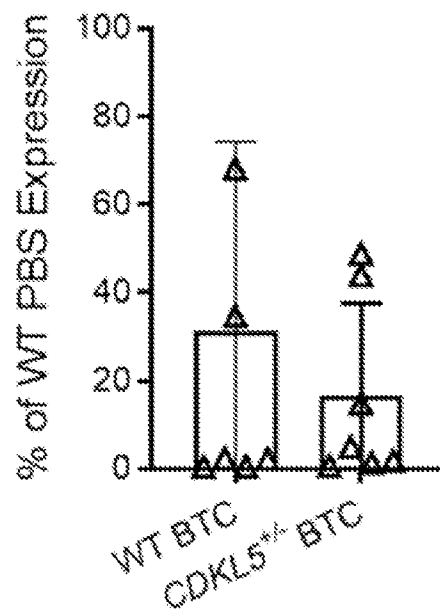


FIG 20

22-01 Biodistribution (DNA)

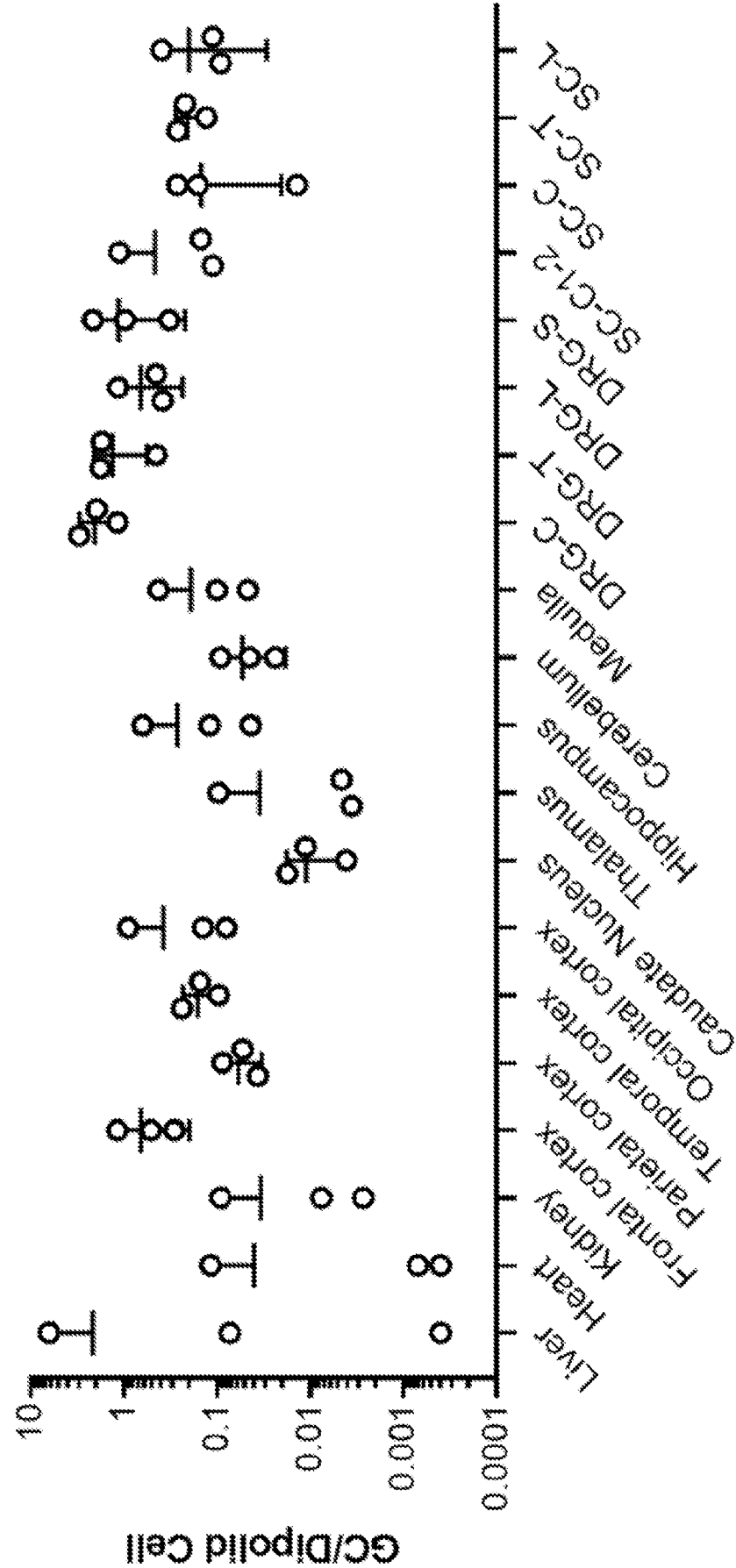


FIG 21

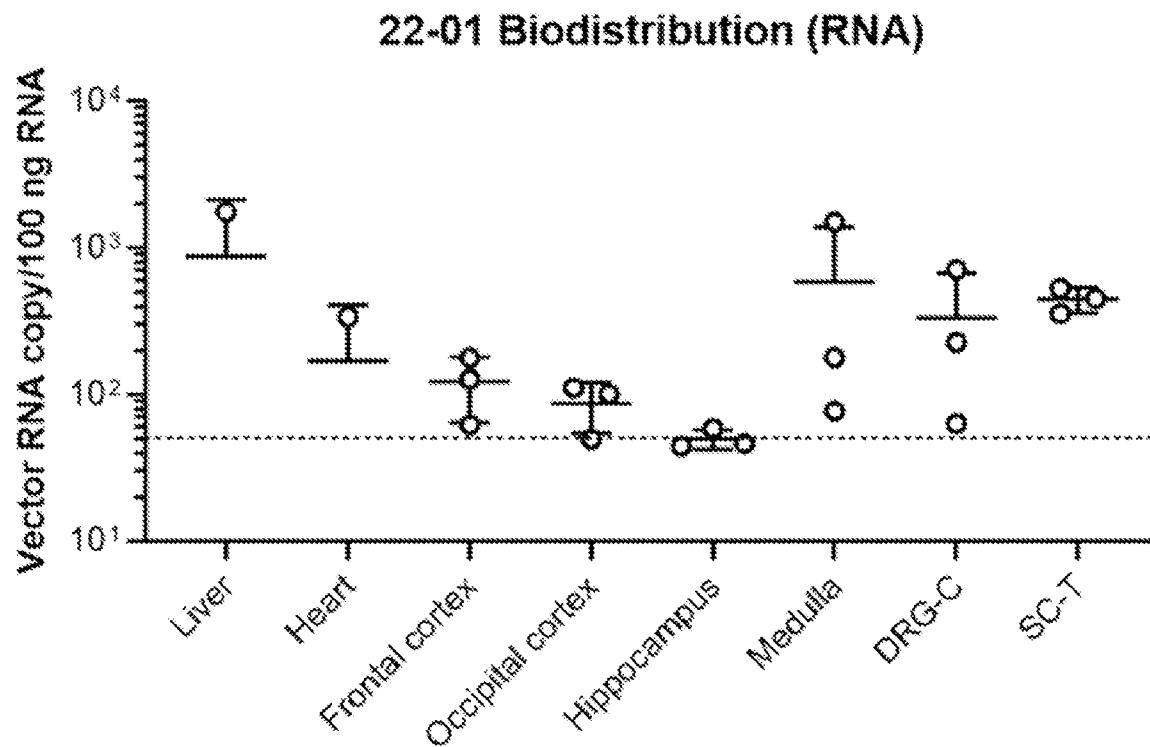


FIG 22

ELISPOT AAVhu68

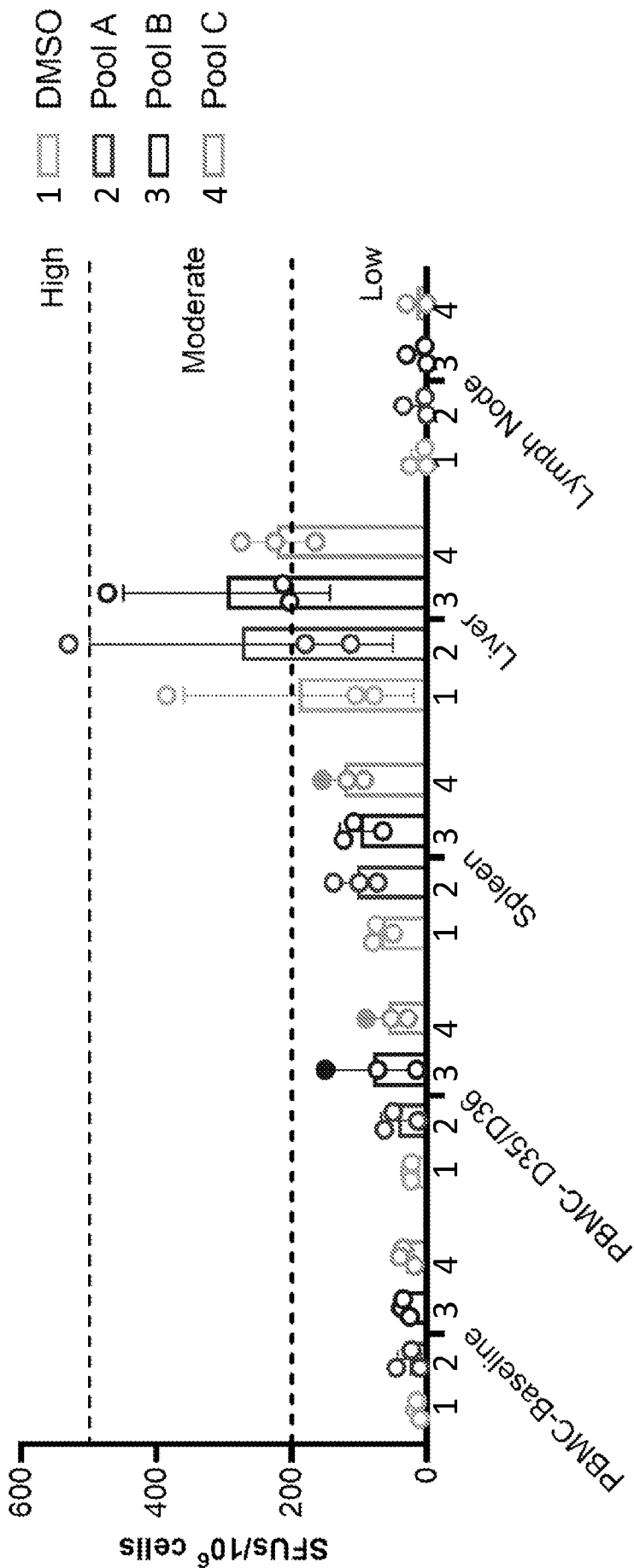
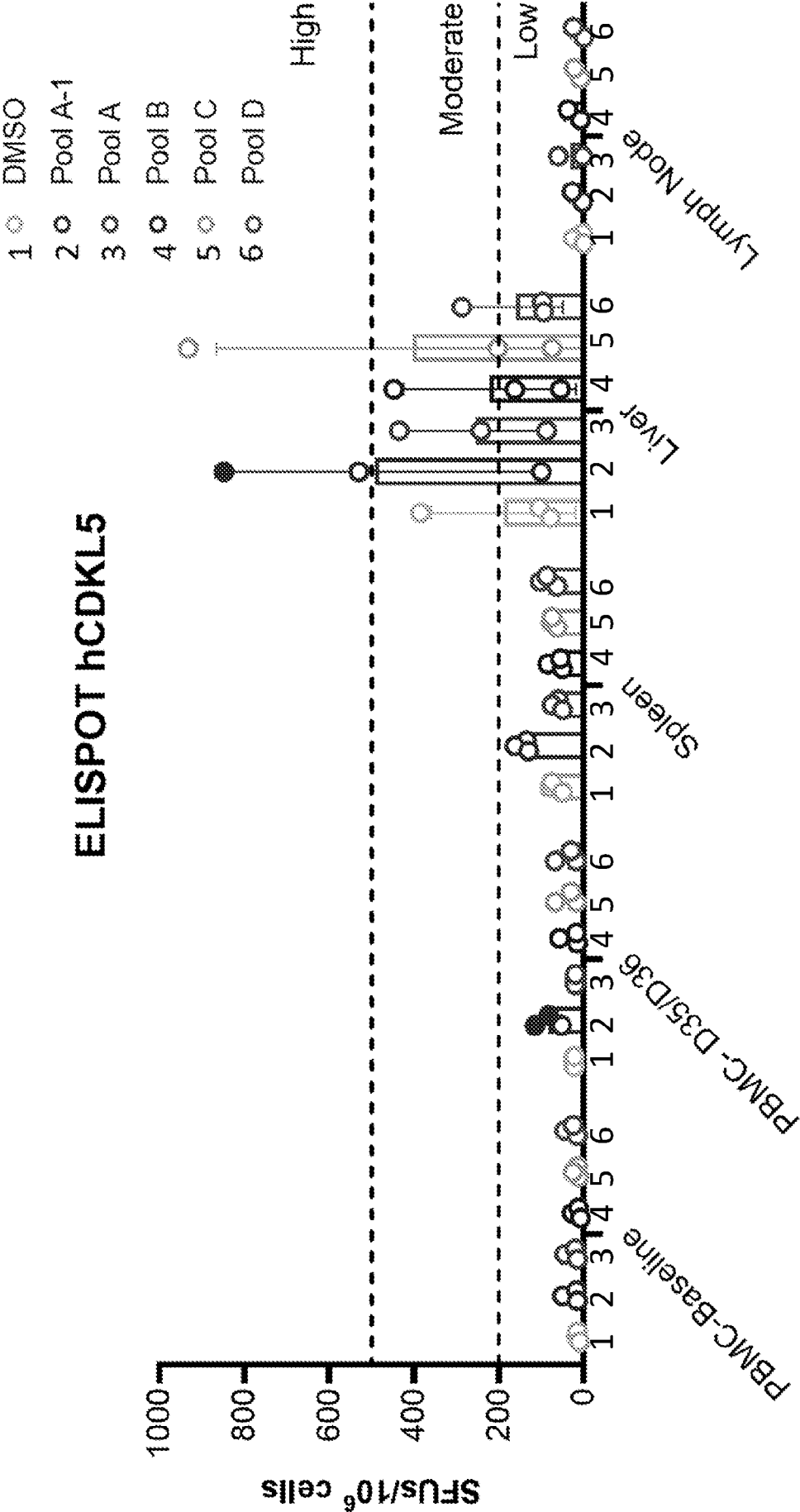


FIG 23



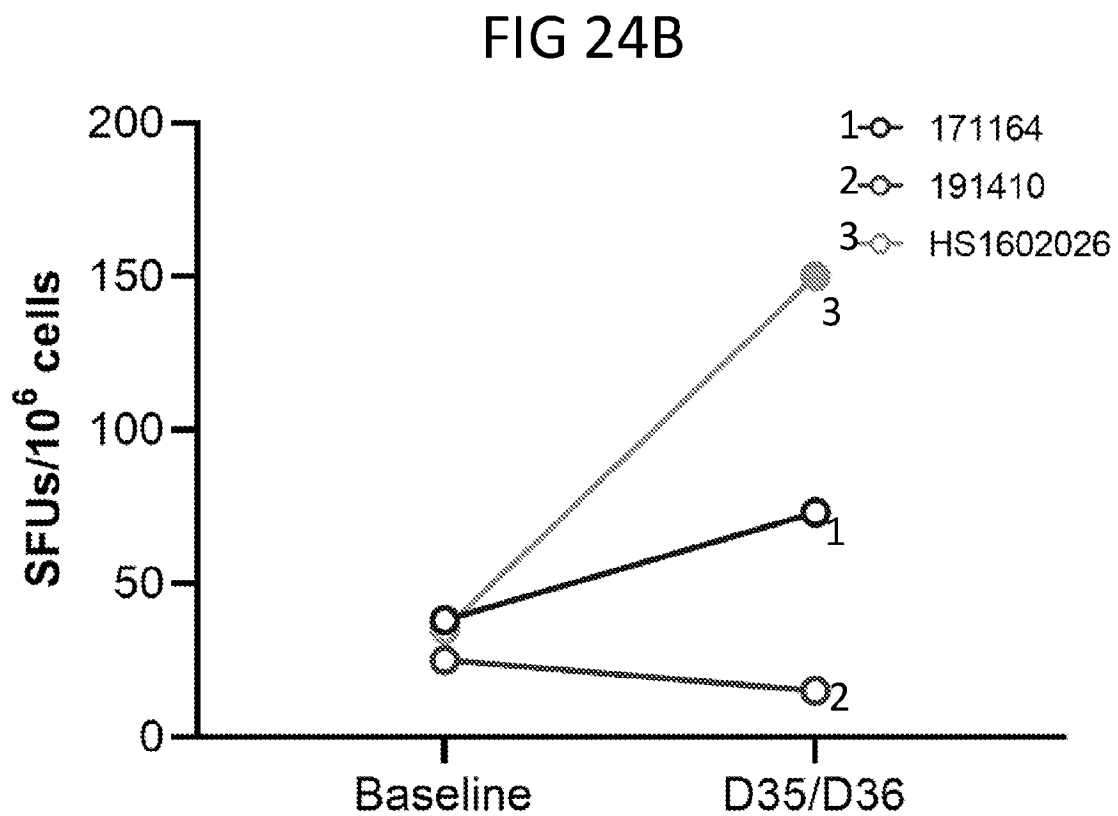
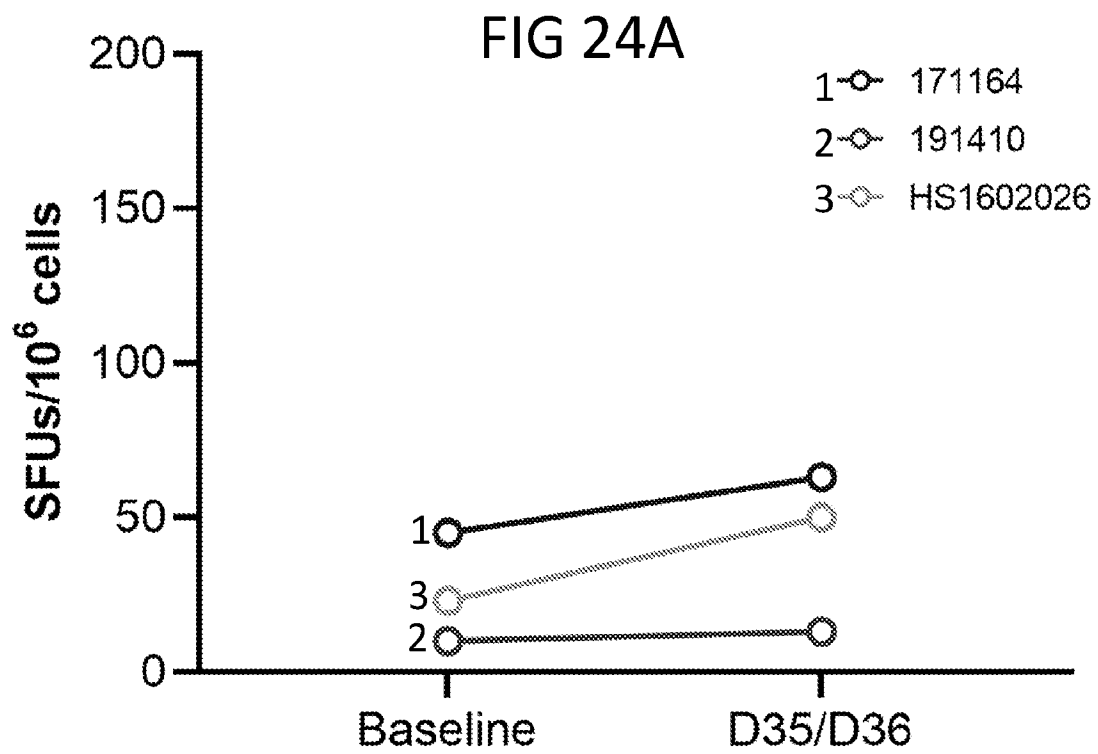


FIG 24C

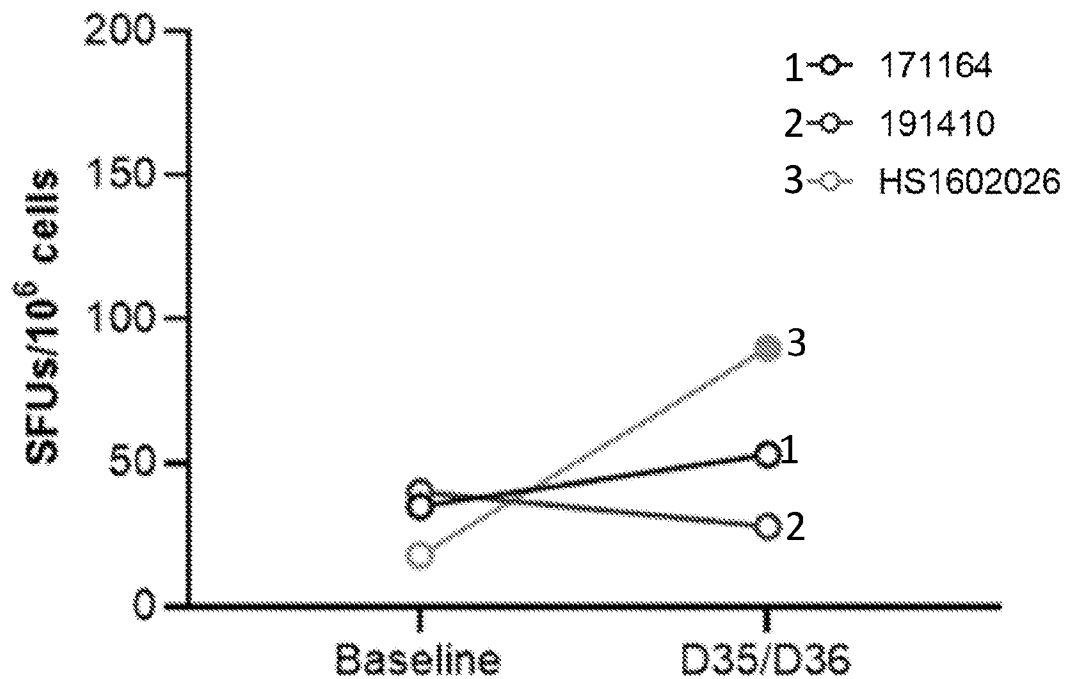


FIG 24D

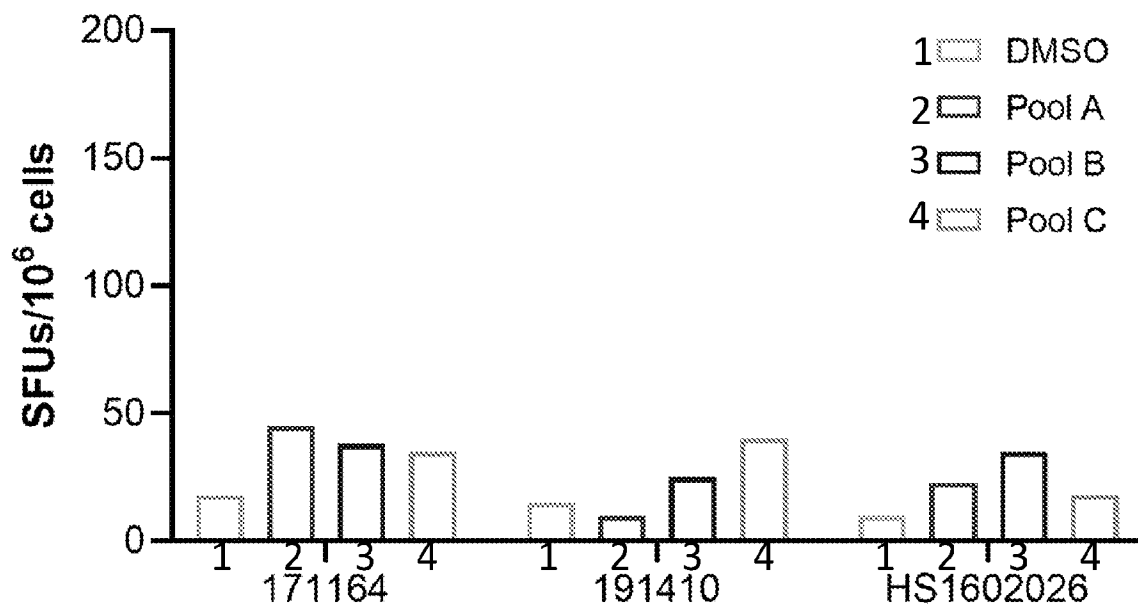
ELISPOT AAVhu68 Baseline PBMC

FIG 24E

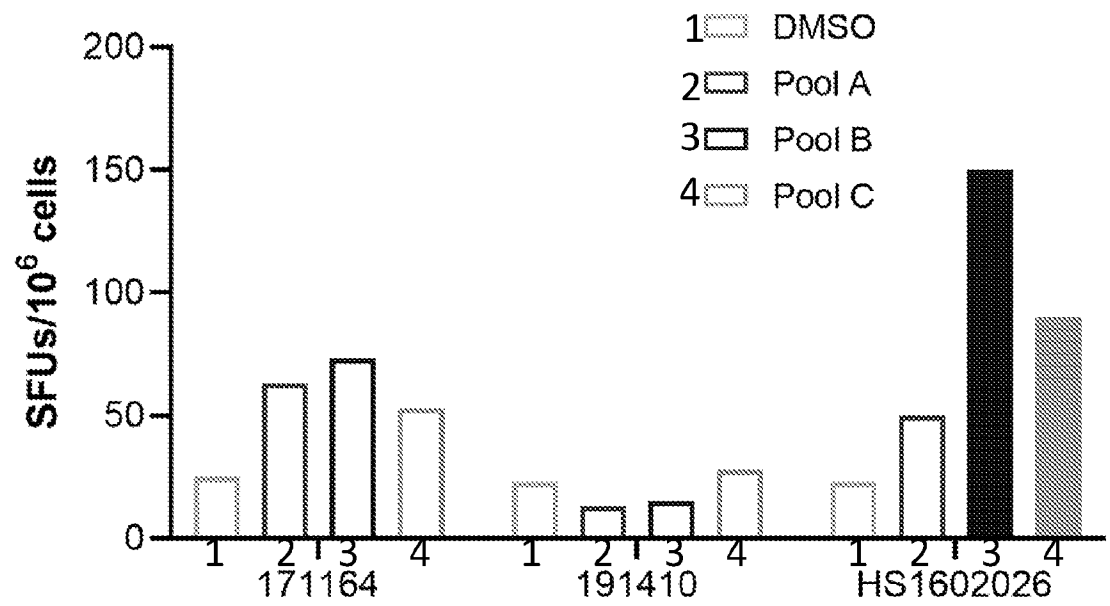


FIG 24F

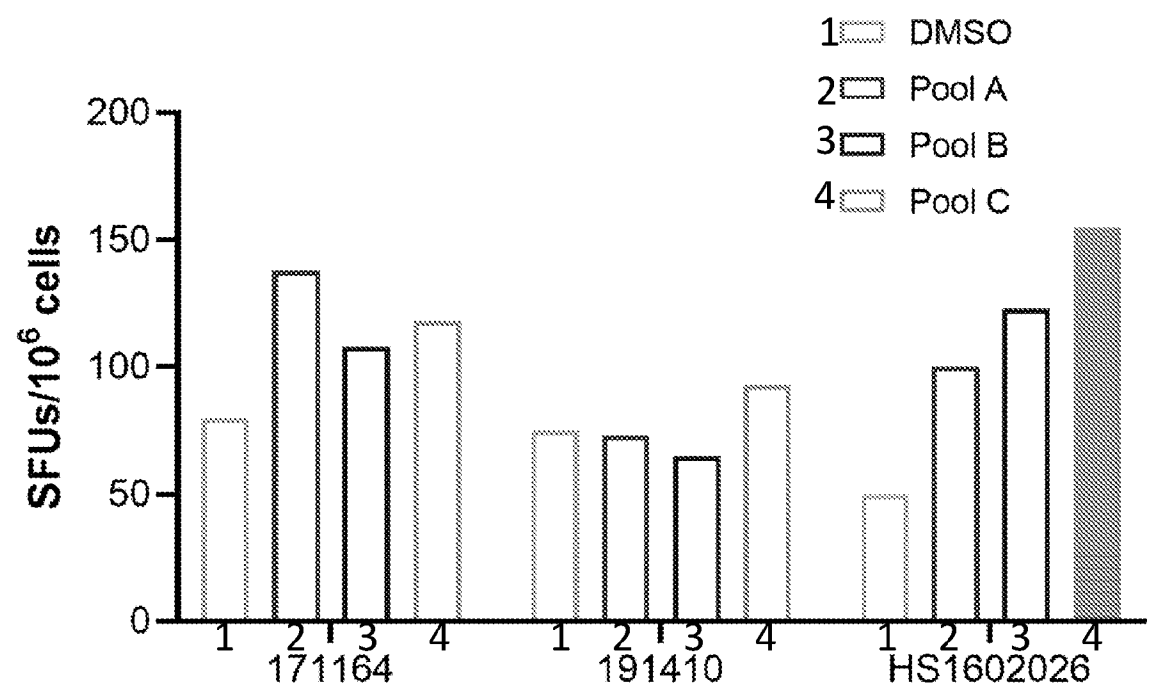


FIG 24G

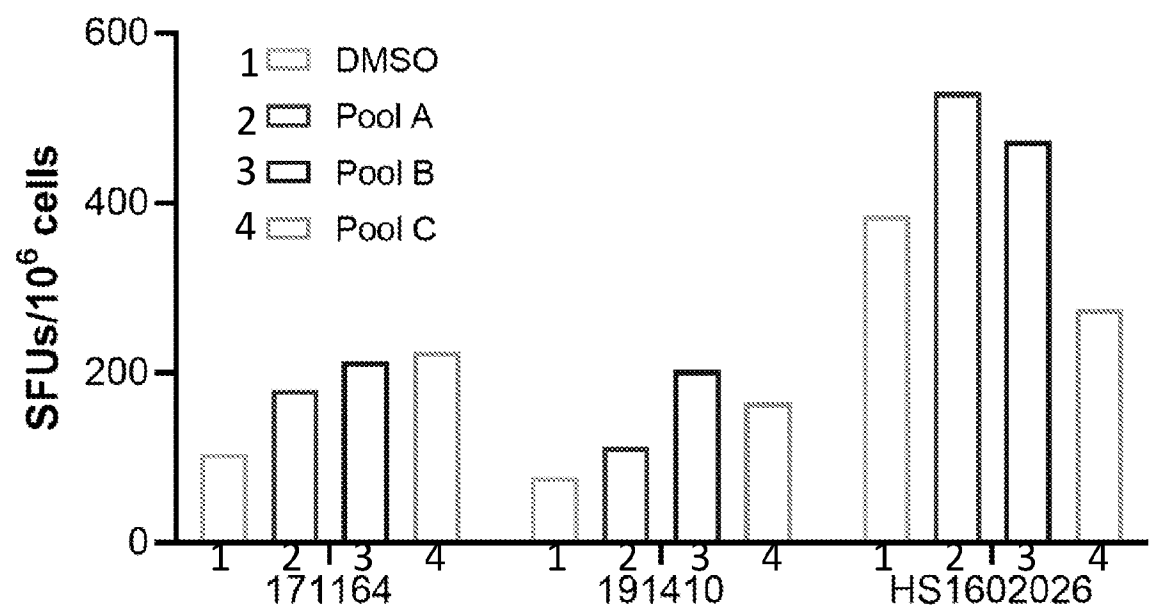


FIG 24H

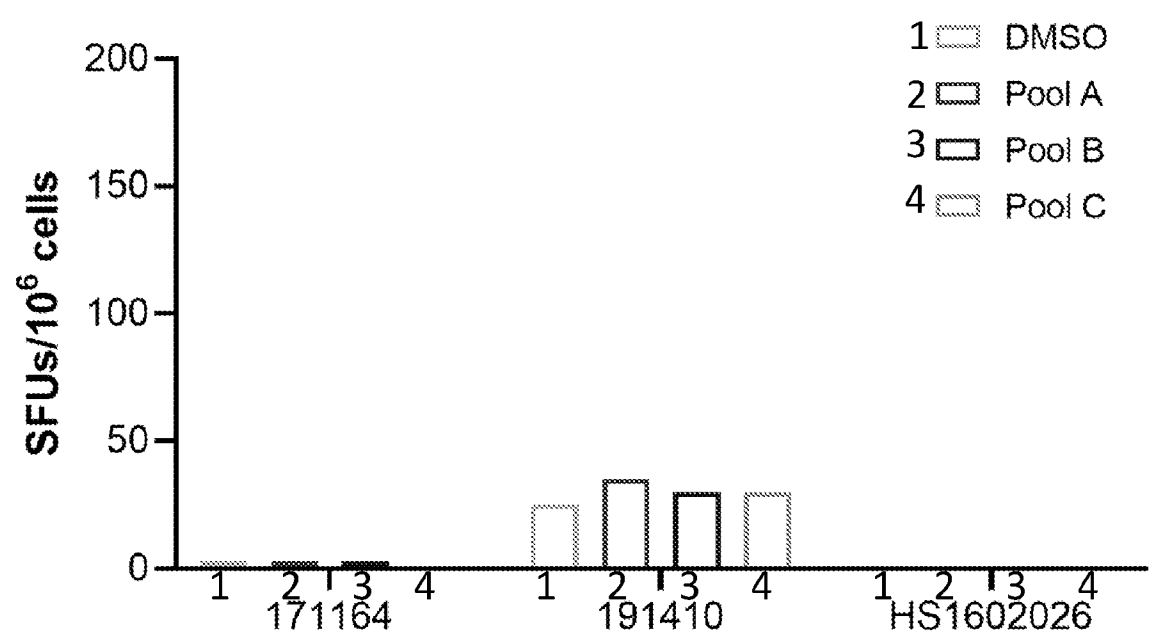


FIG 25A

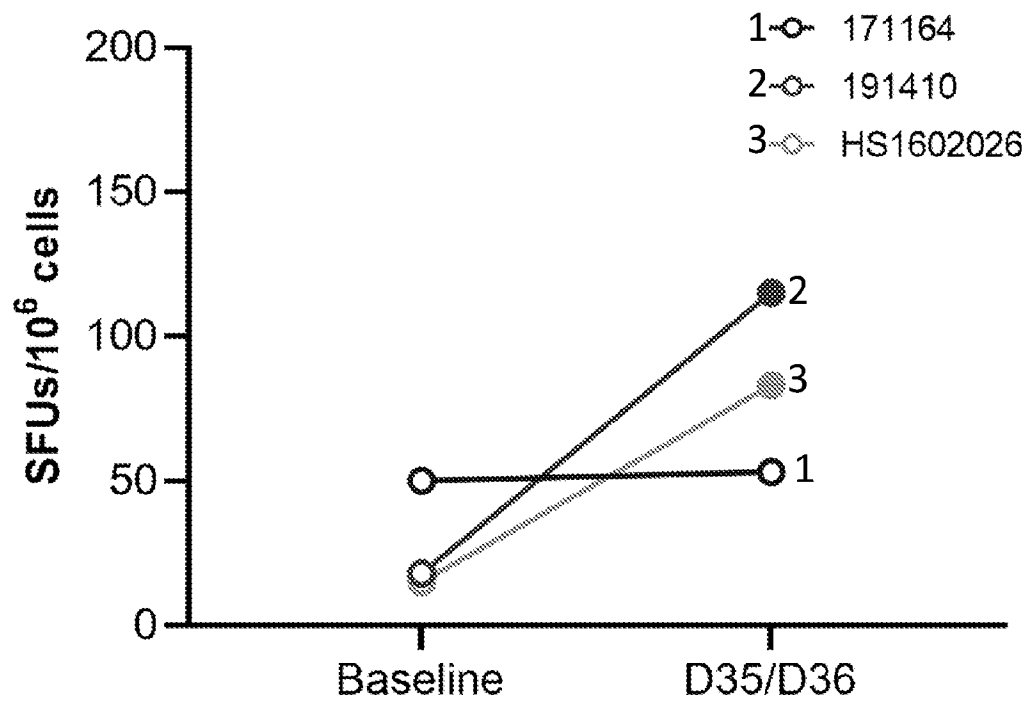


FIG 25B

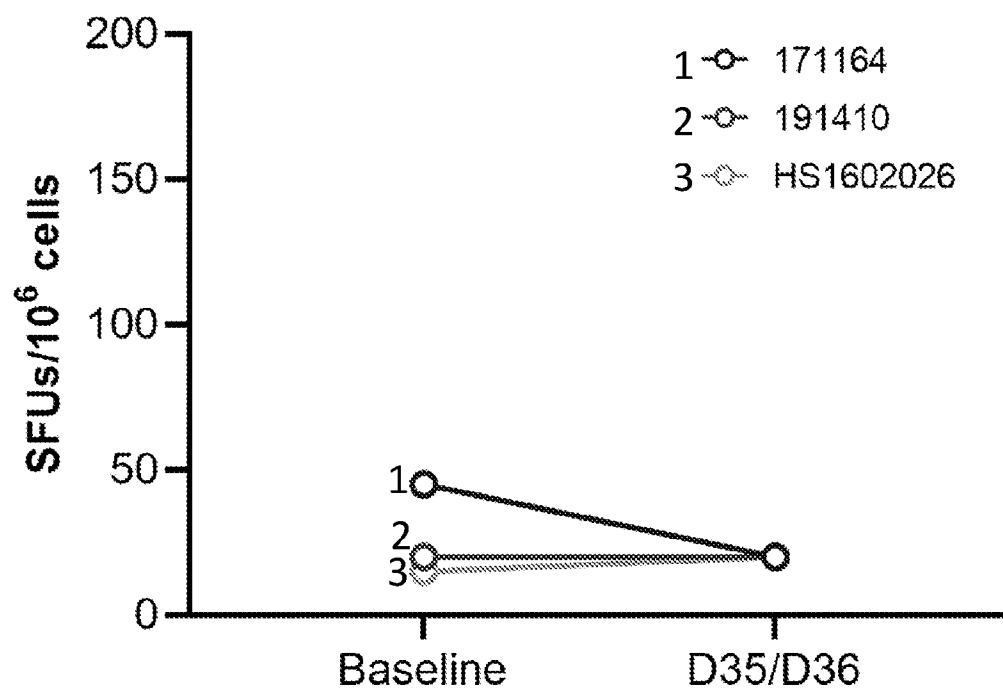


FIG 25C

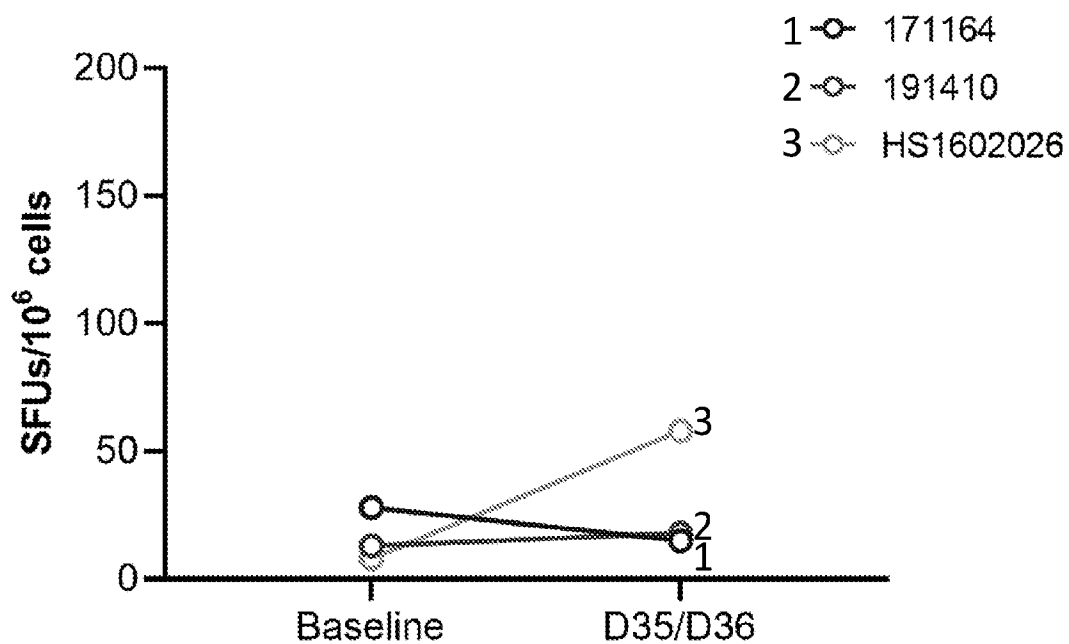


FIG 25D

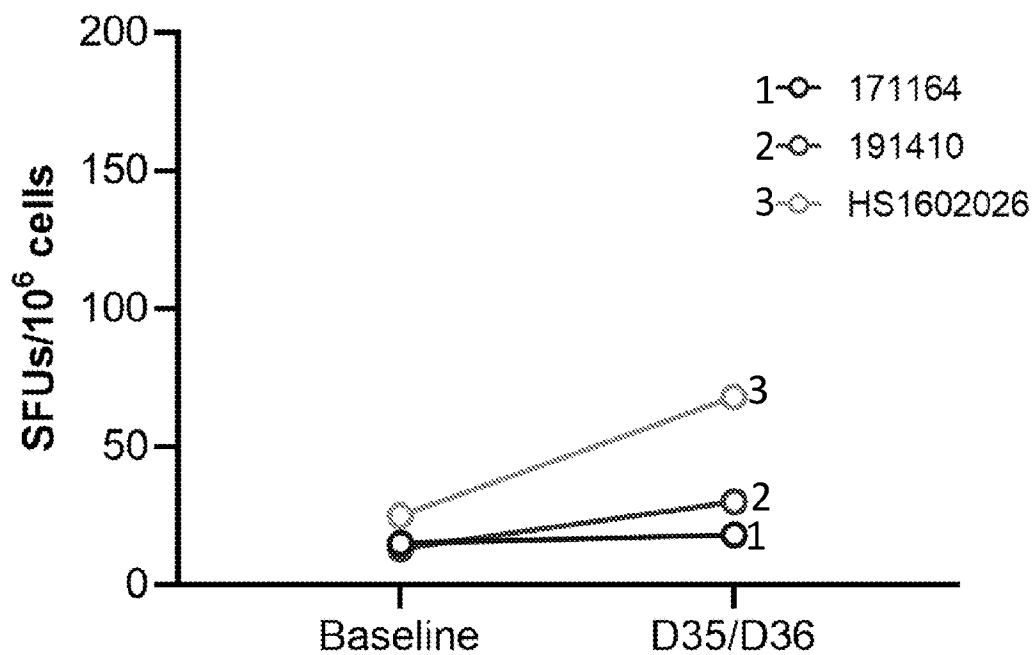


FIG 25E

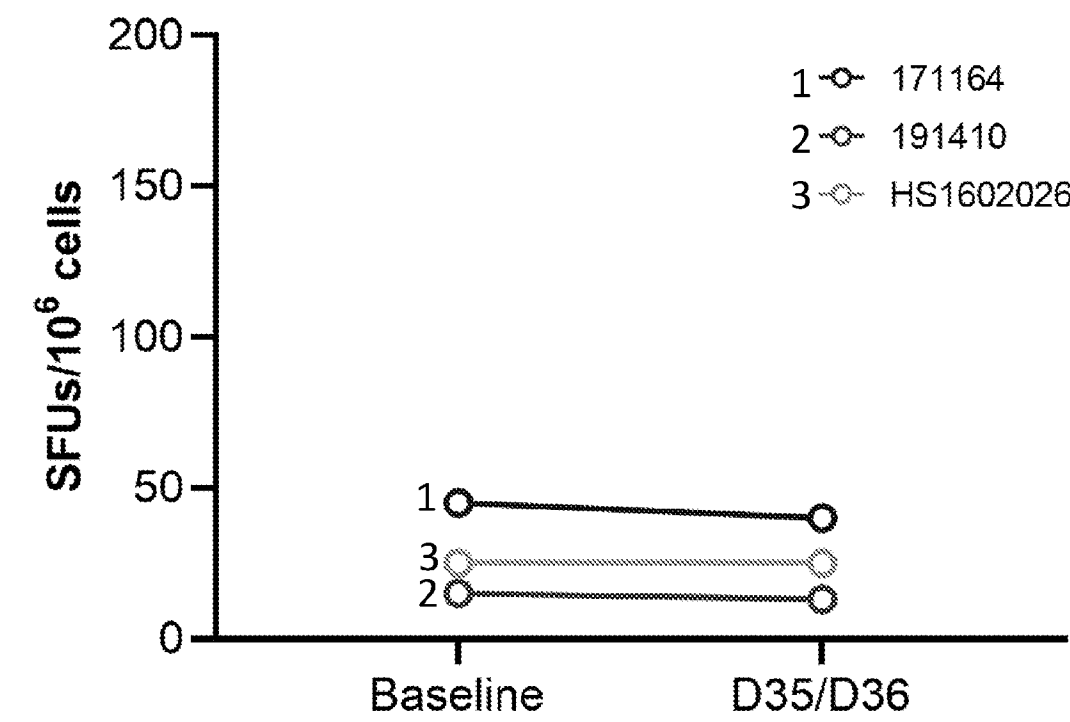


FIG 25F

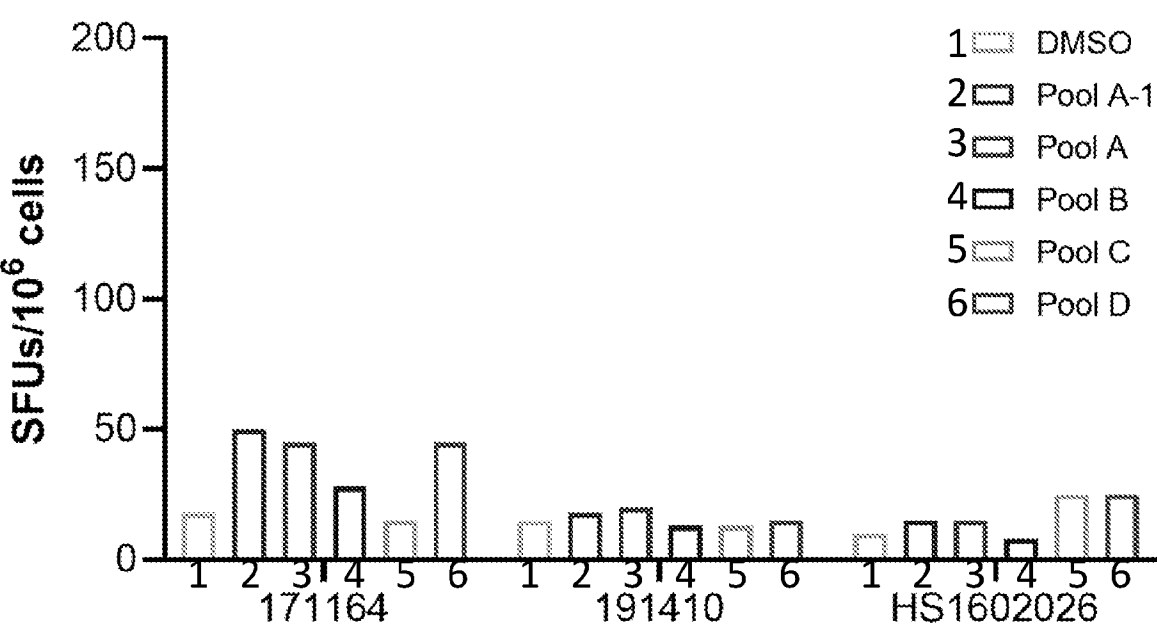


FIG 25G

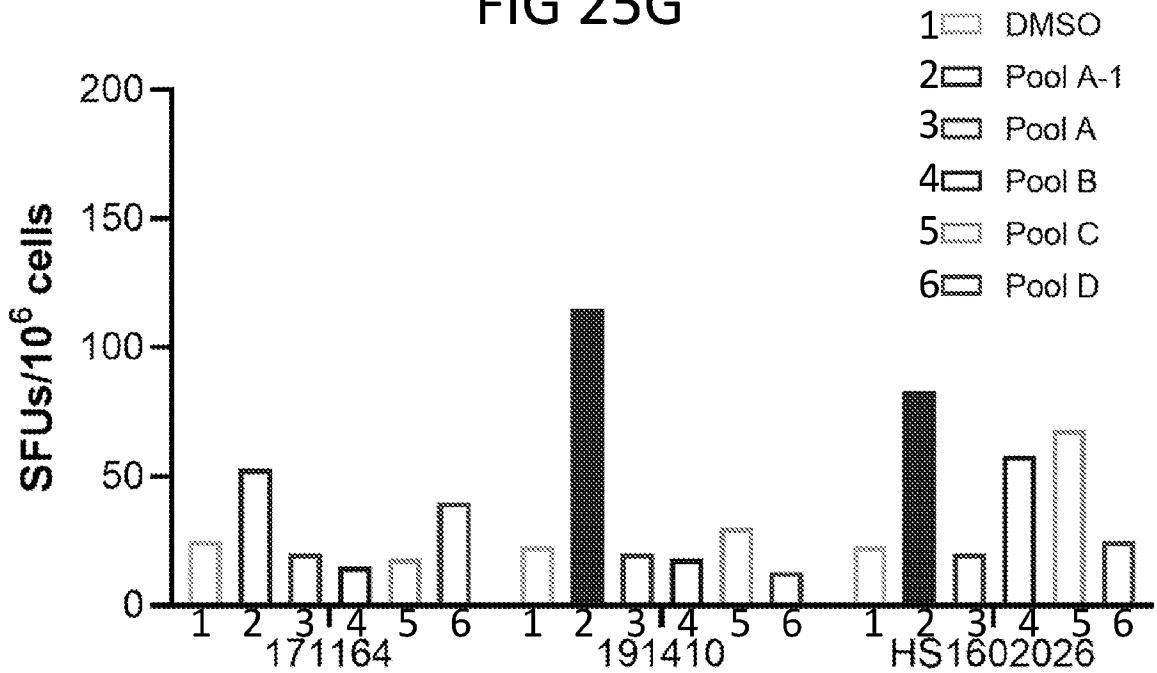


FIG 25H

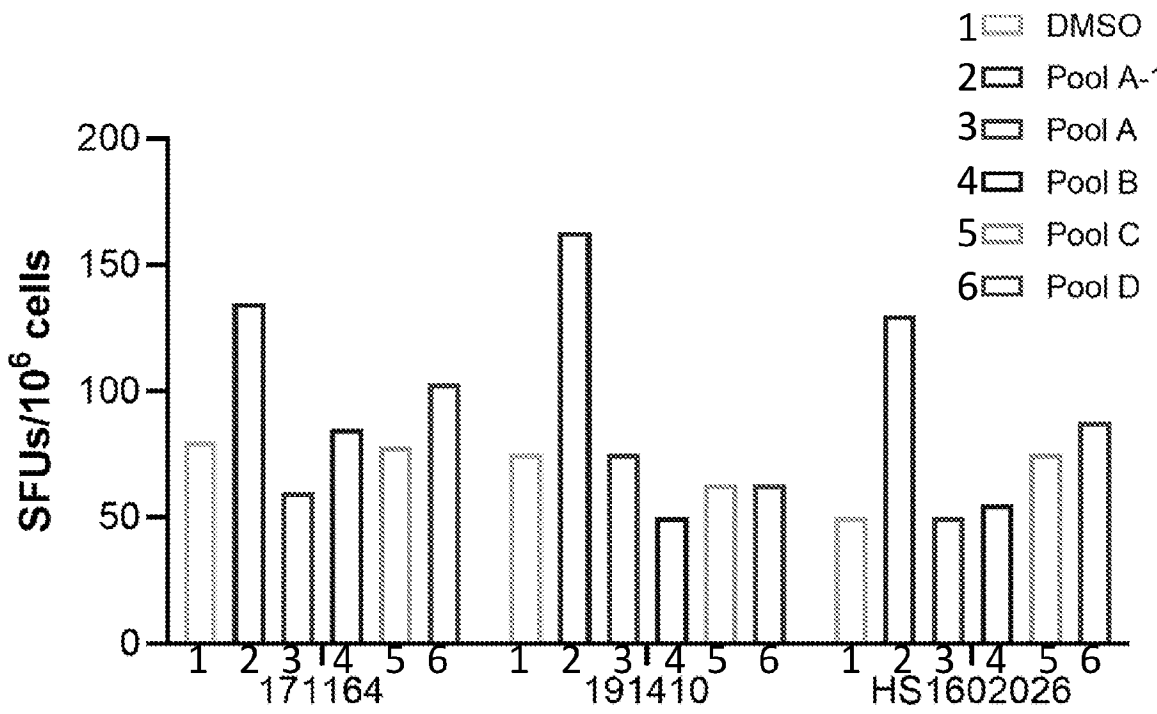


FIG 25I

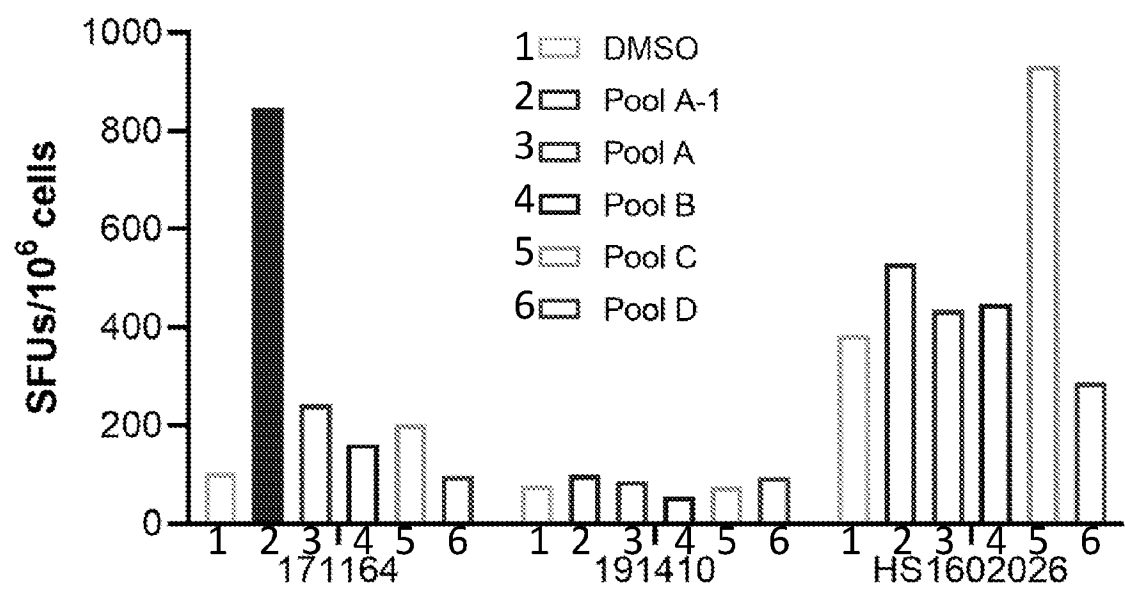


FIG 25J

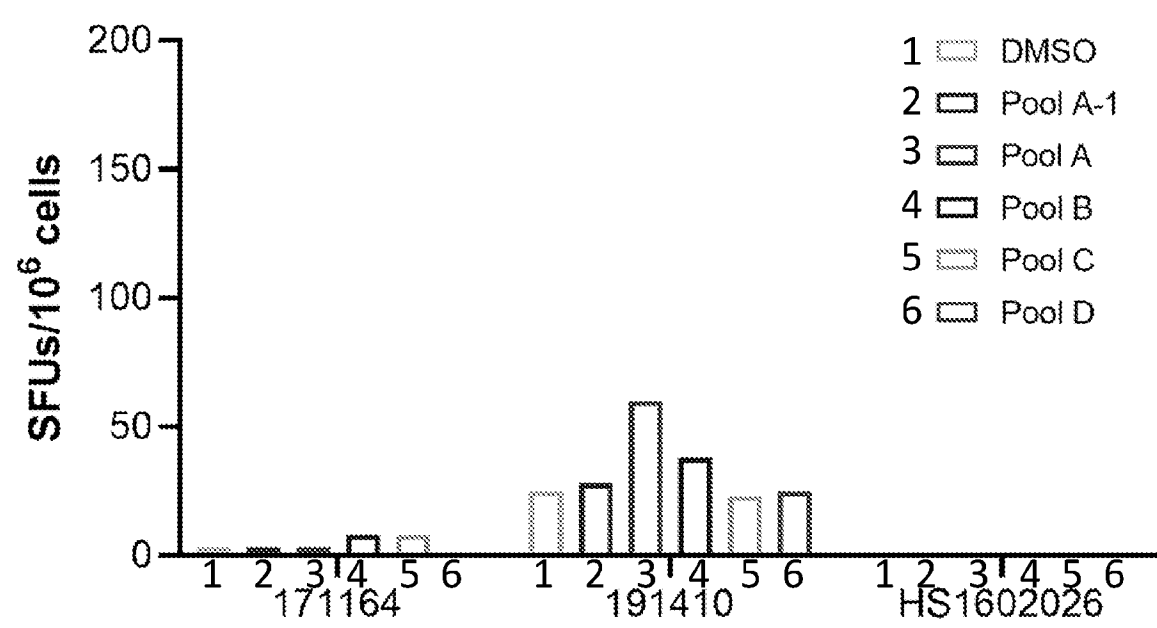


FIG 26A

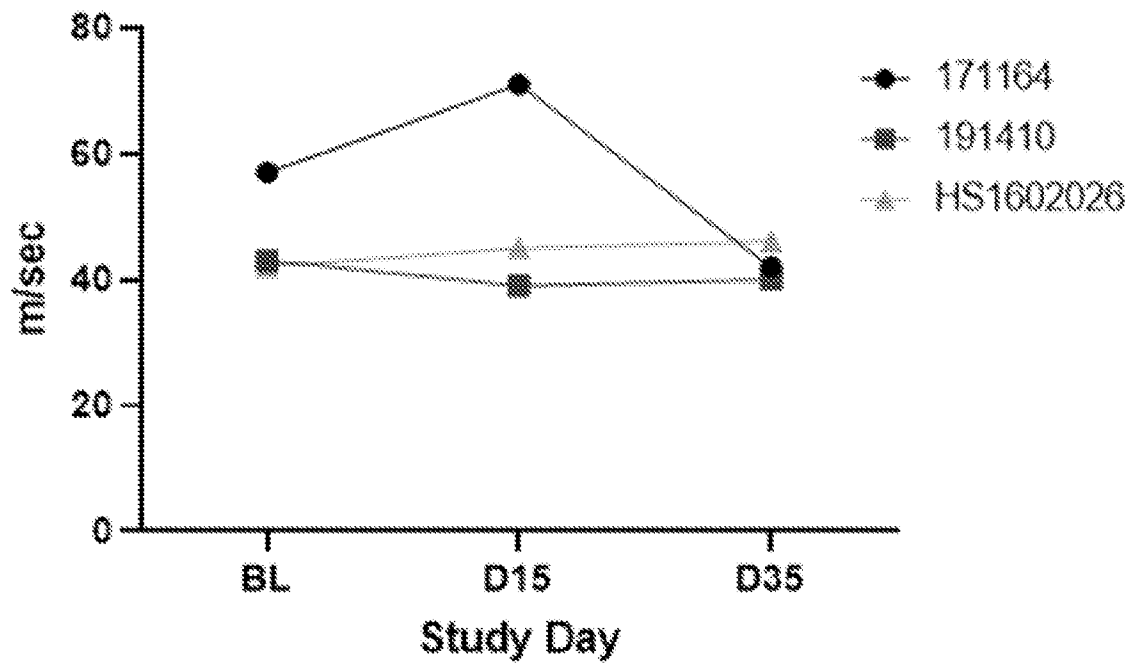


FIG 26B

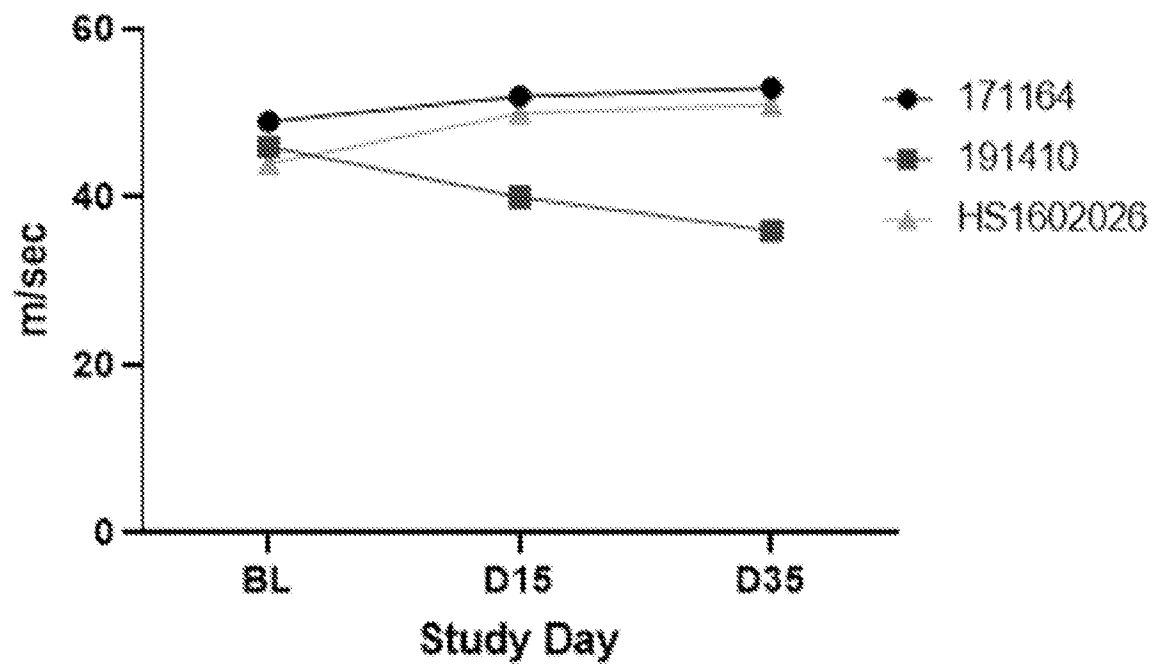


FIG 26C

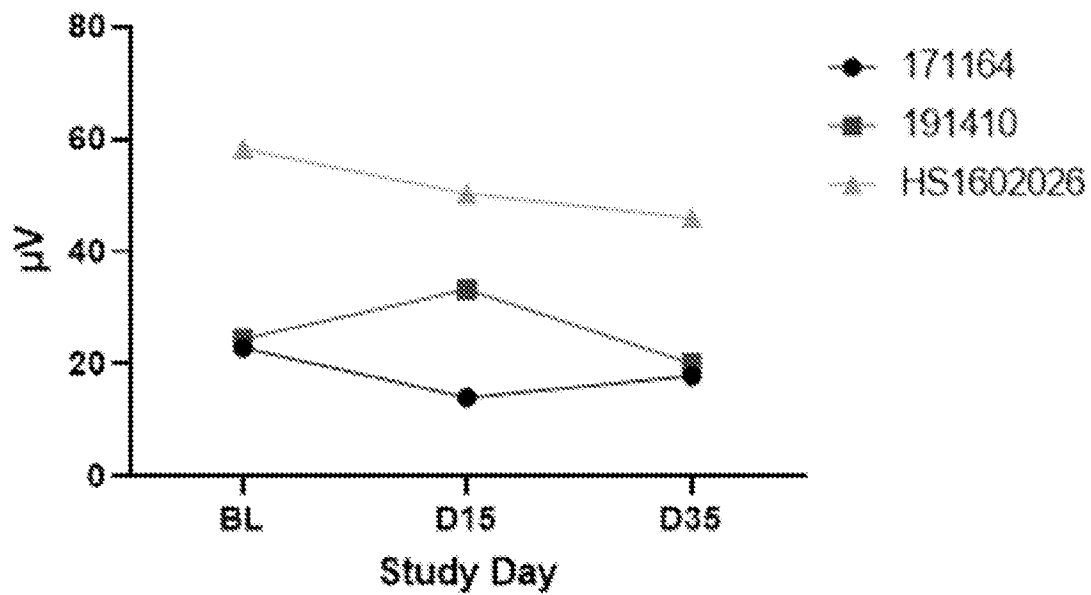


FIG 26D

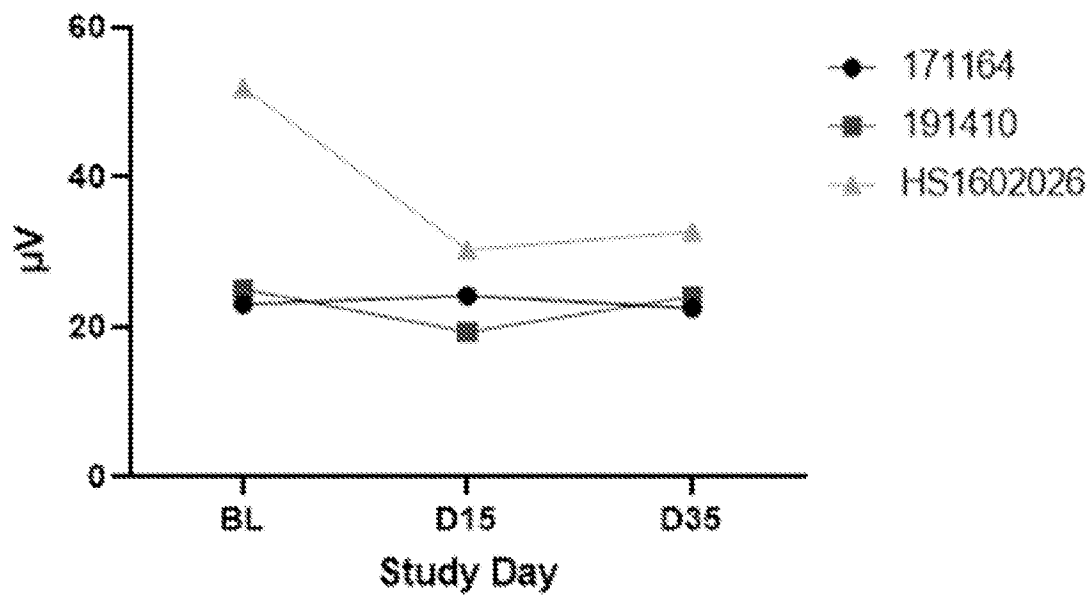


FIG 26E

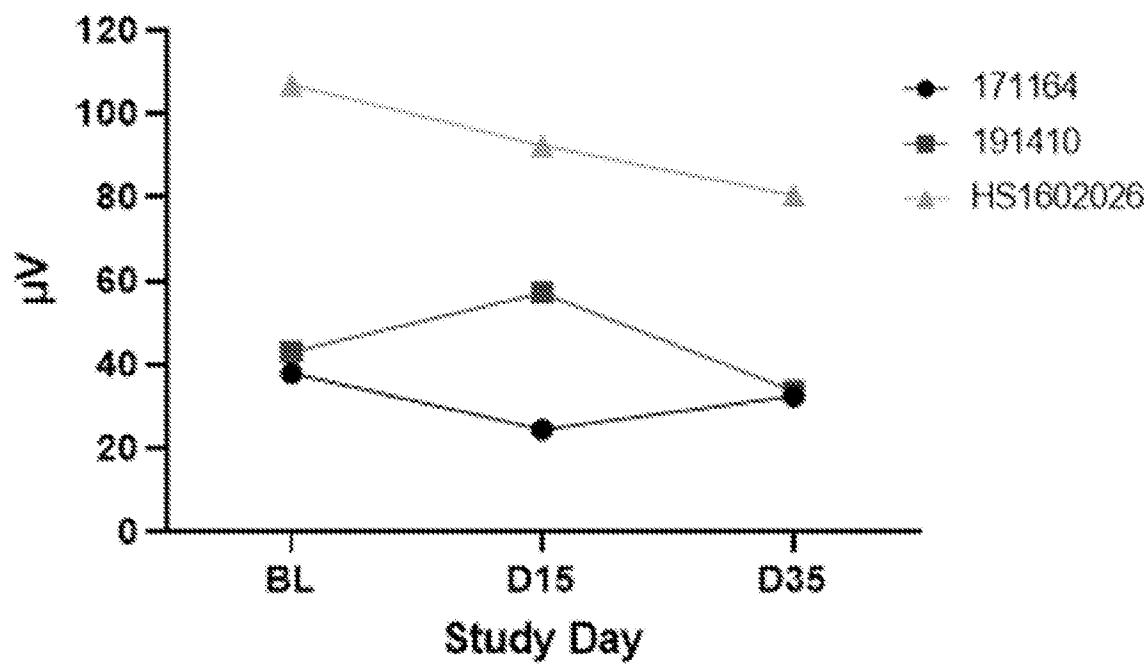


FIG 26F

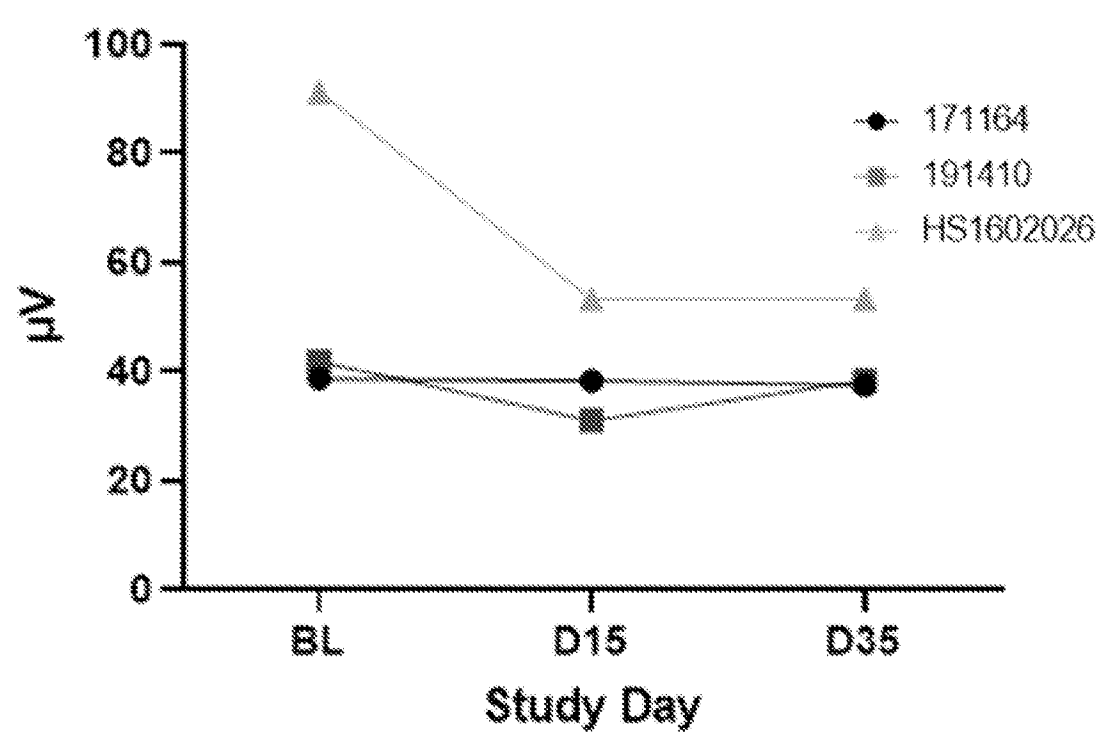


FIG 26G

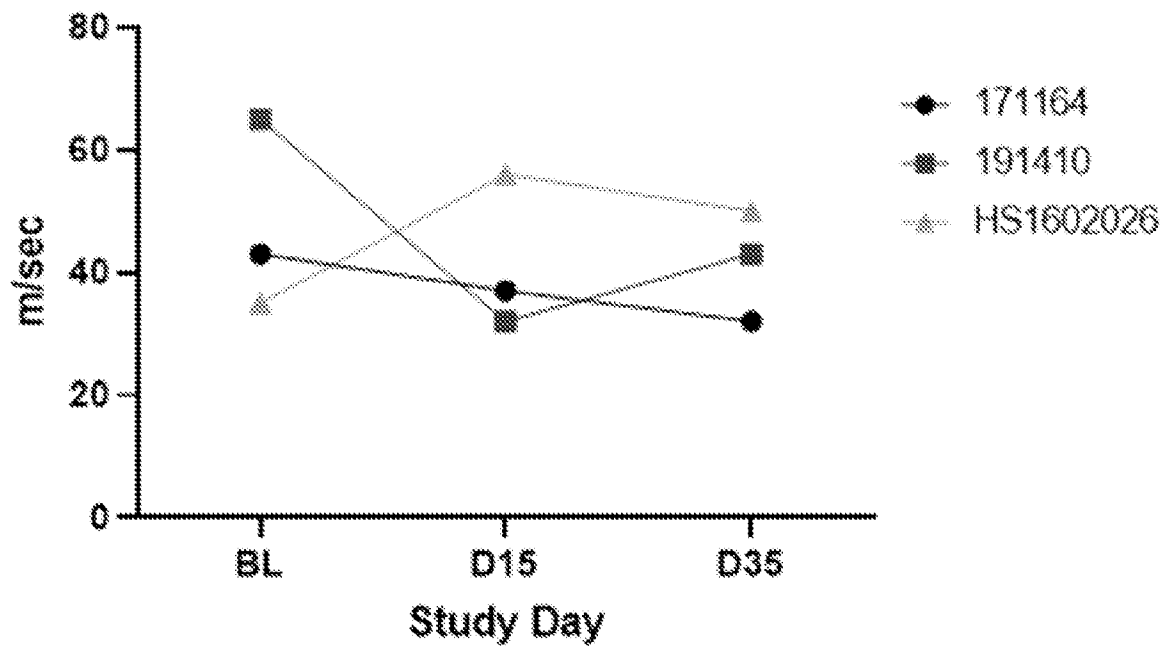


FIG 26H

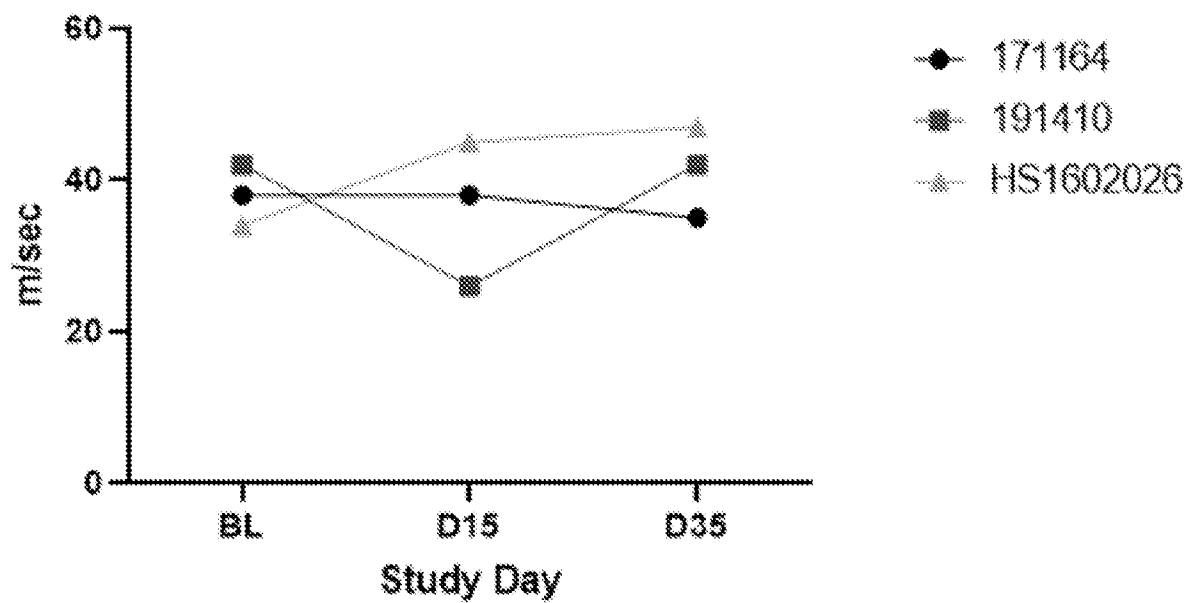


FIG 26I

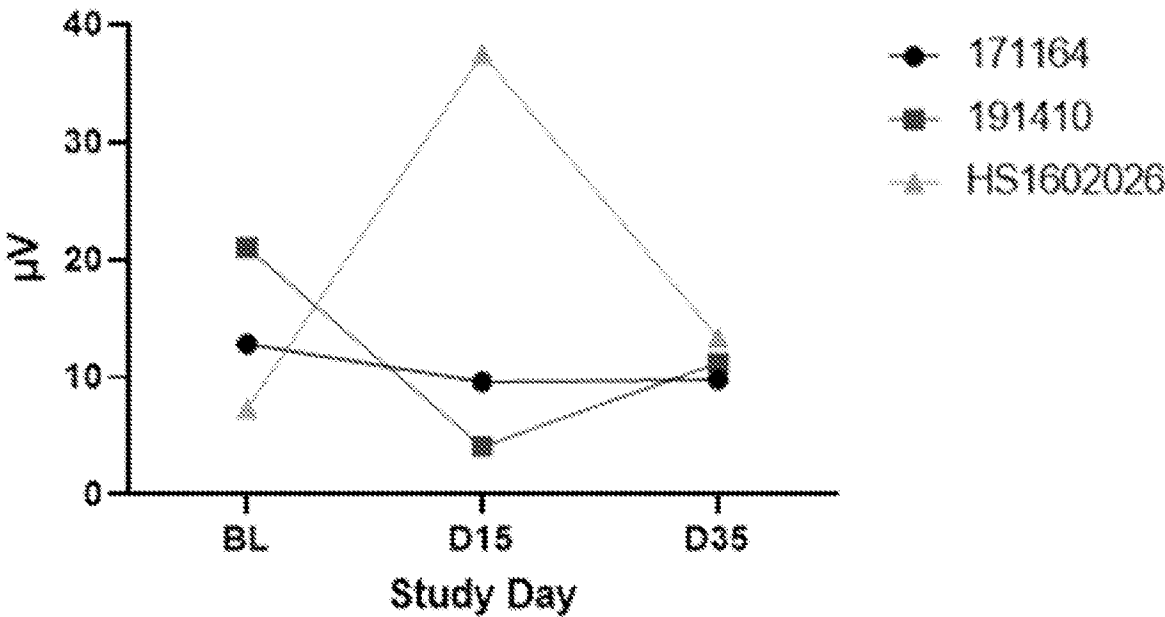


FIG 26J

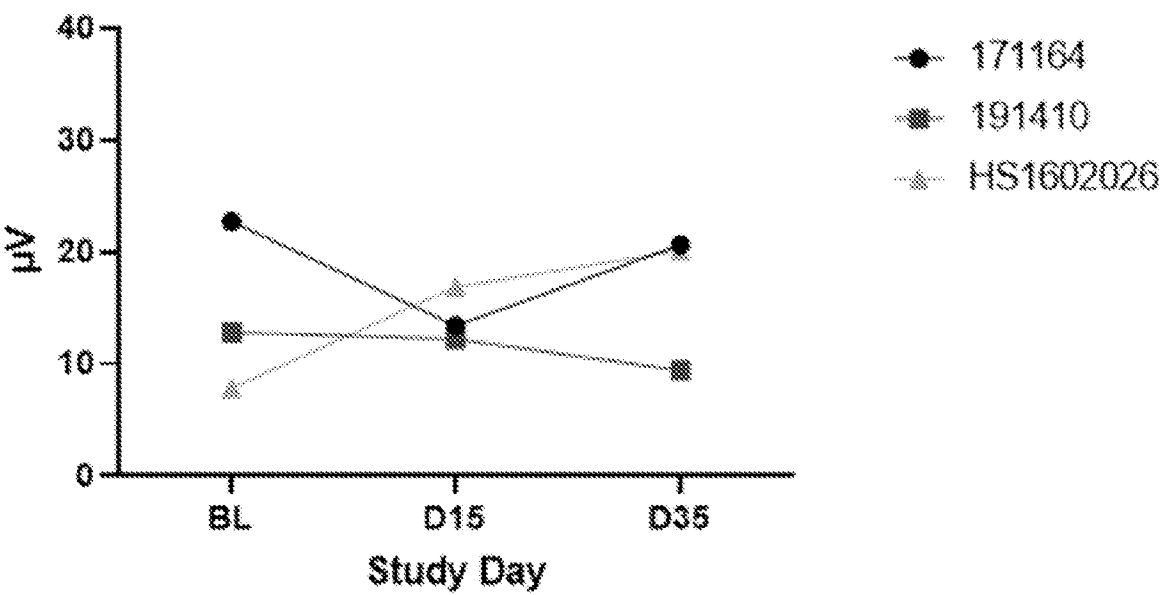


FIG 26K

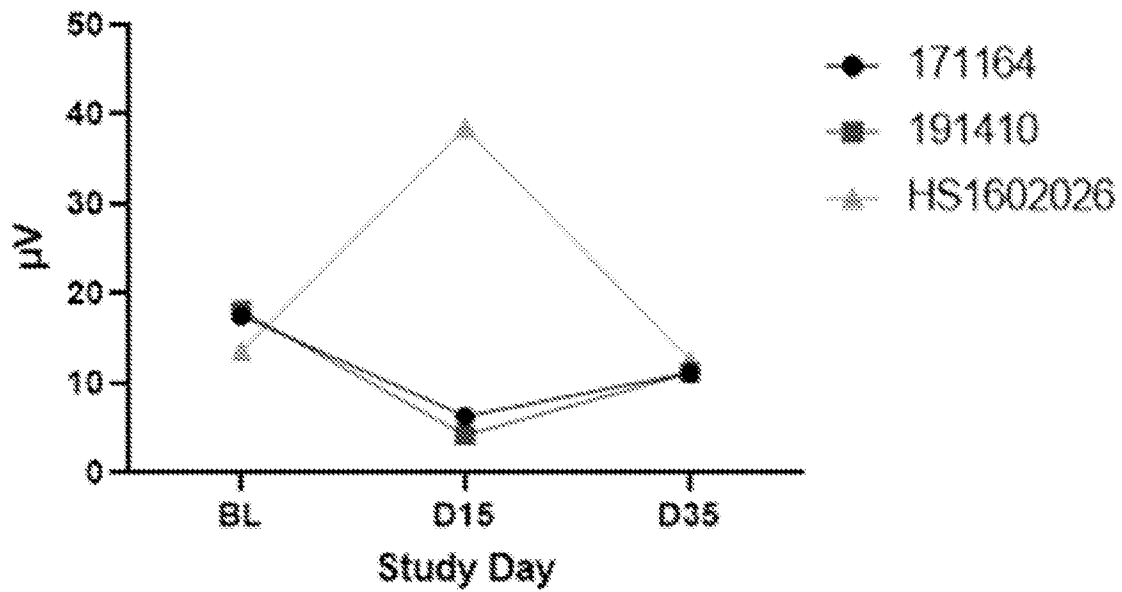
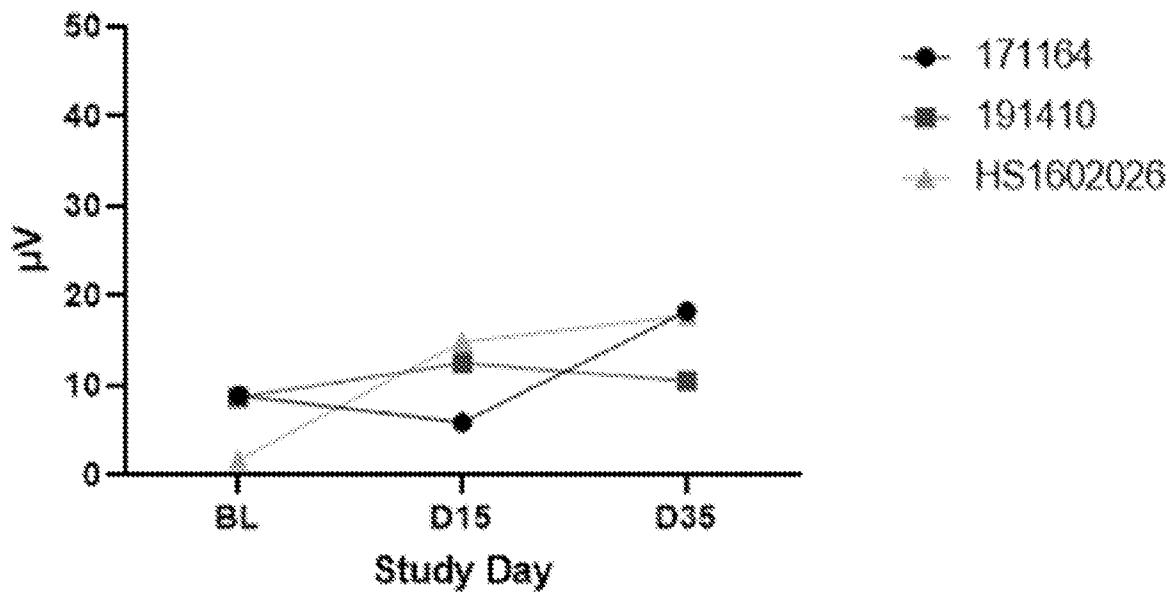


FIG 26L



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US22/79025

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12N 9/12; A61K 38/45; A61K 48/00; C12N 15/52 (2023.01)

ADD.

CPC - INV. C12N 9/1205; A61K 38/45; A61K 48/0066; C12N 15/52

ADD. C12Y 207/11022

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	WO 2021/087282 A1 (AMICUS THERAPEUTICS, INC.) 06 May 2021; paragraphs [0001], [00133]-[00134], [00137], [00148], [00188], [00253], [00266]	1-2, 17 --- 3, 18-19
X --- A	US 2019/0241633 A1 (CUREVAC AG) 08 August 2019; Claim 1	1-2, 17 --- 3, 18-19

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 January 2023 (17.01.2023)

Date of mailing of the international search report

FEB 06 2023

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US22/79025

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US22/79025

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-16, 20-23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.