

INTRATHECAL DELIVERY OF RECOMBINANT ADENO-ASSOCIATED VIRUS
ENCODING METHYL-CPG BINDING PROTEIN 2

[0001] The present application claims the benefit of priority of U. S. Provisional Patent Application No. 62/423,618 filed November 17, 2016.

Incorporation By Reference Of Material Submitted Electronically

[0002] Incorporated by reference in its entirety is a sequence listing in computer-readable form submitted concurrently herewith and identified as follows: ASCII text file named "50215PCT_SeqListing.txt", 24,148 bytes, created November 17, 2017.

Field of the Invention

[0003] The present invention relates to methods and materials for the intrathecal delivery of recombinant Adeno-associated virus 9 (rAAV9) encoding Methyl-CpG binding protein 2 (MECP2). Use of the methods and materials is contemplated, for example, for the treatment of Rett syndrome.

Background

[0004] Rett syndrome is a neurodevelopmental X linked dominant disorder affecting ~1 in 10,000 girls. Hemizygous males usually die of neonatal encephalopathy. Heterozygous females survive into adulthood but exhibit severe symptoms including microcephaly, loss of purposeful hand motions and speech, and motor abnormalities which appear following a period of apparently normal development. Age of onset is around 6-18 months.

[0005] Rett syndrome is classified as Typical (or Classic) Rett or Atypical Rett. Spontaneous mutations gene encoding the transcription factor Methyl-CpG binding protein 2 (MECP2) cause the majority (~90%) of cases in both classifications although Atypical Rett can be caused by mutations in genes other than *MECP2*. The nature of the *MECP2* mutation (e.g. deletion vs. point mutation) and skewed X chromosome inactivation impact disease severity. The MECP2 transcription factor modulates transcription of thousands of genes. Therapeutic efforts have focused on targets downstream of MECP2 including neurotransmitters, growth factors and metabolic pathways. At least nine clinical trials directed toward Rett syndrome have reported positive outcomes across different measures, but the findings have not been independently validated or resulted in new treatments [Katz *et al*, *Trends in Neurosciences*, 39: 100-113 (2016)]. There are currently no approved therapies for Rett Syndrome.

[0006] There are male and female mouse models in which the mice exhibit RTT-like behaviors [Guy *et al.*, *Nature Genetics*, 27: 322-326 (2001); Chen *et al.*, *Nature Genetics* 27: 327-331 (2001); and Katz *et al.*, 5: 733-745 (2012)].

[0007] MECP2 is a 52kD nuclear protein that is expressed in a variety of tissues but is enriched in neurons and has been studied most in the nervous system. There are two isoforms of MECP2 in humans known as MECP2A and B (**Figure 1**) [Weaving *et al.*, *Journal of Medical Genetics*, 42: 1-7 (2005)]. The two isoforms derive from alternatively spliced mRNA transcripts and have different translation start sites. MECP2B includes exons 1, 3 and 4 and is the predominant isoform in the brain. MECP2 reversibly binds to methylated DNA and modulates gene expression [Guy *et al.*, *Annual Review of Cell and Developmental Biology*, 27: 631-652 (2011)] These functions map to the methyl binding domain (MBD) and transcriptional repressor domain (TRD), respectively [Nan & Bird, *Brain & Development*, 23, Suppl 1: S32-37 (2001)]. Originally thought of as a transcriptional repressor, MECP2 can both induce and suppress target gene expression [Chahrour *et al.*, *Science*, 320: 1224-1229 (2008)]. MECP2 is hypothesized to support proper neuronal development and maintenance. In neurons, MECP2 facilitates translation of synaptic activity into gene expression through DNA binding and interaction with different binding partners [Ebert *et al.*, *Nature*, 499: 341-345 (2013) and Lyst *et al.*, *Nature Neuroscience*, 16: 898-902 (2013)]. In astrocytes, MECP2 deficiency is linked to apneic events in mice [Lioy *et al.*, *Nature*, 475: 497-500 (2011)]. MECP2 deficiency can cause reduced brain size, increased neuronal packing density and reduced dendritic complexity [Armstrong *et al.*, *Journal of Neuropathology and Experimental Neurology*, 54: 195-201 (1995)]. Importantly, neuron death is not associated with MECP2 deficiency [Leonard *et al.*, *Nature Reviews, Neurology*, 13: 37-51 (2017)]. MECP2 is also found outside the nervous system though levels vary across tissues (**Figure 2**). A recent study examined the dependence of Rett symptoms in mice on peripheral *Mecp2* expression [Ross *et al.*, *Human Molecular Genetics*, 25: 4389-4404 (2016)]. Peripheral deficiency was associated with hypo-activity, exercise fatigue and bone abnormalities. The majority of RTT-associated behavioral, sensorimotor, gait and autonomic (respiratory and cardiac) phenotypes were absent in mice with peripheral *MECP2* knock out.

[0008] Because *MECP2* is an X-linked gene, one copy of *MECP2* is silenced due to X chromosome inactivation (Xci) in females. On a per cell basis, Xci is believed to be random which leads to *MECP2* chimerism in Rett females. Disease severity is impacted by whether the majority of active X chromosomes contain the intact or mutated *MECP2* gene. This is called skewed Xci. Males do not undergo Xci, therefore MECP2 deficiency is more severe as no cells will have a functional copy of *MECP2*. The nature of the *MECP2* mutation also impacts disease severity. Over 600 different mutations of the *MECP2* gene are described in

the RettBASE database (<http://mecp2.chw.edu.au/>) including deletions, non-sense and point mutations. The most common mutation (~9% of patients) is the T185M allele which affects the methyl binding domain. Other common mutations are shown in **Figure 3** [Leonard, *supra*]. Together these account for over 40% of cases listed in RettBASE. Large scale deletions involving *MECP2* were found in 8-10% of cases [Li *et al.*, *Journal of Human Genetics*, 52: 38-47 (2007) and Hardwick *et al.*, *European Journal of Human Genetics*, 15: 1218-1229 (2007)]. There is genotype-phenotype correlation with R133C, R294X and C-terminal mutations and deletions (downstream of the TRD) causing milder disease. Large deletions and early truncating mutations (R270X, R255X and R168X) are associated with severe Rett syndrome. Table 1 describes consensus Rett diagnostic criteria recently compiled by a group of international Rett clinicians [Neul *et al.*, *Annals of Neurology*, 68: 944-950 (2010)].

<p>Table 1 <u>RTT Diagnostic Criteria 2010</u> -Consider diagnosis when postnatal deceleration of head growth observed.</p>
<p><i>Required for typical or classic RTT</i></p> <ol style="list-style-type: none"> 1. A period of regression followed by recovery or stabilization.* 2. All main criteria and all exclusion criteria 3. Supportive criteria are not required, although often present in typical RTT
<p><i>Required for atypical or variant RTT</i></p> <ol style="list-style-type: none"> 1. A period of regression followed by recovery or stabilization 2. At least 2 out of the 4 main criteria 3. 5 out of 11 supportive criteria
<p>Main Criteria</p> <ol style="list-style-type: none"> 1. Partial or complete loss of acquired purposeful hand skills. 2. Partial or complete loss of acquired spoken language** 3. Gait abnormalities: Impaired (dyspraxic) or absence of ability. 4. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms.
<p>Exclusion Criteria for RTT</p> <ol style="list-style-type: none"> 1. Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems*** 2. Grossly abnormal psychomotor development in first 6 months of life[#]
<p>Supportive Criteria for atypical RTT^{##}</p> <ul style="list-style-type: none"> Breathing disturbances when awake Bruxism when awake Impaired sleep pattern Abnormal muscle tone Peripheral vasomotor disturbances Scoliosis/kyphosis Growth retardation Small cold hands and feet Inappropriate laughing/screaming spells Diminished response to pain Intense eye communication- "eye pointing"
<p>* = Because <i>MECP2</i> mutations are now identified in some individuals prior to any clear evidence of regression, the diagnosis of "possible" RTT should be given to those individuals under 3 years old who have not lost any skills but otherwise have clinical features suggestive of RTT. These individuals should be reassessed every 6-12 months for evidence of regression. If regression manifests, the diagnosis should then be changed to definite RTT. However, if the child does not show any evidence of regression by 5 years, the diagnosis of RTT should be questioned.</p>

**	= Loss of acquired language is based on best acquired spoken language skill, not strictly on the acquisition of distinct words or higher language skills. Thus, an individual who had learned to babble but then loses this ability is considered to have loss of acquired language.
***	= There should be clear evidence (neurological or ophthalmological examination and MRI/CT) that the presumed insult directly resulted in neurological dysfunction.
#	= Grossly abnormal to the point that normal milestones (acquiring head control, swallowing, developing social smile) are not met. Mild generalized hypotonia or other previously reported subtle developmental alterations during the first six months of life is common in RTT and do not constitute an exclusionary criterion.
##	= If an individual has or ever had a clinical feature listed it is counted as a supportive criterion. Many of these features have an age dependency, manifesting and becoming more predominant at certain ages. Therefore, the diagnosis of atypical RTT may be easier for older individuals than for younger. In the case of a younger individual (under 5 years old) who has a period of regression and ≥ 2 main criteria but does not fulfill the requirements of 5/11 supportive criteria, the diagnosis of "probably atypical RTT" may be given. Individuals who fall into this category should be reassessed as they age and the diagnosis revised accordingly.

[0009] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including 145 nucleotide inverted terminal repeat (ITRs). The nucleotide sequence of the AAV serotype 2 (AAV2) genome is presented in Srivastava *et al.*, *J Virol*, 45: 555-564 (1983) as corrected by Ruffing *et al.*, *J Gen Virol*, 75: 3385-3392 (1994). *Cis*-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the ITRs. Three AAV promoters (named p5, p19, and p40 for their relative map locations) drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p19), coupled with the differential splicing of the single AAV intron (at nucleotides 2107 and 2227), result in the production of four rep proteins (rep 78, rep 68, rep 52, and rep 40) from the rep gene. Rep proteins possess multiple enzymatic properties that are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology*, 158: 97-129 (1992).

[0010] AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells, for example, in gene therapy. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects many mammalian cells allowing the possibility of targeting many different tissues *in vivo*. Moreover, AAV transduces slowly dividing and non-dividing cells, and can persist essentially for the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of recombinant genomes feasible.

Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, some or all of the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may be replaced with foreign DNA such as a gene cassette containing a promoter, a DNA of interest and a polyadenylation signal. The rep and cap proteins may be provided *in trans*. Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56° to 65°C for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection. Multiple serotypes of AAV exist and offer varied tissue tropism. Known serotypes include, for example, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13 and AAVrh74. AAV9 is described in U.S. Patent No. 7,198,951 and in Gao *et al.*, *J. Virol.*, 78: 6381-6388 (2004).

[0011] There remains a need in the art for methods and products for delivering MECP2 polynucleotides to, and expressing the polynucleotides in, the central nervous system.

Summary

[0012] The present disclosure provides methods and materials useful for treating Rett syndrome in a patient in need thereof.

[0013] Methods are provided of treating Rett syndrome in a patient comprising the step of intrathecal administration of a recombinant adeno-associated virus 9 (rAAV9) encoding Methyl-CpG binding protein 2 (MECP2) to a patient in need thereof, wherein the rAAV9 comprises a self-complementary genome encoding MECP2B and wherein the sequence of the self-complementary genome is set out in SEQ ID NO: 1. An exemplary rAAV9 provided is the scAAV named AVXS-201.

[0014] Methods are provided which further comprise the step of intrathecal administration of iohexol, iobitridol, iomeprol, iopamidol, iopentol, iopromide, ioversol or ioxilan, or mixtures of two or more thereof, to the patient, and/or which further comprise putting the patient in the Trendelenberg position.

Brief Description of the Drawings

[0015] Figure 1: Diagram of the human MECP2 locus. The picture shows the alternative transcription start sites (arrows), exons (boxes) and splicing pattern of the mature mRNAs. MECP2B is the isoform most abundant in the brain and is encoded by AVXS-201.

[0016] Figure 2: Chart depicts relative MECP2 protein expression levels in various human tissues as detected by immunohistochemistry. Modified from The Human Protein Atlas (www.proteinatlas.org).

[0017] Figure 3: Key functional domains of the MECP2 protein and common mutations found in Rett patients. MBD = methyl-CpG-binding, TRD = Transcriptional Repression Domain, NID = NCOR–SMRT interaction domain (NID), and the nuclear localization signal (NLS)

[0018] Figure 4: Proof of concept with AAV9 mediated restoration of *Mecp2* expression in male Rett mice. A) Cartoon of the recombinant AAV genome. B) Experimental design. C) Kaplan-Meier survival curve showing the increased survival of scAAV9.738.*Mecp2* treated Rett mice compared to animals treated with control vector. D) Vector mediated *Mecp2* restoration improves the behavioral phenotype when measured by the Bird scoring (Box 1).

[0019] Figure 5: scAAV9.738.*Mecp2* treatment of female Rett mice makes physiological levels of *Mecp2* and improves aberrant behavior. A) Experimental design B) Fluorescence intensity measurements from brain sections that were immunolabeled for *Mecp2* from wild type and scAAV9.738.*Mecp2* treated Rett mice. The distribution of intensity measurements is similar between the two groups. C) Bird phenotype scoring shows a reduction in symptoms in animals receiving scAAV9.738.*Mecp2*. D-G) Rotarod, Inverted Grid, Platform and nesting behavioral assessments, respectively, all show improvement in scAAV9.738.*Mecp2* treated versus control treated animals..

[0020] Figure 6: A cartoon depicting the first generation (A) and the revised (B, AVXS-201) recombinant AAV genomes described here. The colors reflect similarities and differences between the constructs. Between scAAV9.738.*Mecp2* and AVXS-201, the promoter was shortened, intervening sequences between key elements were shortened, the murine *Mecp2* alpha cDNA was replaced with a human MECP2B cDNA, and the bovine growth hormone polyadenylation signal was changed to a shorter synthetic element. The overall goal of the changes was to improve packaging efficiency while maintaining physiological expression levels of a clinically relevant MECP2 cDNA.

[0021] Figure 7: Dose response of AVXS-201 in *Mecp2*^{-/-} mice. A) A Kaplan-Meier plot of the different doses used to treat *Mecp2*^{-/-} mice. Median survival for the dose groups are color coded and indicated by the dashed lines. Every cohort that received AVXS-201 had an increase in survival over the control treated null mice. B) The median survival data from each group were plotted to show the dose response curve. The dashed line represents median survival of PBS treated *Mecp2*^{-/-}. These data are consistent with the known effects of MECP2 deficiency and overabundance.

[0022] Figure 8: Bird behavioral scoring for *Mecp2*^{-y} mice treated with AVXS-201. A) Control treated affected mice accumulate deficits with increasing age. Behavioral deficits are attenuated with AVXS-201 treatment regardless of dose. B) The same data as in (A) is re-graphed showing only the control treated and the AVXS-201 1.44x10¹⁰ vg group.

[0023] Figure 9: AVXS-201 treated *Mecp2* null mice recover spontaneous ambulation. Open field analysis shows that AVXS-201 treated null mice traverse (A) greater distances and at increased (B) average velocity compared to control treated nulls. C) Rotarod performance at 3 months of age was also improved with moderate doses of AVXS-201. + = p≤0.001; * = p≤0.05; ⌘ = p≤0.0001 .

[0024] Figure 10: AVXS-201 makes a moderate amount of MECP2 at therapeutic doses. A) Anti-MeCP2 western blots from brain hemisphere homogenates made from male wild type (PBS) or *Mecp2*^{-y} mice. *Mecp2* nulls were either uninjected (KO) or received the indicated doses of AVXS-201. B) Quantification of panel A. The targeted therapeutic dose of 1.44x10¹⁰vg produced 11% of wild type levels of protein.

[0025] Figure 11: AVXS-201 is well tolerated in wild type mice. A) Kaplan-Meier survival plot of male wild type mice that received P1 ICV administration of AVXS-201. B) Bird phenotypic scoring of the treated and wild type mice shows that a wide range of doses are well tolerated. The highest dose (1.13x10¹¹, blue line) produced mild behavioral impairments.

[0026] Figure 12: AVXS-201 treatment in wild type animals does not impair ambulation. Open filed analysis of wild type male mice treated with AVXS-201 show similar (A) distances traveled and (B) average speeds as the control treated wild type mice. C) Rotarod performance was decreased in the wild type animals that received the high dose of AVXS-201. + = p≤0.001, * = p≤0.05

[0027] Figure 13: AVXS-201 produces dose dependent increases in MECP2 protein in wild type brains. A) Anti-MeCP2 western blots show a dose dependent elevation of total MeCP2 protein in various brain regions 3 weeks after P1 ICV injection. (Cb = cerebellum, Med = medulla, Hipp = hippocampus, Ctx = cortex, Mid = midbrain). TG3 indicates samples taken from a severe mouse model of MeCP2 Duplication Syndrome¹. B) Quantification of panel A. High, but not moderate, doses of AVXS-201 double MECP2 expression in select brain regions.

[0028] Figure 14: Intrathecal infusion of AVXS-201 in non-human primates does not impair body weight growth. The three AVXS-201 treated animals are shown in red, and body weight for a control subject is shown in blue.

[0029] Figure 15: Intrathecal infusion of AVXS-201 in non-human primates does not impact hematology values through 18 months post injection. Values for the three AVXS-201 treated animals are shown in red and control subjects are shown in blue.

[0030] Figure 16: Intrathecal infusion of AVXS-201 in non-human primates does not impact serum chemistry through 12-18 months post injection. Liver and electrolyte values are similar between AVXS-201 and control treated subjects. Values for the three AVXS-201 treated animals are shown in red and control subjects are shown in blue.

[0031] Figure 17: Intrathecal infusion of AVXS-201 in non-human primates does not impact serum chemistry through 12-18 months post injection. Cardiac and renal values are similar between AVXS-201 and control treated subjects. Values for the three AVXS-201 treated animals are shown in red and control subjects are shown in blue.

[0032] Figure 18: Similar levels of MeCP2 expression throughout the brains of AVXS-201 treated and control non-human primates. Anti-MeCP2 immunohistochemistry revealed no gross structural abnormalities or obvious differences in MeCP2 expression. OC = Occipital Cortex, TC = Temporal Cortex, LSc = Lumbar spinal cord, Thal = Thalamus, Hipp = Hippocampus, Cb = Cerebellum.

[0033] Figure 19: Western blots of brain regions from control and AVXS-201 injected animals show similar levels of MeCP2. Total MeCP2 levels are shown in green and GAPDH loading controls are shown in red. Quantifications of panels A and B are shown below their respective blots. Dashed lines in the graphs indicate the average normalized values detected across controls. OC = Occipital Cortex, TC = Temporal Cortex, LSc = Lumbar spinal cord, Thal = Thalamus, Hipp = Hippocampus, Cb = Cerebellum. Values are shown as average $\pm$ SEM.

[0034] Figure 20: *In situ* hybridization shows vector derived transcript in all regions examined from brains of AVXS-201 treated animals but not controls. The figure shows probes against Gapdh (red) and vector derived MECP2 mRNA (green) along with nuclear labeling (Dapi, blue). OC = Occipital Cortex, TC = Temporal Cortex, LSc = Lumbar spinal cord, Hipp = Hippocampus, Cb = Cerebellum. Scale bars = 20 μ m.

[0035] Figure 21: *In situ* hybridization shows vector derived transcript in all regions examined from brains of AVXS-201 treated animals but not controls 18 months post injection. The figure shows probes against Gapdh (red) and vector derived MECP2 mRNA (green) along with nuclear labeling (Dapi, blue). OC = Occipital Cortex, TC = Temporal Cortex, CA1 and CA3 = Regions of the Hippocampus, CC = Corpus Callosum, Thal = Thalamus, Cau = Caudate, Put = Putamen, SColl = Superior Colliculus, Med = Medulla, Cb

= Cerebellum, Cerv = cervical spinal cord, Thor = thoracic spinal cord, Lumb = lumbar spinal cord. Scale bars = 20 μ m

Detailed Description

[0036] In one aspect, the invention provides methods for the intrathecal administration (*i.e.*, administration into the space under the arachnoid membrane of the brain or spinal cord) of a polynucleotide encoding MECP2 to a patient comprising administering a rAAV9 with a genome including the polynucleotide. In some embodiments, the rAAV9 genome is a self-complementary genome. In other embodiments, the rAAV9 genome is a single-stranded genome.

[0037] The methods deliver the polynucleotide encoding MECP2 to the brain and spinal cord of the patient (*i.e.*, the central nervous system of the patient). Some target areas of the brain contemplated for delivery include, but are not limited to, the motor cortex and the brain stem. Some target cells of the central nervous system contemplated for delivery include, but are not limited to, nerve cells and glial cells. Examples of glial cells are microglial cells, oligodendrocytes and astrocytes.

[0038] Delivery of polynucleotides encoding MECP2 is indicated, for example, for treatment of Rett Syndrome.

[0039] "Treatment" comprises the step of administering via the intrathecal route an effective dose, or effective multiple doses, of a composition comprising a rAAV of the invention to a subject animal (including a human patient) in need thereof. If the dose is administered prior to development of a disorder/disease, the administration is prophylactic. If the dose is administered after the development of a disorder/disease, the administration is therapeutic. In embodiments of the invention, an effective dose is a dose that alleviates (either eliminates or reduces) at least one symptom associated with the disorder/disease state being treated, improves at least one symptom associated with the disorder/disease state being treated, that slows or prevents progression to a disorder/disease state, that diminishes the extent of disease, that results in remission (partial or total) of disease, and/or that prolongs survival.

[0040] In treatment of Rett syndrome, the methods result in an effect in the subject including, but not limited to, regaining purposeful hand movements, improvement in speech, reduction in apneas, reduction in seizures, reduction in anxiety, increased socialization, increase in IQ, normalization of sleep patterns and/or increased mobility.

[0041] Combination treatments are also contemplated by the invention. Combination as used herein includes both simultaneous treatment or sequential treatment. Combinations of methods of the invention with standard medical treatments for Rett syndrome are specifically contemplated, as are combinations with novel therapies.

[0042] While delivery to an individual in need thereof after birth is contemplated, intrauterine delivery to a fetus is also contemplated.

[0043] In another aspect, the invention provides rAAV genomes. The rAAV genomes comprise one or more AAV ITRs flanking a polynucleotide encoding MECP2. The polynucleotide is operatively linked to transcriptional control DNAs, specifically promoter DNA and polyadenylation signal sequence DNA that are functional in target cells to form a "gene cassette." The gene cassette may include promoters that allow expression specifically within neurons or specifically within glial cells. Examples include neuron specific enolase and glial fibrillary acidic protein promoters. Inducible promoters under the control of an ingested drug may also be used. Examples include, but are not limited to, systems such as the tetracycline (TET on/off) system [Urlinger *et al.*, *Proc. Natl. Acad. Sci. USA* 97(14):7963–7968 (2000)] and the Ecdysone receptor regulatable system [Palli *et al.*, *Eur J. Biochem* 270: 1308-1315 (2003)]. The gene cassette may further include intron sequences to facilitate processing of an RNA transcript when the polynucleotide is expressed in mammalian cells.

[0044] The rAAV genomes of the invention lack AAV rep and cap DNA. AAV DNA in the rAAV genomes (e.g., ITRs) may be from any AAV serotype for which a recombinant virus can be derived including, but not limited to, AAV serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV13 and AAVrh74. The nucleotide sequences of the genomes of the AAV serotypes are known in the art. For example, the AAV9 genome is provided in Gao *et al.*, *J. Virol.*, 78: 6381-6388 (2004).

[0045] In another aspect, the invention provides DNA plasmids comprising rAAV genomes of the invention. The DNA plasmids are transferred to cells permissible for infection with a helper virus of AAV (e.g., adenovirus, E1-deleted adenovirus or herpesvirus) for assembly of the rAAV genome into infectious viral particles with AAV9 capsid proteins. Techniques to produce rAAV particles, in which an AAV genome to be packaged, rep and cap genes, and helper virus functions are provided to a cell, are standard in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (*i.e.*, not in) the rAAV genome, and helper virus functions. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692 which is incorporated by reference herein in its entirety. In

various embodiments, AAV capsid proteins may be modified to enhance delivery of the recombinant vector. Modifications to capsid proteins are generally known in the art. See, for example, US 2005/0053922 and US 2009/0202490, the disclosures of which are incorporated by reference herein in their entirety.

[0046] A method of generating a packaging cell is to create a cell line that stably expresses all the necessary components for AAV particle production. For example, a plasmid (or multiple plasmids) comprising a rAAV genome lacking AAV rep and cap genes, AAV rep and cap genes separate from the rAAV genome, and a selectable marker, such as a neomycin resistance gene, are integrated into the genome of a cell. AAV genomes have been introduced into bacterial plasmids by procedures such as GC tailing (Samulski et al., 1982, Proc. Natl. Acad. Sci. USA, 79:2077-2081), addition of synthetic linkers containing restriction endonuclease cleavage sites (Laughlin et al., 1983, Gene, 23:65-73) or by direct, blunt-end ligation (Senapathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). The packaging cell line is then infected with a helper virus such as adenovirus. The advantages of this method are that the cells are selectable and are suitable for large-scale production of rAAV. Other examples of suitable methods employ adenovirus or baculovirus rather than plasmids to introduce rAAV genomes and/or rep and cap genes into packaging cells.

[0047] General principles of rAAV production are reviewed in, for example, Carter, 1992, Current Opinions in Biotechnology, 1533-539; and Muzyczka, 1992, Curr. Topics in Microbiol. and Immunol., 158:97-129). Various approaches are described in Ratschin et al., Mol. Cell. Biol. 4:2072 (1984); Hermonat et al., Proc. Natl. Acad. Sci. USA, 81:6466 (1984); Tratschin et al., Mol. Cell. Biol. 5:3251 (1985); McLaughlin et al., J. Virol., 62:1963 (1988); and Lebkowski et al., 1988 Mol. Cell. Biol., 7:349 (1988). Samulski et al. (1989, J. Virol., 63:3822-3828); U.S. Patent No. 5,173,414; WO 95/13365 and corresponding U.S. Patent No. 5,658,776 ; WO 95/13392; WO 96/17947; PCT/US98/18600; WO 97/09441 (PCT/US96/14423); WO 97/08298 (PCT/US96/13872); WO 97/21825 (PCT/US96/20777); WO 97/06243 (PCT/FR96/01064); WO 99/11764; Perrin et al. (1995) Vaccine 13:1244-1250; Paul et al. (1993) Human Gene Therapy 4:609-615; Clark et al. (1996) Gene Therapy 3:1124-1132; U.S. Patent. No. 5,786,211; U.S. Patent No. 5,871,982; and U.S. Patent. No. 6,258,595. The foregoing documents are hereby incorporated by reference in their entirety herein, with particular emphasis on those sections of the documents relating to rAAV production.

[0048] The invention thus provides packaging cells that produce replication-deficient, infectious rAAV. In one embodiment packaging cells may be stably transformed cancer cells such as HeLa cells, 293 cells and PerC.6 cells (a cognate 293 line). In another embodiment, packaging cells are cells that are not transformed cancer cells such as low passage 293

3:1124-1132; U.S. Patent. No. 5,786,211; U.S. Patent No. 5,871,982; and U.S. Patent. No. 6,258,595. The foregoing documents are hereby incorporated by reference in their entirety herein, with particular emphasis on those sections of the documents relating to rAAV production.

[0048] The invention thus provides packaging cells that produce replication-deficient, infectious rAAV. In one embodiment packaging cells may be stably transformed cancer cells such as HeLa cells, 293 cells and PerC.6 cells (a cognate 293 line). In another embodiment, packaging cells are cells that are not transformed cancer cells such as low passage 293 cells (human fetal kidney cells transformed with E1 of adenovirus), MRC-5 cells (human fetal fibroblasts), WI-38 cells (human fetal fibroblasts), Vero cells (monkey kidney cells) and FRhL-2 cells (rhesus fetal lung cells).

[0049] Thus, in another aspect, the invention provides rAAV such as rAAV9 (*i.e.*, replication-deficient, infectious encapsidated rAAV9 particles) comprising a rAAV genome of the invention. The genomes of the rAAV lack AAV rep and cap DNA, that is, there is no AAV rep or cap DNA between the ITRs of the genomes. In some embodiments, the rAAV genome is a self-complementary genome. In some embodiments, the rAAV genome is a single-stranded genome.

[0050] rAAV are provided such as a self-complementary AAV9 (scAAV9) named "AVXS-201." Its gene cassette (nucleotides 151-2558 of the AVXS-201 genome set out in SEQ ID NO: 1) has, in sequence, a 549bp promoter fragment (SEQ ID NO: 2) (nucleotides 74085586-74086323 of NC_000086.7 in the reverse orientation) from the mouse *MECP2* gene, an SV40 intron, a human MECP2B cDNA (SEQ ID NO: 3) (CCDS Database #CCDS48193.1), and a synthetic polyadenylation signal sequence (SEQ ID NO: 4). The gene cassette is flanked by a mutant AAV2 inverted terminal repeat (ITR) and a wild type AAV2 inverted terminal repeat that together enable packaging of self-complementary AAV genomes. The genome lacks AAV rep and cap DNA, that is, there is no AAV rep or cap DNA between the ITRs of the genome.

[0051] rAAV are provided such as a scAAV9 named "scAAV9.738.Mecp2." Its gene cassette (nucleotides 198-2890 of the scAAV9.738.Mecp2 genome set out in SEQ ID NO: 5) has, in sequence, a 738bp promoter fragment (SEQ ID NO: 6) (nucleotides 74085586-74086323 of NC_000086.7 in the reverse orientation) from the mouse *MECP2* gene, an SV40 intron, a mouse MECP2 α cDNA (SEQ ID NO: 7) (CCDS Database #CCDS41016.1), and a polyadenylation signal sequence from the bovine growth hormone gene. The gene cassette is flanked by a mutant AAV2 inverted terminal repeat (ITR) and a wild type AAV2 inverted terminal repeat that together enable packaging of self-complementary AAV

[0053] In some embodiments, the MECP2 polypeptide encoded by a rAAV9 of the invention may be a variant MECP2 polypeptide. A variant polypeptide retains MECP2 activity and has an amino acid sequence at least about 60, 70, 80, 85, 90, 95, 97, 98, 99 or 99.5% identical to the amino acid sequence of the MECP2 polypeptide encoded by the MECP2 α cDNA in scAAV9.738.Mecp2 or the MECP2B cDNA in AVXS-201.

[0054] The rAAV may be purified by methods standard in the art such as by column chromatography or cesium chloride gradients. Methods for purifying rAAV vectors from helper virus are known in the art and include methods disclosed in, for example, Clark *et al.*, *Hum. Gene Ther.*, 10(6): 1031-1039 (1999); Schenpp and Clark, *Methods Mol. Med.*, 69: 427-443 (2002); U.S. Patent No. 6,566,118 and WO 98/09657.

[0055] In another aspect, the invention contemplates compositions comprising a rAAV, such as a rAAV9, encoding a MECP2 polypeptide.

[0056] Compositions of the invention comprise rAAV in a pharmaceutically acceptable carrier. The compositions may also comprise other ingredients such as diluents and adjuvants. Acceptable carriers, diluents and adjuvants are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, pluronics or polyethylene glycol (PEG).

[0057] Sterile injectable solutions are prepared by incorporating rAAV in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique that yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

[0058] Titers and dosages of rAAV to be administered in methods of the invention will vary depending, for example, on the particular rAAV, the mode of administration, the treatment goal, the individual, the timing of administration, and the cell type(s) being

targeted, and may be determined by methods standard in the art. Titers of rAAV may range from about 1×10^6 , about 1×10^7 , about 1×10^8 , about 1×10^9 , about 1×10^{10} , about 1×10^{11} , about 1×10^{12} , about 1×10^{13} to about 1×10^{14} or more DNase resistant particles (DRP) per ml.

Dosages may also be expressed in units of viral genomes (vg). These dosages of rAAV may range from about 1×10^9 vg or more, about 1×10^{10} vg or more, about 1×10^{11} vg or more, about 1×10^{12} vg or more, about 6×10^{12} or more, about 1×10^{13} vg or more, about 1.3×10^{13} vg or more, about 1.4×10^{13} vg or more, about 2×10^{13} vg or more, about 3×10^{13} vg or more, about 6×10^{13} vg or more, about 1×10^{14} vg or more, about 3×10^{14} or more, about 6×10^{14} or more, about 1×10^{15} vg or more, about 3×10^{15} or more, about 6×10^{15} or more, about 1×10^{16} or more, about 3×10^{16} or more, or about 6×10^{16} or more. For a neonate, the dosages of rAAV may range from about 1×10^9 vg or more, about 1×10^{10} vg or more, about 1×10^{11} vg or more, about 1×10^{12} vg or more, about 6×10^{12} or more, about 1×10^{13} vg or more, about 1.3×10^{13} vg or more, about 1.4×10^{13} vg or more, about 2×10^{13} vg or more, about 3×10^{13} vg or more, about 6×10^{13} vg or more, about 1×10^{14} vg or more, about 3×10^{14} or more, about 6×10^{14} or more, about 1×10^{15} vg or more, about 3×10^{15} or more, about 6×10^{15} or more, about 1×10^{16} or more, about 3×10^{16} or more, or about 6×10^{16} or more.

[0059] The methods of the invention result in the transduction of target cells (including, but not limited to, nerve or glial cells). The term “transduction” is used to refer to the administration/delivery of a polynucleotide to a target cell either *in vivo* or *in vitro*, via a replication-deficient infectious rAAV of the invention resulting in expression of a functional MECP2 polypeptide by the recipient cell.

[0060] Transduction of cells using rAAV of the invention results in sustained expression of the MECP2 polypeptide encoded by the rAAV. In some embodiments, the target expression level is contemplated to be about 75% to about 125% of the normal (or wild type) physiological expression level in a subject who does not have Rett syndrome. The target expression level may be about 75%, about 80%, about 85%, about 90%, about 95%, about 100%, about 105%, about 110%, about 115%, about 120% or about 125% of the normal expression level.

[0061] In some embodiments of treatment methods of the invention, a non-ionic, low-osmolar contrast agent is also administered to the patient. Such contrast agents include, but are not limited to, iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromide, ioversol, ioxilan, and mixtures of two or more of the contrast agents. In some embodiments, the treatment methods thus further comprise administration of iohexol to the patient. The non-ionic, low-osmolar contrast agent is contemplated to increase transduction of target cells in the central nervous system of the patient. It is contemplated that the transduction of cells is increased when a rAAV of the disclosure is used in combination with a contrast agent as

described herein relative to the transduction of cells when a rAAV of the disclosure is used alone. In various embodiments, the transduction of cells is increased by at least about 1%, or at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 120%, at least about 150%, at least about 180%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 450%, at least about 500% or more when a vector of the disclosure is used in combination with a contrast agent as described herein, relative to the transduction of a vector of the disclosure when not used in combination with a contrast agent. In further embodiments, the transduction of cells is increased by about 10% to about 50%, or by about 10% to about 100%, or by about 5% to about 10%, or by about 5% to about 50%, or by about 1% to about 500%, or by about 10% to about 200%, or by about 10% to about 300%, or by about 10% to about 400%, or by about 100% to about 500%, or by about 150% to about 300%, or by about 200% to about 500% when a vector of the disclosure is used in combination with a contrast agent as described herein, relative to the transduction of a vector of the disclosure when not used in combination with a contrast agent.

[0062] In some embodiments, it is contemplated that the transduction of cells is increased when the patient is put in the Trendelenberg position (head down position). In some embodiments, for example, the patients is tilted in the head down position at about 1 degree to about 30 degrees, about 15 to about 30 degrees, about 30 to about 60 degrees, about 60 to about 90 degrees, or about 90 up to about 180 degrees) during or after intrathecal vector infusion. In various embodiments, the transduction of cells is increased by at least about 1%, or at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 120%, at least about 150%, at least about 180%, at least about 200%, at least about 250%, at least about 300%, at least about 350%, at least about 400%, at least about 450%, at least about 500% or more when a the Trendelenberg position is used as described herein, relative to when the Trendelenberg position is not used.

[0063] In further embodiments, the transduction of cells is increased by about 10% to about 50%, or by about 10% to about 100%, or by about 5% to about 10%, or by about 5% to about 50%, or by about 1% to about 500%, or by about 10% to about 200%, or by about 10% to about 300%, or by about 10% to about 400%, or by about 100% to about 500%, or by about 150% to about 300%, or by about 200% to about 500% when a vector of the disclosure is used in combination with a contrast agent and the Trendelenberg position as

described herein, relative to the transduction of a vector of the disclosure when not used in combination with a contrast agent and Trendelenberg position.

[0064] The disclosure also provides treatment method embodiments wherein the intrathecal administration of a vector of the disclosure and a contrast agent to the central nervous system of a patient in need thereof results in an increase in survival of the patient relative to survival of the patient when a vector of the disclosure is administered in the absence of the contrast agent. In various embodiments, administration of a vector of the disclosure and a contrast agent to the central nervous system of a patient in need thereof results in an increase of survival of the patient of at least about 1%, at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200% or more relative to survival of the patient when a vector of the disclosure is administered in the absence of the contrast agent.

[0065] The disclosure also provides treatment method embodiments wherein the intrathecal administration of a vector of the disclosure and a contrast agent to the central nervous system of a patient in need thereof who is put in the Trendelenberg position results in a further increase in survival of the patient relative to survival of the patient when a vector of the disclosure is administered in the absence of the contrast agent and the Trendelenberg position. In various embodiments, administration of a vector of the disclosure and a contrast agent to the central nervous system of a patient in need thereof put in the Trendelenberg position results in an increase of survival of the patient of at least about 1%, at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200% or more relative to survival of the patient when a vector of the disclosure is administered in the absence of the contrast agent and the Trendelenberg position.

Examples

[0066] The present invention is illustrated by the following.

- Proof of concept studies in female and male Rett mouse models show therapeutic efficacy following intravenous injection of scAAV9.738.Mecp2. (Example 1)
- A second generation gene therapy vector, AVXS-201, shows extension of survival over a wide range of doses following intracerebroventricular (ICV) treatment of *Mecp2*^{-y} mice. The maximal increase in median survival was 477% following AVXS-201 treatment. (Example 2)

- Male *Mecp2*^{-/-} mice treated with AVXS-201 show a durable improvement in behavior as measured by a composite rating developed for Rett mice. (Example 3)
- Phenotypic benefit in *Mecp2*^{-/-} mice treated with AVXS-201 is obtained with moderate levels of protein expression. (Example 4)
- Treatment of wild type mice with AVXS-201 was well tolerated across all doses tested with consistent changes in behavioral scoring only noted in the high dose group. (Example 5 and Example 6)
- Intrathecal dosing in non-human primates indicates AVXS-201 is safe and well tolerated through 18 months post injection. (Example 7)
- AVXS-201 expresses transgene at physiological levels broadly in non-human primate brain and spinal cord following a one-time intrathecal injection. (Example 8)

Example 1

Gene therapy for Rett Syndrome Proof of Concept Studies in Female Rett Mice

[0067] As proof of concept, symptomatic male and female Rett mice were intravenously treated with scAAV9.738.*Mecp2* [Garg *et al.*, *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 33: 13612-13620 (2013)]. The recombinant viral genome of scAAV9.738.*Mecp2* (SEQ ID NO: 5) includes a 738bp promoter fragment from the mouse *Mecp2* gene [Adachi *et al.*, *Human Molecular Genetics*, 14: 3709-3722 (2005)] driving expression of a mouse *Mecp2* α cDNA (CCDS Database # CCDS41016.1) and a bovine growth hormone polyadenylation signal. The gene cassette (nucleotides 198-2890 of SEQ ID NO: 5) is flanked by a mutant AAV2 inverted terminal repeat (ITR) and a wild type AAV2 ITR that enable packaging of self-complementary AAV genomes.

[0068] Self-complementary AAV9 (scAAV9) was produced by transient transfection procedures using a double-stranded AAV2-ITR-based vector, with a plasmid encoding Rep2Cap9 sequence as previously described [Gao *et al.*, *J. Virol.*, 78: 6381–6388 (2004)] along with an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells. Virus was produced in three separate batches for the experiments and purified by two cesium chloride density gradient purification steps, dialyzed against PBS and formulated with 0.001% Pluronic-F68 to prevent virus aggregation and stored at 4°C. All vector preparations were titrated by quantitative PCR using Taq-Man technology. Purity of vectors was assessed by 4–12% sodium dodecyl sulfate-acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA).

[0069] Male mice with an *Mecp2* null allele were treated intravenously with 3x10¹²vg of either scAAV9.738.*Mecp2* or an scAAV9 control vector between 4-6 weeks of age. The animals were followed for survival and assessed weekly for a phenotypic score [Guy *et al.*, *Science*, 315: 1143-1147 (2007)].

Components of the phenotypic scoring from *Guy et al. 2007*.

A. Mobility: The mouse is observed when placed on bench, then when handled gently. 0 = as wild-type. 1 = reduced movement when compared to wild-type: extended freezing period when first placed on bench and longer periods spent immobile. 2 = no spontaneous movement when placed on the bench; mouse can move in response to a gentle prod or a food pellet placed nearby. (Note: mice may become more active when in their own cage environment.)

B. Gait: 0 = as wild-type. 1 = hind legs are spread wider than wild-type when walking or running with reduced pelvic elevation, resulting in a "waddling" gait. 2 = more severe abnormalities: tremor when feet are lifted, walks backwards or 'bunny hops' by lifting both rear feet at once.

C. Hindlimb clasping: Mouse observed when suspended by holding base of the tail. 0 = legs splayed outwards. 1 = hindlimbs are drawn towards each other (without touching) or one leg is drawn in to the body. 2 = both legs are pulled in tightly, either touching each other or touching the body.

D. Tremor: Mouse observed while standing on the flat palm of the hand. 0 = no tremor. 1 = intermittent mild tremor. 2* = continuous tremor or intermittent violent tremor

E. Breathing: Movement of flanks observed while animal is standing still. 0 = normal breathing. 1 = periods of regular breathing interspersed with short periods of more rapid breathing or with pauses in breathing. 2* = very irregular breathing - gasping or panting.

F. General condition: Mouse observed for indicators of general well-being such as coat condition, eyes, body stance. 0 = clean shiny coat, clear eyes, normal stance. 1 = eyes dull, coat dull/ungroomed, somewhat hunched stance. 2* = eyes crusted or narrowed, piloerection, hunched posture.

[0070] Figure 4 shows that the group treated with scAAV9.738.Mecp2 did not reach median survival during the time of the experiment but surpassed control treated animals by more than 10 weeks at the time of publication. Animals treated with scAAV9.738.Mecp2 also had lower behavioral score compared to control treated animals. The experiment was repeated with affected female mice (Figure 5). Animals were IV treated with either scAAV9.738.Mecp2 or control as before with the males. Females were treated between 10-12 months of age when Rett mice are symptomatic. Animals were followed for approximately 6 months post injection and tested for their phenotypic score. Importantly, female Rett mice do not have early lethality like the more severe males [*Guy et al., Nature Genetics*, 27: 322-326 (2001)]. Treatment with scAAV9.738.Mecp2 halted progression of disease and indicated a reversal of disease severity with scores retreating to near 1. This was in stark contrast to control treated animals who finished the experiment with phenotypic scores near 6 indicating a worsening of symptoms (Figure 5C). Data from rotarod, inverted screen test, platform test and nesting ability all support behavioral improvement in animals treated with scAAV9.738.Mecp2 compared to control treated animals. Post mortem analysis of brains from scAAV9.738.Mecp2 treated females showed that the fluorescence intensity measurements of MECP2 expression mirrored that of wild type brains indicating that the gene therapy transgene expression was at approximately physiological levels.

Example 2

AVXS-201 Preclinical Efficacy Studies

[0071] To improve packaging efficiency and to incorporate a clinically relevant human MECP2 cDNA while maintaining physiological levels of gene expression, scAAV9.738.Mecp2 was re-engineered with a shorter promoter, a human MECP2B cDNA,

and a synthetic polyadenylation signal. The re-engineered genome was packaged into AAV9 capsids as described below and the resulting scAAV was subsequently named “AVXS-201” (**Figure 6**). AVXS-201 was originally named “AAV9-P545-MeCP2.”

Promoter region sequence (mouse MeCP2 promoter fragment) (SEQ ID NO: 2)

GTGAACAACGCCAGGCTCCTCAACAGGCAACTTTGCTACTTCTACAGAAAATGATAATA
AAGAAATGCTGGTGAAGTCAAATGCTTATCACAATGGTGAAGTACTCAGCAGGGAGGCT
CTAATAGGCGCCAAGAGCCTAGACTTCCTTAAGCGCCAGAGTCCACAAGGGCCCAAGTT
AATCCTCAACATTCAAATGCTGCCCACAAAACCAGCCCCTCTGTGCCCTAGCCGCCTCT
TTTTTCCAAGTGACAGTAGAACTCCACCAATCCGCAGCTGAATGGGGTCCGCCTCTTTT
CCCTGCCTAAACAGACAGGAACTCCTGCCAATTGAGGGCGTCACCGCTAAGGCTCCGC
CCCAGCCTGGGCTCCACAACCAATGAAGGGTAATCTCGACAAAGAGCAAGGGGTGGG
GCGCGGGCGCGCAGGTGCAGCAGCACACAGGCTGGTTCGGGAGGGCGGGGCGCGAC
GTCTGCCGTGCGGGGTCCCGGCATCGGTTGCGCGCGCGCTCCCTCCTCTCGGAGAGA
GGGCTGTGGTAAAACCCGTCCGGAAC

Coding region sequence (human MeCP2B cds) (SEQ ID NO: 3)

ATGGCCGCGCCGCGCCGCGCCGCGCCGAGCGGAGGAGGAGGAGGAGGCGAGGAGG
AGAGACTGGAAGAAAAGTCAGAAGACCAGGACCTCCAGGGCCTCAAGGACAAACCCCT
CAAGTTTAAAAAGGTGAAGAAAGATAAGAAAGAAGAGAAAGAGGGCAAGCATGAGCCC
GTGCAGCCATCAGCCCACCACTCTGCTGAGCCCGCAGAGGCAGGCAAAGCAGAGACA
TCAGAAGGGTCAGGCTCCGCCCCGGCTGTGCCGGAAGCTTCTGCCTCCCCCAAACAG
CGGCGCTCCATCATCCGTGACCGGGGACCCATGTATGATGACCCACCCCTGCCTGAAG
GCTGGACACGGAAGCTTAAGCAAAGGAAATCTGGCCGCTCTGCTGGGAAGTATGATGT
GTATTTGATCAATCCCCAGGGAAAAGCCTTTTCGCTCTAAAGTGGAGTTGATTGCGTACT
TCGAAAAGGTAGGCGACACATCCCTGGACCCTAATGATTTTGAAGTTCACGGTAACTGGG
AGAGGGAGCCCCTCCCGGCGAGAGCAGAAACCACCTAAGAAGCCCAAATCTCCCAA
GCTCCAGGAAGTGGCAGAGGCCGGGGACGCCCCAAAGGGAGCGGCACCACGAGACC
CAAGGCGGCCACGTGAGAGGGTGTGAGGTGAAAAGGGTCCTGGAGAAAAGTCCTGG
GAAGCTCCTTGTCAAGATGCCTTTTCAAACCTTCGCCAGGGGGCAAGGCTGAGGGGGGT
GGGGCCACCACATCCACCCAGGTCATGGTGATCAAACGCCCCGGCAGGAAGCGAAAA
GCTGAGGCCGACCCTCAGGCCATTCCCAAGAAACGGGGCCGAAAGCCGGGGAGTGTG
GTGGCAGCCGCTGCCGCCGAGGCCAAAAAGAAAGCCGTGAAGGAGTCTTCTATCCGA
TCTGTGAGGAGACCGTACTCCCCATCAAGAAGCGCAAGACCCGGGAGACGGTCAGC
ATCGAGGTCAAGGAAGTGGTGAAGCCCCTGCTGGTGTCCACCCTCGGTGAGAAGAGC
GGGAAAGGACTGAAGACCTGTAAGAGCCCTGGGCGGAAAAGCAAGGAGAGCAGCCCC
AAGGGGCGCAGCAGCAGCGCCTCCTCACCCCCCAAGAAGGAGCACCACCACCATCAC

CACCACTCAGAGTCCCCAAAGGCCCGTGCCACTGCTCCCACCCCTGCCCCACCTC
CACCTGAGCCCGAGAGCTCCGAGGACCCACCAGCCCCCTGAGCCCCAGGACTTGA
GCAGCAGCGTCTGCAAAGAGGAGAAGATGCCCAGAGGAGGCTCACTGGAGAGCGACG
GCTGCCCCAAGGAGCCAGCTAAGACTCAGCCCGCGGTTGCCACCGCCGCCACGGCCG
CAGAAAAGTACAAACACCGAGGGGAGGGAGAGCGCAAAGACATTGTTTCATCCTCCAT
GCCAAGGCCAAACAGAGAGGAGCCTGTGGACAGCCGGACGCCCGTGACCGAGAGAGT
TAGCTGA

PolyA sequence (synthetic) (SEQ ID NO: 4)

AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTGTGTG

[0072] scAAV9 was produced by transient transfection procedures using a double-stranded AAV2-ITR-based vector, with a plasmid encoding Rep2Cap9 sequence as previously described [Gao *et al.*, *supra*] along with an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells. Virus was produced in three separate batches for the experiments and purified by two cesium chloride density gradient purification steps, dialyzed against PBS and formulated with 0.001% Pluronic-F68 to prevent virus aggregation and stored at 4°C. All vector preparations were titered by quantitative PCR using Taq-Man technology. Purity of vectors was assessed by 4–12% sodium dodecyl sulfate-acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA).

[0073] Efficacy and dosing studies were performed in the same strain of Rett mice as in **Figure 4**. Doses between Example 1 and Example 2 are not compared due to improvements in titering methods. Experiments in Example 1 used optical titering of viral preparations while studies in Example 2 and below use the more accurate digital droplet PCR titer. To mimic the proposed clinical delivery route of intrathecal administration, these injections were performed as intracerebroventricular (ICV) in postnatal day 1 pups. Intrathecal delivery was chosen to deliver AVXS-201 directly to the nervous system which is the key site of action for Rett syndrome. Pups were followed for their natural lives and assessed for survival, composite phenotypic score, open field and rotarod behavior. Survival data over a two log dose range is shown in **Figure 7**. The results shown in Figure 7 demonstrate that the combination of vectors and techniques used in the treatment methods of the invention achieve an improved outcome. All doses tested extended median survival over control treated *Mecp2*^{+/−} mice with maximum individual survival observed reaching 500 days (ongoing) compared to 93 days for control treated Rett mice. The highest median lifespan (315d) was achieved with a moderate dose of 1.44x10¹⁰vg per animal. The data show a bell shaped dose response (**Figure 7B**) which distinguishes the results achieved herein from the effects of improper MECP2 dosage (gene copy number) observed previously

[see, for example, Lombardi *et al.*, *The Journal of Clinical Investigation*, 125: 2914-2923 (2015)]. Importantly, even at the highest dose tested, AVXS-201 treatment did not shorten survival of *Mecp2*^{-y} mice relative to control treatment.

[0074] In addition to survival, treated and control mice were scored weekly for Rett phenotypes (phenotypes set out in the previous Example). Untreated males progress rapidly from a score of 0 to an average peak of 5.25 by 10 weeks of age (**Figure 8**). In contrast, phenotypic scores of all the treated groups only reached a score of about 2 by 17 weeks of age with the exception of the 5.56x10¹⁰vg group which reached a score of 5 at 18 weeks. Treated and control animals were also assessed in the open field and rotarod tests (**Figure 9**). Reduced spontaneous movement is a symptom of Rett male mice. Open field analysis to assess spontaneous movement and velocity was performed when groups were 2-3 months of age. Affected animals had a nearly 43% reduction in the total distance traveled compared to wild type mice. Significant increases in distance traveled were noted in all but two of the groups treated with AVXS-201 over control treated *Mecp2* knockout males. Velocity compared to control treated knockouts was also significantly improved. This shows that AVXS-201 treatment of a male Rett mouse model improves exploratory behavior and ambulation. Treated and control animals were tested at 3 months of age for performance on the rotarod which is a measure of motor coordination. Animals were tested on three consecutive days and scores were averaged across days and dose. The resulting data are shown in **Figure 9C**. Control treated *Mecp2*^{-y} mice performed significantly worse on rotarod compared to control treated wild type littermates. Rotarod performance was significantly improved over control treatment in the 7.00x10⁹ and 1.44x10¹⁰vg cohorts.

Example 3

AVXS-201 Expression of MECP2 Protein in the Treated Rett Mouse Brain

[0075] At 3 weeks post injection, PBS treated male wild type, untreated Rett and vector treated Rett animals were euthanized to examine MECP2 protein levels in the brain following postnatal day 1 ICV injection of AVXS-201. One brain hemisphere was homogenized and analyzed by western blot to monitor MECP2 expression. A representative blot and quantification are shown in **Figure 10**. After normalization to the PBS treated wild type brains, the knockout and the 1.75x10⁹vg AVXS-201 dose group had no detectable levels of MECP2. Treatment with 3.50x10⁹vg and 7.00x10⁹vg produced detectable MECP2 levels that reached ~1% and 3.6% of wild type levels, respectively. The most effective dose when measured by increase in median survival (1.44x10¹⁰vg) yielded ~11% of wild type MeCP2 levels. The 5.56x10¹⁰vg dose examined by western blot produced MECP2 levels of ~54% of

wild type while 1.13×10^{11} reached more than 2x wild type levels. These data show that protein expression level *and* distribution throughout the brain are key for predicting the effectiveness of an MECP2 gene therapy.

Example 4

Treatment of Wild Type Mice with AVXS-201 Is Safe and Well Tolerated

[0076] An important concern for an MECP2 replacement therapy is to assess the impact on the cells expressing an intact copy of *MECP2*. AVXS-201 was designed with this consideration in mind by incorporating a fragment of the murine *Mecp2* promoter to support physiological regulation of the MECP2 transgene. To test the safety of AVXS-201, survival and behavior analysis was performed on cohorts of wild type mice that received P1 ICV injections of AVXS-201 just as in the male Rett mice.

[0077] A total of 131 wild type male mice were treated with various ICV doses of AVXS-201 and followed for survival (**Figure 11**). No deaths were recorded in the targeted therapeutic dose (1.44×10^{10} vg) with 21 treated animals alive through P342. No deaths were recorded in the PBS treated group and one death each was recorded in the 3.50×10^9 , 2.78×10^{10} and 1.13×10^{11} vg treated groups. Behavioral scoring using the criteria from Box 1, shows that vector treated groups largely had mean phenotypic scores <1 . Mean aggregate scores >1 were only noted in the two highest dosed groups (5.56×10^{10} and 1.13×10^{11} vg). Open field testing at 2-3 months of age showed no statistical difference between vector and PBS treated wild type males (**Figure 12**). Interestingly, a significant decrease in rotarod performance was detected in the 1.13×10^{11} vg cohort compared to control treated wild type mice at three months of age. These data are suggestive of a toxic effect of MECP2 overexpression at the highest AVXS-201 dose. Together these data indicate that in a “worst-case scenario” of AVXS-201 treatment only transducing wild type cells, there is minimal impact on animal survival and behavior at the targeted therapeutic dose.

Example 5

Physiological Levels of MECP2 are Maintained in Brains of Wild Type Mice Treated with Therapeutic Doses of AVXS-201

[0078] To further investigate the levels associated with symptomatic MECP2 overexpression, wild type male mice received P1 ICV injections of PBS or AVXS-201 at the therapeutic target of 1.44×10^{10} vg or the highest dose tested of 1.13×10^{11} vg. Animals were euthanized 3 weeks post injection, and brains were harvested for western blot. For comparison, tissues were blotted alongside brains from a mouse model of MECP2

overexpression called Tg3. Brains were dissected into separate regions (Cb=cerebellum, Med=medulla, Hipp=hippocampus, Ctx=cortex and Mid=midbrain; **Figure 13**) and the individual regions were homogenized for blotting. Data was normalized to MECP2 levels in PBS treated wild type brains. Treatment with the target therapeutic dose (1.44×10^{10} vg) had MECP2 levels between 1 and 1.5x wild type tissues across all regions examined. The high dose (1.13×10^{11} vg) ranged from 1.31-2.56x wild type levels, but did not reach the 2.31-3.93x levels of Tg3 tissues. These data, along with behavior and survival data shown earlier, give confidence that AVXS-201 expresses protein at near physiological levels when administered at the targeted dose. Importantly, therapeutic dosing does not approach the 2x protein levels associated with MECP2 duplication syndrome. This shows the safety of an MECP2 replacement approach using gene therapy.

Example 6

Body Weight, Hematology and Serum Chemistry are Unremarkable in Non-Human Primates through 18 Months after Intrathecal Injection of AVXS-201

[0079] To investigate the safety and tolerability of AVXS-201 and the associated intrathecal injection procedure, three treated male *cynomolgus macaques* were followed for 18 months post injection. Dosing parameters are shown in **Table 2**.

Table 2	Animal ID	Total Viral Genomes (vg)	Body Weight at Injection (kg)	Vector Genomes/Body Weight (vg/kg)	Duration post Tx
Hematology and Serum Chemistry	15C34	6.0×10^{12}	1.23	4.9×10^{12}	18 mo
	15C40	1.4×10^{13}	1.79	7.8×10^{12}	18 mo
	15C48	1.4×10^{13}	1.83	7.7×10^{12}	18 mo
MECP2 Expression	15C38	1.3×10^{13}	1.68	7.7×10^{12}	6 wk
	15C49	1.0×10^{13}	1.30	7.7×10^{12}	6 wk

[0080] Two animals were treated at the intended therapeutic dose ($\sim 1.44 \times 10^9$ vg equivalent on a per kg of body weight basis), and one received a ~ 2 -fold lower dose ($\sim 7.00 \times 10^8$ vg equivalent on a per kg of body weight basis). The intrathecal injection

procedure was previously described in Meyer *et al.*, *Molecular Therapy: The Journal of the American Society of Gene Therapy*, 23: 477-487 (2015). Briefly, vector was mixed with contrast agent for verifying vector spread. The anesthetized subject was placed in the lateral decubitus position and the posterior midline injection site at ~L4/5 level (below the conus of the spinal cord) was prepared. Under sterile conditions, a spinal needle with stylet was inserted and subarachnoid cannulation was confirmed with the flow of clear CSF from the needle. 0.8 ml of CSF was drained in order to decrease the pressure in the subarachnoid space and immediately after the vector solution was injected. Following injection, animals were kept in the Trendelenburg position and their body was tilted head-down for 10 minutes. Treated animals were dosed at 6 or 12 months of age, body weight, blood counts and serum chemistries were collected monthly for the first 6 months post injection, and every two months thereafter. Body weight is shown in **Figure 14**, blood counts are shown in **Figure 15** and serum chemistries are shown in **Figures 16 and 17** graphed with values from control treated animals from the same colony at the Mannheimer Foundation (Homestead, FL). Overall, body weight, cell counts and serum values from vector treated animals were consistent with control treated animals. No values substantially deviated from controls for more than 2 consecutive observations in a given animal with the exception of amylase which was higher in two vector treated animals at baseline. These data show that AVXS-201 and the intrathecal injection procedure are safe and well tolerated.

Example 7

Histopathological Analysis of Tissues from Non-Human Primates Following Intrathecal Injection of AVXS-201

[0081] In addition to the *in vivo* (Example 6) and post mortem analyses (Example 8) samples of visceral and nervous system tissues from animals 15C38, 15C49 and 15C34 (Table 1) were sent to GEMpath Inc. (Longmont, CO) for paraffin embedding, sectioning and hematoxylin and eosin staining. The remaining animals (Table 8.2) are still in life and will be sent for analysis at study conclusion. Slides were read and reports were prepared by a GEMpath Board Certified Veterinary Pathologist. The tissues sampled and examined are shown in **Table 3**. The pathology reports note that AVXS-201 treatment did not induce lesions in any protocol-specified tissues at the 6 week or 18 month time point.

Table 3

Animal ID	Tissues
15C38 15C49	Adrenal Gland, Brain (amygdala, striatum, hippocampus, occipital cortex, temporal cortex, mid brain, brain stem, cerebellum), Eye and Optic Nerve, Heart, Kidney, Liver, Lung, Lymph Node (inguinal), Pancreas, Spinal Cord (sections from cervical, thoracic, lumbar and sacral regions; some sections had attached dorsal root ganglia), Small Intestine (jejunum and ileum), Skeletal Muscle (diaphragm, gastrocnemius, quadriceps femoris, triceps brachii, transverse abdominal, tibialis anterior), Spleen,

15C34	Testis/Epididymis, Thymus, Urinary Bladder Adrenal Gland, Brain (amygdala, striatum, hippocampus, hypothalamus, visual cortex, motor and somatosensory cortex, associative cortex, auditory cortex, superior and inferior colliculi, cerebellum, deep cerebellar nuclei, pons and medulla oblongata), Eye and Optic Nerve, Heart, Kidney, Liver, Lung, Lymph Node, Pancreas, Spinal Cord (sections from cervical, thoracic, lumbar and sacral regions), Small Intestine (jejunum and ileum), Skeletal Muscle (diaphragm, gastrocnemius, quadriceps femoris, triceps brachii, transverse abdominal, tibialis anterior), Spleen, Testis/Epididymis, Thymus, Urinary Bladder
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Example 8

Physiological Levels of MeCP2 in the Non-Human Primate Brain Following Intrathecal Injection of AVXS-201

[0082] Two 12-month-old, male cynomolgus macaques received intrathecal injections of 7.7×10^{12} vg/kg of AVXS-201 as described above. Animals persisted for six weeks post injection and were euthanized for analysis of MeCP2 expression. Selected brain regions were analyzed for total MeCP2 expression by immunohistochemistry (**Figure 18**). No obvious elevations of MeCP2 were detected in cortical and subcortical regions nor proximal to the injection site (lumbar spinal cord). Importantly, these data also fail to show any gross abnormalities in tissues from animals that received AVXS-201 injection. To further examine transgene expression, brain regions were homogenized and compared against historical control tissue from animals from the same colony (**Figure 19**). Samples of occipital and temporal cortices, hypothalamus, lumbar spinal cord, thalamus, amygdala, hippocampus and cerebellum were analyzed by western blot for total MeCP2 expression. Across all of the regions examined no region showed a $\geq 2x$ level of MeCP2 expression above controls. Elevated MeCP2 was detected in the hypothalamus and amygdala which are regions proximal to 3rd ventricular and lateral ventricular, respectively, but not the cerebellum. Further, the lumbar spinal cord which is proximal to the injection site did not show elevated MeCP2 levels. These data suggest that the combination of viral dose and expression construct are regulating MECP2 expression. Further, *in situ* hybridization (ISH) was performed to detect vector derived transcript and determine distribution in the brain at 6 weeks and 18 months post injection (**Figures 20 and 21**). All regions examined in the brain and spinal cord (occipital cortex, temporal cortex, hippocampus, corpus callosum, thalamus, caudate, putamen, superior colliculus, pons, medulla, cerebellum, cervical, thoracic and lumbar spinal cord) showed expression of vector derived transcript that was not present in tissues from control treated animals. These data show a specificity of the ISH probe for vector derived MECP2 transcript and show that the AVXS-201 promoter construct is functional in NHP nervous system tissue. These data show that AVXS-201 distributes broadly throughout the CNS when administered via lumbar puncture and expresses at physiological levels.

Disclosure from Provisional Patent Application No. 62/423,618

Gene Therapy for Rhett Syndrome

Gene therapy to restore the transcription factor MeCP2 appears to be a feasible strategy for treating Rett syndrome, a progressive neurodevelopmental disorder leading to apparent autistic behavior, loss of motor function, and early death. We have developed an adeno-associated virus serotype 9 (AAV9) expressing human MECP2 under the control of a truncated endogenous promoter. The purpose of this work is to assess the efficacy and safety of this vector in mice (MeCP2 null and wild type) and non-human primates. Through continued research, our goal is to bring this treatment from the bench to the bedside.

AAV9-P545-MeCP2

Promoter region sequence (mouse MeCP2 promoter fragment)

GTGAACAACGCCAGGCTCCTCAACAGGCAACTTTGCTACTTCTACAGAAAATGATA
ATAAAGAAATGCTGGTGAAGTCAAATGCTTATCACAAATGGTGAAGTACTCAGCAGG
GAGGCTCTAATAGGCGCCAAGAGCCTAGACTTCCCTTAAGCGCCAGAGTCCACAAG
GGCCCAGTTAATCCTCAACATTCAAATGCTGCCACAAAACCAGCCCCTCTGTGCC
CTAGCCGCCTCTTTTTTCCAAGTGACAGTAGAACTCCACCAATCCGCAGCTGAATG
GGGTCCGCCTCTTTTCCCTGCCTAAACAGACAGGAAGTCTGCCAATTGAGGGCG
TCACCGCTAAGGCTCCGCCCCAGCCTGGGCTCCACAACCAATGAAGGGTAATCTC
GACAAAGAGCAAGGGGTGGGGCGCGGGCGCGCAGGTGCAGCAGCACACAGGCT
GGTCGGGAGGGCGGGGGCGCGACGTCTGCCGTGCGGGGTCCCGGCATCGGTTGC
GCGCGCGCTCCCTCCTCTCGGAGAGAGGGCTGTGGTAAAACCCGTCCGGAAAAC

Coding region sequence (human MeCP2 cds)

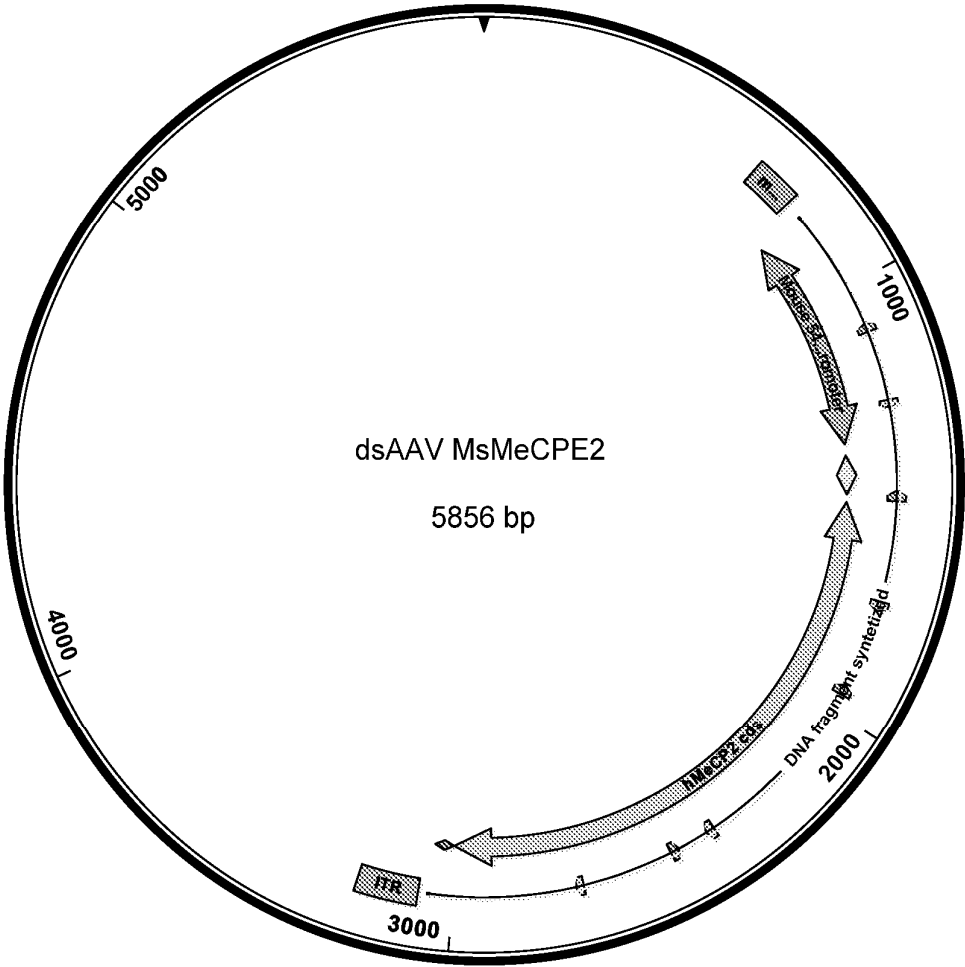
[illegible]

GCTGCCGCCGAGGCCAAAAAGAAAGCCGTGAAGGAGTCTTCTATCCGATCTGTGC
AGGAGACCGTACTCCCCATCAAGAAGCGCAAGACCCGGGAGACGGTCAGCATCG
AGGTCAAGGAAGTGGTGAAGCCCCTGCTGGTGTCCACCCTCGGTGAGAAGAGCG
GGAAAGGACTGAAGACCTGTAAGAGCCCTGGGCGGAAAAGCAAGGAGAGCAGCC
CCAAGGGGGCGCAGCAGCAGCGCCTCCTCACCCCCCAAGAAGGAGCACCACCACC
ATCACCACCACTCAGAGTCCCCAAAGGCCCCCGTGCCACTGCTCCCACCCCTGCC
CCCACCTCCACCTGAGCCCGAGAGCTCCGAGGACCCACCAAGCCCCCCTGAGCC
CCAGGACTTGAGCAGCAGCGTCTGCAAAGAGGAGAAGATGCCCAGAGGAGGCTC
ACTGGAGAGCGACGGCTGCCCAAGGAGCCAGCTAAGACTCAGCCCGCGGTTGC
CACCGCCGCCACGGCCGCAGAAAAGTACAAACACCGAGGGGAGGGAGAGCGCAA
AGACATTGTTTCATCCTCCATGCCAAGGCCAAACAGAGAGGAGCCTGTGGACAGC
CGGACGCCCGTGACCGAGAGAGTTAGCTGA

PolyA sequence (synthetic)

AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTGTGTG

AAV Vector map



Rett Syndrome – Clinical Pathology

X-linked neurodevelopmental Autism Spectrum Disorder (ASD) affecting ~1 in 10,000 girls

Age of onset : 6-18 months of age

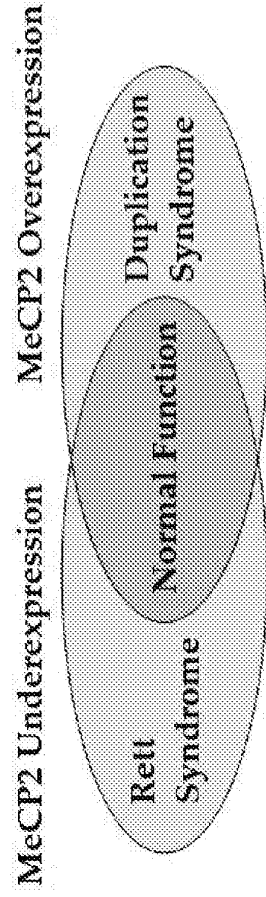
- * Loss of motor function, speech, hand-function

90% of Cases Result from Mutations in Methyl CpG Binding Protein 2 (MeCP2)

- * Transcription factor, regulates thousands of genes



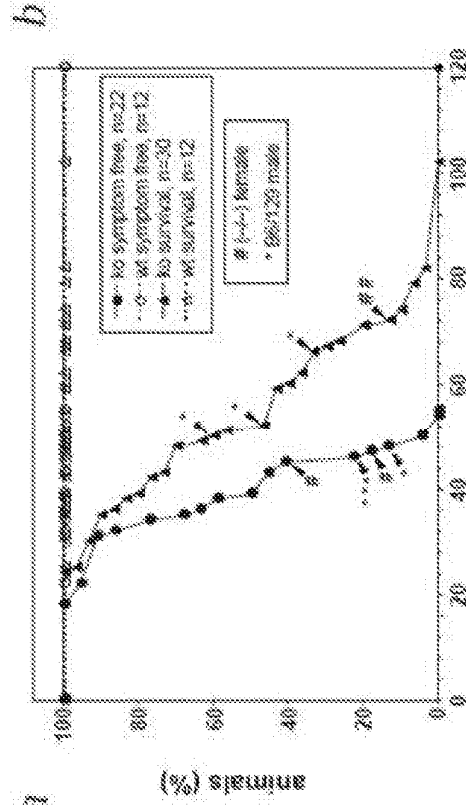
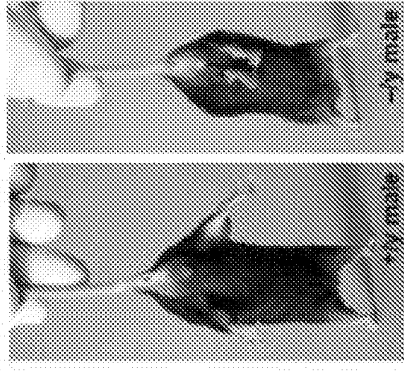
Brookline, 6 years old



Rett Syndrome is Reversible!

Model Expresses a Rett Syndrome- like phenotypes:

- Reduced survival
- Muscle weakness
- Increased anxiety



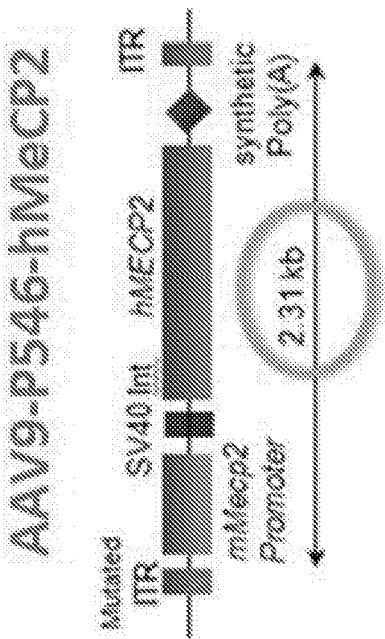
(Guy et al. 2001 Nature)

Re-expression of MeCP2 in null mice ameliorates survival and behavioral phenotypes

Gene Therapy for Rett Syndrome

Adeno-associated virus serotype 9 (AAV9) Mediated Delivery of MeCP2 to the CNS

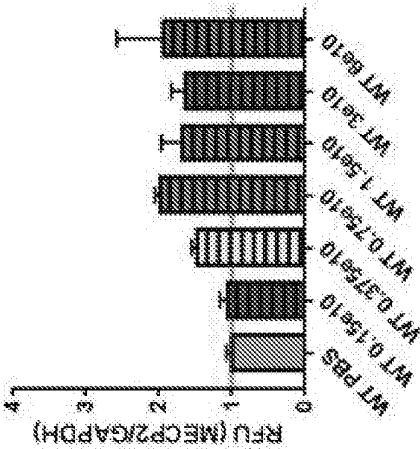
- Endogenous Promoter
- Non-Pathogenic
- Efficient Targeting of Neurons and Astrocytes



Treatment Parameters							
(WT) MeCP2 ^{Y/-} and (KO) MeCP2 ^{Y/+}		Intracerebroventricular (ICV) Injection, directly to the CNS					
Postnatal Day 2 (P2)		From (Lee et al. 2007 Faseb))					
Total Viral Genomes (vg) Injected (e [^] 10)		0.15	0.375	0.75	1.5	3.0	6.0
		AAV9-P546-MeCP2					

Regulation of MecP2 in Wild Type Mice Injected with AAV9-P546-MecP2

MecP2 Protein Expression in Half Brain
of ICV treated MecP2^{Y/+} Mice



MecP2 Protein Expression in Mouse Half-Brain



MeCP2 Protein Expression in Half Brain of ICV treated MeCP2^{Y/-} Mice



MECP2

GAPDH

6.0e+10

3.0e+10

1.5e+10

0.75eV10

0.375e^10

0.15eV10

KO

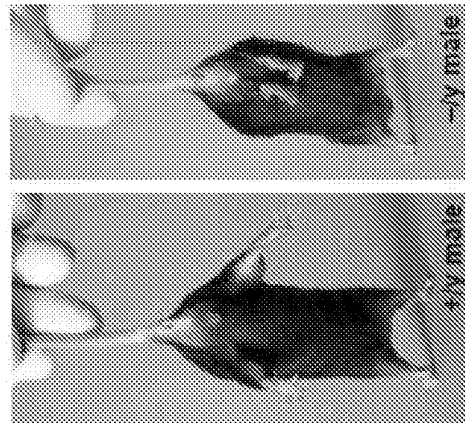
PBS

Effect of P546-MeCP2 delivered directly to the CNS of MeCP2^{Y/-} mice on behavioral scores

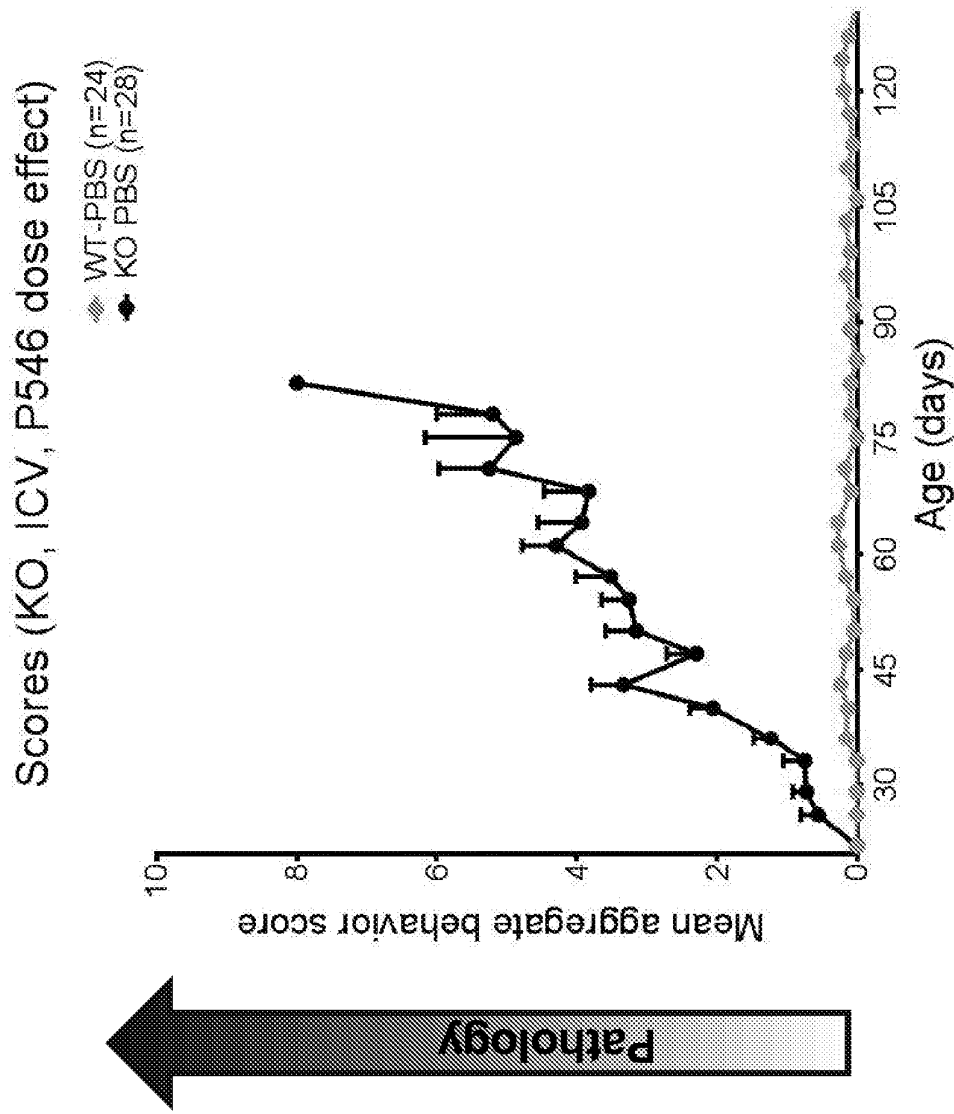
Qualitative Behavioral

Scoring:

- Tremor
- Gait
- Abnormal Respiration
- Mobility
- General Condition
- Hindlimb Clasp



(Guy et al. 2007 Nature)

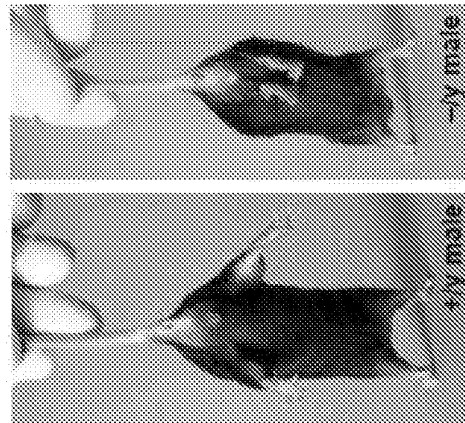


Effect of P546-MeCP2 delivered directly to the CNS of MeCP2^{Y/-} mice on behavioral scores

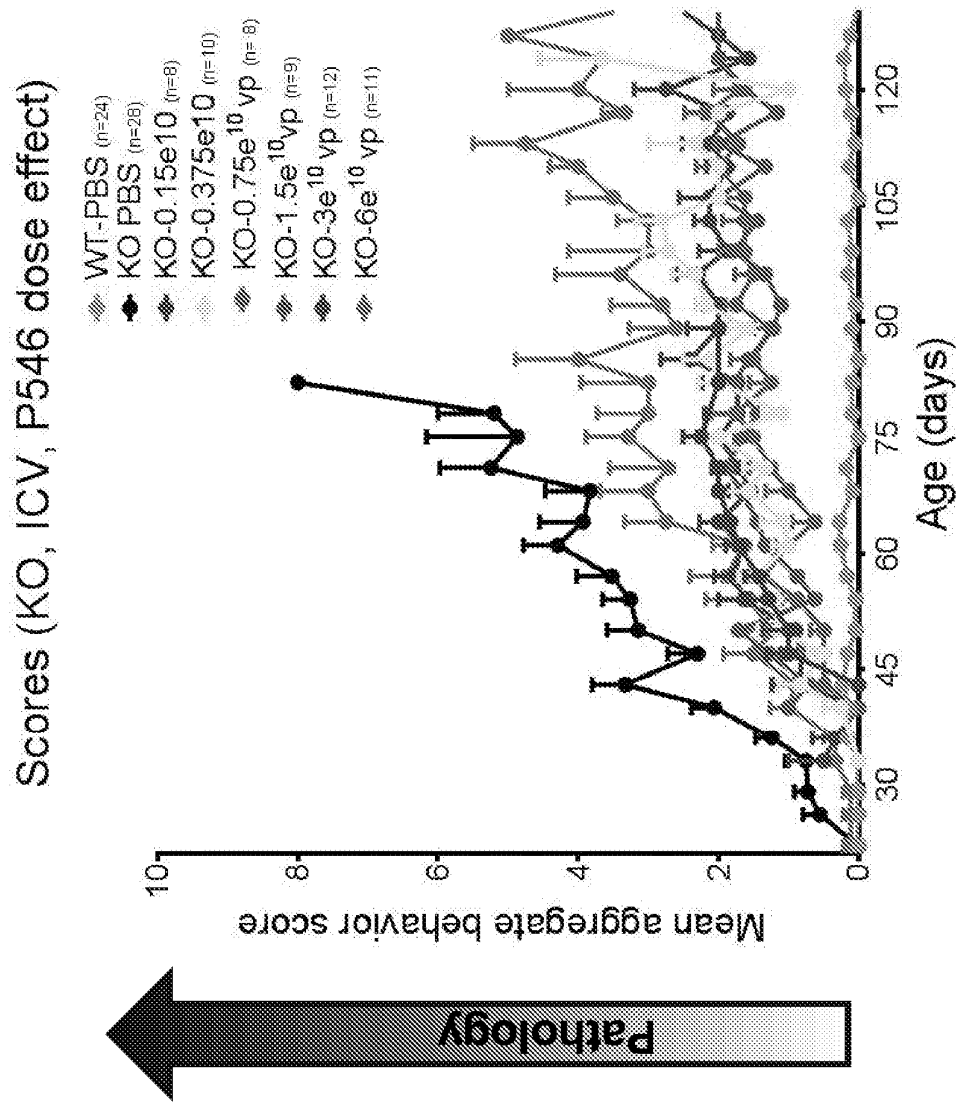
Qualitative Behavioral

Scoring:

- Tremor
- Gait
- Abnormal Respiration
- Mobility
- General Condition
- Hindlimb Clasping

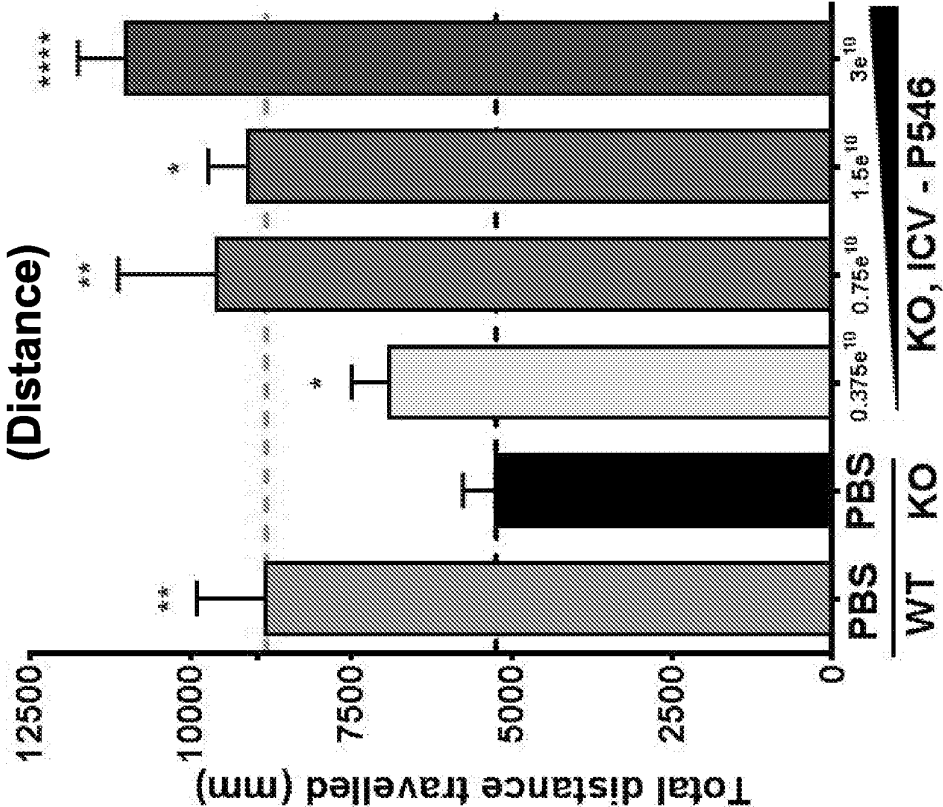


(Guy et al. 2007 Nature)



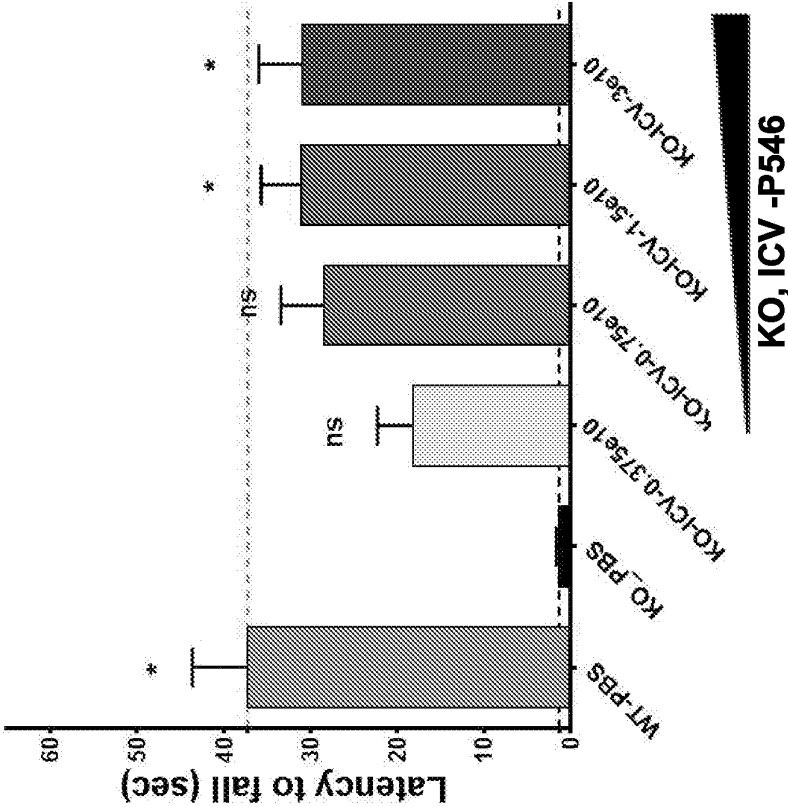
P546-MeCP2 delivered directly to the CNS of *MeCP2^{Y/-}* mice rescues motor activity

Open Field



(7-9 weeks Post Injection)

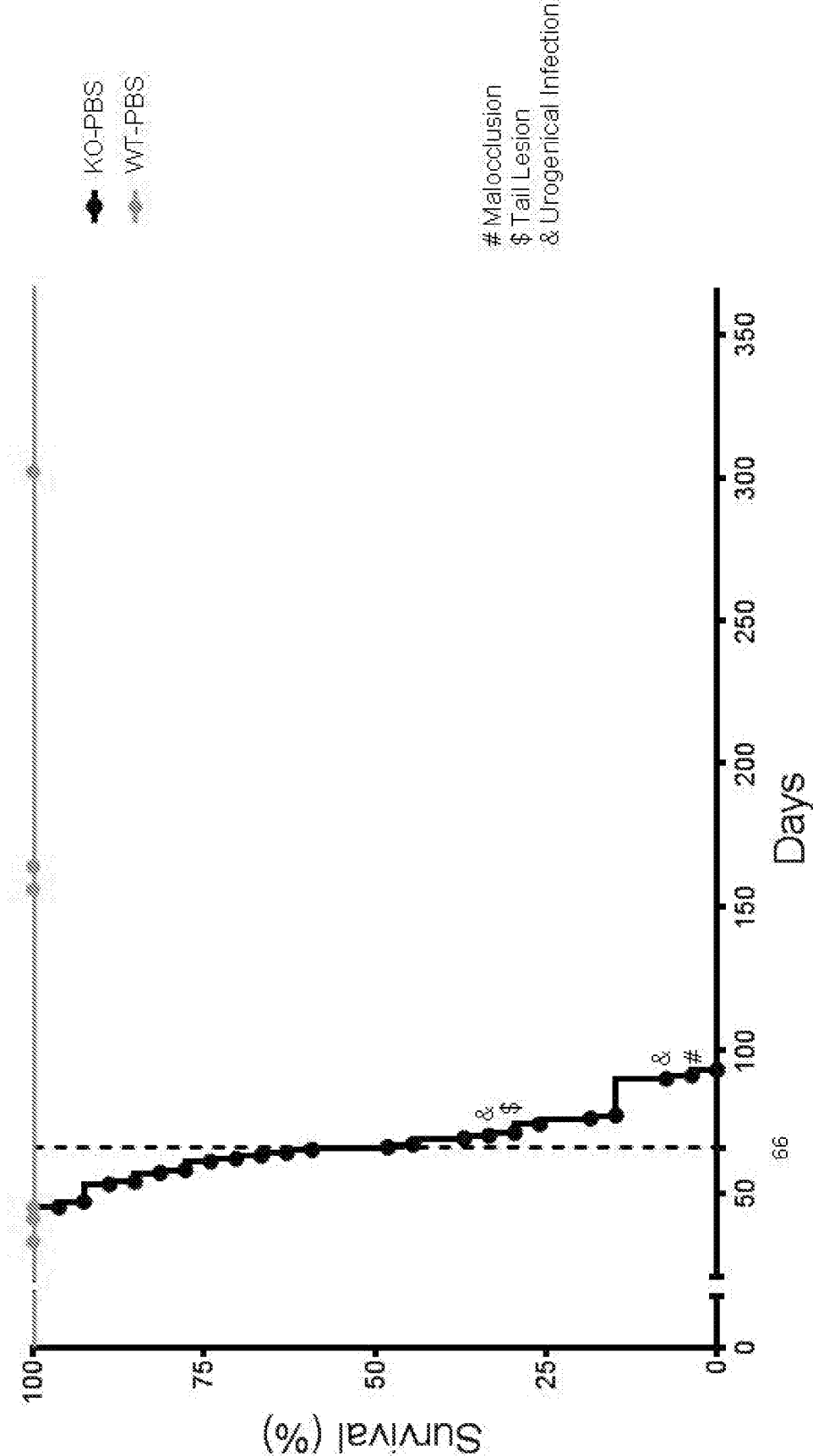
Rotarod Performance



(12 Weeks Post Injection)

Survival of MecP2^{Y/+} vs. MecP2^{Y/-} Mice

Intracerebroventricular, Newborn

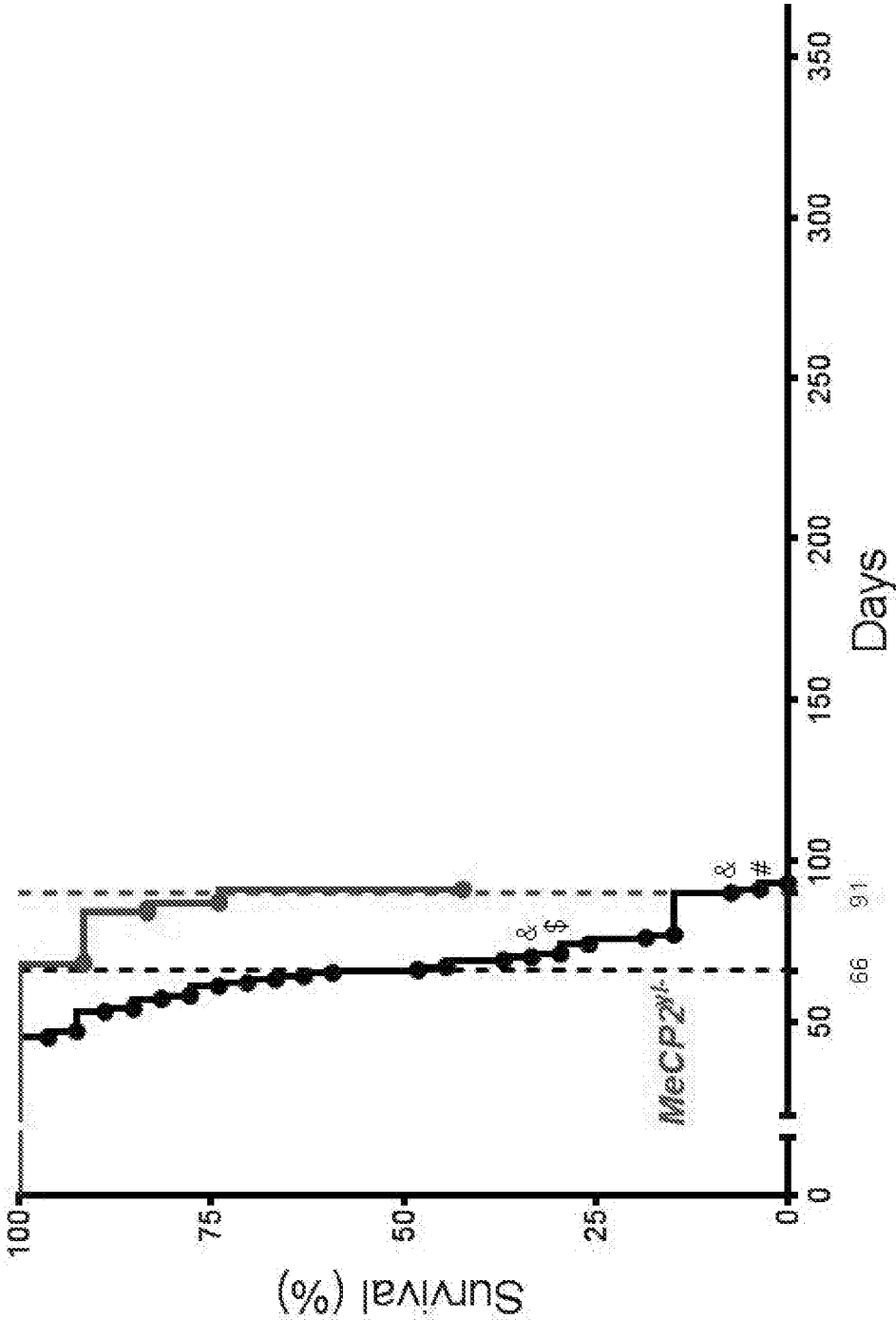




Survival of MeCP2^{Y/+} vs. MeCP2^{Y/-} Mice

Intracerebroventricular, Newborn

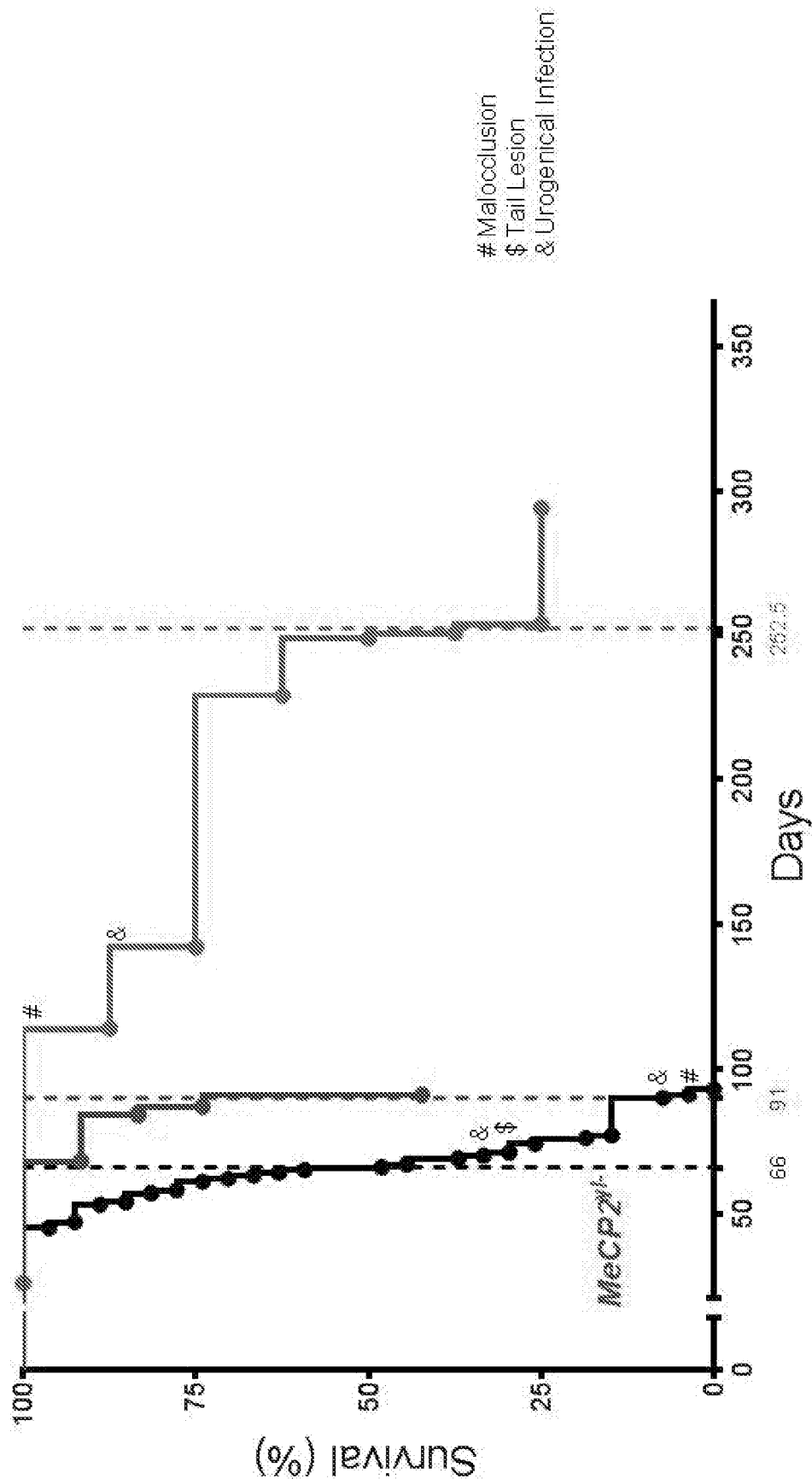
- KO-PBS (n=27)
- ◆ KO-0.15e¹⁰vp (n=12)



Survival of MeCP2^{+/+} vs. MeCP2^{-/-} Mice

Intracerebroventricular, Newborn

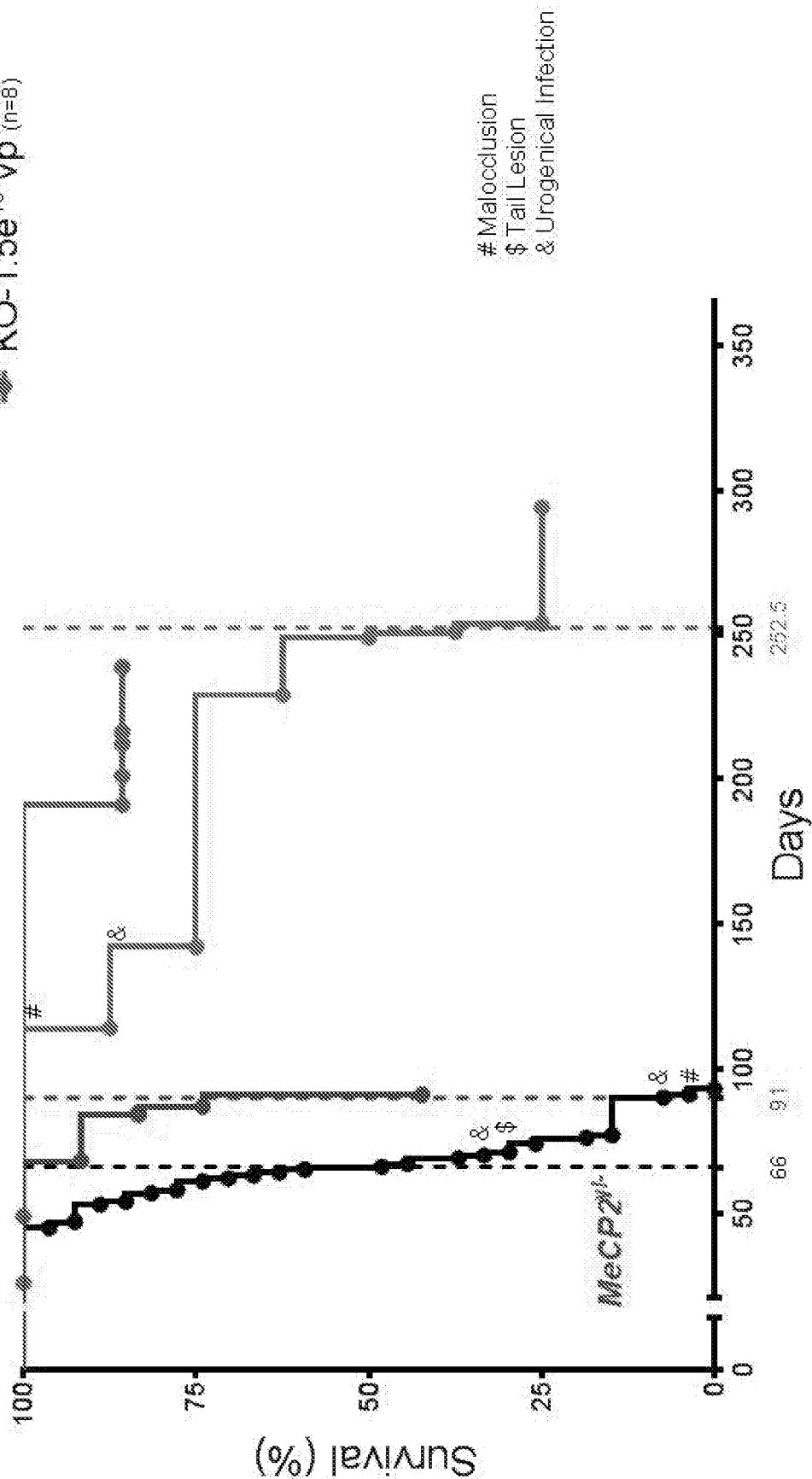
- KO-PBS (n=27)
- ◆ KO-0.15e¹⁰vp (n=12)
- ◆ KO-0.75e¹⁰vp (n=12)



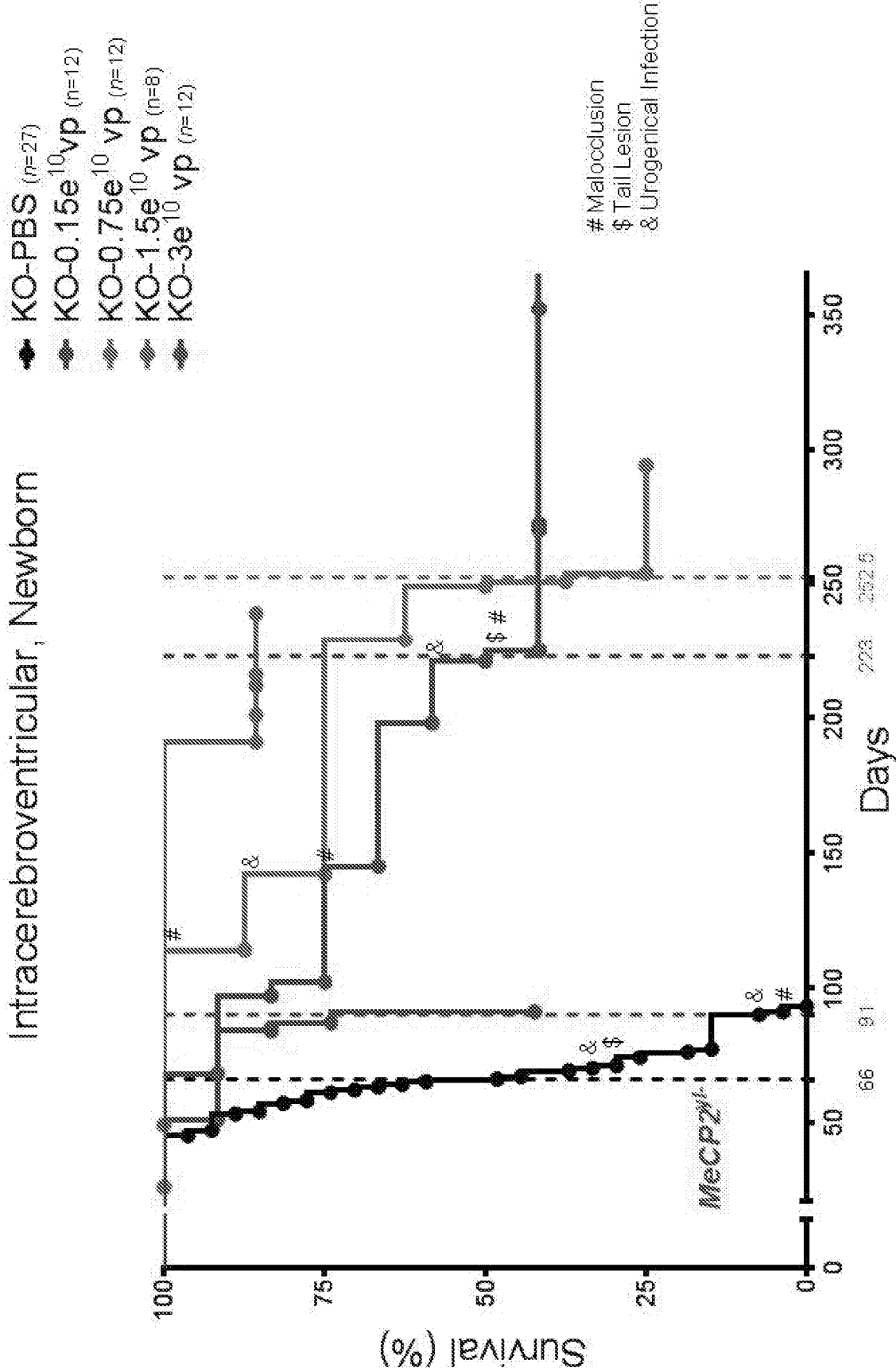
Survival of $\text{MeCP2}^{+/+}$ vs. $\text{MeCP2}^{-/-}$ Mice

Intracerebroventricular, Newborn

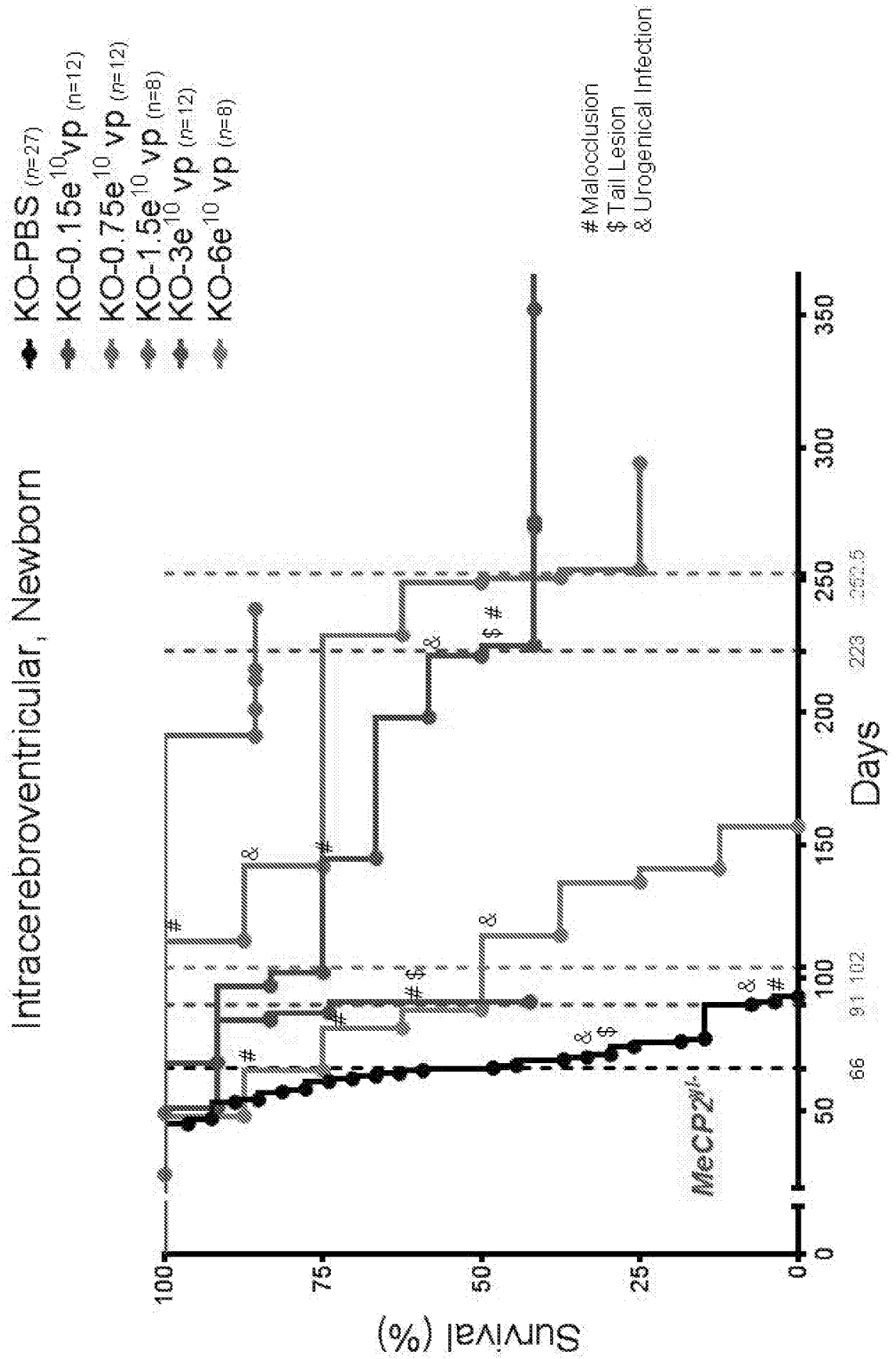
- ◆ KO-PBS ($n=27$)
- ◆ KO- 0.15×10^6 vp ($n=12$)
- ◆ KO- 0.75×10^6 vp ($n=12$)
- ◆ KO- 1.5×10^6 vp ($n=8$)



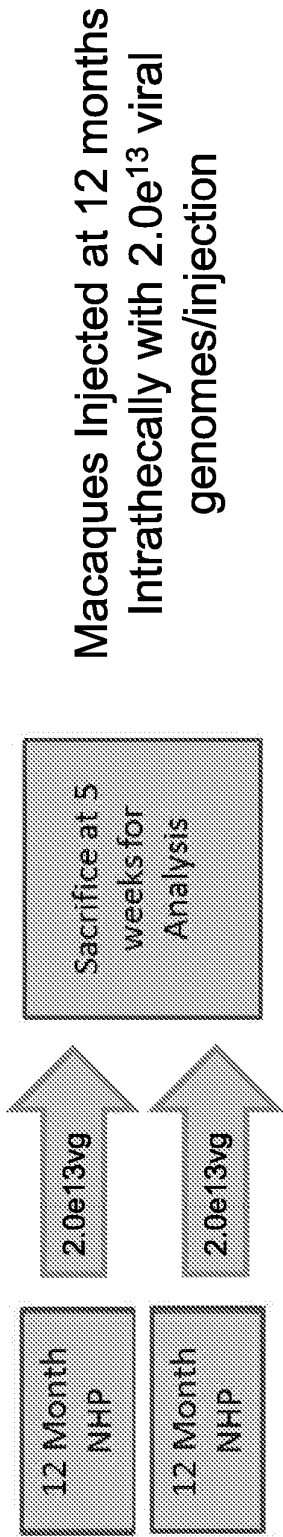
Survival of MeCP2^{Y/+} vs. MeCP2^{Y/-} Mice



Survival of $\text{MeCP2}^{Y/+}$ vs. $\text{MeCP2}^{Y/-}$ Mice



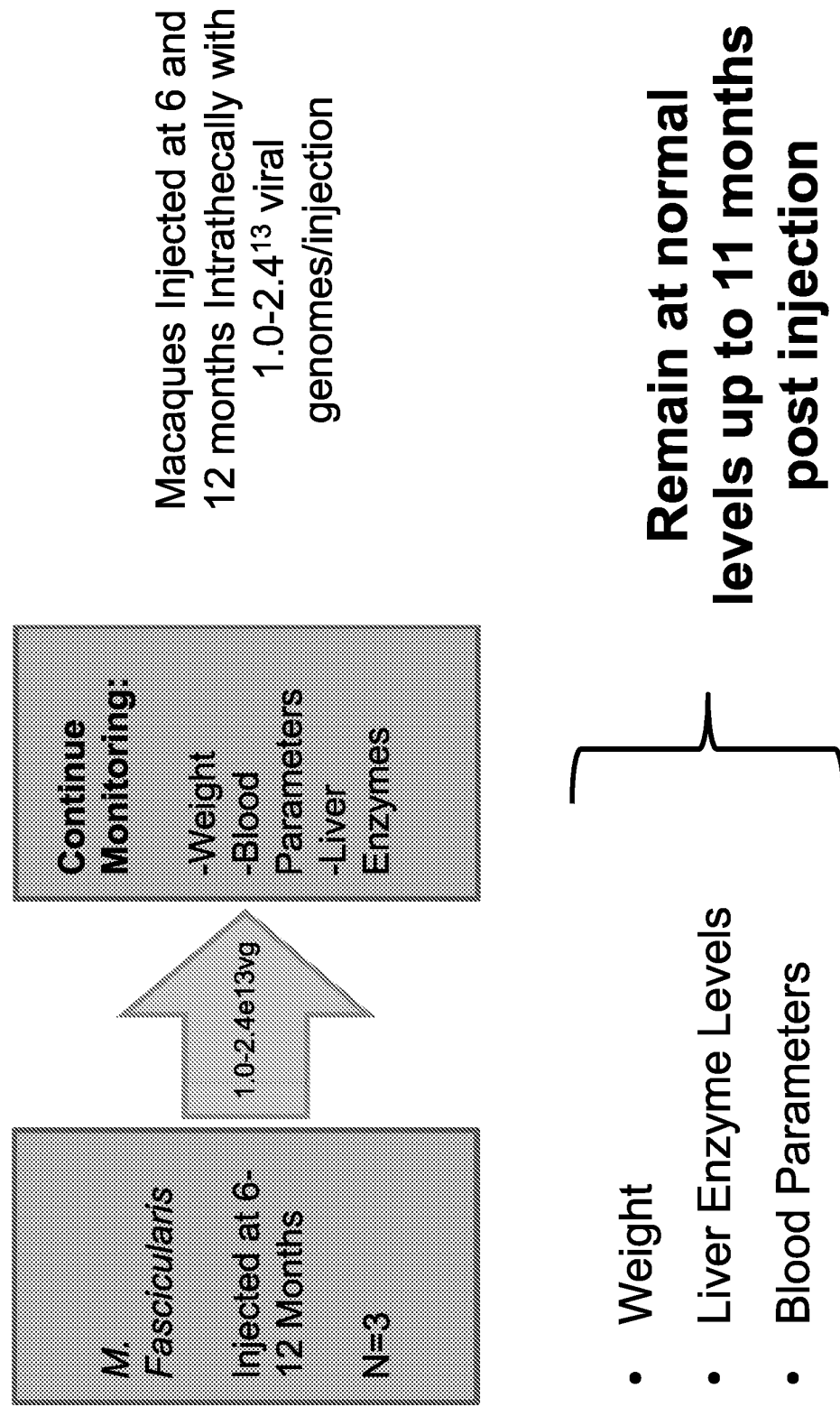
Non-Human Primate (NHP) Safety Study in *Macaca Fascicularis*



Macaques Injected at 12 months
Intrathecally with 2.0e¹³ viral
genomes/injection

MeCP2 Protein Expression in (NHP CNS Tissue) 5 Weeks Post Injection									
Thoracic Spinal Cord		Cervical Spinal Cord		Striatum		Temporal Cortex		Cerebellum	
-	+	+	-	+	+	-	+	+	+
1x		1x		1x		1x		1x	
2.1x		1.3x		2.6x		0.5x		0.8x	
AAV9-P546-MeCP2		AAV9-P546-MeCP2		AAV9-P546-MeCP2		AAV9-P546-MeCP2		AAV9-P546-MeCP2	
Relative MeCP2 Expression		Relative MeCP2 Expression		Relative MeCP2 Expression		Relative MeCP2 Expression		Relative MeCP2 Expression	

Non-Human Primate (NHP) Safety Study in *Macaca Fascicularis*



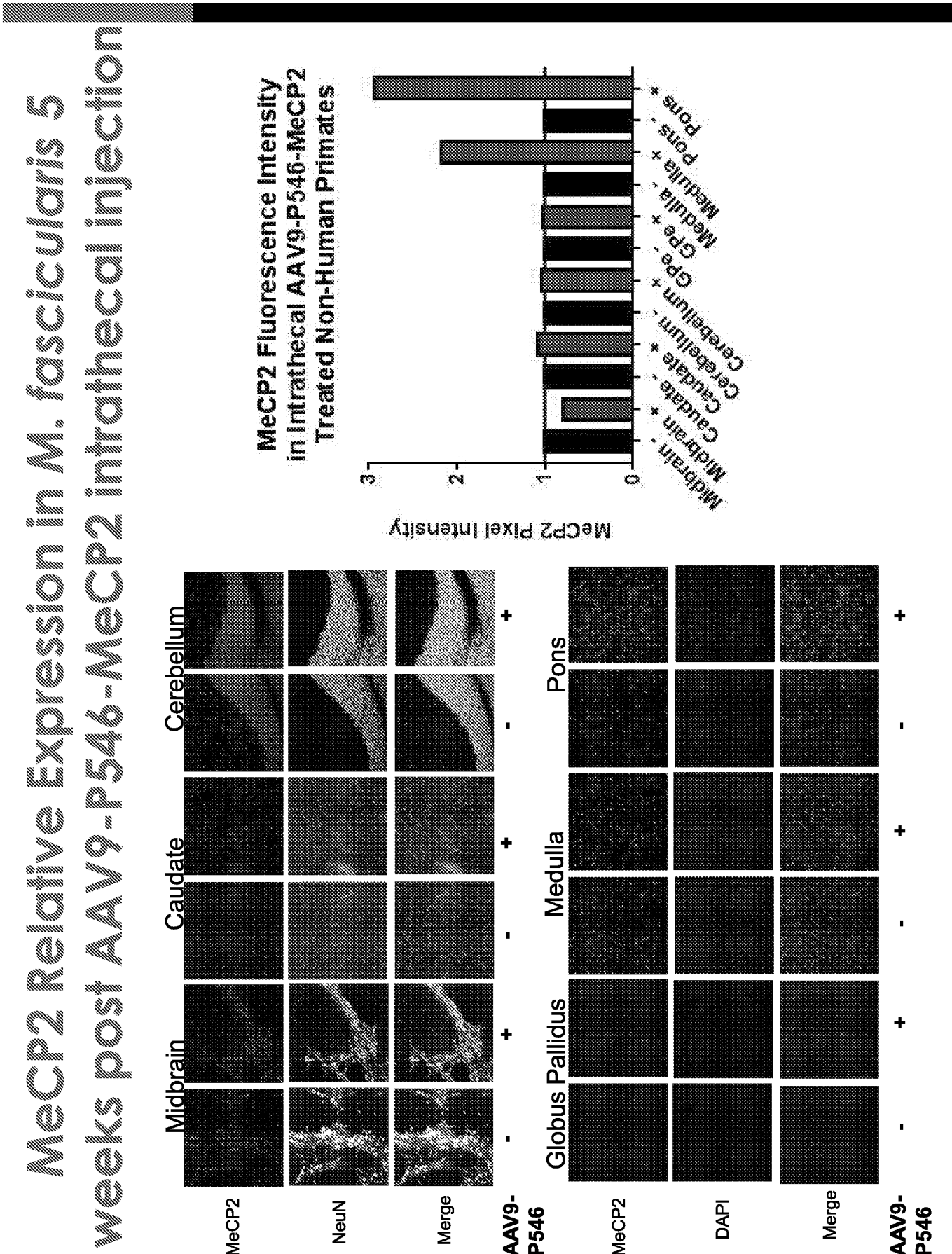
Take Home Message

Rett syndrome is reversible

AAV9-P546-MeCP2 vector allows for controlled MeCP2 expression in mouse and non-human primate CNS

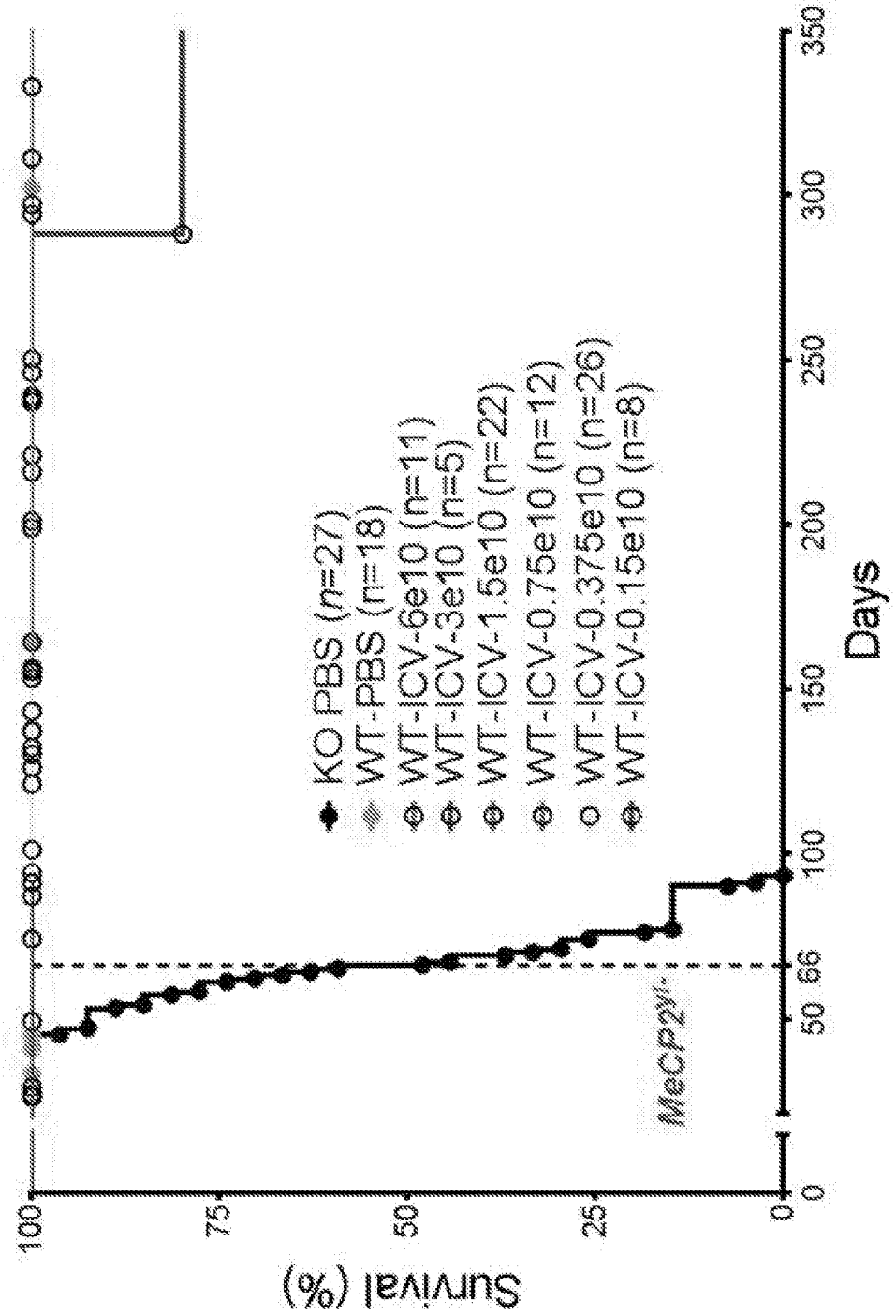
Strong improvement in behavioral phenotype and survival in MeCP2 null mice

Initial Nonhuman primate data shows doses used in previous clinical trials are likely safe

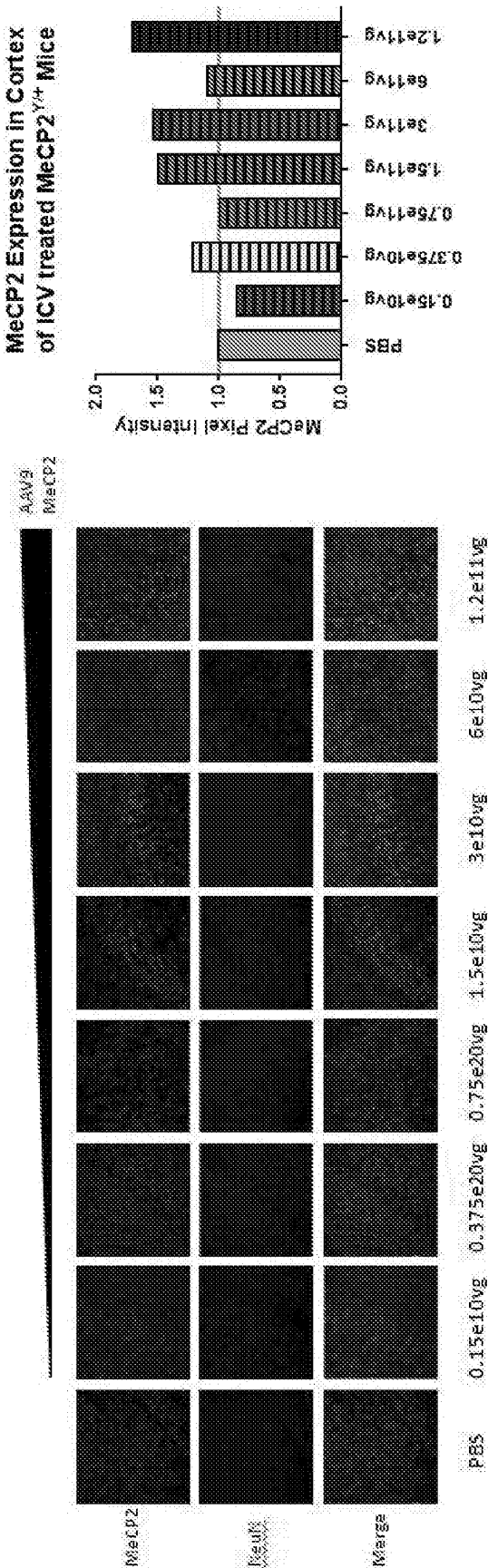


P546-MECP2 DELIVERED DIRECTLY TO THE CNS OF WILD TYPE MICE DOES NOT AFFECT SURVIVAL

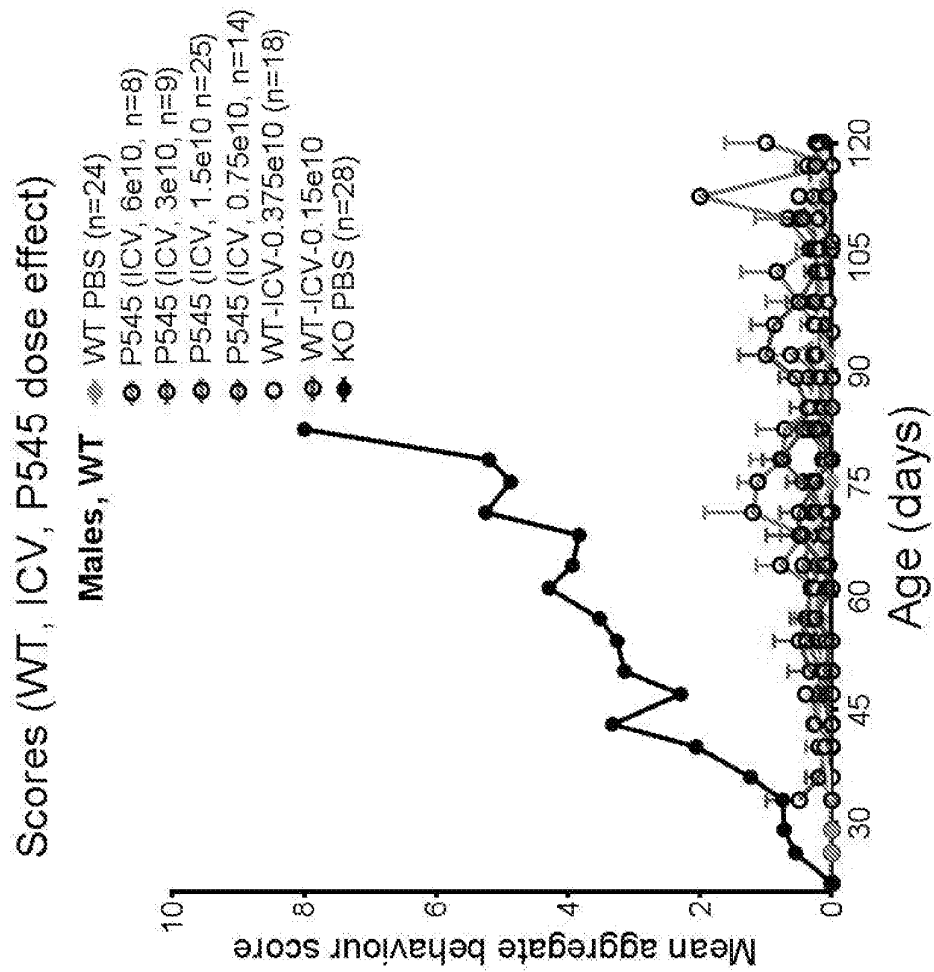
Intracerebroventricular, Newborn



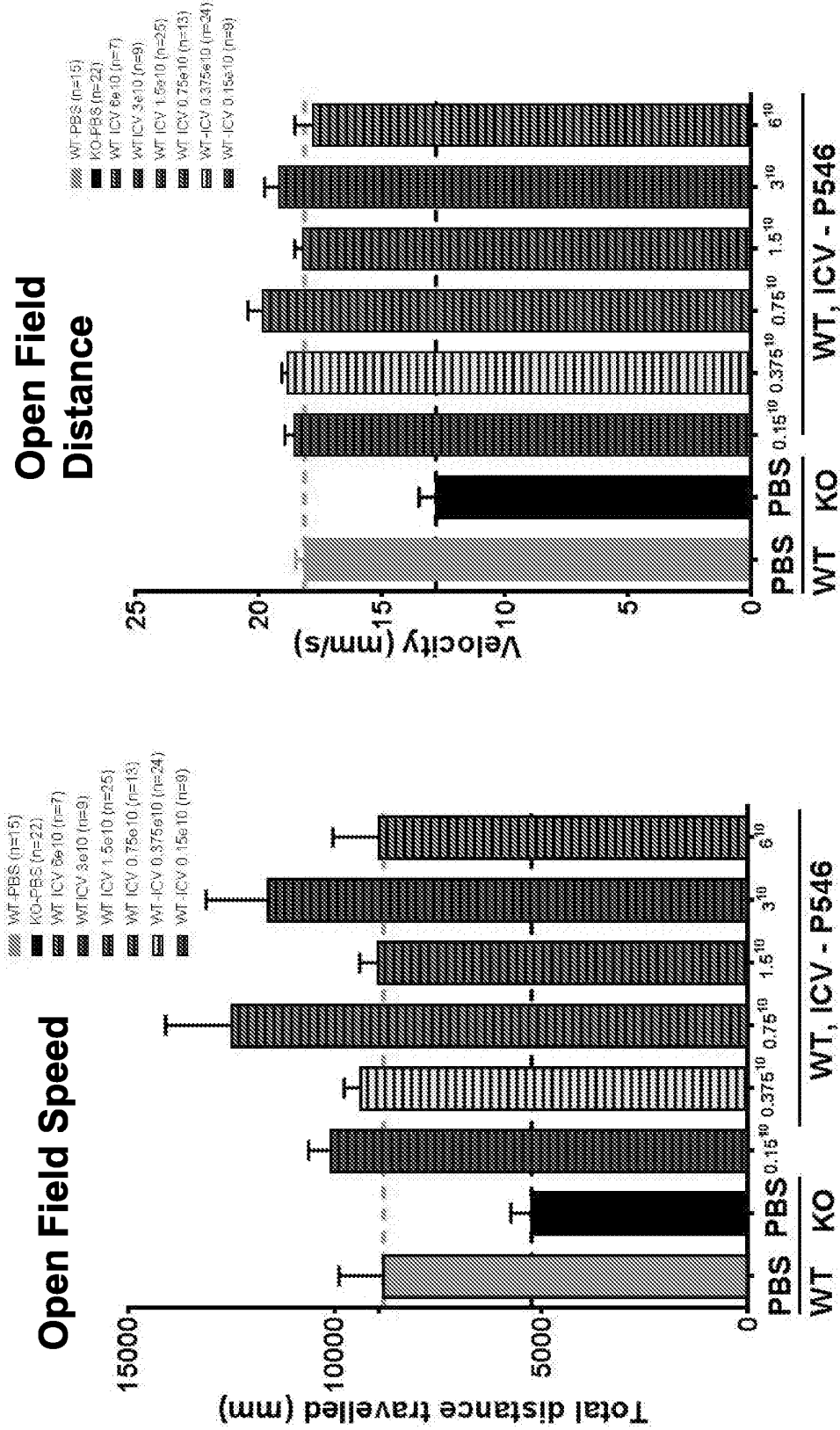
MeCP2 Immunostaining in Mouse Cortex (Move to end)



EFFECT OF P546-MECP2 DELIVERED DIRECTLY TO THE CNS OF MECP2^{Y/+} MICE ON BEHAVIORAL SCORES

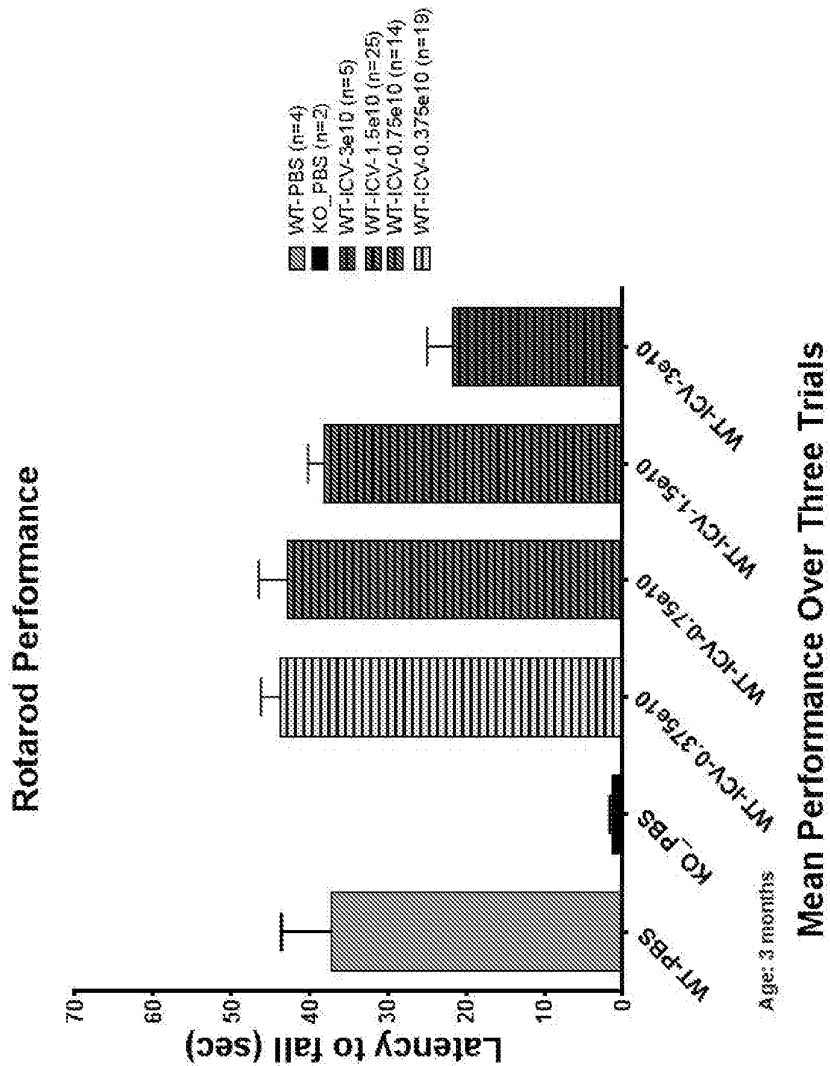


Effect of AAV9-P546-MeCP2 on Wild Type Mouse Open Field Activity



Effect of AAV9-P546-MeCP2 on Wild Type Mouse Rotarod Performance

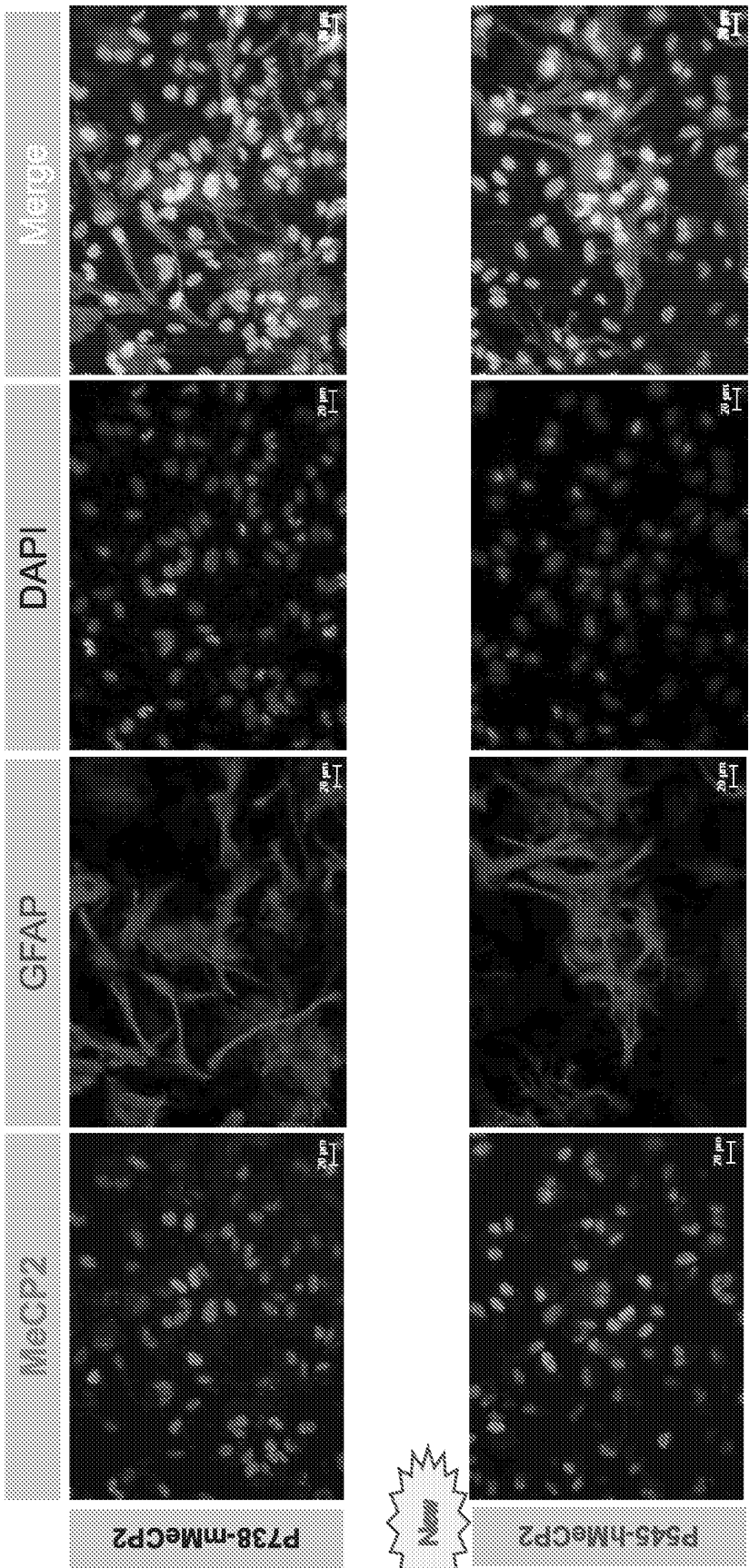
ICV Delivery Wild Type



J. Neurosci., 2013

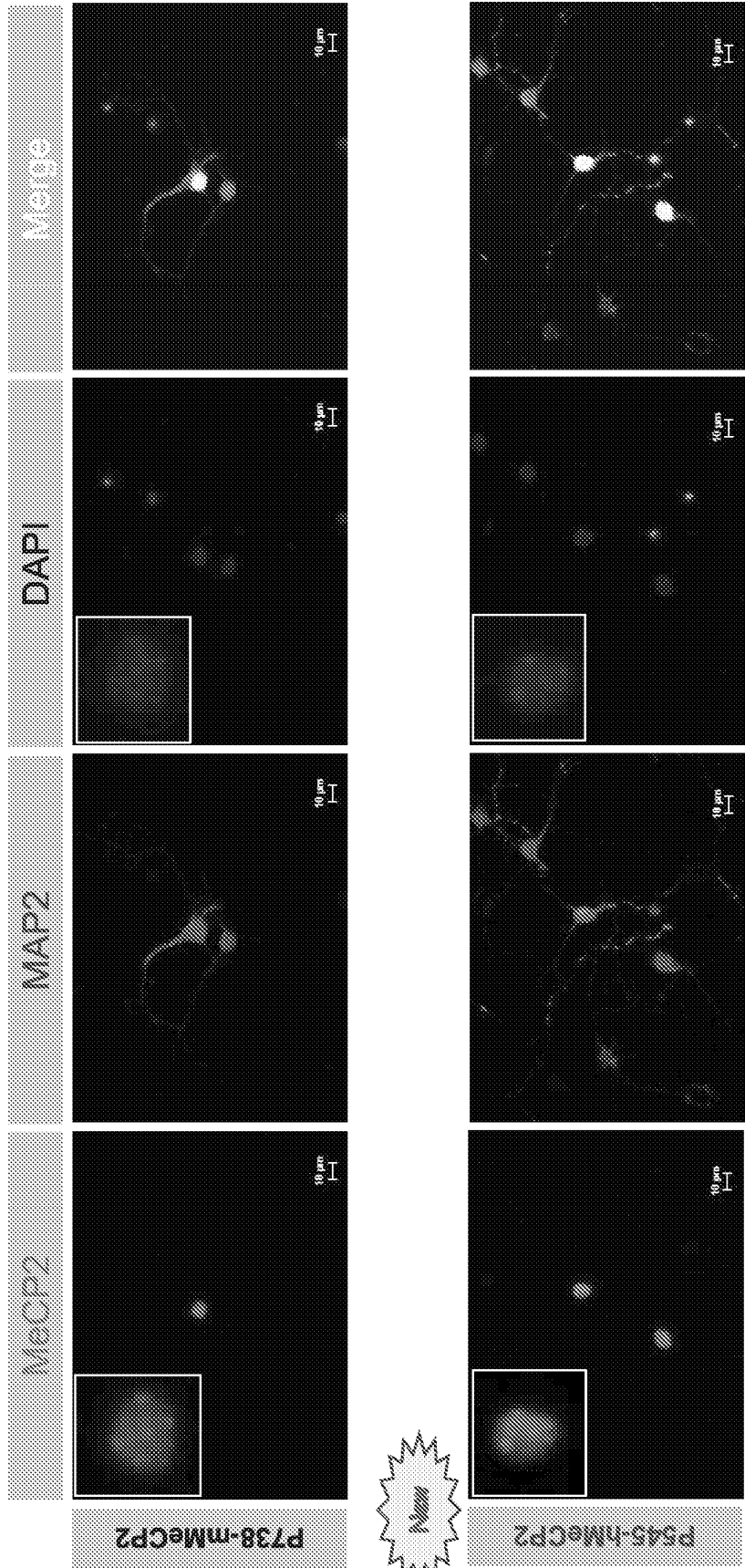


P545-MeCP2 results in robust expression in astrocytes



Non

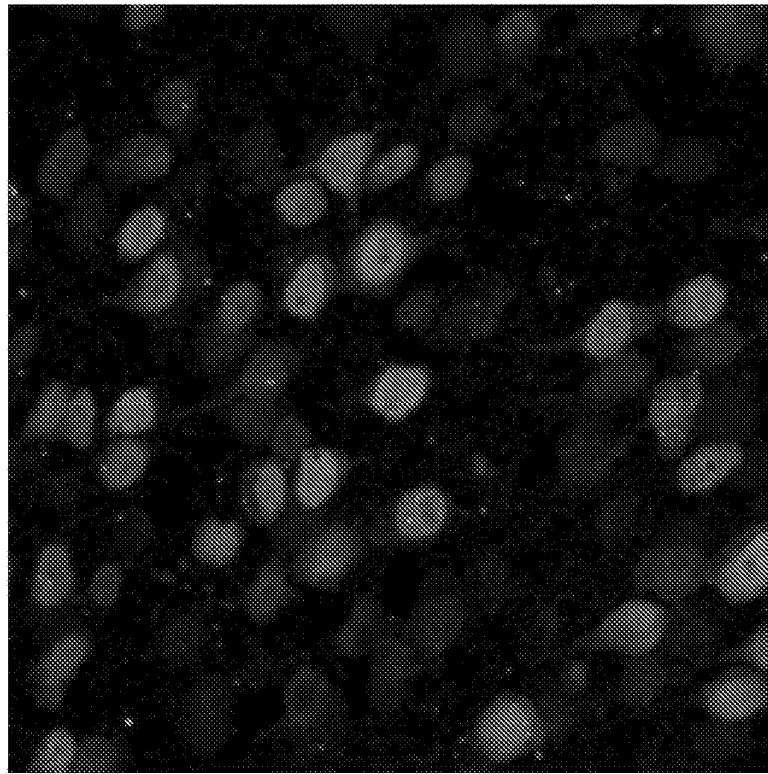
P545-MeCP2 results in robust expression in neurons



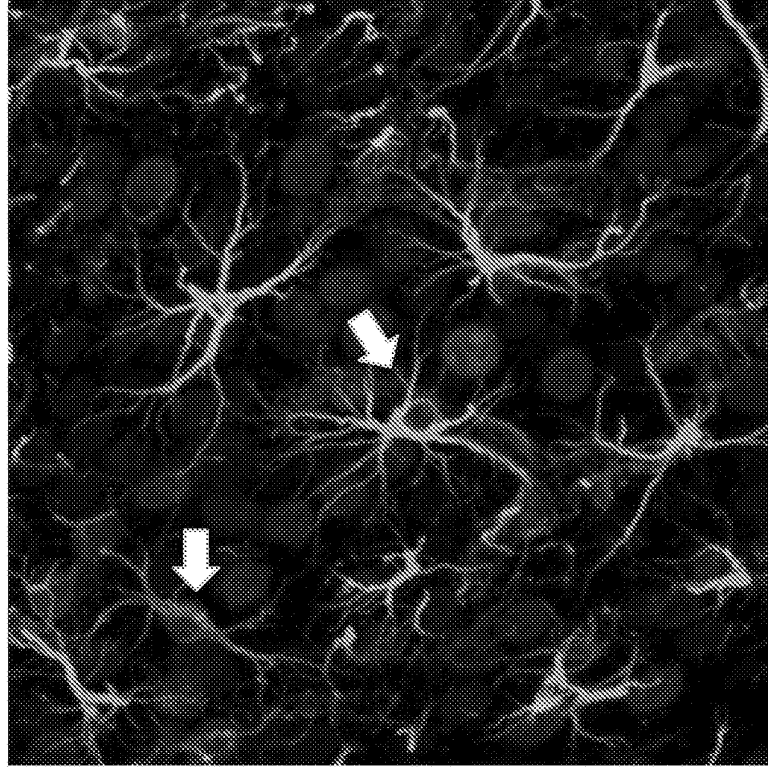
In vivo evaluation of the P545-MeCP2 gene
therapy vector
(AAV9-P545-hMeCP2)

AAV9-P545-MECP2 ALLOWS TARGETING OF BOTH NEURONS AND ASTROCYTES IN MOUSE BRAIN

MeCP2^{Y/-}

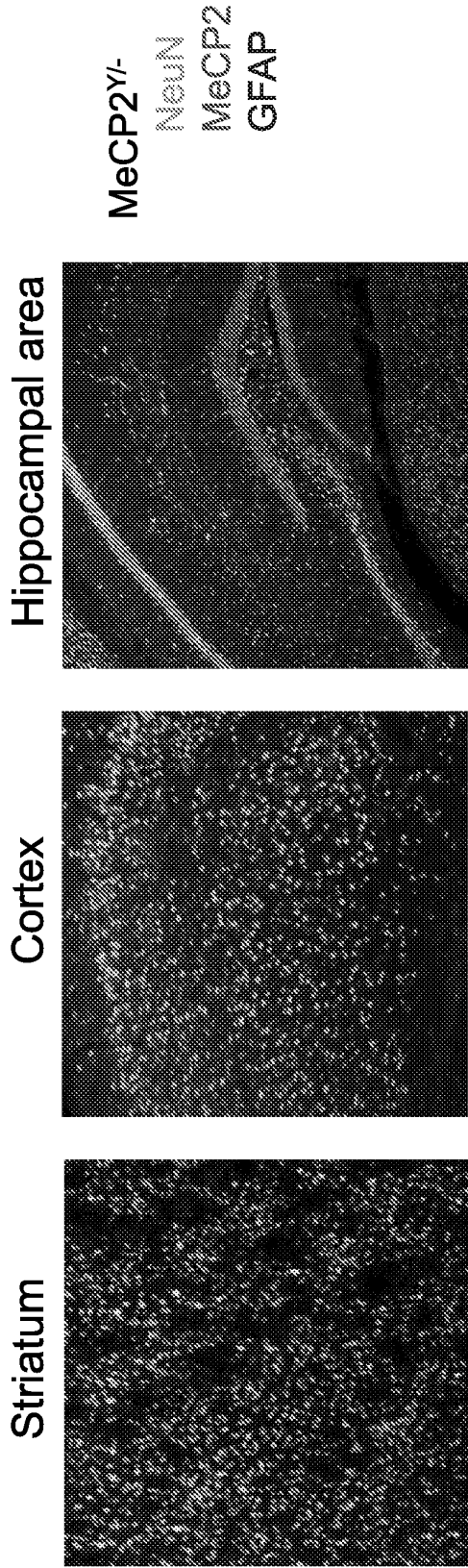


NeuN / MeCP2

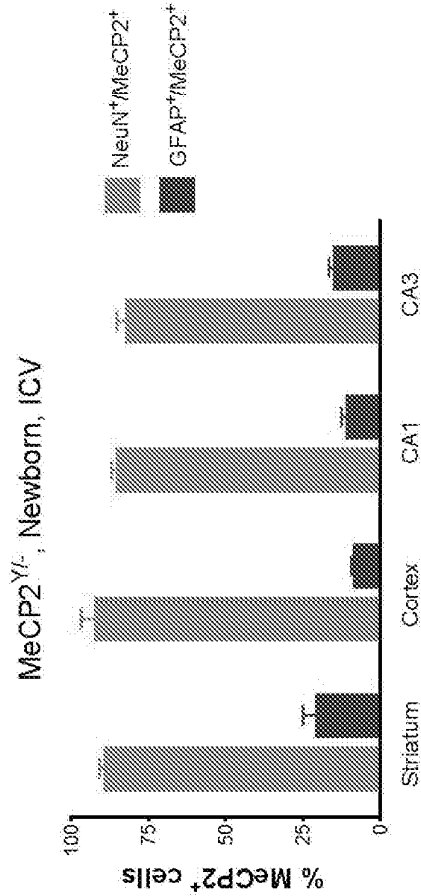


GFAP / MeCP2

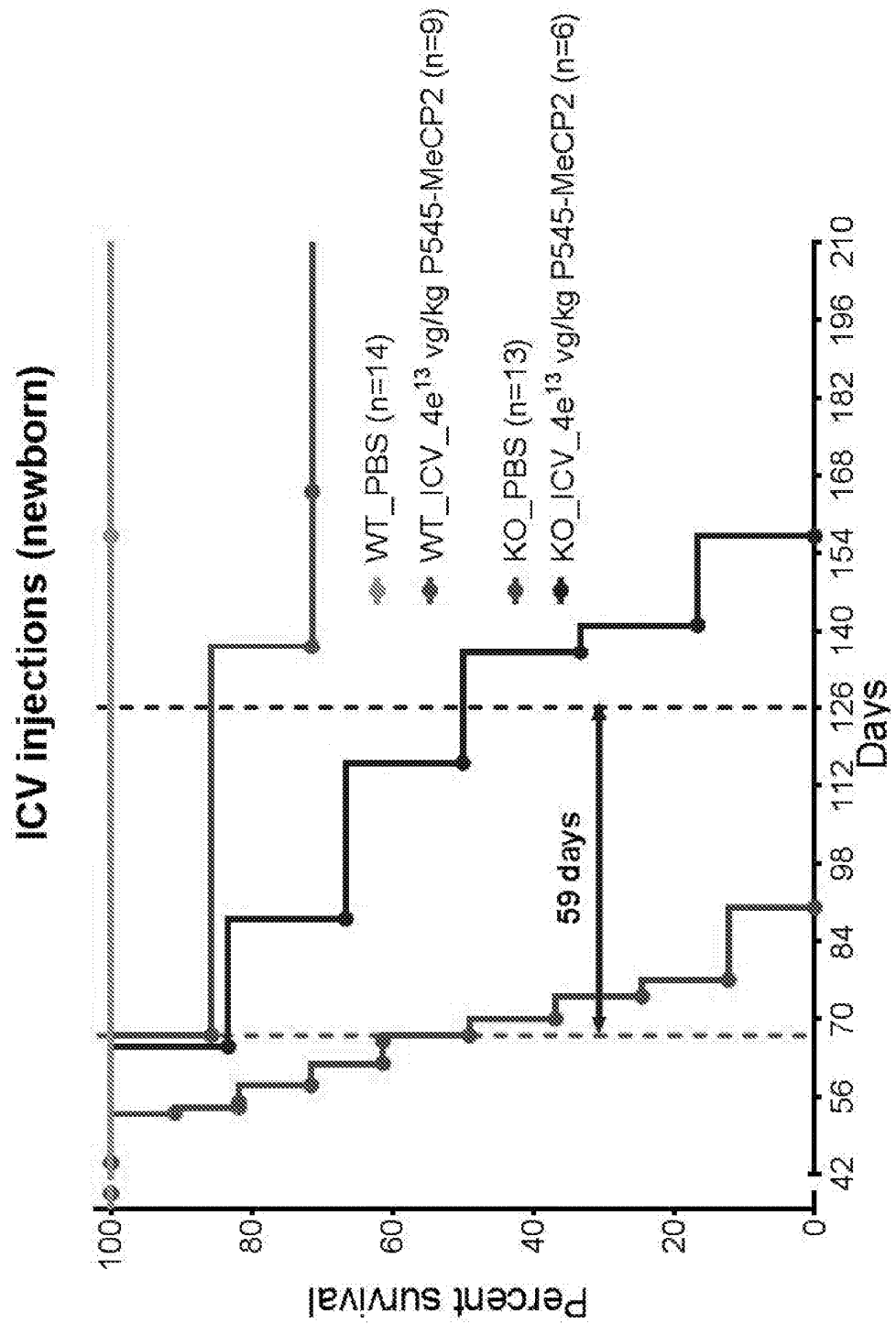
ICV injection of AAV9-P545_MeCP2 in newborn MeCP2^{Y/-} mice results in high transduction of neurons and astrocytes



AAV9-P545_MeCP2 ICV Injection, 88 days old



ICV INJECTIONS OF AAV9-P545-MECP2 GREATLY INCREASES THE LIFESPAN OF MECP2^{Y/-} MICE

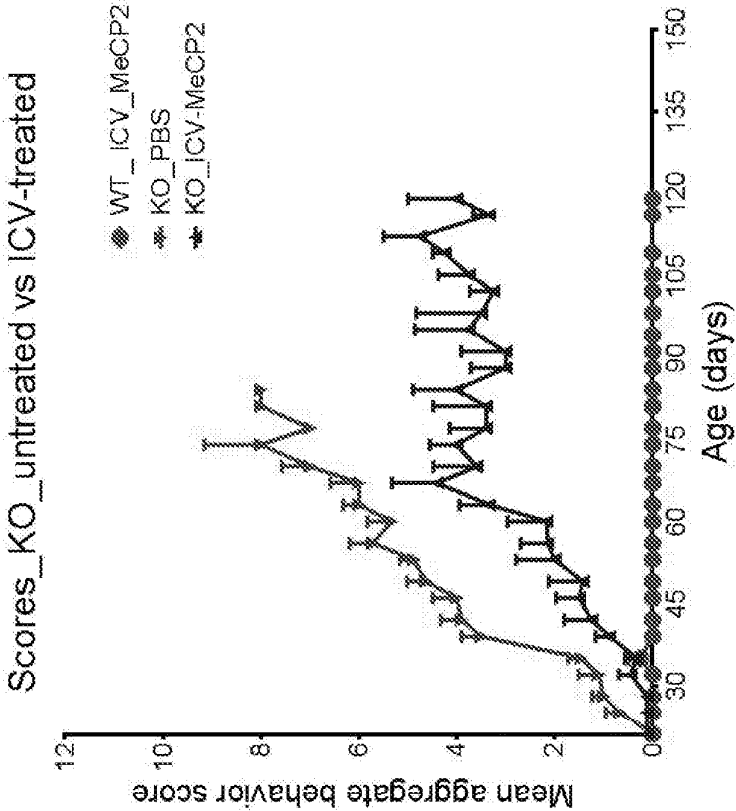


AAV9-P545-MECP2 IMPROVES BEHAVIOR SCORES OF MECP2^{Y/-} MICE AND DOES NOT ALTER BEHAVIOR IN TREATED WT MICE

Score criteria:

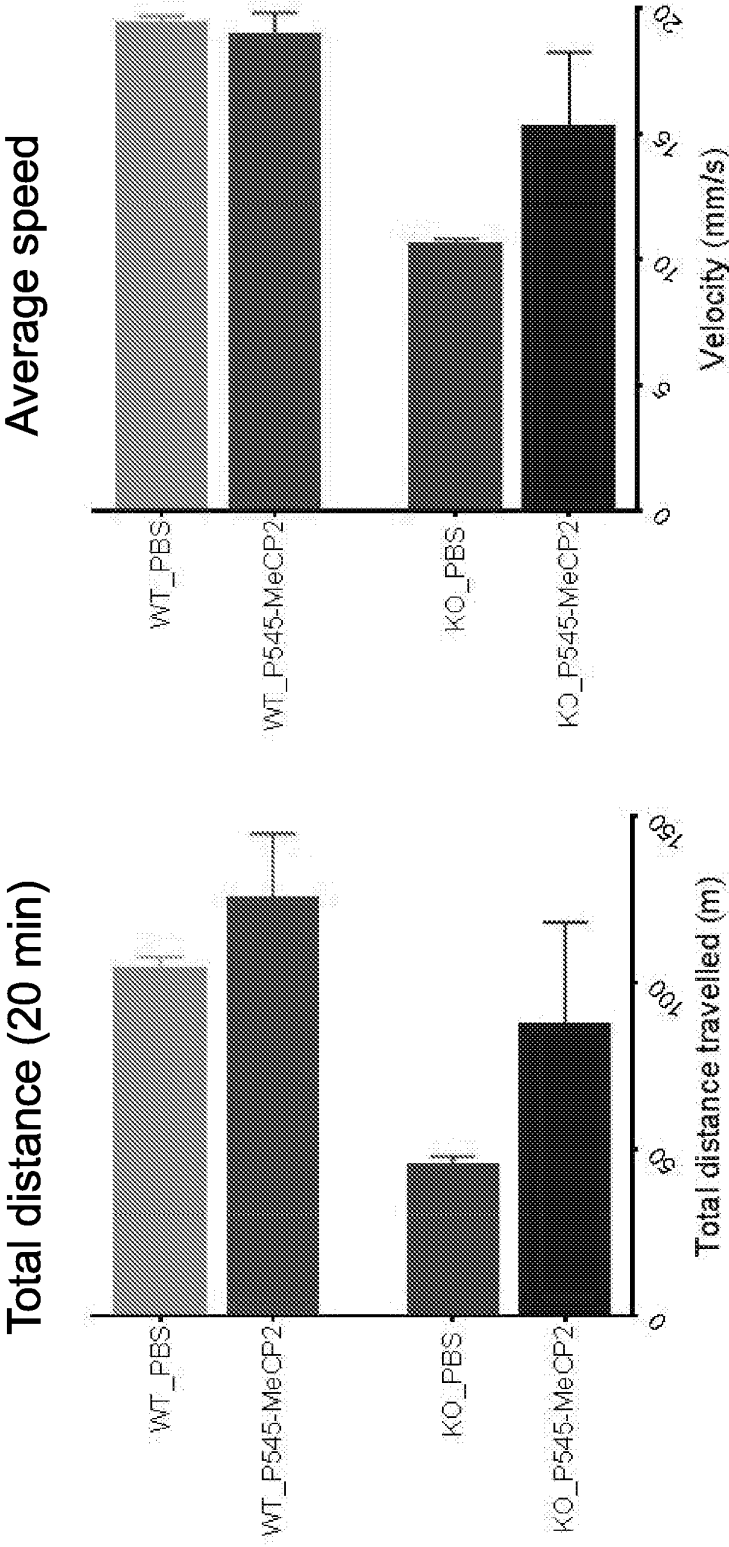
- 0 same as in the WT animal
- 1 where the symptom was present
- 2 when the symptom was severe

- Mobility
- Gait
- Hindlimb clasp
- Tremor
- Breathing
- General condition

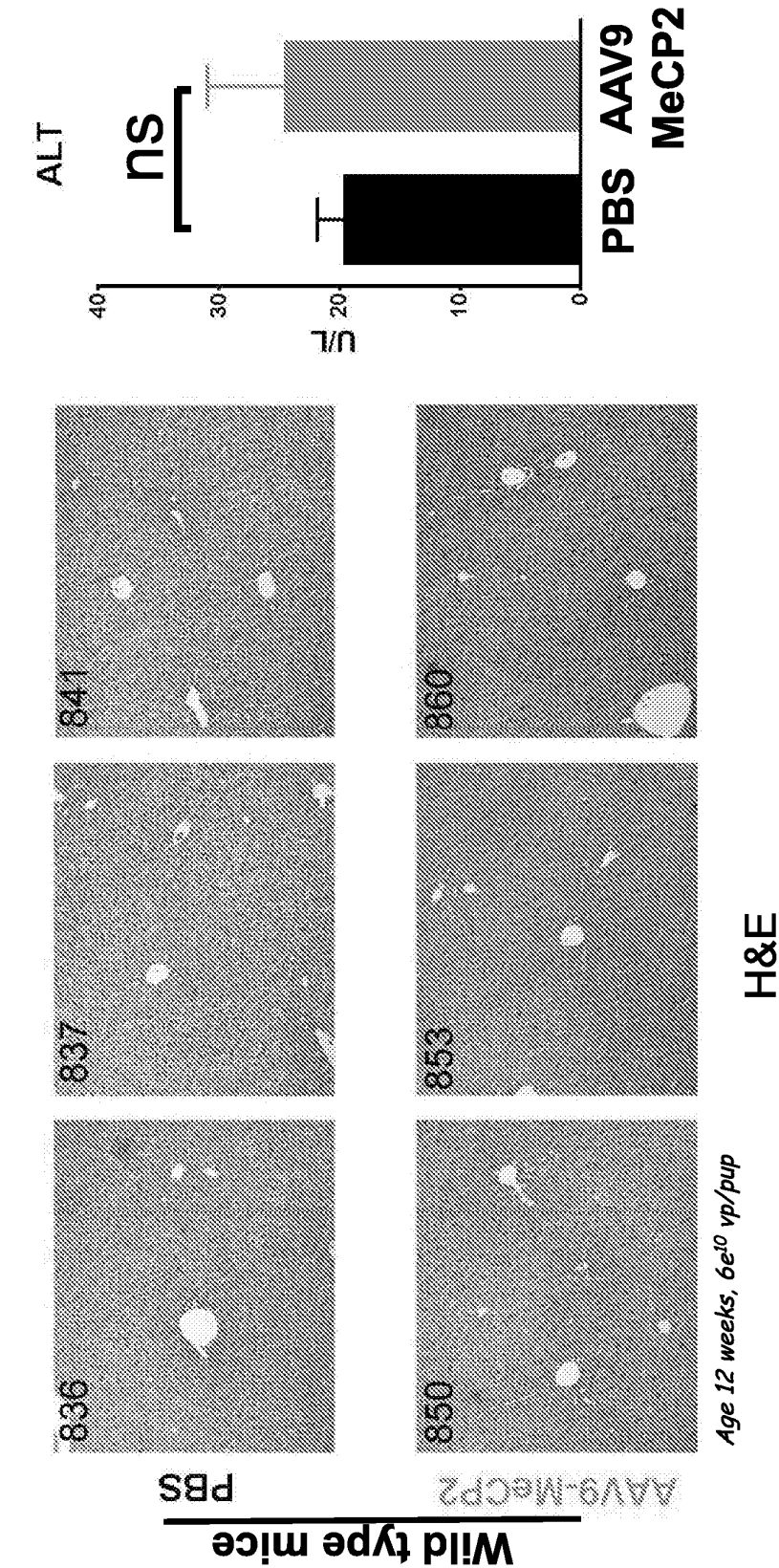


AAV9-P545-MECP2 IMPROVES PERFORMANCE OF MECP2^{Y/-} MICE

Open field analysis



ICV injection of AAV9-P545_MeCP2 in newborn wild type mice does not result in liver toxicity



SUMMARY OF RESULTS

AAV9-P545-MeCP2 CSF delivery in newborn mice

- In MeCP2 KO mice
 - Extends survival, doubling the lifespan of MeCP2 ^{y/-}
 - Improves behavior scores
 - Improves motor performance
- In WT mice
 - Minimal to no impact on survival
 - No "MeCP2" duplication-like symptoms
 - No evidence of liver toxicity to date

[0083] While the present invention has been described in terms of various embodiments and examples, it is understood that variations and improvements will occur to those skilled in the art. Therefore, only such limitations as appear in the claims should be placed on the invention.

[0084] All documents referred to herein are incorporated by reference in their entirety.

CLAIMS

1. A recombinant adeno-associated virus (rAAV) vector comprising a gene cassette comprising a promoter consisting of the polynucleotide sequence of SEQ ID NO: 2, an SV40 intron, a polynucleotide sequence encoding a methyl-CpG-binding protein 2 (MECP2) and a synthetic polyadenylation signal sequence, wherein the gene cassette is flanked by a mutant AAV2 inverted terminal repeat and a wild type inverted terminal repeat.
2. The rAAV vector of claim 1, wherein the rAAV vector is self-complementary (scAAV).
3. The rAAV vector of claim 1 or 2, wherein the rAAV is rAAV9.
4. The rAAV9 vector of claim 3, wherein the polynucleotide sequence encoding MECP2 comprises the polynucleotide sequence of SEQ ID NO: 3.
5. The rAAV vector of any one of claims 1-3, wherein the rAAV vector comprises nucleotides 151-2558 of SEQ ID NO: 1.
6. The rAAV vector of any one of claims 1-5, wherein the polyadenylation signal sequence comprises the polynucleotide sequence of SEQ ID NO: 4.
7. A virus comprising the rAAV vector of any one of claims 1-6.
8. The virus of claim 7, further comprising one or more capsid proteins.
9. A pharmaceutical composition comprising the rAAV vector of any one of claims 1-6 or the virus of any one of claims 7-8.
10. The pharmaceutical composition of claim 9, wherein the composition is formulated for intrathecal (IT) administration.
11. The pharmaceutical composition of any one of claims 9-10 for use in treating Rett syndrome.
12. The pharmaceutical composition according to claim 11, administered by intrathecal (IT) administration.
13. The pharmaceutical composition of claim 11 or 12, further comprising a non-ionic, low-osmolar contrast agent.
14. The pharmaceutical composition of claim 13, wherein the non-ionic, low-osmolar contrast agent comprises one or more of iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromide, ioversol, and ioxilan.
15. The pharmaceutical composition of claim 14, wherein the non-ionic, low-osmolar contrast agent comprises iohexol.

16. Use of an rAAV vector of any one of claims 1-6, the virus of any one of claims 7-8, or the pharmaceutical composition of any one of claims 9-10 for the preparation of medicament for treating Rett syndrome.
17. The use of claim 16, wherein the medicament is formulated for intrathecal (IT) administration.
18. The use of claim 16 or 17, wherein the medicament further comprises a non-ionic, low-osmolar contrast agent.
19. The use of claim 18, wherein the non-ionic, low-osmolar contrast agent comprises one or more of iobitridol, iohexol, iomeprol, iopamidol, iopentol, iopromide, ioversol, and ioxilan.
20. The use of claim 19, wherein the non-ionic, low-osmolar contrast agent comprises iohexol.
21. Use of an i) rAAV vector of any one of claims 1-6, the virus of any one of claims 7-8, or the pharmaceutical composition of any one of claims 9-10, and ii) a non-ionic, low-osmolar contrast agent in the manufacture of a medicament for combination therapy of Rett syndrome.