

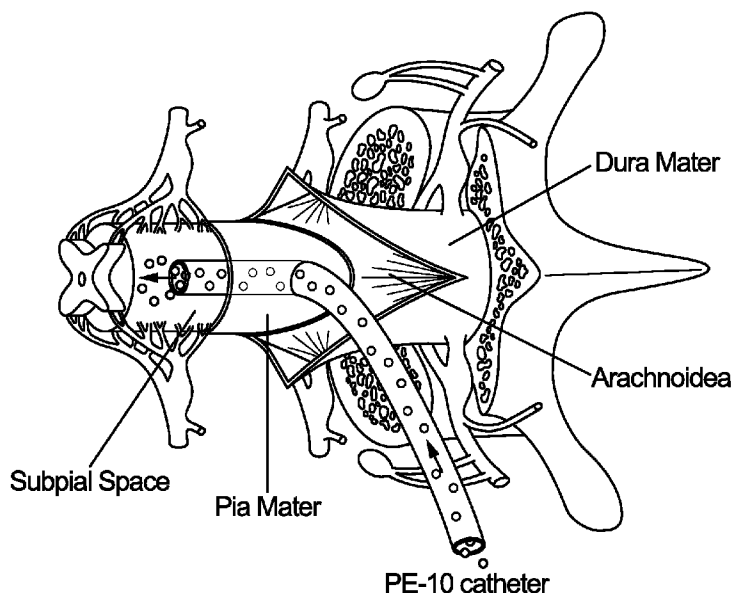


(86) Date de dépôt PCT/PCT Filing Date: 2015/12/15  
(87) Date publication PCT/PCT Publication Date: 2016/08/04  
(45) Date de délivrance/Issue Date: 2021/02/23  
(85) Entrée phase nationale/National Entry: 2017/07/31  
(86) N° demande PCT/PCT Application No.: US 2015/065704  
(87) N° publication PCT/PCT Publication No.: 2016/122791  
(30) Priorité/Priority: 2015/01/30 (US62/110,340)

(51) Cl.Int./Int.Cl. *A61K 31/7088* (2006.01),  
*A61B 17/34* (2006.01), *A61K 38/16* (2006.01),  
*A61M 25/01* (2006.01), *A61M 37/00* (2006.01),  
*A61P 25/28* (2006.01), *C12N 15/113* (2010.01),  
*C12N 15/87* (2006.01)  
(72) Inventeurs/Inventors:  
MARSALA, MARTIN, US;  
MIYANOHARA, ATSUSHI, US  
(73) Propriétaire/Owner:  
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA,  
US  
(74) Agent: OSLER, HOSKIN & HARCOURT LLP

(54) Titre : SYSTEME DE DELIVRANCE DE GENES SOUS-PIAL SPINAL

(54) Title: SPINAL SUBPIAL GENE DELIVERY SYSTEM



(57) Abrégé/Abstract:

Delivery devices, systems, and methods related thereto may be used in humans for spinal delivery of cells, drugs or vectors. Thus, the system enables subpial delivery, which leads to a near complete spinal parenchymal AAV9-mediated gene expression or ASO distribution in both white and grey matter.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(10) International Publication Number  
**WO 2016/122791 A1**

(43) International Publication Date  
4 August 2016 (04.08.2016)

**(51) International Patent Classification:**

*A61M 25/06* (2006.01) *C12N 15/113* (2010.01)  
*A61K 35/76* (2015.01)

**(21) International Application Number:**

PCT/US2015/065704

**(22) International Filing Date:**

15 December 2015 (15.12.2015)

**(25) Filing Language:**

English

**(26) Publication Language:**

English

**(30) Priority Data:**

62/110,340 30 January 2015 (30.01.2015) US

**(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, Fifth Floor, Oakland, California 94607-5200 (US).

**(72) Inventor: MARSALA, Martin;** 1440 Santa Marta Court, Solana Beach, California 92075 (US).

**(74) Agents: NOVOM, Antony et al.;** Wagenknecht Ip Law Group PC, 5677 Oberlin Dr., Ste. 204, San Diego, California 92121 (US).

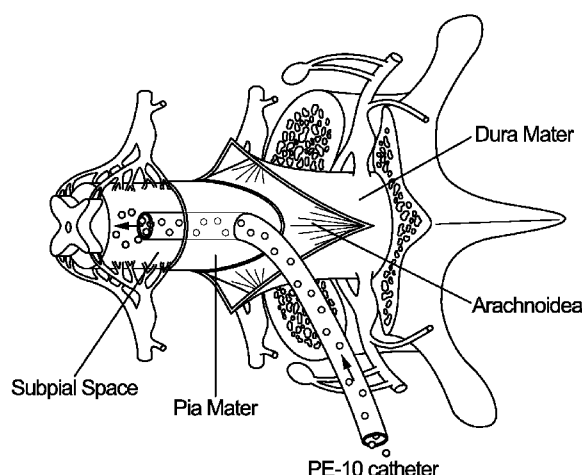
**(81) Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

**(84) Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report (Art. 21(3))

**(54) Title: SPINAL SUBPIAL GENE DELIVERY SYSTEM**

**FIG. 1A**

**(57) Abstract:** Delivery devices, systems, and methods related thereto may be used in humans for spinal delivery of cells, drugs or vectors. Thus, the system enables subpial delivery, which leads to a near complete spinal parenchymal AAV9-mediated gene expression or ASO distribution in both white and grey matter.

## **SPINAL SUBPIAL GENE DELIVERY SYSTEM**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of priority from U.S. Provisional Application No. 62/110,340, filed on January 30, 2015 in the United States Patent and Trademark Office.

### **BACKGROUND OF THE INVENTION**

#### **FIELD OF THE INVENTION**

[0002] The invention relates generally to gene therapy and more specifically to a method and system for delivery genes and oligonucleotides into the subpial space of a mammal to effect spinal trans-parenchymal infection thereof.

#### **BACKGROUND INFORMATION**

[0003] Currently used approaches to delivery vectors or antisense oligonucleotides (ASOs) into spinal parenchyma involve two techniques, each having a substantial limitation as compared to the present invention.

[0004] First, intrathecal delivery is used when vectors or ASO is injected into spinal intrathecal space (*i.e.*, outside of the pial membrane). Using this approach no deep parenchymal transgene expression is seen after AAV9 delivery. Only a subpopulation of A-motoneurons and primary afferents is infected due to the impermeability of the pial membrane to AAV9. While intrathecal delivery of ASO may lead to good penetration of ASO into spinal parenchyma, ASO is seen throughout the entire spinal cord (*i.e.*, from cervical to sacral segments). As such, no segment-restricted distribution of ASO can be achieved by intrathecal delivery.

[0005] Second, a direct spinal parenchymal injection may be used. By using this approach a segment-specific transgene expression or ASO distribution can be achieved in spinal parenchyma. However, a major limitation of this technique is its invasive nature because direct spinal parenchymal needle penetration is required.

[0006] Thus, a need exists for a subpial delivery system that provides near complete spinal parenchymal AAV9-mediated gene expression or ASO distribution in both white and grey matter.

#### SUMMARY OF THE INVENTION

[0007] Effective *in vivo* use of AAV-based vectors to achieve gene-specific silencing or upregulation in the central nervous system has been limited by the inability to provide more than limited deep parenchymal expression in adult animals using delivery routes with the most clinical relevance (*i.e.*, intravenous or intrathecal). Accordingly, the present invention demonstrates that the spinal pia membrane represents a primary barrier limiting effective AAV9 penetration into the spinal parenchyma after intrathecal AAV9 delivery. Thus, the present invention provides a method and system for delivery genes and oligonucleotides into spinal parenchyma of large animals and humans.

[0008] Accordingly, in one aspect, the invention provides a method of spinal trans-parenchymal infection of a nucleic acid molecule in a subject. The method includes administering a nucleic acid molecule to the subpial space of a subject. The subject may be a mammal, such as a human. In various embodiments, the step of administering includes exposing a spinal segment of a vertebra of the subject, creating a pial opening within the spinal segment, advancing a catheter through the pial opening and into subpial space, and delivering the nucleic acid molecule to the subpial space of the subject. The pial opening may be created by puncturing the pia with an L-shaped stainless steel tube and the catheter is advanced through the tube into the subpial space. In various embodiments, the nucleic acid molecule is administered in a mixture containing about 1-10% dextrose. In various embodiments, the nucleic acid molecule is a vector or an antisense oligonucleotide (ASO). The vector may be a lentiviral vector, adenoviral vector, or an adeno-associated vector, such as an AAV9 particle. In certain embodiments, the vector comprises a nucleic acid molecule encoding a protein or functional RNA that modulates or treats a neurodegenerative disorder, such as amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, Parkinson's disease.

**[0009]** In certain embodiments, the nucleic acid molecule is delivered as a single injection. In certain embodiments, the method further includes administering one or more second subpial injections of the nucleic acid molecule into a different spinal segment of the vertebra of the subject. In certain embodiments, the method further includes administering one or more intrathecal injections of the nucleic acid molecule to the subject.

**[0010]** In another aspect, the invention provides a gene delivery system. The system includes an L-shaped guide tube configured to puncture the pia of a subject, a catheter slidably disposed within the guide tube and configured to be advanced into subpial space of a spinal segment of a vertebra of the subject, and a reservoir in fluid communication with the catheter and containing a composition comprising a nucleic acid molecule. In various embodiments, the L-shaped guide tube may be a 16-26G stainless steel tube, and the catheter may be formed from polyethylene tubing, such as PE-5 or PE-10.

**[0011]** In another aspect, the invention provides a method of delivering a nucleic acid molecule to the subpial space of a subject. The method includes exposing a spinal segment of a vertebra of the subject, creating a pial opening within the spinal segment, positioning above the spinal segment the gene delivery system described herein, advancing the catheter through the pial opening and into subpial space, and delivering the nucleic acid molecule to the subpial space of the subject. In various embodiments, the nucleic acid molecule is delivered in a mixture containing about 1-10% dextrose. The nucleic acid molecule is a vector or an antisense oligonucleotide (ASO). The vector is a lentiviral vector, adenoviral vector, or an adeno-associated vector, such as an AAV9 particle. The subject may be a mammal, such as a human being.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0012]** Figures 1A-1J are pictorial diagrams showing subpial AAV9 delivery and macroscopically defined spinal cord surface transgene expression. Figure 1A shows a schematic diagram of a spinal cord, meninges and a subpially placed PE-10 catheter in fig. Figure 1B shows a catheter guiding tube (18G) with a sharp pia-penetrating tip (insert), which is used to penetrate the pia and to advance the PE-10 catheter into the subpial space. Figures 1C-1E are pictorial diagrams showing the progression of placement of the catheter

into the subpial space: the dura mater is first cut open (Figure 1C) and the catheter is advanced into the subpial space (Figures 1D and 1E). An air bubble that was injected into the subpial space can be seen (Figure 1D-asterisk). Figures 1F and 1G show surface GFP fluorescence densitometry showing an intense signal in both pig and rat spinal cords with the most intense GFP fluorescence seen at the epicenter of lumbar subpial injection. The presence of intense RFP fluorescence in the spinal cord parenchyma detected macroscopically in pig thoracic spinal cord (Figures 1H and 1J). A clear high level of RFP expression in ventral roots can also be seen (Figure 1H-insert). No fluorescence in the control non-injected spinal cord can be identified (Figure 1I).

**[0013]** Figures 2A-2D are pictorial diagrams showing insertion of the PE-10 catheter into the subpial space and GFP expression throughout the spinal parenchyma and in axons projecting distally from AAV9 injected segments.

**[0014]** Figures 3A-3G are pictorial diagrams showing effective parenchymal AAV9-mediated transgene expression after a single bolus subpial AAV9-UBI-RFP injection in an adult pig. Figures 3A and 3B show horizontal spinal cord sections taken from mid-thoracic spinal cord of a pig injected with AAV9-UBI-RGF six weeks previously. Intense RFP expression can be seen throughout the whole region including the white and gray matter. Staining with NeuN antibody (green) shows that virtually all neurons are also RFP positive. Figures 3C-3G show images of a transverse spinal cord section taken from the subpially-injected region showing transversally-cut RFP+ axons in the dorsal (DF) lateral (LF) and ventral (VF) funiculus (box inserts). RFP expression can also be seen in GFAP-stained astrocytes (insert; RFP/GFAP). High density RFP+ terminal boutons surrounding RFP-expressing  $\alpha$ -motoneuron (Figures 3D and 3E) and interneurons (Figures 3F and 3G) can be seen. (Scale bars: Figures 3A-3C = 500  $\mu$ m; Figures 3D, 3F = 30  $\mu$ m).

**[0015]** Figures 4A-4C are pictorial diagrams showing potent GFP expression in descending motor axons in lumbar spinal cord after mid-thoracic subpial AAV9 injection in a pig. Figures 4A and 4B show a transverse spinal cord section taken from the lumbar spinal cord after subpial AAV9-UBI-GFP injection into the mid-thoracic subpial space six weeks previously. Intense GFP expression in transversal cut axons in lateral (LF) and

ventral (VF) funiculus can be seen (white asterisks). A relatively lower density of GFP+ axons in the dorsal funiculus was identified (DF). Correspondingly, a high density of GFP+ motor axons projecting into the gray matter localized between the dorsal horn (DH) and ventral horn (VH) can also be seen. Figure 4C shows a higher resolution confocal image showing very fine arborization of GFP+ axons and terminal boutons in the central gray matter. (Scale bars: Figure 4A = 1000  $\mu$ m; Figure 4C = 30  $\mu$ m), (DH-dorsal horn, VH-ventral horn, DF-dorsal funiculus, LF-lateral funiculus, VF-ventral funiculus).

**[0016]** Figures 5A-5L are pictorial diagrams showing retrograde transport-mediated GFP expression in brain motor centers after subpial mid-thoracic AAV9 delivery in adult pig. Figures 5A-5E show retrogradely-labeled pyramidal neurons in the motor cortex of a pig at six weeks after a mid-thoracic, single AAV9-UBI-GFP injection. Figures 5F-5J show a comparable level of GFP expression in neurons localized in the brain stem. Figure 5K shows the presence of large retrogradely-labeled motor GFP+ axons in the medulla oblongata (medullary pyramids). Figure 5L shows a high density of anterogradely-labeled sensory afferents in formatio reticularis. (Scale bars: Figures 5A-5E and 5G-5J = 50  $\mu$ m; Figures 5K and 5L = 50  $\mu$ m).

**[0017]** Figures 6A-6G are pictorial diagrams showing retrograde-transport-mediated GFP expression in brain motor centers after subpial cervical AAV9 delivery in adult rat. Figures 6A-6D show bilateral retrogradely-GFP-labeled pyramidal neurons in the motor cortex of rat at eight weeks after upper-cervical single AAV9-UBI-GFP injection. Figures 6E-6G show bilateral neuronal GFP expression in the nucleus ruber. (Scale bars: Figures 6A, 6B, 6E, and 6F = 50  $\mu$ m; Figures 6C and 6D = 50  $\mu$ m; Figure 6G = 20  $\mu$ m).

**[0018]** Figures 7A-7G are pictorial diagrams showing differential regional spinal transgene expression after intrathecal AAV9-UBI-GFP vs. subpial AAV9-UBI-RFP delivery in a rat. Figure 7A shows that lumbar intrathecal injection of AAV9-UBI-GFP led to the preferential GFP expression in the dorsal funiculus (DF), dorsal root (DR) and ventral root entry zone (white box insert No. 2). Subpial AAV9-UBI-RFP injection into the upper cervical spinal cord led to clear descending motor tracts labeling. Figure 7B shows expression of GFP in dorsal root ganglion cells (L4) after lumbar intrathecal AAV9-UBI-

GFP injection. Figures 7C and 7D show that intense GFP expression after intrathecal AAV9-UBI-GFP injection is seen in the dorsal root (DR) and in primary afferents boutons in the deeper dorsal horn (white asterisk), but no expression in dorsal horn NeuN+ neurons can be identified. Figure 7E shows that no co-localization of GFP and RFP in dorsal funiculus (DF) can be seen (white insert from Figure 7A, No. 1). Figure 7F shows GFP expression in glial cells localized in the ventral root entry zone resulting from intrathecal AAV9-UBI-GFP injection (white insert from Figure 7A, No. 2). Figure 7G shows that some retrogradely-labeled GFP expressing  $\alpha$ -motoneurons surrounded by GFP+ primary Ia afferents can be seen in animals injected with AAV9-UBI-GFP. (Scale bars: Figure 7A = 500  $\mu$ m; Figures 7B-7G = 30  $\mu$ m), (DR-dorsal root, DH-dorsal horn, VH-ventral horn, DF-dorsal funiculus).

**[0019]** Figure 8 is a pictorial schematic diagram showing subpial AAV9 delivery and resulting transgene (GFP) expression throughout the CNS after a single subpial AAV9-UBI-GFP injection. The AAV9-UBI-GFP virus is delivered into the subpial space using PE-10 catheter in an adult pig. Subpial delivery of AAV9-UBI-GFP leads to a diffusion and resulting uptake of virus into segmental neurons (*i.e.*, interneurons and  $\alpha$ -motoneurons) and ascending and descending axons which are trans-passing through the subpially-injected segments. Resulting transgene expression is then seen in: i) segmental neurons, ii) dorsal root ganglion cells (retrograde infection), iii) motor axons innervating skeletal muscles (anterograde infection), iv) pyramidal neurons in motor cortex (retrograde infection), and v) brain terminals of spinothalamic neurons (anterograde infection).

**[0020]** Figures 9A-9F are pictorial diagrams showing effective parenchymal AAV9-mediated transgene expression after single bolus lumbar subpial AAV9-UBI-GFP injection in adult rat. Figures 9A-9D show widespread GFP expression in neurons and white matter tracts in lower thoracic and upper lumbar spinal cord after L1 subpial AAV9-UBI-GFP injection. Virtually all neurons in the horizontally cut section (Figure 9A) show GFP expression. In transversally cut sections GFP+ neurons can be seen throughout the whole gray matter between laminae I-IX (Figures 9B-9D). Numerous NeuN-stained neurons expressing GFP in the superficial dorsal horn (Laminae I-III) and in the ventral horn can be seen. Figures 9E and 9F show a high density of GFP+ descending motor fibers in the



lumbar spinal cord after upper cervical AAV9-UBI-GFP injection. Figure 9F shows a high density confocal image depicting easily recognizable GFP+ terminal boutons in the gray matter. (Scale bars: Figure 9A = 1000  $\mu$ m; Figures 9B-9D = 30  $\mu$ m; Figure 9E = 50  $\mu$ m; Figure 9F = 100  $\mu$ m), (WM-white matter, GM-gray matter, DH-dorsal horn, VH-ventral horn, DF-dorsal funiculus).

### DETAILED DESCRIPTION OF THE INVENTION

[0021] The present invention provides a method and system for delivery genes and oligonucleotides into spinal parenchyma of large animals and humans.

[0022] Before the present compositions and methods are described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0023] As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, references to “the method” includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0024] The term “comprising,” which is used interchangeably with “including,” “containing,” or “characterized by,” is inclusive or open-ended language and does not exclude additional, unrecited elements or method steps. The phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. The present disclosure contemplates embodiments of the invention compositions and methods corresponding to the scope of each of these phrases. Thus, a composition or method

comprising recited elements or steps contemplates particular embodiments in which the composition or method consists essentially of or consists of those elements or steps.

**[0025]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

**[0026]** As used herein, the term “pia mater” refers to the innermost layer of the meninges, the membranes surrounding the brain and spinal cord (Figure 1A). Pia mater is a thin fibrous tissue that is impermeable to fluid. This allows the pia mater to enclose cerebrospinal fluid. By containing this fluid the pia mater works with the other meningeal layers to protect and cushion the brain. Spinal pia mater encloses the surface of the medulla spinalis, or spinal cord, and is attached to it through a connection to the anterior fissure. Accordingly, the term “subpial” refers to being situated or occurring beneath the pia mater.

**[0027]** As used herein, the term “parenchyma” refers to the functional tissue of an organ as distinguished from the connective and supporting tissue. Thus, the term “spinal parenchyma” refers to the various known anatomical tissues of the spinal cord, including, but not limited to the grey matter, white matter, the dura mater, arachnoid mater, pia mater, posterior and anterior funiculi, posterior and anterior spinocerebellar tracts, etc.

**[0028]** The term “subject” as used herein refers to any individual or patient to which the subject methods are performed. Generally the subject is human, although as will be appreciated by those in the art, the subject may be an animal. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, etc., and primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of subject.

**[0029]** As used herein, “treatment” refers to a clinical intervention made in response to a disease, disorder or physiological condition manifested by a patient/subject or to which a

patient/subject may be susceptible. The aim of treatment includes, but is not limited to, the alleviation or prevention of symptoms, slowing or stopping the progression or worsening of a disease, disorder, or condition and/or the remission of the disease, disorder or condition. “Treatments” refer to one or both of therapeutic treatment and prophylactic or preventative measures. Subjects in need of treatment include those already affected by a disease or disorder or undesired physiological condition as well as those in which the disease or disorder or undesired physiological condition is to be prevented.

**[0030]** As used herein, the terms “nucleic acid” and “polynucleotide” are interchangeable and refer to any nucleic acid, whether composed of phosphodiester linkages or modified linkages such as phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphoramidate, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate or sultone linkages, and combinations of such linkages. The terms “nucleic acid” and “polynucleotide” also specifically include nucleic acids composed of bases other than the five biologically occurring bases (adenine, guanine, thymine, cytosine and uracil).

**[0031]** The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

**[0032]** The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline,  $\alpha$ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an  $\alpha$  carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*,

homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

**[0033]** Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

**[0034]** “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

**[0035]** As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino

acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

**[0036]** A “polynucleotide” is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5’ to the 3’ end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated “bp”), nucleotides (“nt”), or kilobases (“kb”). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term “base pairs”. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired.

**[0037]** The term “gene” is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, “gene” refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory sequences. “Genes” also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. “Genes” can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. An “allele” is one of several alternative forms of a gene occupying a given locus on a chromosome.

**[0038]** As used herein, a “vector” is capable of transferring gene sequences to target cells. Typically, “vector construct,” “expression vector,” and “gene transfer vector,” mean

any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

**[0039]** Viral vectors can be particularly useful for introducing a polynucleotide useful in performing a method of the invention into a target cell. Viral vectors have been developed for use in particular host systems, particularly mammalian systems and include, for example, retroviral vectors, other lentivirus vectors such as those based on the human immunodeficiency virus (HIV), adenovirus vectors (AV), adeno-associated virus vectors (AAV), herpes virus vectors, vaccinia virus vectors, and the like (see Miller and Rosman, *BioTechniques* 7:980-990, 1992; Anderson *et al.*, *Nature* 392:25-30 *Suppl.*, 1998; Verma and Somia, *Nature* 389:239-242, 1997; Wilson, *New Engl. J. Med.* 334:1185-1187 (1996)). In one aspect of the invention, a lentivirus or an adenovirus vector is utilized. Adenoviruses are double-stranded DNA viruses, where both strands of DNA encode genes. The genome encodes about thirty proteins. In another aspect of the invention, an adeno-associated virus vector is utilized.

**[0040]** The term "adenovirus" refers to over 40 adenoviral subtypes isolated from humans, and as many from other mammals and birds. See, Strauss, "Adenovirus infections in humans," in *The Adenoviruses*, Ginsberg, ed., Plenum Press, New York, N.Y., pp. 451-596 (1984). Recombinant adenovirus vectors, such as those based on the human adenovirus 5 (as described by McGrory W J, et al., *Virology* 163: 614-617, 1988) are missing essential early genes from the adenovirus genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products in trans. In place of the missing adenovirus genomic sequences, a transgene of interest can be cloned and expressed in tissue/cells infected with the replication-defective adenovirus. Although adenovirus-based gene transfer does not generally result in stable integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), adenovirus vectors can be propagated in high titer and transfect non-replicating cells; and, although the transgene is not passed to daughter cells, this is suitable for gene transfer to adult cardiac myocytes, which do not actively divide. Retrovirus vectors provide stable gene transfer, and high titers are now

obtainable via retrovirus pseudotyping (Burns, et al., *Proc. Natl. Acad. Sci. (USA)* 90: 8033-8037, 1993), but current retrovirus vectors are generally unable to efficiently transduce nonreplicating cells

**[0041]** Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M. S., *Adenoviridae and Their Replication*, in Fields, B., et al. (eds.) *Virology*, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham, F., et al., pp. 109-128 in *Methods in Molecular Biology*, Vol. 7: Gene Transfer and Expression Protocols, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller, N., et al., *FASEB Journal* 9: 190-199, 1995; Schreier, H., *Pharmaceutica Acta Helvetiae* 68: 145-159, 1994; Schneider and French, *Circulation* 88:1937-1942, 1993; Curiel D. T., et al., *Human Gene Therapy* 3: 147-154, 1992; Graham, F. L., et al., WO 95/00655 (5 Jan. 1995); Falck-Pedersen, E. S., WO 95/16772 (22 Jun. 1995); Deneffe, P. et al., WO 95/23867 (8 Sep. 1995); Haddada, H. et al., WO 94/26914 (24 Nov. 1994); Perricaudet, M. et al., WO 95/02697 (26 Jan. 1995); Zhang, W., et al., WO 95/25071 (12 Oct. 1995). A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996).

**[0042]** An adeno-associated virus (AAV) is a small (26 nm) replication-defective, nonenveloped virus that depends on the presence of a second virus, such as adenovirus or herpes virus, for its growth in cells. AAV is not known to cause disease and induces a very mild immune response. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Aspects of the invention provide methods for delivering a transgene to the spinal tissue in a subject using recombinant AAV-based gene transfer.

**[0043]** Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter, B., *Handbook of Parvoviruses*, vol. I, pp. 169-228, 1990; Berns, *Virology*, pp. 1743-1764 (Raven Press 1990); Carter, B., *Curr. Opin. Biotechnol.*, 3: 533-539, 1992; Muzyczka, N., *Current Topics in Microbiology and Immunology*, 158: 92-129, 1992; Flotte, T. R., et al., *Am. J. Respir.*

*Cell Mol. Biol.* 7:349-356, 1992; Chatterjee et al., *Ann. NY Acad. Sci.*, 770: 79-90, 1995; Flotte, T. R., et al., WO 95/13365 (18 May 1995); Trempe, J. P., et al., WO 95/13392 (18 May 1995); Kotin, R., *Human Gene Therapy*, 5: 793-801, 1994; Flotte, T. R., et al., *Gene Therapy* 2:357-362, 1995; Allen, J. M., WO 96/17947 (13 Jun. 1996); and Du et al., *Gene Therapy* 3: 254-261, 1996. See also, US Pat. No. 8,865,881.

[0044] An "effective amount" of an AAV is an amount sufficient to infect a sufficient number of cells of a target tissue in a subject. An effective amount of an AAV may be an amount sufficient to have a therapeutic benefit in a subject, *e.g.*, to extend the lifespan of a subject, to improve in the subject one or more symptoms of disease, *e.g.*, a symptom of a neurodegenerative disease. The effective amount may depend on a variety of factors such as, for example, the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among subject and tissue. An effective amount may also depend on the mode of administration. For example, targeting a CNS tissue by intravascular injection may require different (*e.g.*, higher) doses, in some cases, than targeting CNS tissue by intrathecal or intracerebral injection. In some cases, multiple doses of an AAV are administered. An effective amount may also depend on the particular AAV used. For example, dosages for targeting a CNS tissue may depend on the serotype (*e.g.*, the capsid protein) of the AAV. For example, the AAV may have a capsid protein of an AAV serotype selected from the group consisting of: AAV1, AAV2, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh.10, rh.39, rh.43 and CSp3. In certain embodiments, the effective amount of AAV is  $10^{10}$ ,  $10^{11}$ ,  $10^{12}$ ,  $10^{13}$ , or  $10^{14}$  genome copies per kg. In certain embodiments, the effective amount of AAV is  $10^{10}$ ,  $10^{11}$ ,  $10^{12}$ ,  $10^{13}$ ,  $10^{14}$ , or  $10^{15}$  genome copies per subject.

[0045] Depending on the host cell/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, and the like can be used in the expression vector (Bitter *et al.*, *Meth. Enzymol.* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like can be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells, for example, a human or



mouse metallothionein promoter, or from mammalian viruses, for example, a retrovirus long terminal repeat, an adenovirus late promoter or a vaccinia virus 7.5K promoter, can be used. Promoters produced by recombinant DNA or synthetic techniques can also be used to provide for transcription of the inserted GDF receptors coding sequence.

**[0046]** As used herein a “reporter gene” or “reporter sequence” refers to any sequence that produces a protein product that is easily measured, preferably although not necessarily in a routine assay. Suitable reporter genes include, but are not limited to, sequences encoding proteins that mediate antibiotic resistance (*e.g.*, ampicillin resistance, neomycin resistance, G418 resistance, puromycin resistance), sequences encoding colored or fluorescent or luminescent proteins (*e.g.*, green fluorescent protein (GFP), enhanced green fluorescent protein, red fluorescent protein (RFP), luciferase), and proteins which mediate enhanced cell growth and/or gene amplification (*e.g.*, dihydrofolate reductase). Epitope tags include, for example, one or more copies of FLAG, His, myc, Tap, HA or any detectable amino acid sequence. “Expression tags” include sequences that encode reporters that may be operably linked to a desired gene sequence in order to monitor expression of the gene of interest.

**[0047]** As used herein, the terms “transformed” or “transfected” are used interchangeably and refer to the process by which exogenous DNA or RNA is transferred or introduced into an appropriate host cell. Additionally, nucleic acids encoding other heterologous proteins may be introduced into the host cell. Such transfected cells include stably transfected cells wherein the inserted DNA is rendered capable of replication in the host cell. Typically, stable transfection requires that the exogenous DNA be transferred along with a selectable marker nucleic acid sequence, such as for example, a nucleic acid sequence that confers antibiotic resistance, which enables the selection of the stable transfectants. This marker nucleic acid sequence may be ligated to the exogenous DNA or be provided independently by simultaneous cotransfection along with the exogenous DNA. Transfected cells also include transiently expressing cells that are capable of expressing the RNA or DNA for limited periods of time. The transfection procedure depends on the host cell being transfected. It can include packaging the polynucleotide in a virus as well as direct uptake of the polynucleotide. Transformation can result in incorporation of the

inserted DNA into the genome of the host cell or the maintenance of the inserted DNA within the host cell in plasmid form. Methods of transformation/transfection are well known in the art and include, but are not limited to, direct injection, such as microinjection, viral infection, particularly replication-deficient adenovirus infection, electroporation, lipofection, and calcium phosphate-mediated direct uptake.

**[0048]** As used herein, a nucleic acid sequence (*e.g.*, coding sequence) and regulatory sequences are said to be operably linked when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. If it is desired that the nucleic acid sequences be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably linked to a nucleic acid sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide. Similarly two or more coding regions are operably linked when they are linked in such a way that their transcription from a common promoter results in the expression of two or more proteins having been translated in frame. In some embodiments, operably linked coding sequences yield a fusion protein. In some embodiments, operably linked coding sequences yield a functional RNA (*e.g.*, shRNA, miRNA).

**[0049]** For nucleic acids encoding proteins, a polyadenylation sequence may be inserted following the transgene sequences and before the 3' AAV ITR sequence. An AAV construct useful in the present invention may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene. One possible intron sequence is derived from SV-40, and is referred to as the SV-40 T intron sequence. Another vector element that may be used is an internal ribosome entry site (IRES). An IRES sequence is used to produce more than one polypeptide from a single gene transcript. For example, an

IRES sequence would be used to produce a protein that contain more than one polypeptide chains. Selection of these and other common vector elements are conventional and many such sequences are available. In some embodiments, a Foot and Mouth Disease Virus 2A sequence is included in polyprotein; this is a small peptide (approximately 18 amino acids in length) that has been shown to mediate the cleavage of polyproteins (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459). The cleavage activity of the 2A sequence has previously been demonstrated in artificial systems including plasmids and gene therapy vectors (AAV and retroviruses) (Ryan, M D et al., EMBO, 1994; 4: 928-933; Mattion, N M et al., J Virology, November 1996; p. 8124-8127; Furler, S et al., Gene Therapy, 2001; 8: 864-873; and Halpin, C et al., The Plant Journal, 1999; 4: 453-459; de Felipe, P et al., Gene Therapy, 1999; 6: 198-208; de Felipe, P et al., Human Gene Therapy, 2000; 11: 1921-1931; and Klump, H et al., Gene Therapy, 2001; 8: 811-817).

**[0050]** The precise nature of the regulatory sequences needed for gene expression in host cells may vary between species, tissues or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, enhancer elements, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region that includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

**[0051]** Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected

by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothioneine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al, Science, 268:1766-1769 (1995), see also Harvey et al, Curr. Opin. Chem. Biol., 2:512-518 (1998)), the RU486-inducible system (Wang et al, Nat. Biotech., 15:239-243 (1997) and Wang et al, Gene Ther., 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al, J. Clin. Invest., 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

**[0052]** In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, etc.) are well known in the art. Exemplary tissue-specific regulatory sequences include, but are not limited to the following tissue specific promoters: neuronal such as neuron-specific enolase (NSE) promoter (Andersen *et al.*, Cell. Mol. Neurobiol., 13:503-15 (1993)), neurofilament light-chain gene promoter (Piccioli et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991)), and the neuron-specific vgf gene promoter (Piccioli et al., Neuron, 15:373-84 (1995)). In some embodiments, the tissue-specific promoter is a promoter of a gene selected from: neuronal nuclei (NeuN), glial fibrillary acidic protein (GFAP), adenomatous polyposis coli (APC), and ionized calcium-binding adapter molecule 1 (Iba-1). Other appropriate tissue specific promoters will be apparent to the skilled artisan. In some embodiments, the promoter is a chicken Beta-actin promoter.

**[0053]** In some aspects, the invention provides an AAV vector for use in methods of preventing or treating one or more gene defects (*e.g.*, heritable gene defects, somatic gene alterations) in a mammal, such as for example, a gene defect that results in a polypeptide

deficiency or polypeptide excess in a subject, and particularly for treating or reducing the severity or extent of deficiency in a subject manifesting a CNS-associated disorder linked to a deficiency in such polypeptides in cells and tissues. In some embodiments, the methods involve administration of an AAV vector that encodes one or more therapeutic peptides, polypeptides, shRNAs, microRNAs, antisense nucleotides, etc., in a pharmaceutically-acceptable carrier to the subject in an amount and for a period of time sufficient to treat the CNS-associated disorder in the subject having or suspected of having such a disorder. “Antisense inhibition” refers to the production of antisense RNA transcripts capable of suppressing the expression of protein from an endogenous gene or a transgene.

**[0054]** Thus, the AAV vector may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TH), GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1) and amino acid decarboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCH, which generates the TH cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, *e.g.*, shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (*e.g.*, Mucopolysaccharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, *e.g.*, HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, *e.g.*, when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a  $\beta$ -endorphin may be

useful for treating pain. Other examples of transgenes that may be used in the AAV vectors of the invention will be apparent to the skilled artisan (*See, e.g., Costantini L C, et al., Gene Therapy (2000) 7, 93-109*).

**[0055]** Over the past decade several experimental and/or clinical studies reported on the successful use of AAV-based vectors (particularly AAV9) for CNS-targeted gene delivery. These studies established unequivocally the value of AAV-based-delivery vectors as a tool to achieve a potent gene upregulation or silencing in targeted CNS regions and have provided evidence that this therapeutic approach can effectively be used in treatment of numerous neurodegenerative disorders including ALS, SMA, muscle spasticity and chronic pain. Despite these encouraging data and extensive pre-clinical animal studies, a detailed mechanism on how the AAV vectors penetrate in brain or spinal cord parenchyma after using different routes of AAVs delivery (systemic, intrathecal) have not been fully understood. These data are critical for development of new and more effective AAV delivery protocols which would be equally potent in young and in fully developed adult animal and human subjects. In general, the preclinical animal studies can be categorized into several groups based on the developmental stage of when the animal is employed or the route the AAV is delivered (*e.g., systemic or intrathecal*). Depending on the parameters used in the individual studies, the level of transgene expression and the specific cell populations (neuronal and/or glial) that are being infected varies greatly.

**[0056]** The early studies demonstrated that the systemic-vein (iv) injection of AAV9-GFP in neonatal mice leads to widespread CNS GFP expression, including dorsal root ganglia, spinal motoneurons (MN) and neurons in brain (neocortex, hippocampus, cerebellum). Using adult mice, iv-delivered AAV9-GFP leads to a preferential astrocyte infection throughout the entire CNS, but only limited neuronal expression is seen (Foust, *et al.* (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature biotechnology* 27: 59-65). Comparable data were reported demonstrating widespread spinal MN GFP expression after systemic (iv) delivery of AAV9 in neonatal mice (Duque, *et al.* (2009). Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Molecular therapy: the journal of the American Society of Gene Therapy* 17: 1187-1196). In addition, the same group

demonstrated successful transgene expression in spinal MN once AAV9 was delivered iv in adult mice or cats. Similar to these two studies, Gray, *et al.* have shown CNS neuronal GFP expression after iv AAV9 administration in adult mice, however, only limited transduction efficiency was seen in juvenile non-human primates; compared previous studies, where a clear shift from neuronal to glial expression was seen in the brain (Gray, *et al.* (2011). Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Molecular therapy: the journal of the American Society of Gene Therapy* 19: 1058-1069).

**[0057]** Given the apparent limited neuronal expression after systemic iv AAV9 delivery in adult animals, the use of intrathecal route (to bypass the blood brain barrier) was explored in several studies. Using one-year-old, 2 kg BW, non-human primates (*Cynomolgus* macaques), it was demonstrated that a single lumbar-intrathecal injection of AAV9-CB-GFP led to 50-75% MN transduction in the entire spinal cord at two weeks after AAV9 injection. Similarly, in another study using juvenile 2- to 3-year-old non-human primates (*Cynomolgus* macaques) or young (2-month-old) pigs, potent MN transduction was seen throughout the entire spinal cord after intra-cisternal or combined cisternal-lumbar intrathecal AAV9 delivery. In addition, in the same study GFP expression was observed in dorsal root ganglion cells, motor cortex and in Purkinje cells in the cerebellar cortex. In a more recent study, comparable lumbar MN GFP expression was seen in non-human primates (*Cynomolgus* macaques) after intra-cisternal AAV9-GFP injection.

**[0058]** Interestingly, in all studies that employ a cisternal or lumbo-sacral intrathecal delivery route, the available histological data showed a very specific spinal transgene expression pattern. It is characterized by intense expression in  $\alpha$ -motoneurons, however, the ventral horn interneurons that are residing in close proximity to the GFP-expressing  $\alpha$ -motoneurons appear to be transgene negative. Similarly, potent transgene expression is seen in dorsal root ganglion cells and primary afferents. However, small interneurons localized in the superficial dorsal horn or in the intermediate zone show no transgene expression.

[0059] This high level of transgene expression selectivity and lack of infection in deeper gray matter cells indicate the presence of a well-developed regulatory-barrier system (in addition to the blood-brain barrier), which prevents the penetration of virus into deeper spinal compartments from the intrathecal space. Based on a well described anatomical organization of spinal meninges, it had been hypothesized that the pia mater represents a key barrier that regulates the penetration of AAV into the spinal parenchyma after intrathecal AAV delivery. It was therefore speculated that the high level of transgene expression seen in MN and DRGs cells reported after intrathecal delivery is likely mediated by the preferential retro- and anterograde infection of axons projecting into and out of the spinal parenchyma and trans-passing the intrathecal space (*i.e.*, ventral and dorsal roots).

[0060] Accordingly, to address this issue, the present invention provides a subpial vector delivery method in mammals, and demonstrates that this delivery route leads to potent trans-spinal transgene expression infecting the entire population of neurons in the gray matter of subpially injected segments. In addition, near complete infection of descending and ascending axons was achieved, and corresponded with the transgene expression in brain centers (*i.e.*, motor cortex, nucleus ruber).

[0061] Thus, the method and delivery system provided herein permit spinal subpial gene therapy, such as AAV9 vector or antisense oligonucleotide (ASO) delivery, into spinal parenchyma in large animals or in humans. To deliver AAV9 or ASO into the subpial space a new delivery system was designed to include a guiding tube bended at 90° and catheter (*e.g.*, PE-5 or PE-10), which permits precise guidance and placement of the subpial catheter into the dorsal subpial space of targeted spinal cord segments. After placement of the catheter, the AAV9 or ASO is infused for a certain amount of time before being removed. In various embodiments, the AAV9 or ASO is infused for approximately 2-3 minutes.

[0062] As used herein, the term “PE-10” refers to polyethylene tubing having an inner diameter of approximately 0.010 inches. In certain embodiments, the inner diameter of the PE-10 tubing will be about 0.011 inches. Likewise, the term “PE-5” refers to polyethylene tubing having an inner diameter of approximately 0.005 inches. In certain embodiments, the inner diameter of the PE-5 tubing will be about 0.008 inches.



[0063] Accordingly, the claimed system provides subpial delivery (*i.e.*, bypassing the pial membrane), which provides near complete spinal parenchymal AAV9-mediated gene expression or ASO distribution in both white and grey matter of the subject being treated. Currently available non-invasive techniques do not permit a comparable level of spinal parenchymal transgene expression or well controlled segment-specific gene silencing.

[0064] To place the subpial catheter in a mammalian subject, several sequential procedural steps may be followed to minimize potential spinal injury associated with instruments/catheter manipulation in the vicinity of the exposed "dura-free" spinal cord. In various embodiments, the use of caudal and cranial spinal clamps (placed just above and below the laminectomy) minimized spinal cord pulsation during catheter placement. Also in various embodiments, an "L" shaped catheter stainless steel guiding tube (*e.g.*, a 16-26 G stainless steel tube bended at 90°) mounted on an XYZ manipulator (as described in, for example, US Pub. No. 2015/0224331) is used for subpial catheter placement.

[0065] In certain embodiments, the pia is first punctured using a bent 30G needle. Once the tip of the penetrating 30G needle is in the subpial space for about 1-1.5 mm, the pia may be slightly lifted by 1-2 mm. The subpial catheter is then placed into the subpial space by advancing the catheter from the guiding tube. After the catheter is advanced into the targeted length, the penetrating needle tip of the guiding tube is removed from the subpial space. Once the vector injection is completed (typically over 2-5 min, and in some embodiments, over about 3 min), the catheter is pulled out of the subpial space and the dura is closed. By using this technical approach, placement of the subpial catheter may be accomplished within about 3-5 min from the moment of dura opening.

[0066] The subpial catheter described herein has been successfully placed in 17 pigs using this technique, achieving consistent and injury-free subpial catheter placement. In adult rats an identical technique is used, however, a PE-5 catheter is used instead. The data obtained using these adult rats and pigs demonstrates *i)* potent spinal parenchymal transgene expression in white and gray matter including neurons and glial cells after single bolus subpial AAV9 delivery, *ii)* delivery to almost all descending motor axon throughout the

length of the spinal cord after cervical or thoracic subpial AAV9 injection, *iii*) potent retrograde transgene expression in brain motor centers (motor cortex and brain stem), and *iv*) safety of this approach by defining normal neurological function for up to three months after AAV9 delivery. Thus, subpial delivery of AAV-9 enables gene-based therapies with a wide range of experimental and clinical utilizations in adult mammals.

[0067] In one embodiment, a 16-26 G stainless steel tube bended at 90° is used as a guiding cannula for a PE-10 catheter. The guiding tube is positioned just above the spinal pia and the PE-catheter is then advanced into subpial space through a small pial opening (Figures 1A-1E). AAV9 or ASO is then infused into subpial space. In certain embodiments, the AAV9 is delivered in a mixture containing between 1-10 % of dextran (10,000-30,000 MW) to permit a longer lasting deposition of AAV9 particles in spinal parenchyma. After AAV9-GFP delivery a consistent GFP expression may be seen throughout the spinal parenchyma at the level of injection and in axons projecting distally (into lumbar spinal cord) from AAV9 injected segments.

[0068] As demonstrated herein, a single subpial AAV9 injection led to potent parenchymal transgene expression spreading rostral-caudally for multiple segments. Thus, subpial AAV9 delivery leads to a wide-spread transgene expression in neurons throughout the gray matter and ascending and descending axons in subpial-injected segments. For example, in an adult pig spinal cord, the transgene spread was consistently seen in distances of about 10-15 cm from the point of administration. Expression was identified in neurons and glial cells in all gray matter laminae and in axons in ventral, lateral and dorsal funiculi confirming a near complete penetration of subpial-injected AAV9 vector throughout the spinal parenchyma. By analyzing transverse lumbar spinal cord sections in pigs which received a mid-thoracic AAV9-UBI-GFP injection (*i.e.*, about 30 cm distance from the site of AAV9 delivery), virtually all descending motor axons appeared to be labeled at six weeks after AAV9 injection. Higher resolution confocal microscopy revealed a dense network of fine axonal arborizations with terminal boutons throughout the gray matter. Consistent with the level and distribution of infected axons in the white matter, retrogradely infected GFP-expressing neurons in the motor cortex and in the brain stem were identified.

Similarly, centrally projecting sensory axons were identified in the reticular formation and in the thalamus.

**[0069]** By comparing the transgene expression pattern after subpial vs. intrathecal AAV9 delivery in rats, the present invention demonstrates a substantially different regional-cellular expression. As such, the pia mater represents a primary barrier for effective parenchymal penetration of AAV9 after intrathecal delivery.

**[0070]** First, after intrathecal delivery, the expression was only seen in regions and neuronal-glial pools which are morphologically associated with dorsal and ventral root entry zone. Thus, potent transgene expression was seen in primary afferents and was clearly present in the dorsal funiculi, primary afferents in the dorsal horn and Ia afferents projecting to the ventral horn. This transgene expression in primary afferents corresponded with a potent expression in dorsal root ganglion cells. Similarly, a clear expression was seen around the ventral root entry zone with some retrogradely-labeled  $\alpha$ -motoneurons in the ventral horn. In contrast, however, the transgene expression was virtually absent in all other neurons between laminae I-VII and X, and no descending motor axons were labeled in the lateral or ventral funiculi. Similarly, the axons of the corticospinal tract in rats (localized on the base of dorsal funiculi), which were surrounded by GFP-expressing primary afferents, showed no transgene expression. Without being bound by theory, these data jointly suggest that the spinal parenchymal GFP expression (whether in neurons or projecting primary afferents) may be caused by retrograde or anterograde transgene expression, and not by an uptake of AAV9 into the spinal parenchyma from the intrathecal space. This observation is consistent with the data from other laboratories, which demonstrate potent  $\alpha$ -motoneuron GFP expression after single intrathecal or cisternal AAV9-GFP injection in adult non-human primates or juvenile pigs. However, no interneuronal GFP expression is seen in the interneurons residing just in the close vicinity of GFP+  $\alpha$ -motoneurons (Meyer, *et al.* (2015). Improving single injection CSF delivery of AAV9-mediated gene therapy for SMA: a dose-response study in mice and nonhuman primates. *Molecular therapy: the journal of the American Society of Gene Therapy* 23: 477-487; Foust, *et al.* (2013). Therapeutic AAV9-mediated suppression of mutant SOD1 slows disease progression and extends survival in models of inherited ALS. *Molecular therapy: the journal of the American*

*Society of Gene Therapy* 21: 2148-2159; Passini, *et al.* (2014). Translational fidelity of intrathecal delivery of self-complementary AAV9-survival motor neuron 1 for spinal muscular atrophy. *Human gene therapy* 25: 619-630; Bell, *et al.* (2015). Motor Neuron Transduction after Intracisternal Delivery of AAV9 in a Cynomolgus Macaque. *Human gene therapy methods* 26: 43-44).

**[0071]** In contrast, as described above, subpial AAV9 delivery was associated with potent transgene expression in the gray matter neurons (*i.e.*,  $\alpha$ -motoneurons and interneurons), and in virtually all descending motor axons and primary afferents of injected segments. These data clearly demonstrate that the pia mater represents the primary barrier preventing the penetration of AAV9 into other spinal cord compartments that are distant from the ventral and dorsal root entry zone. By bypassing the pial membrane and depositing the AAV9 into the subpial space a trans-parenchymal infection of white and gray matter can be effectively achieved in adult rodents or large animals.

**[0072]** Thus, the claimed methods and system can be used in subjects to increase axonal sprouting after spinal trauma by upregulating the expression level of neurotrophic genes in descending motor axons. Additionally, such local delivery of ASO enables a segment-restricted silencing of genes associated with the development of chronic pain or muscle spasticity to be targeted, but without a supraspinal side effect that is otherwise seen after intrathecal ASO delivery.

**[0073]** The potency of subpial-induced infection and the neuronal cell populations that are being infected in the spinal cord and brain in an adult animals has several potential clinical and experimental implications. First, in cases when a specific gene is to be silenced-downregulated, a single cervical subpial injection of the silencing AAV9 construct will lead to effective gene silencing in cervical neurons and glial cells, in descending motor axons throughout the whole length of spinal cord and in the majority of ascending sensory fibers. Given a well characterized neurodegenerative pattern in ALS patients and experimental models of ALS and which includes progressive degeneration of upper motor neuron and projecting descending motor axons, lower motor neuron and spinal interneurons, the ability to achieve widespread mutated gene silencing will likely provide a substantial

advantage in achieving the most potent therapeutic effect. In addition, a single cervical subpial injection can be combined with one or more additional subpial injections into the lumbar enlargement to target the lumbar neuronal/glial population and/or with lumbar intrathecal injection to target  $\alpha$ -motoneuronal pools throughout the thoracic and lumbar spinal cord. Second, increased expression of therapeutic genes (growth factors for example) associated with axonal sprouting can be readily achieved in descending motor tracts as well as ascending sensory fibers, and tested for its treatment potency in spinal trauma studies, for example. In this case the AAV9 vector can be administered from a single laminectomy site just at the injury epicenter with a subpial catheter advanced rostrally and caudally to target the distal end of severed motor axons and proximal ends of ascending sensory axons, respectively. Third, near complete descending motor tract labeling which can be achieved from cervical subpial AAV9-GFP injection will permit the study of axonal sprouting and synapse formation between labeled motor axons of the subject and spinally-grafted cells. Such data systematically characterizing the level of axonal sprouting and/or the development of synaptic contacts in cell-grafted large animal models of spinal injury are currently not available.

**[0074]** As described herein, an advantage of subpial AAV9 delivery, if compared to intrathecal delivery, appears to be superior spinal trans-parenchymal transgene expression. In addition, a comparable high level of transgene expression is achieved in fully adult rats or minipigs. Because the dimension of the spinal cord in adult 35-40 kg pigs is similar to that of humans, it is expected that a similar parenchymal AAV9 uptake will also be achieved in adult humans.

**[0075]** One of the relative limitations of the subpial delivery technique described herein is the requirement to perform local laminectomy to gain access to the dorsal surface of the subpially-injected spinal cord. The requirement for laminectomy can limit its repetitive use (which is in contrast to the potential for repetitive intrathecal delivery). However, the degree of transgene expression that can be achieved after subpial AAV9 delivery appears to balance, if not super pass, this limitation should a clear and more potent therapeutic effect be seen once subpially-based gene delivery is used in disease modifying studies. In addition, it has recently been determined that a continuing high level of spinal GFP

expression remains at 12 months post-administration in the lumbar spinal cord in naïve-control rats. This would indicate that a single subpial delivery of a therapeutic gene can potentially lead to a long-lasting effect before additional gene delivery needs to be considered.

**[0076]** Using exemplary mammalian subjects (*e.g.*, adult pigs and rats), the present invention demonstrates that the subpial spinal cord AAV9 delivery technique provided herein permits widespread transgene expression in spinal parenchyma, descending and ascending axons and does not require direct spinal cord tissue needle penetration (See Figure 8). In addition to spinal regional transgene expression, robust retrograde expression in brain motor centers was seen. This technique can potentially be used in pre-clinical and human clinical studies targeted to upregulate or downregulate the gene of interest in specific spinal cord segments and/or in projecting motor and ascending sensory axons. The extent of transgene expression in these exemplary adult animals suggests that present invention may be successfully used in the adult patient population to target a variety of spinal neurodegenerative disorders and/or CNS-related disorders. Exemplary neurodegenerative disorders, CNS-related disorders, diseases, or injuries include, but are not limited to, multiple sclerosis (MS), progressive multifocal leukoencephalopathy (PML), encephalomyelitis (EPL), central pontine myelolysis (CPM), adrenoleukodystrophy, Alexander's disease, Pelizaeus Merzbacher disease (PMZ), Globoid cell Leucodystrophy (Krabbe's disease) and Wallerian Degeneration, optic neuritis, transverse myelitis, amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, Parkinson's disease, Parkinson's-plus diseases (*e.g.*, multiple system atrophy, progressive supranuclear palsy, and corticobasal degeneration), surgical resection, spinal cord injury or trauma, CNS injury resulting from tumor resection, transverse myelitis, optical myelitis, Guillain-Barré syndrome (GBS), stroke, traumatic brain injury, post radiation injury, neurologic complications of chemotherapy, acute ischemic optic neuropathy, vitamin E deficiency, isolated vitamin E deficiency syndrome, Bassen-Kornzweig syndrome, Marchiafava-Bignami syndrome, metachromatic leukodystrophy, trigeminal neuralgia, glossopharyngeal neuralgia, myasthenia gravis, epilepsy, Bell's palsy, muscular dystrophy, progressive muscular atrophy, primary lateral sclerosis (PLS), pseudobulbar palsy, progressive bulbar

palsy, spinal muscular atrophy, progressive bulbar palsy, inherited muscular atrophy, invertebrate disk syndromes (*e.g.*, herniated, ruptured, and prolapsed disk syndromes), cervical spondylosis, plexus disorders, thoracic-outlet destruction syndromes, peripheral neuropathies, porphyria, mild cognitive impairment, and chronic pain syndrome.

[0077] In some embodiments, the AAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high AAV concentrations are present (*e.g.*,  $\sim 10^{13}$  GC/ml or more). Methods for reducing aggregation of AAVs are well known in the art and, include, for example, addition of surfactants, pH adjustment, salt concentration adjustment, etc. (*See, e.g.*, Wright F R, *et al.*, Molecular Therapy (2005) 12, 171-178).

[0078] Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens. Typically, these formulations may contain at least about 0.1% of the active ingredient or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active ingredient in each therapeutically-useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0079] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable

under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

**[0080]** For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

**[0081]** Sterile injectable solutions are prepared by incorporating the active AAV in the required amount in the appropriate solvent with various of the other ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients 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 freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

**[0082]** The agents, compositions, and/or systems described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate



their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components of the invention and instructions for use. Specifically, such kits may include compositions including the AAV for administration, as described herein, along with instructions describing the intended application and the proper use of the composition. In certain embodiments, the kits may further include a separate container containing a guiding tube (*e.g.*, 18 or 23G) bended at 90° and a catheter (*e.g.*, PE-5 or PE-10) suitable for a particular application and for a method of subpial administration of the composition. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments. The kit may further include one or more or all of the components required to administer the composition subpially to a subject, such as a syringe, caudal and/or cranial spinal clamps, XYZ manipulator, etc.

[0083] As used herein, “instructions” can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the invention. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, etc.), Internet, and/or web-based communications, etc. The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflect approval by the agency of manufacture, use or sale for animal administration.

[0084] The following examples are intended to illustrate but not limit the invention.

## EXAMPLE 1

### Materials and Methods

[0085] *Animals and general surgical preparation* – Adult Sprague-Dawley rats (male and female, 250-350 grams; n=16) or adult minipigs resulting from cross-breeding of Minnesota and Gottingen strains (both sexes; 30-40 kg; n=6) were used. Rats were anesthetized with 5% isoflurane and maintained at 2-3% of isoflurane during surgery depending on breathing rate and paw pinch response. The back of the rat was then shaved and cleaned with 2% chlorohexadine. After skin incision, the paravertebral muscle

surrounding the cervical, thoracic or lumbar spinal vertebrae was removed and animals mounted into a spinal immobilization frame (Stoelting) using Cunningham's spinal clamps as previously described (Kakinohana, *et al.* (2004). Region-specific cell grafting into cervical and lumbar spinal cord in rat: a qualitative and quantitative stereological study. *Experimental neurology* 190: 122-132). To expose the spinal cord a dorsal laminectomy of corresponding vertebra was performed using a dental drill. The dura was then cut opened using a scalpel blade.

**[0086]** Minipigs were premedicated with intramuscular azaperonum (2 mg/kg) and atropine (1 mg/kg; Biotika, SK) and then induced with ketamine (20 mg/kg; IV). After induction, animals were intubated with a 2.5F tracheal tube. Anesthesia was maintained with 1.5% isoflurane in 50%/50% air/oxygen mixture at a constant 2 L/min flow rate. Oxygen saturation was monitored throughout the procedure using a pulse oximeter (Nellcor Puritan Bennett Inc., Ireland). After induction of anesthesia, animals were placed into a spinal immobilization apparatus as described previously (Usvald, *et al.* (2010). Analysis of dosing regimen and reproducibility of intraspinal grafting of human spinal stem cells in immunosuppressed minipigs. *Cell transplantation* 19: 1103-1122). A dorsal laminectomy of Th5 or L2-L4 vertebrae, corresponding to Th5 and L3-L6 spinal segments, respectively, was then performed and epidural fat removed using cotton swabs. The dura was cut open and secured to the surrounding tissue using 6.0 Prolene (Figures 1C-1E).

**[0087]** *Placement of subpial catheter and subpial AAV9 injection* – To place the subpial catheter, an L-shape catheter guiding tube (18 or 23G) was constructed (Figure 1B). The guiding tube was mounted into an XYZ (Stoelting) manipulator and advance to the surface of the exposed spinal segment. A 30G needle previously bent into 45° was used to puncture the pia. The subpial catheter (PE-10 for pig and PE-5 for rat) was then advanced into the subpial space from the guiding tube by manually pushing the catheter from the other end of the guiding tube. In rats, the catheter was advance into the subpial space for about 1-1.5 cm, and in pigs for about 3-6 cm. The virus was then injected into the subpial space over 3 min using a 50 or 250 µl Hamilton syringe. After injection the catheter was removed, dura closed using 6.0 Prolene (dura is closed in pig only), and animals allowed to recover.

**[0088]** *Preparation of AAV9 for in vivo injection* – 1.2 kb ubiquitin-C (UBC) promoter was made by oligonucleotide synthesis, linked with either eGFP or DsRed (RFP) and SV40 polyA signal, and cloned into a self-complementary double-strand DNA genome AAV (scAAV) vector plasmid (Xu, *et al.* (2012). *In vivo* gene knockdown in rat dorsal root ganglia mediated by self-complementary adeno-associated virus serotype 5 following intrathecal delivery. *PLoS one* 7: e32581). Helper virus-free scAAV9 vectors expressing either eGFP or RFP driven by UBC promoter were produced by transient transfection of HEK293T cells with the vector plasmid, pRep2-Cap9 and pAd-Helper plasmids (Xiao, *et al.* (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 72: 2224-2232). Plasmid pRep2-Cap9 was obtained from the Vector Core of U. Penn. AAV vectors in the cell lysates prepared at 72 hrs after transfection were purified as previously described and titered by Q-PCR (Xu, *et al.*, *supra*). The final titers were adjusted to  $1.0 \times 10^{13}$  genome copies per ml (gc/ml). Just before injection the virus was mixed with dextran (10,000 MW) 1:1 to a final dextran concentration of 2.5%. The volume subpial injectate was 30  $\mu$ l in rats and 200  $\mu$ l in pigs.

**[0089]** *Perfusion fixation, postmortem in situ GFP fluorescence imaging and immunofluorescence staining of spinal cord and brain sections* – Animals (rats and pigs) were deeply anesthetized with pentobarbital and transcardially perfused with 200 ml (rat) or 2000 ml (pig) of heparinized saline followed by 250 ml (rat) or 4000 ml (pig) of 4% paraformaldehyde in PBS. The spinal cords and brains were dissected and post-fixed in 4% formaldehyde in PBS overnight at 4°C and then cryoprotected in 30% sucrose PBS until transverse or longitudinal sections (30- $\mu$ m-thick) were cut on a cryostat and stored in PBS. Before sectioning the whole spinal cord was imaged *in situ* using an IVIS Spectrum optical imaging system (Xenogen, Alameda, CA). Sequences were acquired at excitation wavelength 465 nm and emission wavelength 520 nm. Medium binning was used, and the exposure time was 3 seconds. Images were analyzed using Living Image 4.3.1 (Xenogen, Alameda, CA) software. The signals were calculated using fixed volume ROIs. Prepared sections were immunostained overnight at 4°C with the following primary antibodies made in PBS with 0.2% Triton X-100: rabbit anti-glial fibrillary acidic protein (GFAP; 1:500, Origene, Rockville, Maryland, USA) and mouse anti-neuronal nuclei antigen (NeuN,

1:1000, Chemicon). After incubation with primary antibodies, sections were washed three times in PBS and incubated with fluorescent-conjugated secondary donkey anti-rabbit and donkey anti-mouse antibodies (Alexa Fluor 488, 546 or 647, 1:1000, Invitrogen), respectively, and DAPI for general nuclear staining. Sections were then mounted on slides, dried at room temperature and covered with a Prolong anti-fade kit (Invitrogen). Fluorescence images were captured using a Zeiss Imager M2 microscope and confocal images were taken using an Olympus FV1000 microscope.

[0090] The consistency, high level of spinal parenchymal transgene expression, and feasibility of this approach represent a major technological improvement over current approaches, with a potential direct clinical application aimed at spinal gene upregulation or silencing in general. Currently used approaches employ spinal intrathecal or invasive direct intraparenchymal AAV injection with its apparent limitations such as a low level of parenchymal transgene expression or its invasive nature required to achieve a more robust infection effect. In addition, this approach provides an unparalleled level of motor and sensory fibers labeling at segments distant from the site of AAV9 delivery making it a very robust tool to study the extent of synaptic connectivity and to identify factors (*i.e.*, genes, neurotrophic factors) that regulate axonal sprouting in targeted spinal segments in both rodents and large animal species.

[0091] An example demonstrating the principle of spinal subpial AAV9 delivery and resulting regional parenchymal expression as well as axonal labeling in segments distant from the site of AAV9 delivery in the adult pig at 2 months post-AAV9 delivery is provided herein.

## EXAMPLE 2

### Parenchymal AAV9-Mediated Transgene Expression After Single Subpial Bolus

[0092] Initially, the potency of single bolus subpial AAV9-UBI-GFP or AAV9-UBI-RFP delivery in rats and pigs was tested. Animals received 20  $\mu$ l (rat) or 200  $\mu$ l (pig) of AAV9 vector in 2.5% dextran solution delivered into subpial space of cervical (C4-6), thoracic (Th6-9) or lumbar (L2-L5) segments. At 6-8 weeks after AAV9 delivery spinal cords were dissected from 4% paraformaldehyde-fixed animals and imaged *in situ* using

Avis fluorescence system. Transverse or horizontal spinal cord sections were then cut from AAV9-injected segments and analyzed for the presence of GFP or RFP and co-stained with neuronal (NeuN) and glial (GFAP) antibodies. In both rat and pig spinal cord an intense GFP or RFP expression was seen on the surface of the spinal cord and was readily identified by visual inspection as yellow-green or red areas. Figures 1H and 1J show the presence of RFP (red color) in transversely cut pig spinal cord and in the ventral roots (Figure 1H, insert) in comparison to naive spinal cord (Figure 1I). Correspondingly, the spinal surface densitometric analysis of AAV9-UBI-GFP-injected animals showed a wide spread of GFP signal extending for up to 5-10 cm from the epicenter of subpial AAV9 delivery (Figures 1F and 1G).

**[0093]** Using horizontally cut thoracic sections taken from pigs previously injected subpially with AAV9-UBI-RFP in the mid-thoracic level (the same spinal cord as shown in Figure 1H), extensive parenchymal RFP expression was seen. The RFP expression was readily identified in the majority of interneurons and  $\alpha$ -motoneurons and extended throughout 4-6 spinal segments (Figures 3A and 3B). Similarly, intense RFP expression in axo-dendritic arbor was seen throughout the whole RFP-expressing gray matter (Figures 3A and 3B, white asterisks).

**[0094]** Analysis of transverse spinal cord sections taken from the epicenter of subpial AAV9 injection confirmed an intense RFP expression throughout the white and gray matter. In white matter, a punctate-like RFP expression was seen in the majority of transversally-cut axons (Figure 3C, white asterisks, box inserts) and were identified in dorsal, lateral and ventral funiculi (Figure 3C, DF, LF, VF; box inserts). In the gray matter, numerous interneurons distributed between laminae I-IX and ventral  $\alpha$ -motoneurons in ventral horns showed intense RFP expression in soma and in axo-dendritic arbor. A high density of RFP expression was also seen in the terminal boutons thorough the gray matter (Figures 3D-3G). Similarly, confocal analysis showed the presence of RFP signal in astrocytes (Figure 3C, insert: RFP/GFAP).

**[0095]** A similar neuronal GFP expression pattern was seen in the rat lumbar spinal cord after subpial AAV9-UBI-GFP injection at the L1-2 level. A high density of GFP+ neurons

throughout the L1-L5 lumbar segments and localized in the whole gray matter between laminae I-IX was identified (Figures 9A-9D).

### **EXAMPLE 3**

#### **GFP Expression in Distant Spinal Segments**

[0096] Next, the extent of descending spinal tract GFP expression in lumbar spinal cord was characterized after subpial injection of AAV9-UBI-GFP into the subpial space of mid-thoracic (Th6-7) or lower cervical segments in both rats and pigs. At 3-6 weeks after subpial AAV9 delivery, an intense GFP expression was seen throughout the whole lumbar spinal cord. Using transverse lumbar (L2-L6) spinal cord sections taken from pigs high intensity GFP expression in transversely-cut axons in the lateral and ventral funiculi was readily identified without additional GFP immunostaining (Figure 4A, white asterisks). In these regions a similar density of GFP+ axons was seen throughout the whole white matter. In comparison to lateral and ventral funiculi, a relatively lower number of GFP+ axons was seen in the dorsal funiculi. (Figure 4A, DF). Consistent with the degree of axonal labeling seen in the white matter of lateral and ventral funiculi a dense network of GFP+ axons terminating in the gray matter was seen (Figures 4A and 4B). These axons were identified between laminae III-X. Only few GFP+ axons in the lamina I-III were seen. High power confocal microscopy showed a high density of fine GFP+ axons with numerous terminal boutons in the gray matter (Figure 4C).

[0097] A comparable GFP expression pattern in descending motor axons in lumbar gray matter was seen in rats previously receiving upper cervical subpial injection of AAV9-UBI-GFP (Figures 9E and 9F).

### **EXAMPLE 4**

#### **Retrograde Transgene Expression in Brain Motor Regions After Spinal Subpial Delivery**

[0098] Analysis of transgene (GFP) expression in brain motor centers (motor cortex, nucleus ruber and formatio reticularis) at six weeks after subpial AAV9-UBI-GFP delivery in pigs showed intensely GFP-labeled pyramidal neurons in the motor cortex (Figures 5A-

5E). Similarly, numerous GFP+ neurons localized in the brain stem were identified (Figures 5F-5J). Consistent with the presence of GFP+ neurons in the motor cortex, a high number of GFP+ corticospinal axons in the ventral region of the medulla oblongata (medullary pyramids) was seen (Figure 5K). In addition, a high density of anterogradely labeled GFP+ spinoreticular terminals was seen throughout the reticular formation (Figure 5L) as well as spinothalamic terminals in the thalamic nuclei (not shown).

[0099] Comparably, as seen in pigs, a high density of GFP+ pyramidal neurons localized bilaterally in motor cortex was seen in rats (Figures 6A-6D). A similar high level of GFP expression was also seen in the nucleus ruber and was easily identified by the presence of bilaterally localized GFP+ nuclear-neuronal clusters (Figures 6E-6G).

## EXAMPLE 5

### Differential Regional Spinal Transgene Expression

#### After Intrathecal vs. Subpial AAV9 Delivery

[00100] The distribution of spinal transgene expression was then compared once the AAV9 was injected into the lumbar (L1-L2) intrathecal space (AAV9-UBI-GFP) and into the subpial space of thoracic Th7 segment (AAV9-UBI-RFP) in the same animal (rat). Subpial AAV9 injection was performed three weeks after lumbar intrathecal AAV9 injection and the expression pattern was analyzed in the transverse lumbar spinal cord sections at three weeks after subpial AAV9 injection. Intrathecal injection of AAV9-UBI-GFP led to intense GFP expression in the dorsal funiculus, primary afferents in dorsal horn (lamina II-III) and in the medial part of lamina V-VII. Several GFP+ Ia afferents terminating in the ventral horn in the vicinity of CHAT+  $\alpha$ -motoneurons were also identified. Consistent with a high GFP expression in primary afferents, a high number of GFP+ neurons were found in dorsal root ganglion cells (Figure 7B). A clear presence of increased GFP expression was also consistently seen around the ventral root entry zone (Figures 7A and 7F). In this region, several GFP expressing glial cells were seen. Similarly, few GFP+ cells were identified in the dorsal root entry zone. In the ventral gray matter, some  $\alpha$ -motoneurons showed GFP expression (Figure 7G). Except for these regions and cell groups which showed GFP expression, near complete lack of neuronal or glial GFP expression was seen in other deeper regions of the gray matter including dorsal horn,

intermediate zone and in white matter of lateral and ventral funiculi (Figure 7A).

Interestingly, even the neurons localized in the superficial dorsal horn and which reside in a close vicinity to the intrathecal space (but separated by pia mater) showed a complete lack of GFP expression (Figures 7C and 7D).

**[00101]** In contrast to the GFP expression pattern seen after intrathecal AAV9-UBI-GFP delivery, the RFP expression resulting from cervical subpial AAV9 injection showed a substantially different regional expression pattern if analyzed in the same lumbar spinal cord sections. The dsRED expression was identified in the majority of axons in white matter and was present in lateral and ventral funiculi. Numerous axons projecting into the gray matter of the dorsal horn, intermediate zone (Lamina VII) and ventral horn were also seen (Figure 7A). Confocal microscopy showed that virtually all RFP+ fibers either in the white or gray matter were GFP negative. Interestingly, numerous RFP+ corticospinal axons residing on the base of dorsal funiculi were found in close vicinity of the GFP-labeled primary afferents but no colocalization of RFP+GFP was identified in either of these fibers (Figure 7E).

**[00102]** Although the invention has been described with reference to the above example, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.



The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. Use of a pharmaceutical composition comprising a nucleic acid molecule for inducement of spinal trans-parenchymal infection in a subject, wherein the pharmaceutical composition is formulated for administration to the subpial space.
2. The use of claim 1, wherein the pharmaceutical composition is formulated for administration via a catheter.
3. The use of claim 1, wherein the pharmaceutical composition comprises about 1-10% dextrose.
4. The use of claim 1, wherein the nucleic acid molecule is a vector or an antisense oligonucleotide (ASO).
5. The use of claim 4, wherein the vector is a lentiviral vector, adenoviral vector, or an adeno-associated vector.
6. The use of claim 5, wherein the vector is an AAV9 particle.
7. The use of claim 6, wherein the vector comprises a nucleic acid molecule encoding a protein or functional RNA that modulates or treats a neurodegenerative disorder.
8. The use of claim 7, wherein the neurodegenerative disorder is amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, or Parkinson's disease.
9. The use of claim 1, wherein the pharmaceutical composition is formulated for administration in a single injection.
10. The use of claim 2, wherein the pharmaceutical composition is formulated for administration in multiple injections.
11. The use of claim 1, wherein the subject is a mammal.
12. The use of claim 11, wherein the subject is human.
13. A gene delivery system for inducement of spinal trans-parenchymal infection in a subject comprising:
  - (a) an L-shaped guide tube configured to puncture the pia of the subject;

(b) a catheter slidably disposed within the guide tube and configured to be advanced into subpial space of a spinal segment of a vertebra of the subject; and

(c) a reservoir in fluid communication with the catheter and containing a pharmaceutical composition comprising a nucleic acid molecule for inducement of spinal trans-parenchymal infection in a subject, wherein the pharmaceutical composition is formulated for administration to the subpial space.

14. The gene delivery system of claim 13, wherein the L-shaped guide tube is a 16-26G stainless steel tube.

15. The gene delivery system of claim 13, wherein the catheter is formed from polyethylene tubing.

16. Use of the gene delivery system of claim 14 for subpial delivery of a nucleic acid molecule to a subject.

17. Use of a pharmaceutical composition comprising a vector or an antisense oligonucleotide (ASO) for the treatment of a neurodegenerative disorder in a subject in need thereof via inducement of spinal trans-parenchymal infection in the subject, wherein the pharmaceutical composition is formulated for administration to the subpial space.

18. The use of claim 17, wherein the pharmaceutical composition is formulated for administration via a catheter.

19. The use of claim 17, wherein the neurodegenerative disorder is amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, or Parkinson's disease.

20. The use of claim 17, wherein the pharmaceutical composition is formulated for administration to the subpial space in multiple injections.

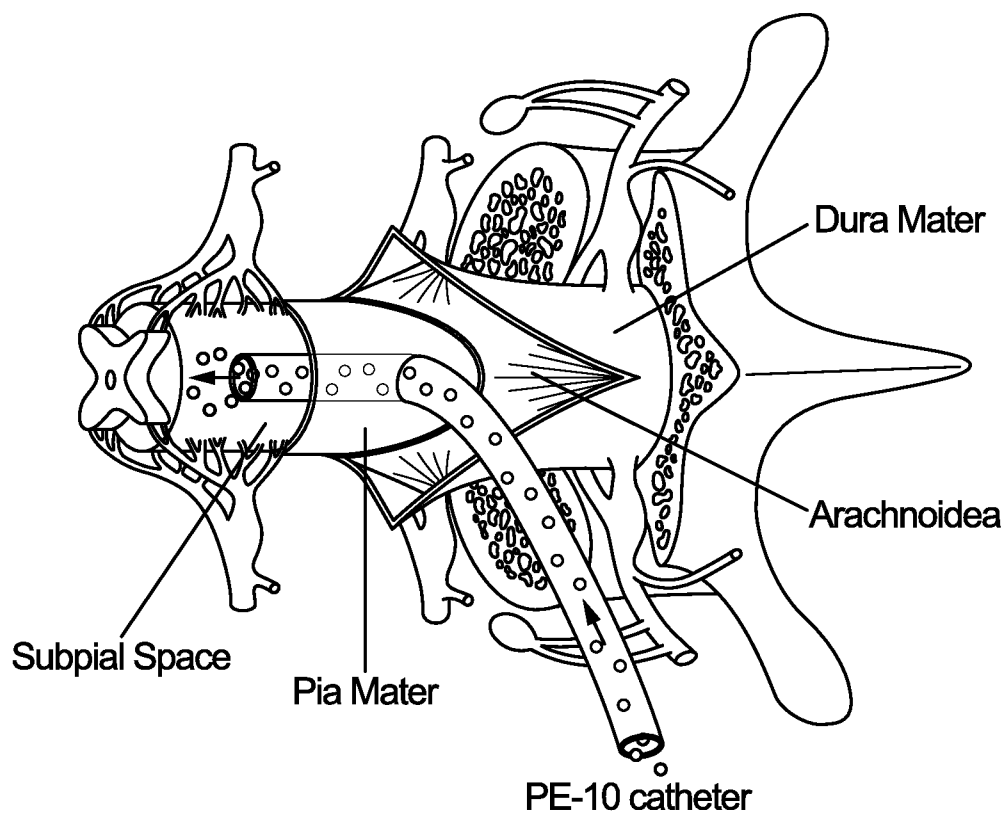
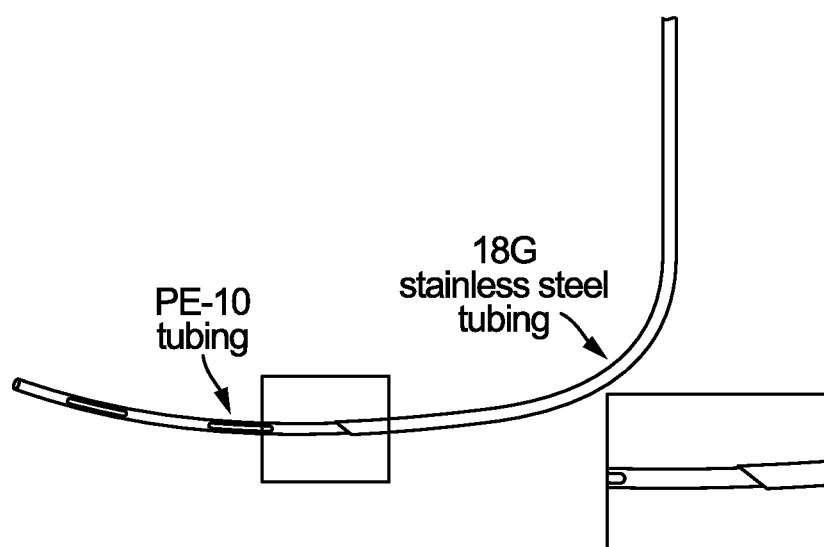
21. The use of claim 17, wherein the subject is a mammal.

22. The use of claim 21, wherein the subject is human.

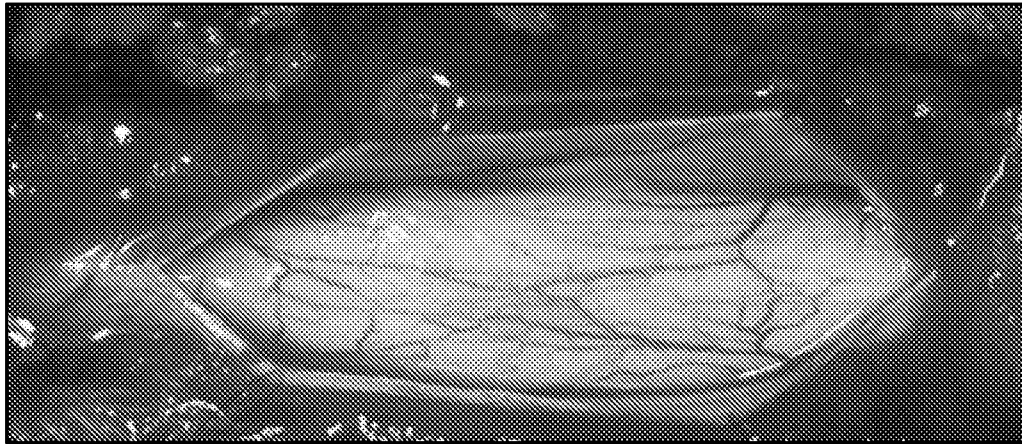
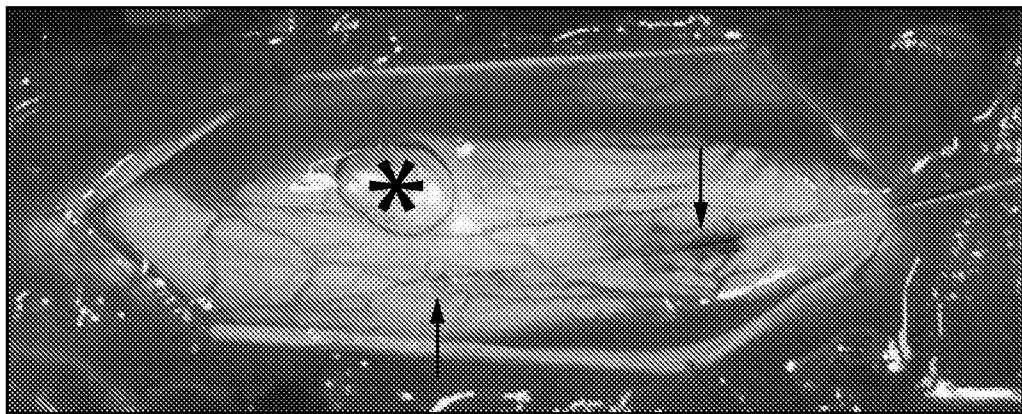
23. Use of a vector or an antisense oligonucleotide (ASO) in the manufacture of a medicament for the treatment of a neurodegenerative disorder in a subject in need thereof via inducement of spinal trans-parenchymal infection in the subject, wherein the medicament is formulated for administration to the subpial space.

24. The use of claim 23, wherein the medicament is formulated for administration via a catheter.
25. The use of claim 23, wherein the neurodegenerative disorder is amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, or Parkinson's disease.
26. The use of claim 23, wherein the medicament is formulated for administration to the subpial space in multiple injections.
27. The use of claim 23, wherein the subject is a mammal.
28. The use of claim 27, wherein the subject is human.

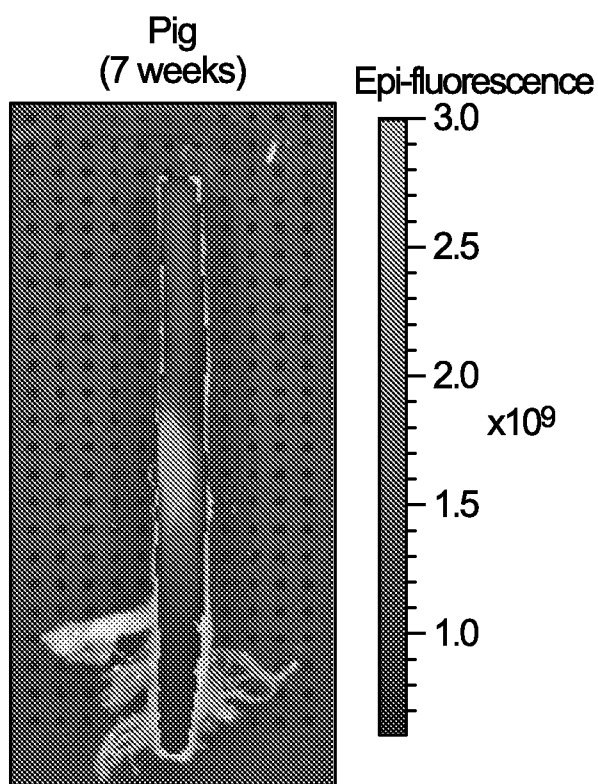
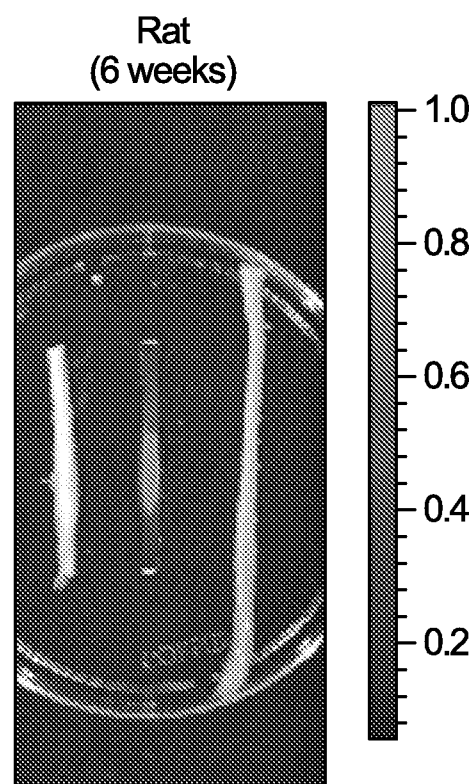
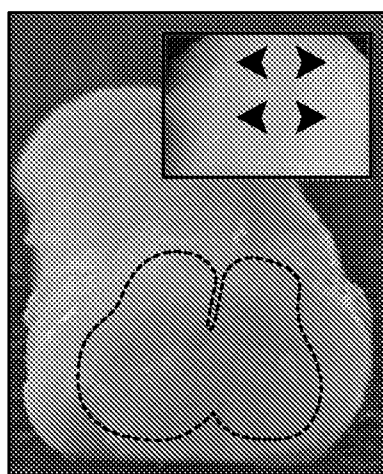
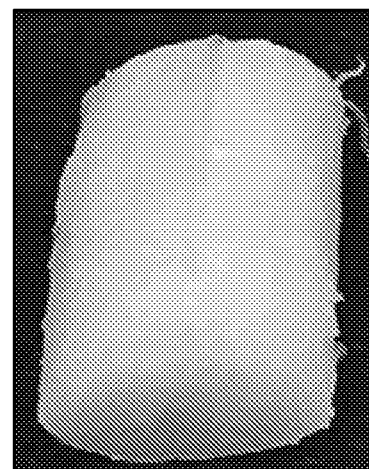
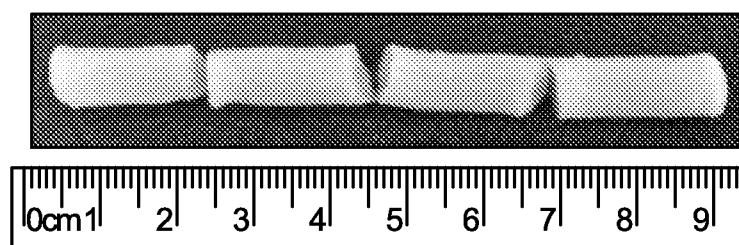
1/11

**FIG. 1A****FIG. 1B**

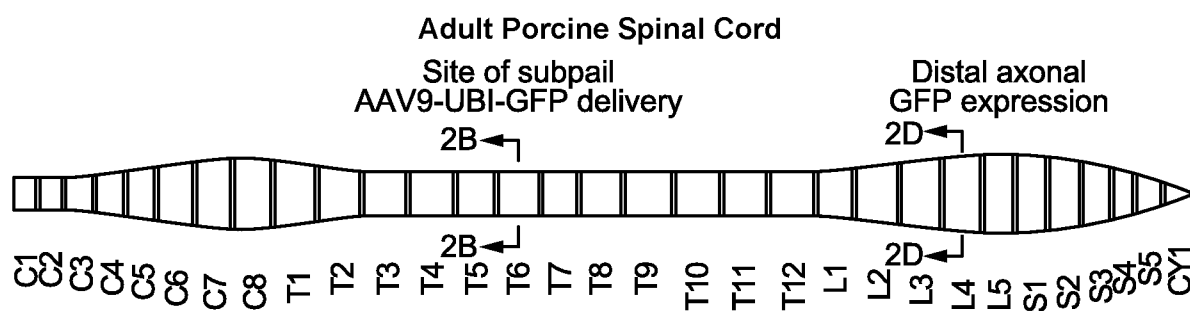
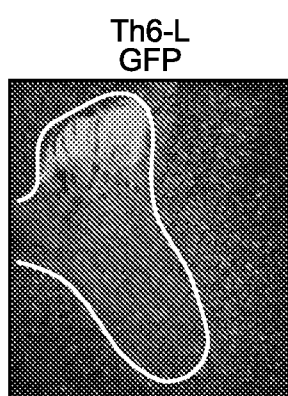
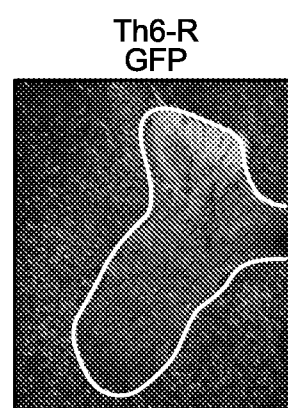
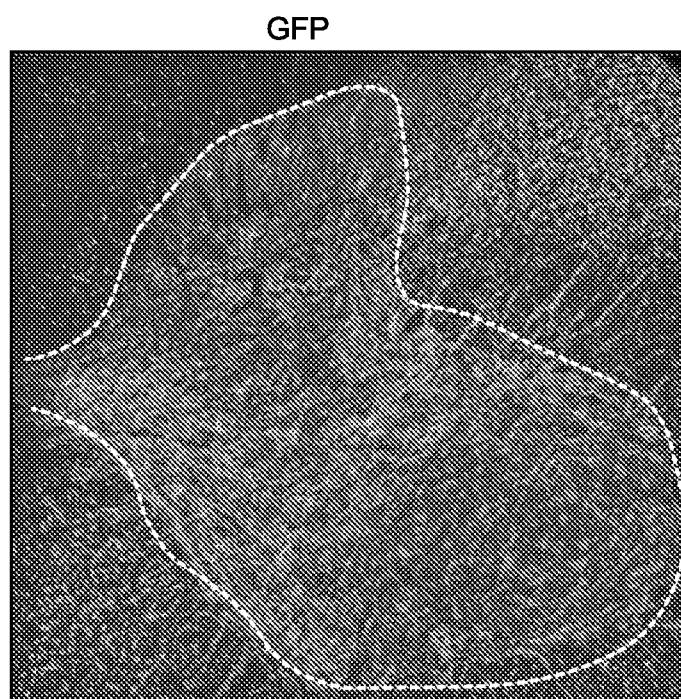
2/11

**FIG. 1C****FIG. 1D****FIG. 1E**

3/11

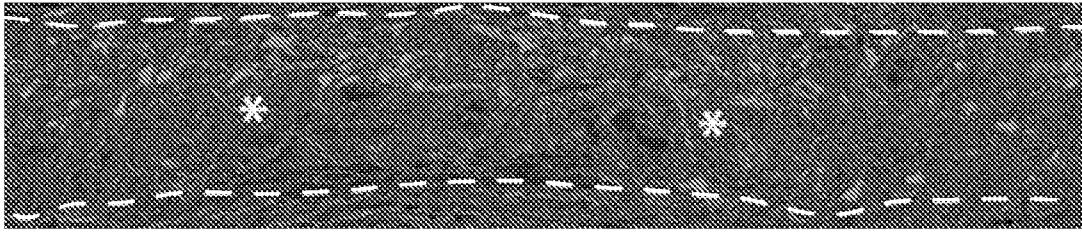
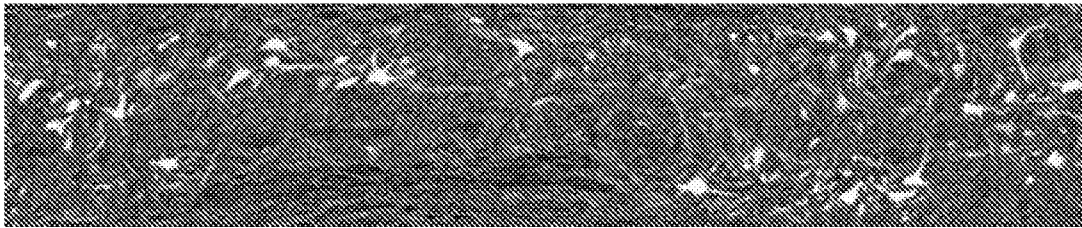
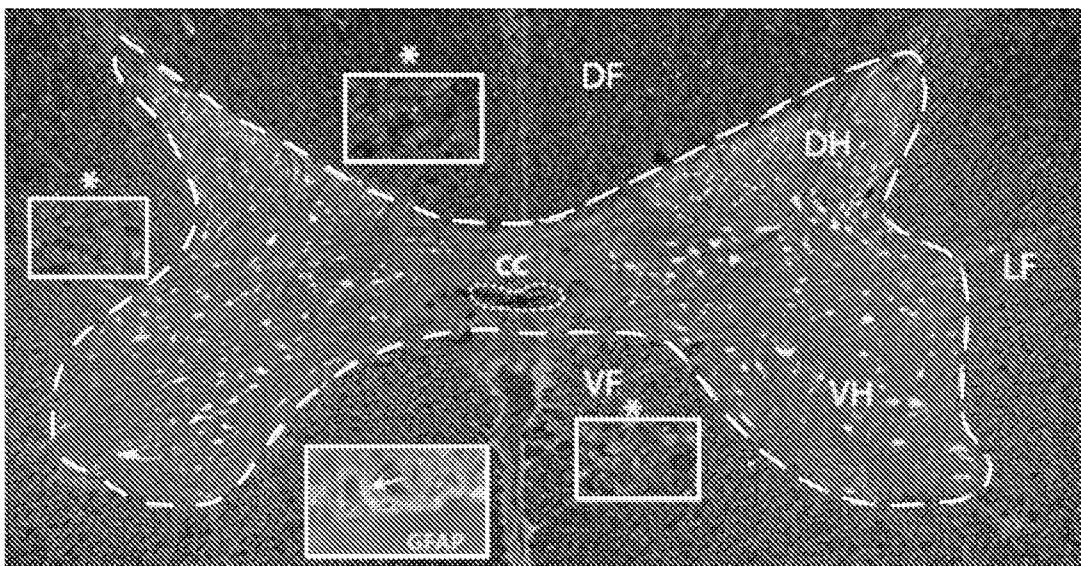
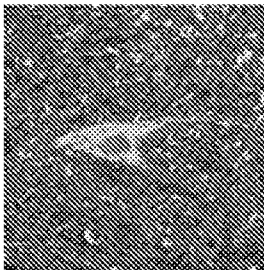
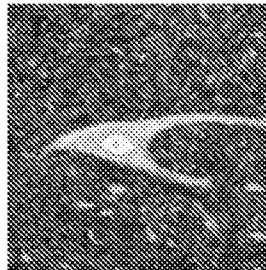
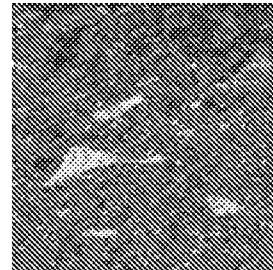
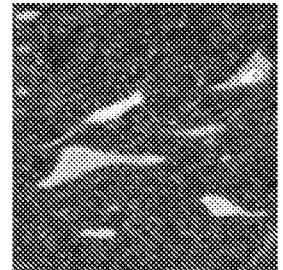
**FIG. 1F****FIG. 1G****FIG. 1H****FIG. 1I****FIG. 1J**

4/11

**FIG. 2A****FIG. 2B****FIG. 2C****FIG. 2D**

5/11

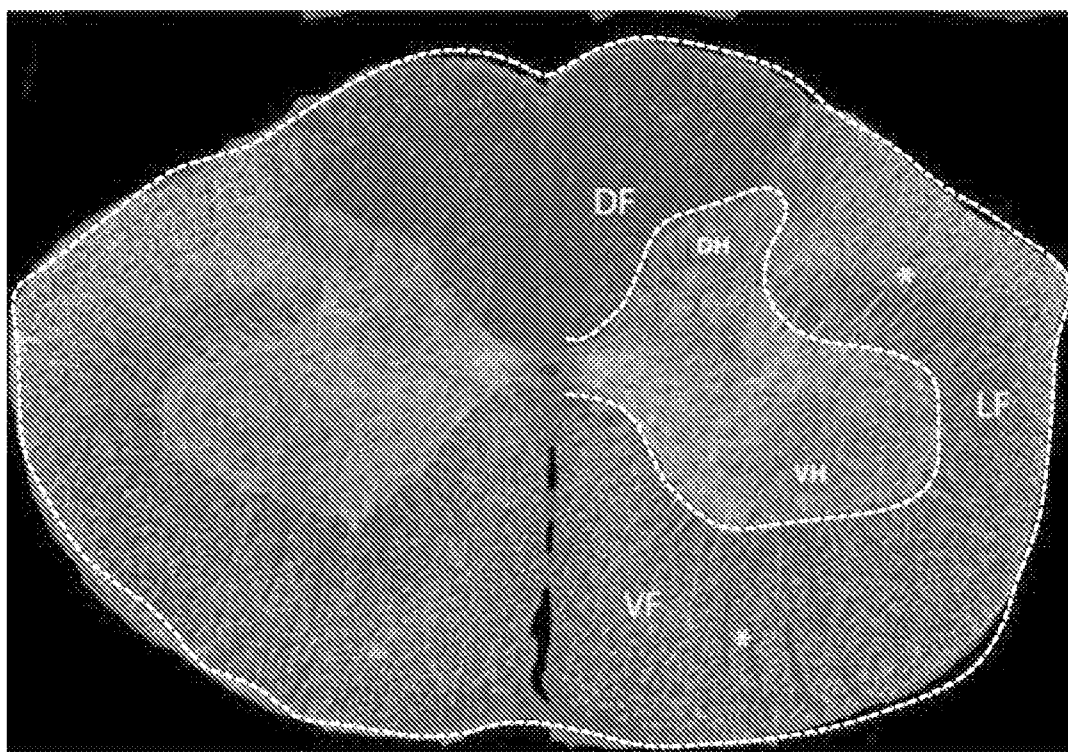
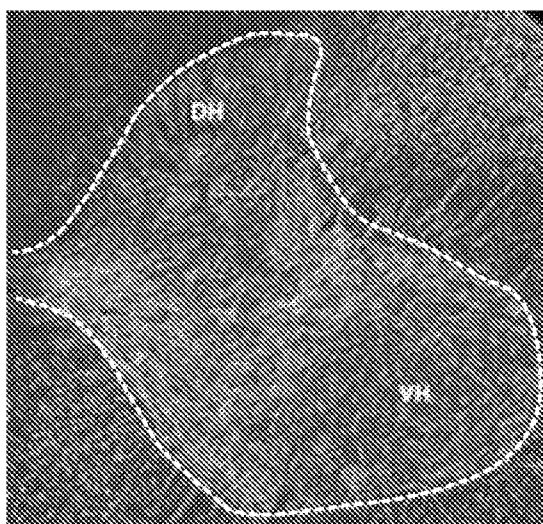
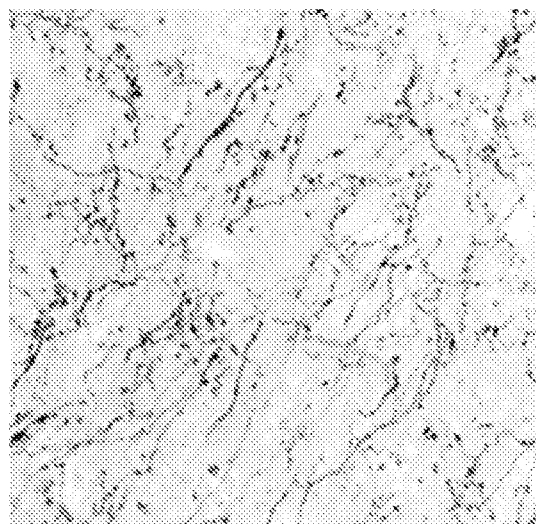
RFP / NeuN

**FIG. 3A****FIG. 3B****FIG. 3C****FIG. 3D****FIG. 3E****FIG. 3F****FIG. 3G**



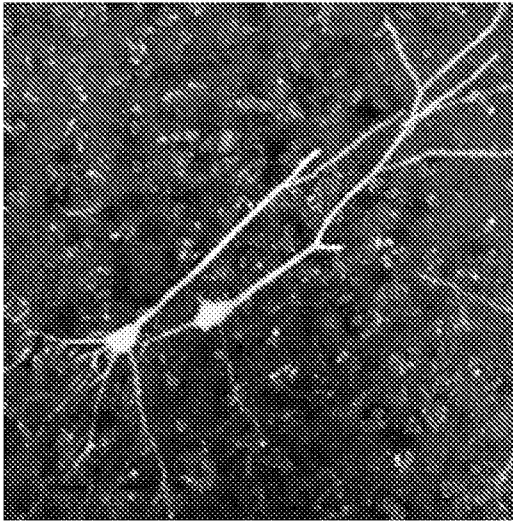
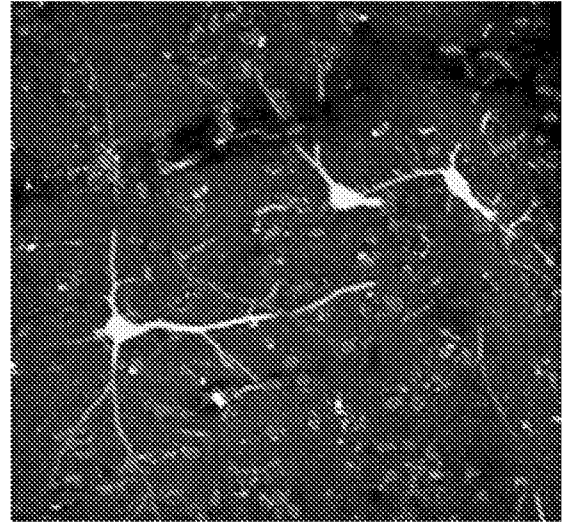
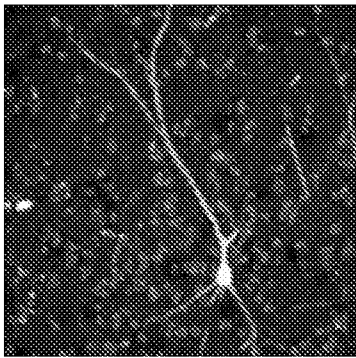
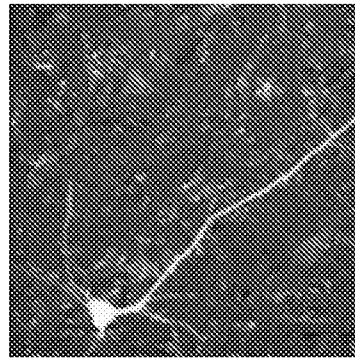
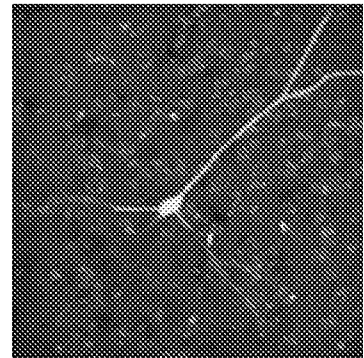
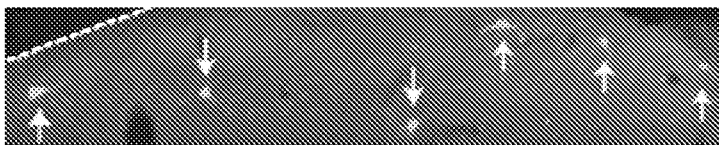
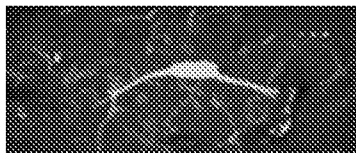
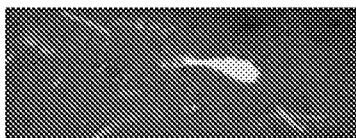
6/11

GFP / NeuN

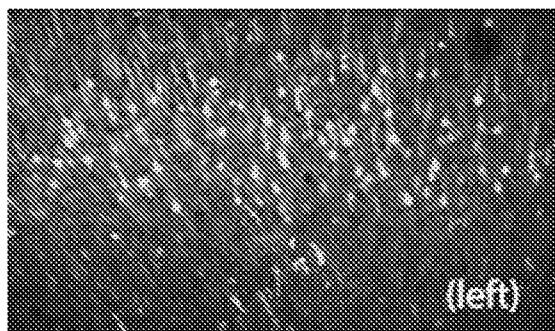
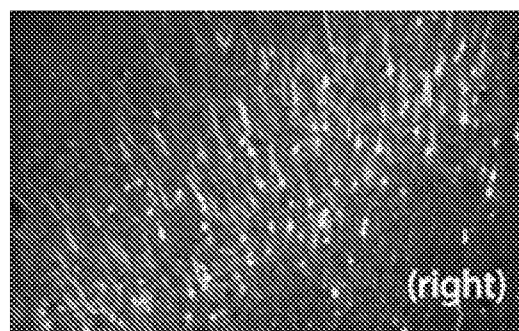
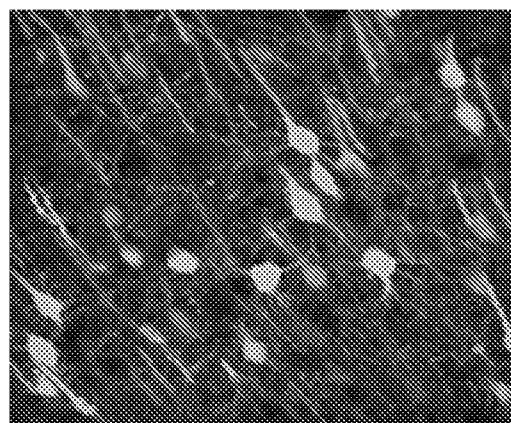
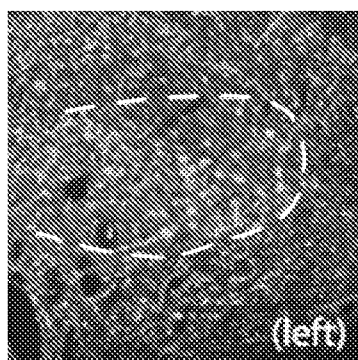
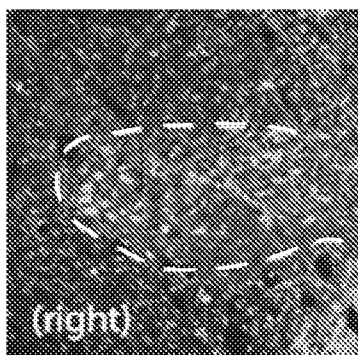
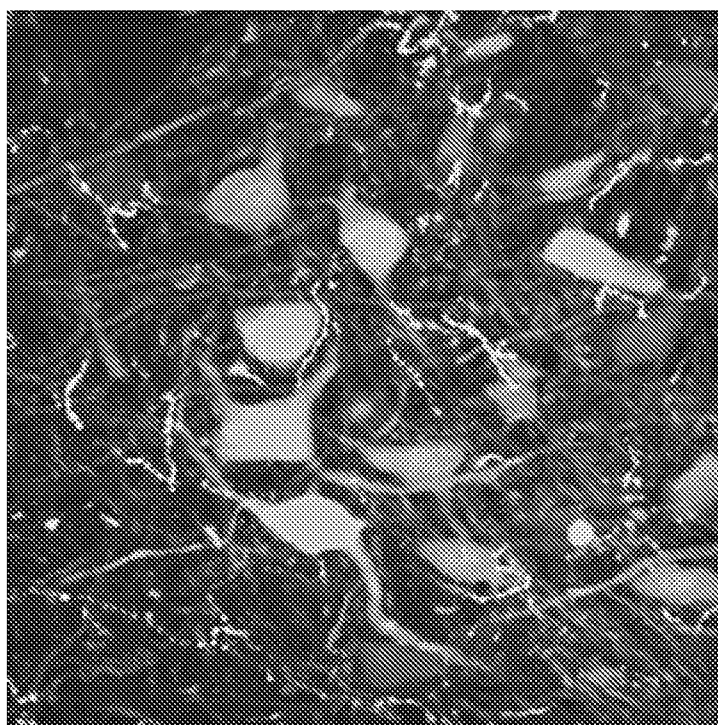
**FIG. 4A****FIG. 4B****FIG. 4C**

7/11

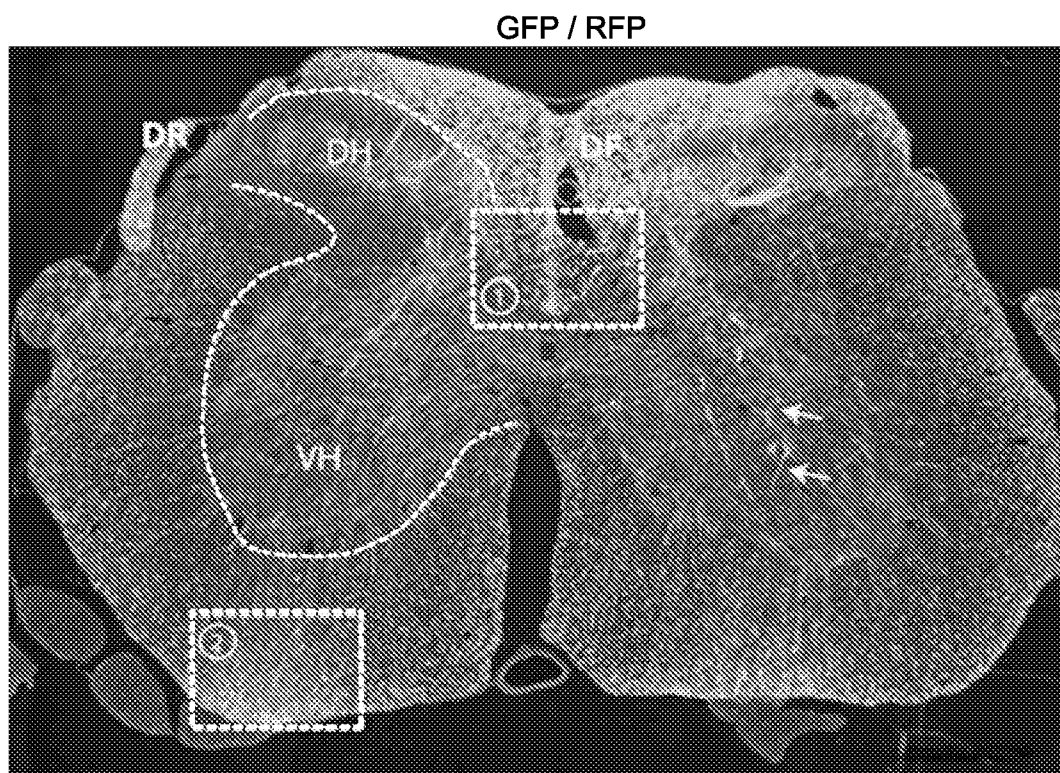
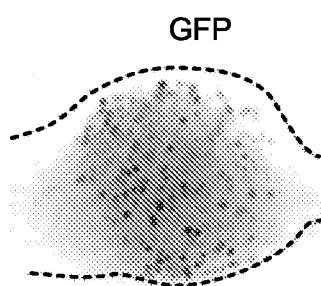
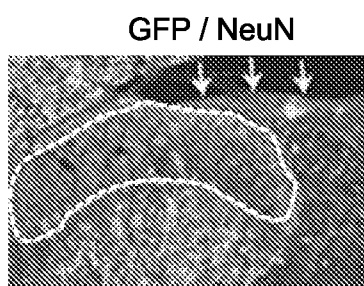
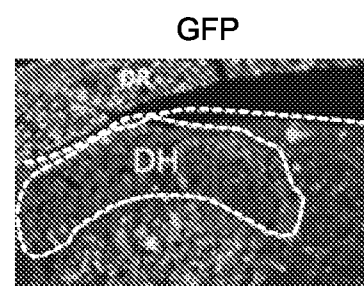
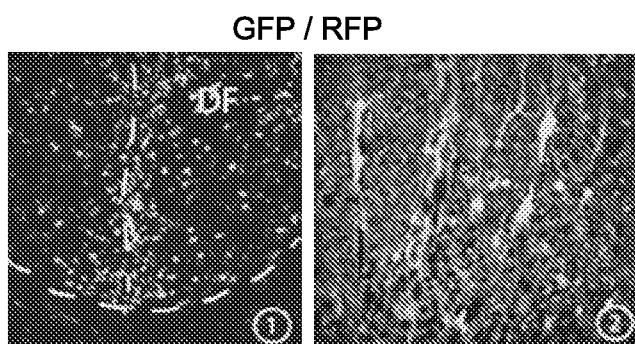
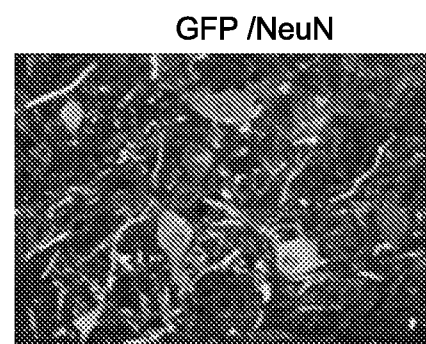
GFP / DAPI

**FIG. 5A****FIG. 5B****FIG. 5C****FIG. 5D****FIG. 5E****FIG. 5F****FIG. 5K****FIG. 5G****FIG. 5I****FIG. 5H****FIG. 5J****FIG. 5L**

8/11

**FIG. 6A****FIG. 6B****FIG. 6C****FIG. 6D****FIG. 6E****FIG. 6F****FIG. 6G**

9/11

**FIG. 7A****FIG. 7B****FIG. 7C****FIG. 7D****FIG. 7E****FIG. 7F****FIG. 7G**



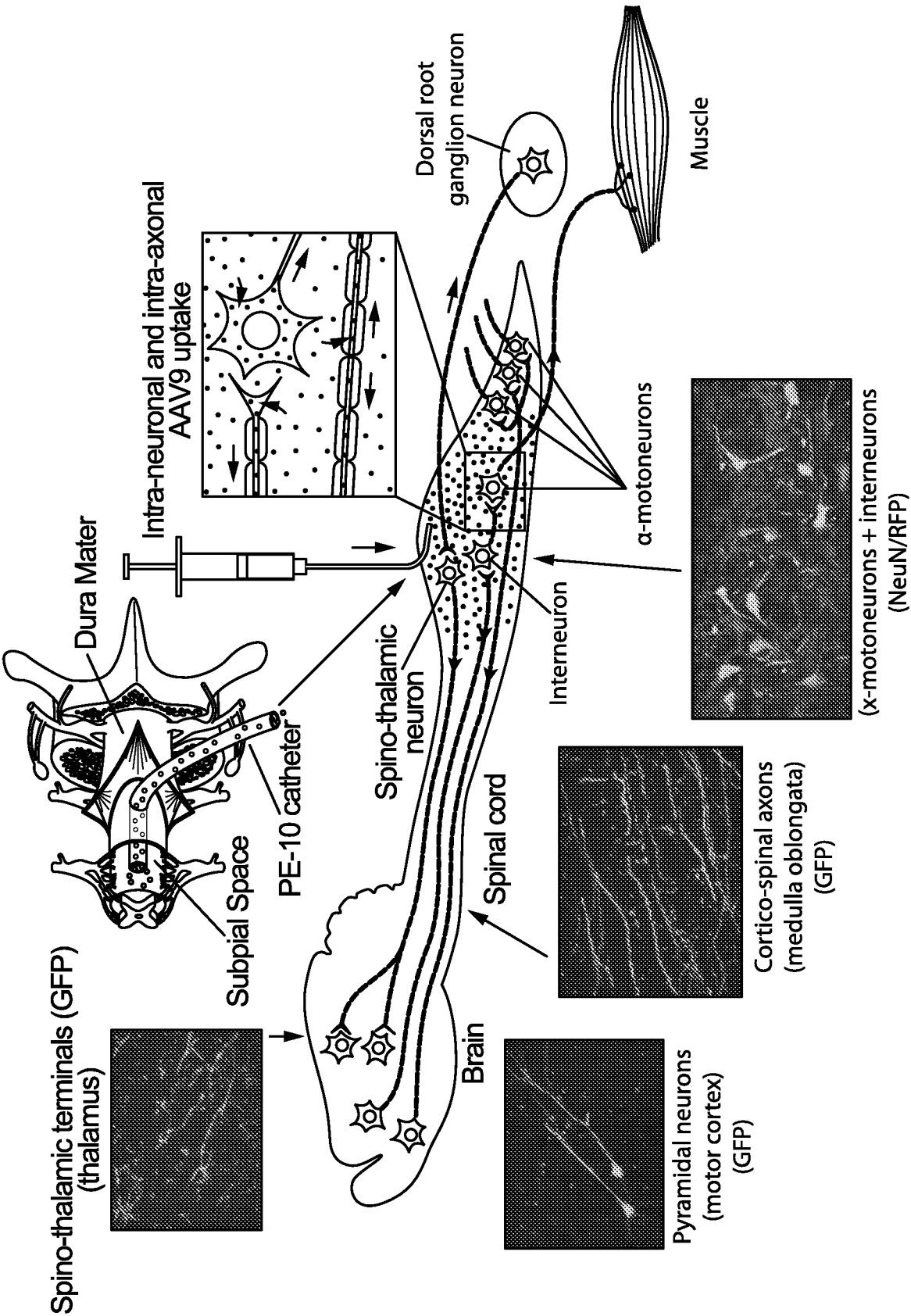
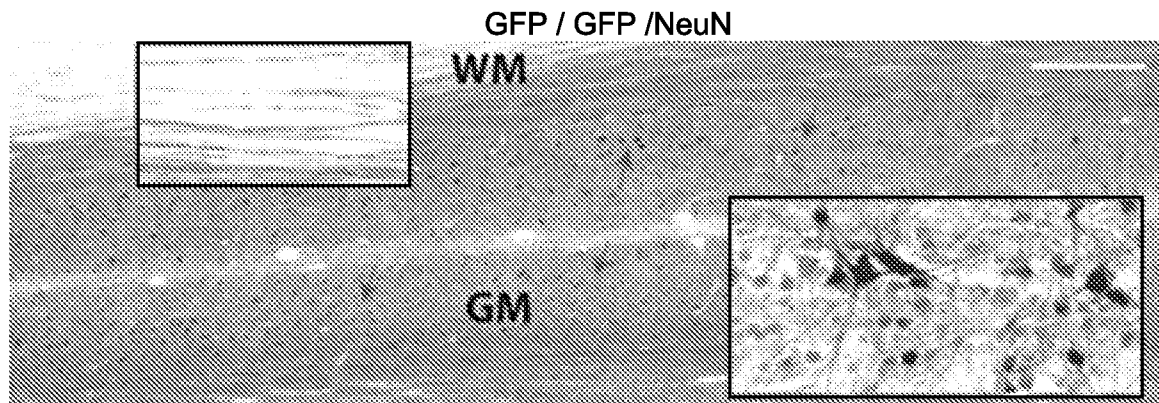
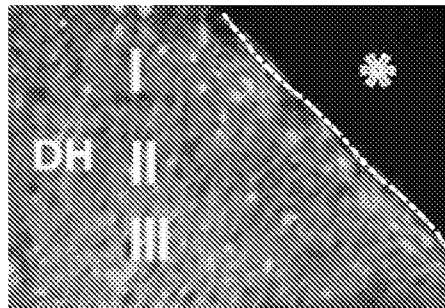
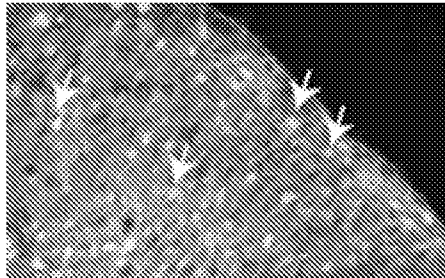
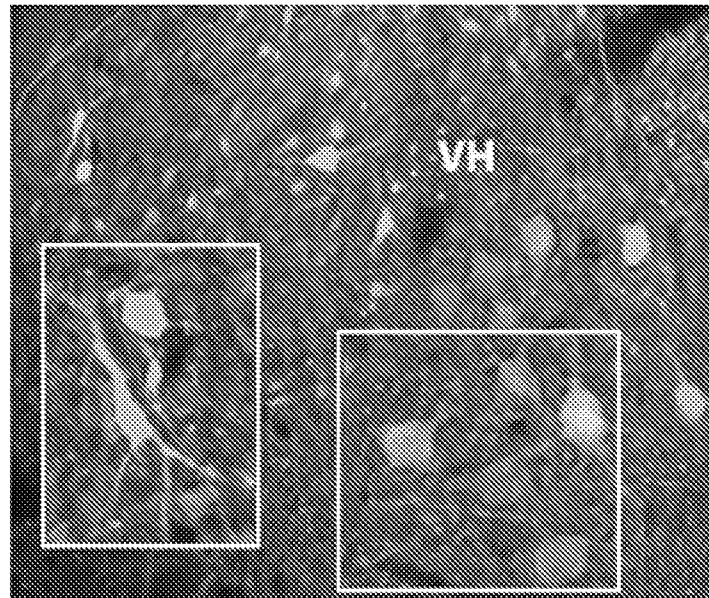
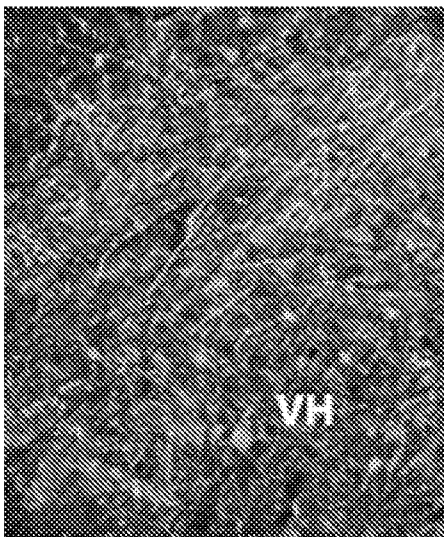
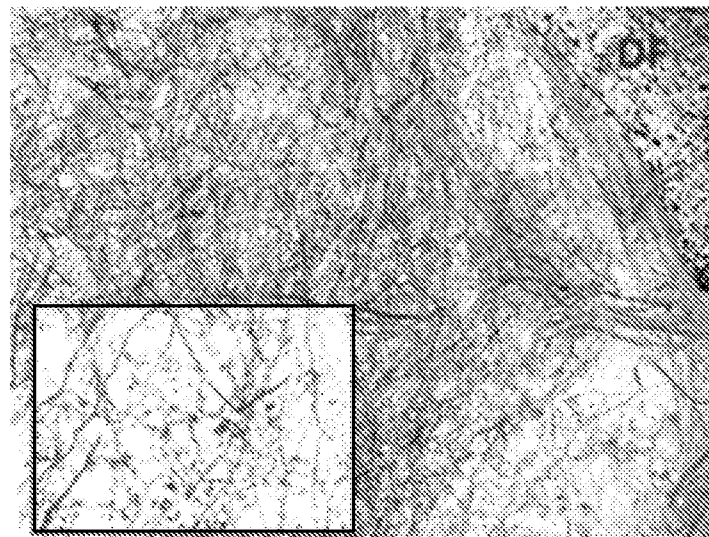


FIG. 8

11/11

**FIG. 9A****FIG. 9B****FIG. 9C****FIG. 9D****FIG. 9E****FIG. 9F**

