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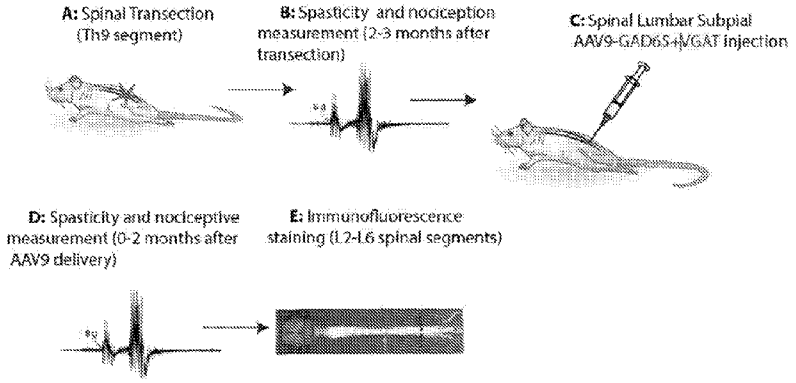

FIG. 1

(57) Abstract: The present invention provides a therapy for treating loss of GABA-mediated pre-synaptic inhibition after spinal injury. The therapeutic regimen includes spinal segment-specific upregulation of GAD65 (glutamate decarboxylase) and VGAT (vesicular GABA transporter) to modulate chronic spasticity in patients after spinal traumatic or ischemic injury.

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METHOD AND COMPOSITION FOR TREATING NEURONAL HYPER-EXCITABILITY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Serial No. 62/314,128, filed on March 28, 2016, the entire content of which is herein incorporated by reference.

GRANT INFORMATION

[0002] This invention was made with government support under Grant No. NS051644-02A2, awarded by the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0003] The invention relates generally to treating spinal injury and more specifically to a combined therapeutic regimen to modulate chronic spasticity in patients after spinal traumatic or ischemic injury.

BACKGROUND INFORMATION

[0004] Spinal cord injury (traumatic or ischemic) may lead to the development of clinically-defined spasticity and rigidity. One of the underlying mechanisms leading to the appearance of spasticity after spinal injury is believed to be the loss of local segmental inhibition and the resulting: i) increase in tonic motoneuron firing, ii) increase in primary afferent input during muscle stretch, and/or iii) exacerbated responses to peripheral sensory stimulation (*i.e.*, allodynia). Loss of gamma-aminobutryic acid (GABA)-mediated presynaptic, recurrent and reciprocal postsynaptic inhibition as well as the loss of its inhibitory effect in flexor afferent pathways has been shown to represent one of the key mechanisms.

[0005] Interestingly, however, previous studies have shown a significant increase in spinal parenchymal GAD67 expression in lumbar spinal segments in Th12 transected cats. Similarly, an increased density of inhibitory boutons apposing α-motoneuron membranes has been shown in adult rats with midthoracic spinal cord transection performed at postnatal day 5. These data suggest that a static increase in GABA synthesizing enzymes in spinal interneurons or increase in the number of inhibitory contacts with α-motoneurons after spinal trauma, in the absence of a specific inhibitory neuron-driven activity, is not sufficient to prevent the development of spasticity/hypereflexia. In addition to the role of decreased inhibition, several other potential mechanisms have been shown to contribute to the development of spasticity after spinal trauma, including: i) progressive increase in α-motoneuronal 5-HT_{2C} receptor activity which became spontaneously active in the absence of brain-derived serotonin, or ii) the down regulation of the potassium-chloride co-transporter KCC2 in motoneurons and resulting switch to GABA-mediated depolarization. Jointly, these data indicate that the mechanism leading to the development of spasticity after spinal injury (traumatic or ischemic) is complex and can vary depending on the model used as well as the age of experimental animals when the injury is induced.

[0006] Clinical pharmacological-treatment studies show that the use of systemic or spinally-administered baclofen ($GABA_B$ receptor agonist) represents the most potent anti-spasticity pharmacological treatment. While effective in modulating spasticity of different etiologies including spinal trauma, amyotrophic lateral sclerosis or central stroke, major side effects such as general sedation and progressive tolerance development often limit its chronic use. The use of systemically-administered GABA-mimetic compounds such as tiagabine (GABA reuptake inhibitor) shows only a weak or no anti-spasticity effect in clinically-acceptable doses, which correlates with a relatively modest potentiation of brain or spinal parenchymal GABA release after systemic delivery. In addition, currently available spinal drug delivery systems (such as epidural or intrathecal delivery) do not permit a spinal segment-restricted therapeutic effect. Because the origin of spasticity affecting individual muscle groups can be somatotopically mapped to specific spinal segments, the development of segment-targeted anti-spasticity

treatments would represent a clear advantage over current therapeutic approaches by reducing unwanted side effects. Accordingly, there is a need for novel antispasticity treatments.

SUMMARY OF THE INVENTION

[0007] The present invention is based on the observation that a combined treatment composed of spinal segment-specific upregulation of GAD65 (glutamatedecarboxylase) and VGAT (vesicular GABA transporter) in rats with ischemia-induced spasticity leads to an antispasticity effect, and that such a combined treatment results in decreased muscle spasticity.

[0008] Accordingly, the invention provides a method of treating spasticity in a subject. The method includes upregulation of GAD65 (glutamate decarboxylase) gene and VGAT (vesicular GABA transporter) gene, thereby treating spasticity in the subject. Upregulation of the GAD65 gene and VGAT gene may be spinal-specific upregulation of the GAD65 gene and VGAT gene, by administering to the subject a viral vector comprising a polynucleotide encoding GAD65 and VGAT, wherein GAD65 and VGAT are expressed, thereby decreasing spasticity. The GAD65 gene and VGAT gene may be overexpressed. The vector may be a lentiviral vector, adenoviral vector, or an adeno-associated vector (AAV). The AAV may be AAV type 9 (AAV9). In various embodiments, the viral vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

[0009] In another aspect, the invention provides a method of treating spasticity in a subject. The method includes administering to a subject in need thereof a therapeutically effective amount of a viral vector comprising a polynucleotide encoding GAD65 gene and VGAT gene, thereby treating spasticity in the subject. The vector may be a lentiviral vector, adenoviral vector, or an adeno-associated vector (AAV), and may be administered directly into the spine of the subject. The AAV may be AAV type 9 (AAV9). In various embodiments, the vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the

subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

[0010] In another aspect, the invention provides a treatment regimen for treating a subject having a spinal cord injury. The treatment regimen includes administering a viral vector comprising a polynucleotide encoding GAD65 and VGAT, wherein GAD65 and VGAT are expressed, thereby decreasing spasticity. Upregulation of GAD65 and VGAT includes administering a viral vector encoding GAD65 and VGAT, wherein GAD65 and VGAT are expressed and decrease spasticity. The vector may be a lentiviral vector, adenoviral vector, or an adeno-associated vector, and may be administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject. In various embodiments, the vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

[0011] In another aspect, the present invention provides an expression cassette comprising a promoter or regulatory sequence functionally linked to a polynucleotide encoding GAD65 and VGAT. Also provided are a vector, such as an AAV9, that includes a regulatory sequence such as a promoter functionally linked to a polynucleotide encoding GAD65 and VGAT.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 is a pictorial diagram showing an exemplary methodology for performing the methods of the invention.

[0013] Figure 2 is a pictorial diagram showing the distribution of transgene expression achieved after lumbar subpial AAV9-UBI-GFP delivery. A wide-spread GFP expression in interneurons through the gray matter can be seen. AAV9 virus encoding GAD65 (gutamate-decarboxylase 65) and VGAT (vesicular GABA transporter) is injected into targeted segments using subpial delivery method.

[0014] Figures 3A-3D are graphical diagrams showing potent anti-spasticity and anti-nociceptive effect after lumbar subpial AAV9-UBI-GAD65+VGAT delivery in chronic spinal transection-induced spastic rat.

[0015] Figures 4A-4D are pictorial diagrams showing induction of a mixed inhibitory-excitatory neurotransmitter phenotype in spinal excitatory interneurons by lumbar subpial AAV9-UBI-GAD65 + VGAT delivery. At 8 weeks immunofluorescence analysis of GAD65/VGAT gene-injected segments showed a significant upregulation of both genes and appearance of mixed inhibitory/excitatory neurotransmitter phenotype (coexpression of GAD65 or VGAT with VGLUT2 (vesicular glutamate transporter), (Figs. 4A and 4B). No coexpression in animals injected with control AAV9 was seen (Figs. 4C and 4D). These data confirmed an effective induction of inhibitory drive in GAD65/VGAT over-expressing neurons which likely mediate decrease in muscle spasticity.

[0016] Figure 5 is a pictorial diagram showing a significant increase in number of a mixed inhibitory-excitatory interneurons and projecting DRG neurons in lumbar spinal cord in spastic rats after lumbar subpial AAV9-UBI-GAD65+VGAT delivery. The table shows quantitative analysis of GAD65 and VGAT expression.

DETAILED DESCRIPTION OF THE INVENTION

[0017] The present invention is based on the observation that a combined treatment composed of spinal segment-specific upregulation of GAD65 (glutamatedecarboxylase) gene and VGAT (vesicular GABA transporter) gene in rats with ischemia-induced spasticity leads to an antispasticity effect, and that such a combined treatment results in decreased muscle spacticity.

[0018] 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.

[0019] 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.

[0020] 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.

[0021] 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.

[0022] 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.

[0023] A "therapeutic effect," as used herein, encompasses a therapeutic benefit and/or a prophylactic benefit as described herein.

[0024] As used herein, the terms "reduce" and "inhibit" are used together because it is recognized that, in some cases, a decrease can be reduced below the level of detection of a particular assay. As such, it may not always be clear whether the expression level or activity is "reduced" below a level of detection of an assay, or is completely "inhibited." Nevertheless, it will be clearly determinable, following a treatment according to the present methods.

[0025] As used herein, "treatment" or "treating" means to administer a composition to a subject or a system with an undesired condition. The condition can include a disease or disorder. "Prevention" or "preventing" means to administer a composition to a subject or a system at risk for the condition. The condition can include a predisposition to a disease or disorder. The effect of the administration of the composition to the subject (either treating and/or preventing) can be, but is not limited to, the cessation of one or more symptoms of the condition, a reduction or prevention of one or more symptoms of the condition, a reduction in the severity of the condition, the complete ablation of the condition, a stabilization or delay of the development or progression of a particular event or characteristic, or minimization of the chances that a particular event or characteristic will occur.

[0026] 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.

[0027] 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, α-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 α 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.

[0028] 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.

[0029] As used herein, a "regulatory gene" or "regulatory sequence" is a nucleic acid sequence that encodes products (*e.g.*, transcription factors) that control the expression of other genes.

[0030] As used herein, a "protein coding sequence" or a sequence that encodes a particular protein or polypeptide, is a nucleic acid sequence that is transcribed into mRNA (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' terminus (N-terminus) and a translation stop nonsense codon at the 3' terminus (C-terminus). A coding sequence can include, but is not limited to, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic DNA, and synthetic nucleic acids. A transcription termination sequence will usually be located 3' to the coding sequence.

[0031] As used herein, a "promoter" is defined as a regulatory DNA sequence generally located upstream of a gene that mediates the initiation of transcription by directing RNA polymerase to bind to DNA and initiating RNA synthesis. A promoter can be a constitutively active promoter (*i.e.*, a promoter that is constitutively in an active/"ON" state), it may be an inducible promoter (*i.e.*, a promoter whose state, active/"ON" or inactive/"OFF", is controlled by an external stimulus, *e.g.,* the presence of a particular compound or protein), it may be a spatially restricted promoter (*i.e.*, transcriptional control element, enhancer, etc.)(*e.g.*, tissue specific promoter, cell type specific promoter, etc.), and it may be a temporally restricted promoter (*i.e.,* the promoter is in the "ON" state or "OFF" state during specific stages of embryonic development or during specific stages of a biological process.

[0032] As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene. A "gene" may also include non-translated sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into heterogenous nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

[0033] As used herein, the terms "functionally linked" and "operably linked" are used interchangeably and refer to a functional relationship between two or more DNA segments, in particular gene sequences to be expressed and those sequences controlling their expression.

For example, a promoter/enhancer sequence, including any combination of cis-acting transcriptional control elements is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Promoter regulatory sequences that are operably linked to the transcribed gene sequence are physically contiguous to the transcribed sequence.

[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] The following eight groups each contain amino acids that are conservative substitutions for one another:

[0037] 1) Alanine (A), Glycine (G);

[0038] 2) Aspartic acid (D), Glutamic acid (E);

[0039] 3) Asparagine (N), Glutamine (Q);

[0040] 4) Arginine (R), Lysine (K);

[0041] 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

[0042] 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

[0043] 7) Serine (S), Threonine (T); and

[0044] 8) Cysteine (C), Methionine (M) (see, *e.g.*, Creighton, Proteins (1984)).

[0045] A conservative substitution may include substitution such as basic for basic, acidic for acidic, polar for polar, etc. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram (Livingstone C. D. and Barton G. J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" Comput. Appl Biosci. 9: 745-756; Taylor W. R. (1986) "The classification of amino acid conservation" J. Theor. Biol. 119; 205-218). Conservative substitutions may be made, for example, according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

TABLE 1: Grouping of amino acids

Characteristic	Set	Characteristic	Sub-set
Hydrophobic	F W Y H K M I L V A G C	Aromatic Aliphatic	F W Y H I L V
Polar	W Y H K R E D C S T N Q	Charged Positive	H K R E D H K R
		Charged Negative	E D
Small	V C A G S P T N D	Tiny	A G S

[0046] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (*e.g.*, a polypeptide of the invention), which does not comprise additions or deletions, for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0047] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. The invention provides polypeptides that are substantially identical to the polypeptides, respectively, exemplified herein, as well as uses thereof including, but not limited to, use for treating or preventing neurological diseases or disorders, *e.g.,* neurodegenerative diseases or disorders, and/or treating SCI. Optionally, the identity exists over a region that is at least about

50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length, or the entire length of the reference sequence.

[0048] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0049] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, *e.g.*, Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

[0050] Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) Nuc. Acids Res. 25:3389-3402, and Altschul et al. (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves

first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0051] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0052] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, and complements thereof. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs). In various embodiments, nucleic acids are isolated when purified away from other cellular components or other contaminants (*e.g.*, other nucleic acids or proteins present in the cell) by standard techniques including, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well-known in the art. See *e.g.*, F. Ausubel, et al., ed. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York. In various embodiments, a nucleic acid is, for example, DNA or RNA and may or may not contain intronic sequences. In a preferred embodiment, the nucleic acid is a cDNA molecule.

[0053] As used herein “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water and emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents.

[0054] As used herein, the term "neuron" include a neuron and a portion or portions thereof (*e.g.*, the neuron cell body, an axon, or a dendrite). The term "neuron" as used herein denotes nervous system cells that include a central cell body or soma, and two types of extensions or projections: dendrites, by which, in general, the majority of neuronal signals are conveyed to the cell body, and axons, by which, in general, the majority of neuronal signals are conveyed from the cell body to effector cells, such as target neurons or muscle. Neurons can convey information from tissues and organs into the central nervous system (afferent or sensory neurons) and transmit signals from the central nervous systems to effector cells (efferent or motor neurons). Other neurons, designated interneurons, connect neurons within the central

nervous system (the brain and spinal column). Certain specific examples of neuron types that may be subject to treatment or methods according to the invention include cerebellar granule neurons, dorsal root ganglion neurons, and cortical neurons.

[0055] The term "neuronal degeneration" is used broadly and refers to any pathological changes in neuronal cells, including, without limitation, death or loss of neuronal cells, any changes that precede cell death, and any reduction or loss of an activity or a function of the neuronal cells. The pathological changes may be spontaneous or may be induced by any event and include, for example, pathological changes associated with apoptosis. The neurons may be any neurons, including without limitation sensory, sympathetic, parasympathetic, or enteric, *e.g.*, dorsal root ganglia neurons, motor neurons, and central neurons, *e.g.*, neurons from the spinal cord. Neuronal degeneration or cell loss is a characteristic of a variety of neurological diseases or disorders, *e.g.,* neurodegenerative diseases or disorders. In some embodiments, the neuron is a sensory neuron. In some embodiments, the neuron is a motor neuron. In some embodiments, the neuron is a damaged spinal cord neuron.

[0056] As used herein, “spasticity” refers to a condition in which certain muscles are continuously contracted. This contraction causes stiffness or tightness of the muscles and can interfere with normal movement, speech, and gait. Spasticity mostly occurs in disorders of the central nervous system (CNS) affecting the upper motor neurons in the form of a lesion, such as spastic diplegia, or upper motor neuron syndrome, and can also be present in various types of multiple sclerosis, where it occurs as a symptom of the progressively-worsening attacks on myelin sheaths and is thus unrelated to the types of spasticity present in neuromuscular cerebral palsy rooted spasticity disorders. Without being bound by theory, spasticity develops when an imbalance occurs in the excitatory and inhibitory input to α motor neurons caused by damage to the spinal cord and/or central nervous system. The damage causes a change in the balance of signals between the nervous system and the muscles, leading to increased excitability in muscles. Spasticity is found in conditions where the brain and/or spinal cord are damaged or

fail to develop normally; these include cerebral palsy, multiple sclerosis, spinal cord injury, and acquired brain injury including stroke.

[0057] As used herein, "neurodegenerative disorder" or a "neurological disorder" refers to a disorder which causes morphological and/or functional abnormality of a neural cell or a population of neural cells. The neurodegenerative disorder can result in an impairment or absence of a normal neurological function or presence of an abnormal neurological function in a subject. For example, neurodegenerative disorders can be the result of disease, injury, and/or aging. Non-limiting examples of morphological and functional abnormalities include physical deterioration and/or death of neural cells, abnormal growth patterns of neural cells, abnormalities in the physical connection between neural cells, under- or over production of a substance or substances, *e.g.,* a neurotransmitter, by neural cells, failure of neural cells to produce a substance or substances which it normally produces, production of substances, *e.g.*, neurotransmitters, and/or transmission of electrical impulses in abnormal patterns or at abnormal times. Neurodegeneration can occur in any area of the brain of a subject and is seen with many disorders including, for example, head trauma, stroke, ALS, multiple sclerosis, Huntington's disease, Parkinson's disease, and Alzheimer's disease.

[0058] As used herein, the term “nociception” refers to the sensory nervous system’s response to certain harmful or potentially harmful stimuli. In nociception, intense chemical (*e.g.*, chili powder in the eyes), mechanical (*e.g.*, cutting, crushing), or thermal (heat and cold) stimulation of sensory nerve cells called nociceptors produces a signal that travels along a chain of nerve fibers via the spinal cord to the brain. Nociception triggers a variety of physiological and behavioral responses and usually results in a subjective experience of pain in sentient beings.

[0059] Gamma-aminobutyric acid (GABA) and glutamate are the primary inhibitory and excitatory neurotransmitters in mammals. The balance between GABA and glutamate controls diverse processes such as neurogenesis, movement, circadian clocks, tissue development and blood glucose regulation. GABA is synthesized from glutamate by the 65 kDa and 67 kDa

isoforms of the pyridoxal phosphate (PLP) dependent enzyme Glutamic Acid Decarboxylase (GAD65 and GAD67). Human GAD65 and GAD67 have been isolated and cloned by Bu et al. (1992) Proc Natl Acad Sci 89:2115-2119. Human GAD65 cDNA encodes a Mr 65,000 polypeptide, with 585 amino acid residues (Genbank Accession No. NM000818; M81882), Human GAD67 encodes a Mr 67,000 polypeptide, with 594 amino acid residues (Genbank Accession No. NM013445; M81883); each of which is incorporated herein by reference). See also, US Pub. No. 2016/0081956, incorporated herein by reference).

[0060] Additional nucleic acid and amino acid sequences for human GAD65 are known in the art. See, for example, GenBank Accession No.: Q05329, human Glutamate decarboxylase 2 (GAD2/GAD65), which provides the amino acid sequence (SEQ ID NO: 3):

```
        10         20         30         40         50
MASPGSGFWS FGSEDGSGDS ENPGTARAWC QVAQKFTGGI GNKLCALLYG
        60         70         80         90        100
DAEKPAESGG SQPPRAAARK AACACDQKPC SCSKVDVNYA FLHATDLLPA
       110        120        130        140        150
CDGERPTLAF LQDVMNILLQ YVVKSFDRST KVIDFHYPNE LLQEYNWELA
       160        170        180        190        200
DQPQNLEEIL MHCQTTLKYA IKTGHPRYFN QLSTGLDMVG LAADWLTSTA
       210        220        230        240        250
NTNMFTYEIA PVFVLLEYVT LKKMREIIGW PGGSGDGIFS PGGAISNMYA
       260        270        280        290        300
MMIARFKMFP EVKEKGMAAL PRLIAFTSEH SHFSLKKGAA ALGIGTDSVI
       310        320        330        340        350
LIKCDERGKM IPSDLERRIL EARQKGFVPF LVSATAGTTV YGAFDPLLAV
       360        370        380        390        400
ADICKKYKIW MHVDAAWGGG LLMSRKHKWK LSGVERANSV TWNPHKMMGV
       410        420        430        440        450
PLQCSALLVR EEGLMQNCNQ MHASYLFQQD KHYDLSYDTG DKALQCGRHV
       460        470        480        490        500
DVFKLWLMWR AKGTTGFEAH VDKCLELAEY LYNIIKNREG YEMVFDGKPQ
       510        520        530        540        550
HTNVCFWYIP PSLRTLEDNE ERMSRLSKVA PVIKARMMEY GTTMVSYQPL
       560        570        580
GDKVNFFRMV ISNPAATHQD IDFLIEEIER LGQDL
```

See also, for example, GenBank Accession No.: X69936, Homo sapiens mRNA for glutamate decarboxylase (GAD2/GAD65), which provides the nucleic acid sequence (SEQ ID NO: 2):

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   1 atgtccccta tacatcacca tcaccatcac ctggttccgc gtggatccga agcttcgaat
  61 tctggctttt ggtctttcgg gtcggaagat ggctctgggg attccgagaa tcccggcaca
 121 gcgcgagcct ggtgccaagt ggctcagaag ttcacgggcg gcatcggaaa caaactgtgc
 181 gccctgctct acggagacgc cgagaagccg gcggagagcg gcgggagcca accccgcggg
 241 gccgccgccc ggaaggccgc ctgcgcctgc gaccagaagc cctgcagctg ctccaaagtg
 301 gatgtcaact acgcgtttct ccatgcaaca gacctgctgc cggcgtgtga tggagaaagg
 361 cccactttgg cgtttctgca agatgttatg aacattttac ttcagtatgt ggtgaaaagt
 421 ttcgatagat caaccaaagt gattgatttc cattatccta atgagcttct ccaagaatat
 481 aattgggaat tggcagacca accacaaaat ttggaggaaa ttttgatgca ttgccaaaca
 541 actctaaaat atgcaattaa aacagggcat cctagatact tcaatcaact ttctactggt
 601 ttggatatgg ttggattagc agcagactgg ctgacatcaa cagcaaatac taacatgttc
 661 acctatgaaa ttgctccagt atttgtgctt ttggaatatg tcacactaaa gaaaatgaga
 721 gaaatcattg gctggccagg gggctctggc gatgggatat tttctcccgg tggcgccata
 781 tctaacatgt atgccatgat gatcgcacgc tttaagatgt tcccagaagt caaggagaaa
 841 ggaatggctg ctcttcccag gctcattgcc ttcacgtctg aacatagtca tttttctctc
 901 aagaagggag ctgcagcctt agggattgga acagacagcg tgattctgat taaatgtgat
 961 gagagagggg aaatgattcc atctgatctt gaaagaagga ttcttgaagc caaacagaaa
1021 gggtttgttc ctttcctcgt gagtgccaca gctggaacca ccgtgtacgg agcatttgac
1081 ccctcttag ctgtcgctga catttgcaaa aagtataaga tctggatgca tgtggatgca
1141 gcttggggtg ggggattact gatgtcccga aaacacaagt ggaaactgag tggcgtggag
1201 agggccaact ctgtgacgtg gaatccacac aagatgatgg gagtcccttt gcagtgctct
1261 gctctcctgg ttagagaaga gggattgatg cagaattgca accaaatgca tgcctcctac
1321 ctctttcagc aagataaaca ttatgacctg tcctatgaca ctggagacaa ggccttacag
1381 tgcggacgcc acgttgatgt ttttaaacta tggctgatgt ggagggcaaa ggggactacc
1441 gggtttgaag cgcatgttga taaatgtttg gagttggcag agtatttata caacatcata
1501 aaaaaccgag aaggatatga gatggtgttt gatgggaagc ctcagcacac aaatgtctgc
1561 ttctggtaca ttcctccaag cttgcgtact ctggaagaca atgaagagag aatgagtcgc
1621 ctctcgaagg tggctccagt gattaaagcc agaatgatgg agtatggaac cacaatggtc
1681 agctaccaac ccttgggaga caaggtcaat ttcttccgca tggtcatctc aaacccagcg
1741 gcaactcacc aagacattga cttcctgatt gaagaaatag aacgccttgg acaagatttta
1801 taa
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[0061] GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes. This binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This action results in a negative change in the transmembrane potential, usually causing hyperpolarization. Two general classes of GABA receptor are known: $GABA_A$ in which the receptor is part of a ligand-gated ion channel complex, and $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open or close ion channels via intermediaries (G proteins).

[0062] Loss of GABA-mediated pre-synaptic inhibition after spinal injury plays a key role in the progressive increase in spinal reflexes and the appearance of spasticity. Clinical studies show that the use of baclofen ($GABA_B$ receptor agonist), while effective in modulating

spasticity is associated with major side effects such as general sedation and progressive tolerance development. The present study provides an assessment as to whether a combined therapy composed of spinal segment-specific upregulation of GAD65 (glutamate decarboxylase) gene and VGAT (vesicular GABA transporter) gene will lead to an antispasticity effect.

[0063] VGAT (vesicular GABA transporter) (also known as vesicular inhibitory amino acid transporter (VIAAT)) is a protein that in humans is encoded by the SLC32A1 gene (also known as the VGAT gene). VGAT is highly concentrated in the nerve endings of GABAergic neurons in the brain and spinal cord but also in glycinergic nerve endings. Caudhry, et al., J. Neurosci., 18(23):9733-9750 (1998), incorporated herein by reference. Nucleic acid and amino acid sequences for human VGAT are known in the art. See, for example, GenBank Accession No.: Q9H598, human Vesicular inhibitory amino acid transporter (VIAAT/VGAT), which provides the amino acid sequence (SEQ ID NO: 3):

```
        10         20         30         40         50
MATLLQSKLS NVATSVSNKS QARMSGMFAR MGFQAATDEE AVGFAHCDDL
        60         70         80         90        100
DFEHRQGLQM DILKAEGEFC GDEGAEAFVE GDIHYQRGSG APLPPSGSRD
       110        120        130        140        150
QVGGGGEPGG HDKPKITANE AGNNVTNAIQ GMFVLGLPYA ILMGGVLGLF
       160        170        180        190        200
LIIFAAVVCC YTGKILIACL YEENEDGEVV RVRDSYVAIA NACCAPRFPT
       210        220        230        240        250
LGGRVVNVAQ IIELVMTCIL YVVVSGNLMY NSFPGLPVSQ KSWSIIATAV
       260        270        280        290        300
LLPCAFLVNL KAVSEPSLLC TLAHFVINIL VIAYCLSSAR DWAWEKVKPY
       310        320        330        340        350
IDVKKFPISI GIIVFSYTSQ IFLPSLEGNM QQPSEFHCMN NWTHIAACVL
       360        370        380        390        400
NGLFALVAYL TWADETNEVI TINLPGSIRA VVNIFLVAKA LLSYPLPFEA
       410        420        430        440        450
AVEVLEKSLF QEGSRAFFPA CYSGDGRLKS WGLTLRCALV VFTLLMAIYV
       460        470        480        490        500
PHFALLMGLT GSLTGAGLCF LLPSLFHLRL LWRKLLMHQV PFDVAIFVIG
       510        520
GICSVSGFVH SLEGLIEAIR TNAED
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See also, for example, GenBank Accession No.: NM_080552, Homo sapiens solute carrier family 32 member 1 (SLC32A1), mRNA, which provides the nucleic acid sequence (SEQ ID NO: 4):

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   1 gctcgcgccc cgcggcagct ccgcagtgca ctagccacca ccgccgccgc cgccgctccg
  61 ccagcctgc tgccagcttg cccggtccag cgctgagaga gcctcgaacg ccagctgcga
 121 gggtcatgag ccagagagcc ccggggcgcc gcgcggagag caagcggaga tagcgacttt
 181 gcgccccca gccctcgcct tcttgcatcg cgttccccgc atcctcgggt cctttctgtcc
 241 tttccgctgt ccccaccgcc gccatggcca ccttgctccg cagcaagctg tccaacgtgg
 301 ccacgtccgt gtccaacaag tcccaggcca agatgagcgg catgttcgcc aggatgggtt
 361 ttcaggcggc cacggatgag gaggcggtgg gcttcgcgca ttgcgacgac ctcgactttg
 421 agcaccgcca gggcctgcag atggacatcc tgaaagccga gggagagccc tgcggggacg
 481 agggcgctga agcgcccgtc gagggagaca tccattatca gcgaggcagc ggagctcctc
 541 tgccgccctc cggctccaag gacccaggtgg gaggtggtgg cgaattcggg ggccacgaca
 601 agcccaaaat cacggcgtgg gaggcaggct ggaacgtgac caacgccatc cagggcatgt
 661 tcgtgctggg cctaccctac gccatcctgc acggcggcta cctggggttg tttctcatca
 721 tcttcgccgc cgttgtgtgc tgctacaccg gcaagatcct catcgcgtgc ctgtacgagg
 781 agaatgaaga cggcgaggtg gtgcgcgtgc gggactcgta cgtggccata gccaacgcct
 841 gctgcgccct gcgcttccca acgctgggcg gccgagtggt gaacgtagcg cagatcatcg
 901 agctggtgat gacgtgcatc ctgtacgtgg tggtgagtgg caacctcatg tacaacagct
 961 tccccgggct gcccgtgtcg cagaagtcct ggtccattat cgccacggcc gtgctgctgc
1021 cttgcgcctt ccttaagaac ctcaaggccg tgtccaagtt cagtctgctg tgcactctgg
1081 ccgacttcgt catcaatatc ctggtcatag cctactgtct atcgcgggcg cgcgactggg
1141 cctgggagaa ggtcaagttc tacatcgacg tcaagaagtt ccccatctcc attggcatca
1201 tcgtgttcag ctacacgtct cagatcttcc tgcctttgct ggagggcaat atgcagcagc
1261 ccagcgagtt ccactgcatg atgaactgga cgcacatcgc agcctgcgtg ctcaagggcc
1321 tcttcgcgct cgtcgcctac ctcacctggg ccgacgagac caaggaggtc atcacggata
1381 acctgcccgg ctccatccgc gccgtggtca acatctttct ggtggccaag gcgctgttgt
1441 cctatcctct gccattcttt gccgctgtcg aggtgctgga gaagtcgctc ttccaggaag
1501 gcagccgcgc cttttccccg gcctgctaca gcggcgacgg gcgcctgaag tcctgggggc
1561 tgacgctgcg ctgcgcgctc gtcgtcttca cgctgctcat ggccatttat gtgccgcact
1621 tcgcgctgct catgggcctc accggcagcc tcacgggcgc cggcctctgt ttcttgctgc
1681 ccagcctctt tcacctgcgc ctgctctggc gcaagctgct gtggcaccaa gtcttcttcg
1741 acgtcgccat cttcgtcatc ggcggcatct gcagcgtgtc cggcttcgtg cactccctcg
1801 agggcctcat cgaagcctac cgaaccaacg cggaggacta gggcgcaagg gcgagcccca
1861 gccgcgcttc tgcgctctct cccttctccc ctcacccccc ccccaccagc ccagtgcgcc
1921 ctgccgcccc gcttgggagg ccaagcttta aacatctctg gttcctagtt tctgattatt
1981 cggggatggg ggggatgggg gggacaggg attcacgatc catcgcgtct gcgtttctgt
2041 tgtcctttct tttccacaac accctggttt tgggggggag cggggtgcat ttgcgggcag
2101 ggttctctgt ccttccaagt gggcccccga cactttggtt ccagtcatcg agggggttgg
2161 gaagggaggg agagggggcg cagctcgcag gcgtggcaac ttgaccttgg gggaatattt
2221 cacatccatc cagagctcgg aatctacagc gtccagccat ttccagcaag agcgcttccc
2281 attcgggaga cgtttcaacc ctgcagcggg aaaggctgac tgggaaatcc attttgggtg
2341 ggcaatttcc ttcaacgaag ccggaaggcg agaagccgcg gcgggccag cttgcctgcc
2401 ggttttcagg aatctaaact ctcatcttgt gcaatttatc aggtgtggaa ctgttctact
2461 gtgcgtgtgg tgtgctcgtg gtgaataaga tgaaatgtat atcagaaaaa aatctatctc
2521 taatttagag tgcggtacat aattatatcc gcaaataaag aagagacaaa ggctaaaaaa
2581 a
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[0064] Accordingly, in one aspect, the invention provides a vector comprising a nucleotide sequence encoding GAD65 and VGAT. Also within the scope of the invention is a polypeptide encoded by nucleotide sequence that has at least 60% homology to GAD65 or a functional fragment thereof. A polypeptide encoded by nucleotide sequence that about 70% homology,

about 75% homology, about 80% homology, about 85% homology, about 90% homology, about 95% homology, about 99% homology to GAD65 or a fragment thereof. Also within the scope of the invention is a polypeptide encoded by nucleotide sequence that has at least 60% homology to VGAT or a fragment thereof. A polypeptide encoded by nucleotide sequence that about 70% homology, about 75% homology, about 80% homology, about 85% homology, about 90% homology, about 95% homology, about 99% homology to VGAT or a fragment thereof.

[0065] Decreased or completely lost activity of a facilitatory supraspinal input into spinal GABA-ergic inhibitory interneurons and resulting decrease in local segmental inhibition has been postulated as one of the key mechanisms leading to the development of muscle spasticity in patients with spinal cord injury (SCI). Comparably, loss of spinal inhibitory interneurons, as seen after transient episodes of spinal cord ischemia leads to development of functionally defined muscle spasticity and rigidity. Independent of the insult nature (*e.g.*, spinal trauma or ischemia), clinical and experimental animal pharmacology studies have shown a comparable and potent antispasticity effect after systemic or spinal treatment with most commonly used antispasticity agent baclofen ($GABA_B$ receptor agonist). The primary site of baclofen-mediated hyperpolarizing action is believed to be at presynaptic Ia afferents.

[0066] One of the major limitations of systemic baclofen treatment, however, is the lack of a localized spinal segment-restricted effect and relatively high doses required to achieve clinically relevant relief of spasticity frequently produce unwanted systemic side effects such as sedation. Direct spinal delivery of baclofen using chronic intrathecal catheter provides a more site-restricted effect with less pronounced systemic activity, however it requires surgical intervention and ensuing complications associated with chronic intrathecal catheterization such as cerebrospinal fluid leak or infection has been described. More importantly, limits of effective long-term use of IT baclofen include the development of baclofen tolerance (*i.e.*, progressive escalation of dose to achieve consistent anti-spasticity effect) and withdrawal after an abrupt termination of baclofen treatment.

[0067] Preferential expression of GAD65 gene in infected astrocytes (as opposed to neurons) appears to provide a specific advantage with respect to expected GABA mediated anti-spasticity effect (see, *e.g.,* WO2014/116652, incorporated herein by reference). As has been shown *in vitro*, infection of primary astrocytes led to a Ca^{2+} independent increase in extracellular GABA concentration. Accordingly, it is expected that astrocyte-mediated GABA release in the spinal parenchyma will be independent of the functionality and connectivity of local neuronal inhibitory circuitry and will specifically exert its hyperpolarizing effect on $GABA_B$ receptor expressed on Ia afferents and/or α-motoneurons. The biological activity of astrocyte-produced GABA was confirmed by its depolarization-inducing effect on preferentially $GABA_A$ receptor-expressing cultured hNT neurons.

[0068] The use of a dual GAD65 and VGAT gene therapy represents a novel approach previously not tested in the context of spinal or brain delivery with the goal to increase regional neuronal inhibition. The core of this discovery is that both genes need to be upregulated in order to achieve a functionally relevant inhibition of otherwise hyperexcitable neurons. The potency of this treatment effect indicates that sufficient quantities of releasable GABA is available in the synaptic cleft to induce inhibition of postsynaptic membrane, leading to a decrease in α-motoneuron excitability and resulting suppression of muscle spasticity.

[0069] Accordingly, in another aspect, the invention provides a method of treating spasticity in a subject by spinal-specific upregulation of the GAD65 gene and VGAT gene. In various embodiments, upregulation of GAD65 and VGAT includes administering a viral vector encoding GAD65 and VGAT, and expressing the GAD65 and VGAT in the spinal cord of the subject, thereby decreasing spasticity in the subject.

[0070] 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), each of which is incorporated herein by reference). In one aspect of the invention, a lentivirus, an AV, or an AAV is utilized. Adenoviruses represent the largest nonenveloped viruses, because they are the maximum size able to be transported through the endosome (*i.e.,* envelope fusion is not necessary). The virion also has a unique "spike" or fibre associated with each penton base of the capsid that aids in attachment to the host cell. AAV is a dependent parvovirus that by definition requires co-infection with another virus (typically an adenovirus or herpesvirus) to initiate and sustain a productive infectious cycle. In the absence of such a helper virus, AAV is still competent to infect or transduce a target cell by receptor-mediated binding and internalization, penetrating the nucleus in both non-dividing and dividing cells.

[0071] Once in the nucleus, the virus uncoats and the transgene is expressed from a number of different forms--the most persistent of which are circular monomers. AAV will integrate into the genome of 1-5% of cells that are stably transduced (Nakai *et al.*, *J. Virol.* 76: 11343-349, 2002). Expression of the transgene can be exceptionally stable. Because progeny virus is not produced from AAV infection in the absence of helper virus, the extent of transduction is restricted only to the initial cells that are infected with the virus. It is this feature which makes AAV a suitable gene therapy vector for the present invention.

[0072] 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); Denefle, 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).

[0073] 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.

[0074] As used herein, the term "adeno-associated virus" (AAV), includes but is not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, and any other AAV now known. In one embodiment, the AAV is AAV type 2. In another embodiment, the AAV is AAV type 9.

[0075] 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). As defined above, reference to a "promoter" or "promoter sequence" is to be taken in its broadest context and includes a DNA regulatory region capable of binding RNA polymerase in a cell and initiating

transcription of a polynucleotide or polypeptide coding sequence such as messenger RNA, ribosomal RNAs, small nuclear of nucleolar RNAs or any kind of RNA transcribed by any class of any RNA polymerase. "Promoters" contemplated herein may also include the transcriptional regulatory sequences of a classical genomic gene, including the Goldberg-Hogness box which is required for accurate transcription initiation in eukaryotic cells, with or without a CAT box sequence and additional regulatory elements (*i.e.*, upstream activating sequences, enhancers and silencers).

[0076] Placing a sequence under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations, generally promoter position may be a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, *i.e.*, the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, *i.e.*, the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

[0077] Exemplary promoters useful in the methods and treatment regimens of the present invention include, but are not limited to, human ubiquitin promoter and human synapsin promoter. However, other known tissue-specific or cell-specific promoters may be used.

[0078] Suitable host cells for producing recombinant AAV particles include, but are not limited to, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of a exogenous nucleic acid molecule. Thus, a "host cell" as used herein generally refers to a cell which has been transfected with an exogenous nucleic acid molecule. The host cell includes any eukaryotic cell or cell line so long as the cell or cell line is

not incompatible with the protein to be expressed, the selection system chosen or the fermentation system employed.

[0079] The AAV vectors can be formulated into preparations for injection or administration by dissolving, suspending or emulsifying them in appropriate, pharmaceutically acceptable carriers or diluents. Examples of such pharmaceutically acceptable carriers or diluents include an aqueous or nonaqueous solvent, such as oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

[0080] If a viral vector specific for the cell type is not available, the vector can be modified to express a receptor (or ligand) specific for a ligand (or receptor) expressed on the target cell, or can be encapsulated within a liposome, which also can be modified to include such a ligand (or receptor). A peptide agent can be introduced into a cell by various methods, including, for example, by engineering the peptide to contain a protein transduction domain such as the human immunodeficiency virus TAT protein transduction domain, which can facilitate translocation of the peptide into the cell. In addition, there are a variety of biomaterial-based technologies such as nano-cages and pharmacological delivery wafers (such as used in brain cancer chemotherapeutics) which may also be modified to accommodate this technology.

[0081] In addition to cell integrating gene transfer after the use of lentiviral vectors, there are reports of successful GAD65 gene overexpression after AAV-GAD65 injections into subthalamic nuclei. In those studies, persistent GAD65 expression was seen up to 4-5 months after AAV-GAD65 injections. More importantly, recent systematic data demonstrate a high efficiency of AAV-based gene delivery into rat or minipig striatum even after a limited number of AAV injections (1-2 injections). Thus, in another embodiment, the present invention employs an AAV-based, genome-non-integrating GAD65-encoding and VGAT-encoding vector to achieve segment-specific GAD65 and VGAT expression.

[0082] By combining spinal delivery of GAD65 and VGAT, a significant and functionally relevant increase in spinal spasticity inhibition was achieved. The potency of spinal inhibition was tested in a well-characterized model of spinal trauma-induced muscle spasticity in rat. This animal model is characterized by the presence of highly developed spinal hyperreflexia and resulting muscle spasticity clearly present at chronic stages after spinal injury. Chronic spastic animals which received spinal subpial injection of GAD65+VGAT (delivered in AAV9-UBI vector) showed a significant suppression of spasticity response seen at 5 weeks after gene delivery and this significant treatment effect continue for at least the 8-th week. Immunofluoresence analysis showed the appearance of a mixed inhibitory-excitatory neurotransmitter phenotype in spinal interneurons as evidenced by colocalization of GAD65 and VGAT expression with glutamatergic markers VGLUT1 and VGLUT2. In animals injected with control GFP vector no anti-spasticity effect was seen and no co-localization of GAD65 and VGAT expression with glutamatergic markers VGLUT1 and VGLUT2 was detected.

[0083] Administering the instant combinational therapy can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. As used herein, the term "administration" or "administering" is defined to include an act of providing a compound or pharmaceutical composition of the invention to a subject in performing the methods of the invention. Exemplary routes of administration include, but are not limited to, intravenously, intraarticularly, intracisternally, intraocularly, intraventricularly, intrathecally, subpially, intramuscularly, intraperitoneally, intradermally, intracavitarily, and the like, as well as combinations of any two or more thereof. In certain embodiments, the AAV may be delivered directly into the spinal parenchyma, intrathecal space of the spine, into the spinal subpial space of the subject, and/or into the peripheral spastic muscle to achieve spinal upregulation of the GAD65 gene and VGAT gene. See, *e.g.,* WO2016/122791, incorporated herein by reference.

[0084] The term "therapeutically effective amount" or "effective amount" means the amount of the compound or composition that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician, *e.g.*, spinal upregulation of the GAD65 gene and VGAT gene. Thus, the term "therapeutically effective amount" is used herein to denote any amount of a formulation that causes a substantial improvement in a disease condition when applied to the affected areas repeatedly over a period of time. The amount will vary with the condition being treated, the stage of advancement of the condition, and the type and concentration of formulation applied. Appropriate amounts in any given instance will be readily apparent to those skilled in the art or capable of determination by routine experimentation. For example, a "therapeutically effective amount" of, *e.g.*, an AAV encoding the GAD65 gene and VGAT gene or a composition comprising the AAV encoding the GAD65 gene and VGAT gene, with respect to the subject method of treatment, refers to an amount of the AAV in a preparation which, when applied as part of a desired treatment regimen brings about upregulation of the GAD65 gene and VGAT gene.

[0085] Determining a therapeutically or prophylactically effective amount of the delivery vector can be done based on animal data using routine computational methods. Appropriate doses will depend, among other factors, on the specifics of the transfer vector chosen, on the route of administration, on the mammal being treated (*e.g.*, human or non-human primate or other mammal), age, weight, and general condition of the subject to be treated, the severity of the disorder being treated, the location of the area within the heart being treated and the mode of administration. Thus, the appropriate dosage may vary from patient to patient. An appropriate effective amount can be readily determined by one of skill in the art.

[0086] Dosage treatment may be a single dose schedule or a multiple dose schedule. Moreover, the subject may be administered as many doses as appropriate. One of skill in the art can readily determine an appropriate number of doses. However, the dosage may need to be adjusted to take into consideration an alternative route of administration, or balance the

therapeutic benefit against any side effects. Such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed.

[0087] Optionally, AAV-mediated delivery according to the invention may be combined with delivery by other viral and non-viral vectors. Such other viral vectors including, without limitation, adenoviral vectors, retroviral vectors, lentiviral vectors. herpes simplex virus (HSV) vectors, and baculovirus vectors may be readily selected and generated according to methods known in the art. Similarly, non-viral vectors, including, without limitation, liposomes, lipid-based vectors, polyplex vectors, molecular conjugates, polyamines and polycation vectors, may be readily selected and generated according to methods known in the art. When administered by these alternative routes, the dosage is desirable in the range described above.

[0088] In another aspect, the invention also provides a treatment regimen for treating a subject having a spinal cord injury. The treatment regimen includes administering a spinal-specific upregulation of the GAD65 gene and VGAT gene. As discussed in detail above, upregulation of GAD65 and VGAT may include administering a viral vector encoding GAD65 and VGAT, wherein GAD65 and VGAT is expressed and treats the spinal cord injury.

[0089] In addition, the methods of the invention can be used in the treatment of nerve damage, such as peripheral neuropathy, which is caused by exposure to toxic compounds, including heavy metals (*e.g.,* lead, arsenic, and mercury) and industrial solvents, as well as drugs including chemotherapeutic agents (*e.g.,* vincristine and cisplatin), dapsone, HIV medications (*e.g.*, Zidovudine, Didanosine, Stavudine, Zalcitabine, Ritonavir, and Amprenavir), cholesterol lowering drugs (*e.g.*, Lovastatin, Indapamid, and Gemfibrozil), heart or blood pressure medications (*e.g.,* Amiodarone, Hydralazine, Perhexiline), and Metronidazole.

[0090] The methods of the invention can also be used to treat injury to the nervous system caused by physical, mechanical, or chemical trauma. Thus, the methods can be used in the treatment of peripheral nerve damage caused by physical injury (associated with, *e.g.*, burns,

wounds, surgery, and accidents), ischemia, prolonged exposure to cold temperature (*e.g.*, frost-bite), as well as damage to the central nervous system due to, *e.g.*, stroke or intracranial hemorrhage (such as cerebral hemorrhage). Likewise, the methods of the invention can be used in the treatment of chronic pain/nociception caused by such trauma.

[0091] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

[0092] AAV9 virus encoding GAD65 (glutamate-decarboxylase 65) and VGAT (vesicular GABA transporter) is injected into targeted segments using subpial delivery method (Figure 1). Animals (rats) with spinal injury-induced muscle spasticity were used. The distribution of transgene expression achieved after lumbar subpial AAV9-UBI-GFP delivery is shown in Figure 2. A wide-spread GFP expression in interneurons through the gray matter can be seen.

[0093] After GAD65 and VGAT gene delivery spasticity response was measured for up to 8 weeks after gene delivery. In control spastic animals a control AAV9-UBI-GFP was used. Figures 3A-3D show a progressive decrease in spasticity response in animals injected with AAV9-UBI-GAD65+VGAT. A significant anti-spasticity effect continue for minimum of 8 weeks after gene delivery (Figures 3A and 3B). Measurement of rate-dependent depression (represents an index of altert spinal inhibition) show a significant recovery if compared to cotrol AAV9-injected animals (Figure 3C).

[0094] At 8 weeks immunofluorescence analysis of GAD65/VGAT gene-injected segments showed a significant upregulation of both genes and appearance of mixed inhibitory/excitatory neurotransmitter phenotype (co-expression of GAD65 or VGAT with VGLUT2 (vesicular glutamate transporter), (Figures 4A and 4B). No co-expression in animals injected with control AAV9 was seen (Figures 4C and 4D). These data confirmed an effective induction of

inhibitory drive in GAD65/VGAT over-expressing neurons which likely mediate decrease in muscle spasticity.

[0095] 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.

What is claimed is:

1. A method of treating spasticity in a subject comprising upregulating GAD65 (glutamate decarboxylase) gene and VGAT (vesicular GABA transporter) gene, thereby treating spasticity in the subject.

2. The method of claim 1, wherein the upregulation of the GAD65 gene and VGAT gene is spinal-specific upregulation of the GAD65 gene and VGAT gene.

3. The method of claim 1, wherein upregulation of the GAD65 gene and the VGAT gene comprises administering to the subject a viral vector comprising a polynucleotide encoding GAD65 and VGAT, wherein GAD65 and VGAT are expressed, thereby decreasing spasticity.

4. The method of claim 3, wherein the GAD65 and VGAT are overexpressed.

5. The method of claim 3, wherein the vector is a lentiviral vector, adenoviral vector (AV), or an adeno-associated vector (AAV).

6. The method of claim 5, wherein the vector is a lentiviral vector.

7. The method of claim 5, wherein the vector is an AAV.

8. The method of claim 7, wherein the AAV is AAV9.

9. The method of claim 3, wherein the viral vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

10. A method of treating spasticity in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a viral vector comprising a polynucleotide encoding GAD65 and VGAT, thereby treating spasticity in the subject.

11. The method of claim 10, wherein the vector is a lentiviral vector, an AV, or an AAV.

12. The method of claim 11, wherein the vector is an AAV.

13. The method of claim 12, wherein the AAV is AAV9.

14. The method of claim 10, wherein the vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

15. A treatment regimen for treating a subject having a spinal cord injury comprising administering a viral vector comprising a polynucleotide encoding GAD65 and VGAT, wherein GAD65 and VGAT are expressed, thereby treating the spinal cord injury.

16. The treatment regimen of claim 15, wherein the GAD65 and VGAT are overexpressed.

17. The treatment regimen of claim 15, wherein the vector is a lentiviral vector, an AV, or an AAV.

18. The treatment regimen of claim 17, wherein the vector is a lentiviral vector.

19. The treatment regimen of claim 17, wherein the vector is an AAV.

20. The treatment regimen of claim 19, wherein the AAV is AAV9.

21. The treatment regimen of claim 15, wherein the vector is administered directly into the spinal parenchyma of the subject, into the intrathecal space of the subject, into the spinal subpial space of the subject, or into a peripheral spastic muscle of the subject.

22. A vector comprising a promoter functionally linked to a polynucleotide encoding GAD65 and VGAT.

23. The vector of claim 22, wherein the vector is a viral vector selected from the group consisting of a lentiviral, adenoviral, and AAV vector.

24. The vector of claim 23, wherein the vector is AAV9-UBI-GAD65+VGAT.

25. An isolated mammalian host cell containing the vector according to claim 22.

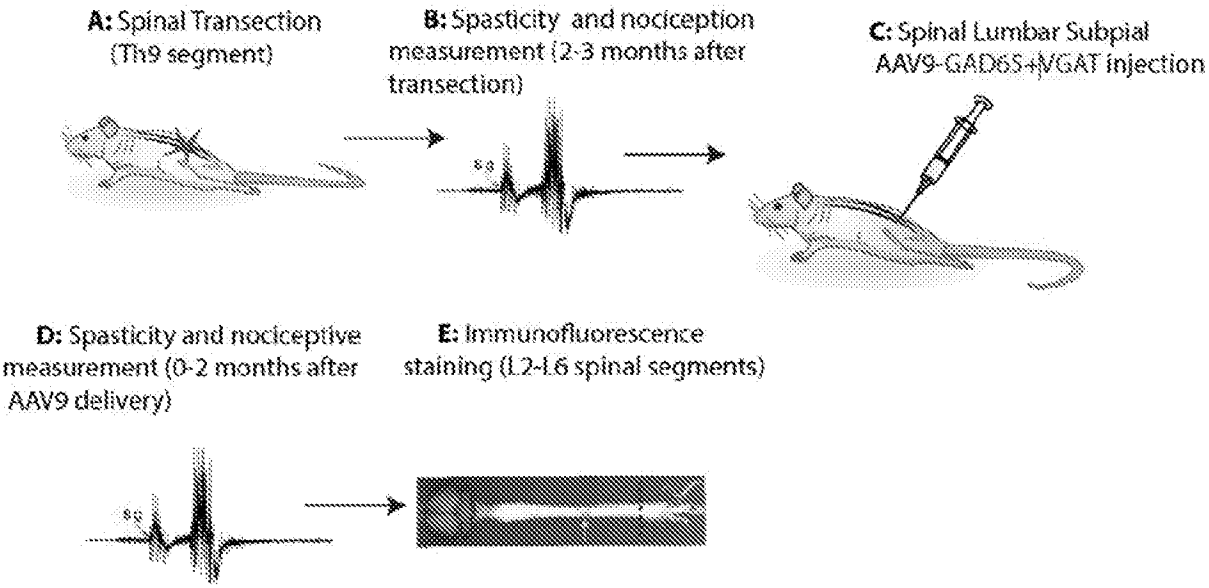

A: Spinal Transection (Th9 segment)
B: Spasticity and nociception measurement (2-3 months after transection)
C: Spinal Lumbar Subpial AAV9-GAD65+VGAT injection
D: Spasticity and nociceptive measurement (0-2 months after AAV9 delivery)
E: Immunofluorescence staining (L2-L6 spinal segments)

FIG. 1

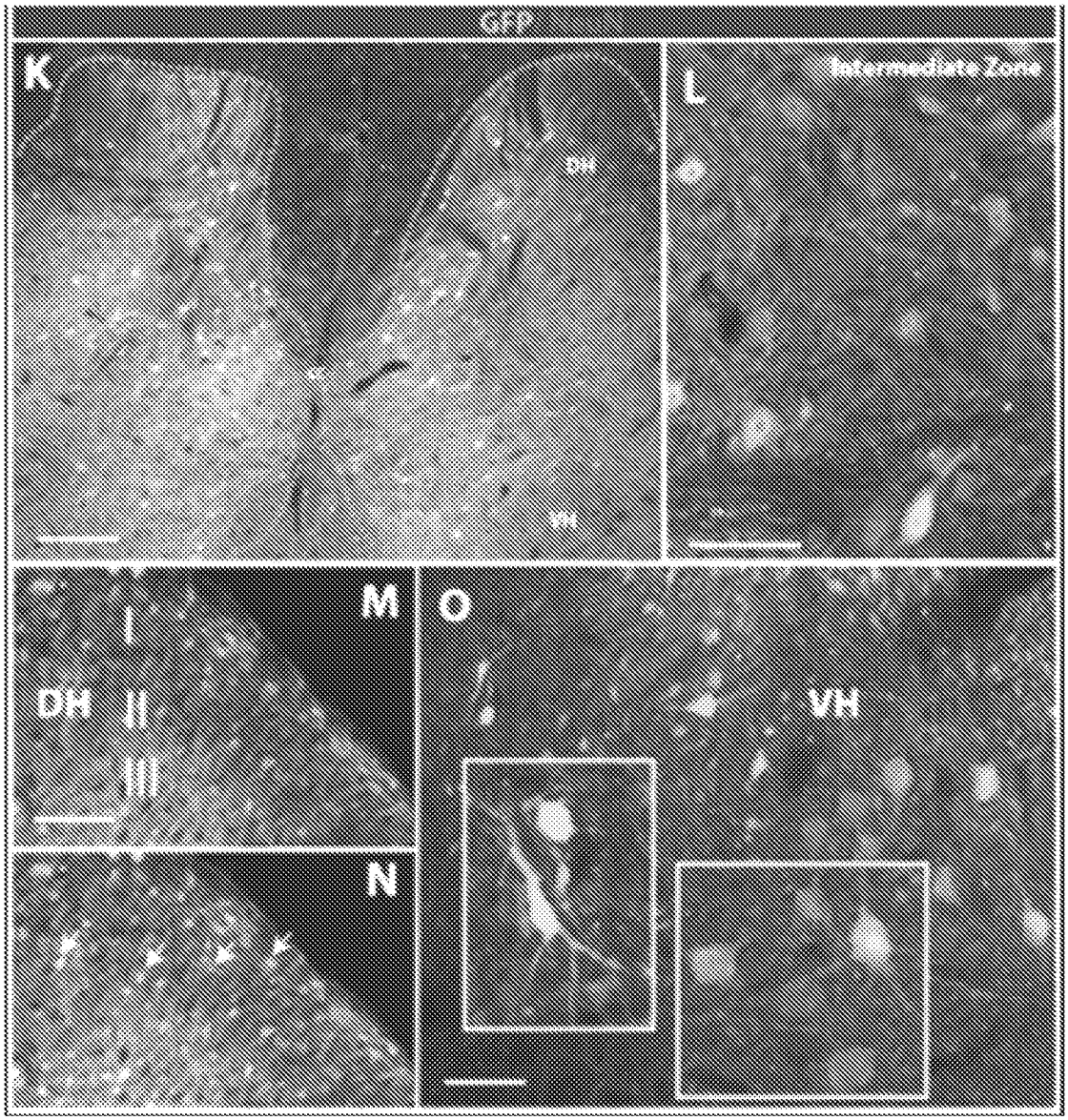


FIG. 2

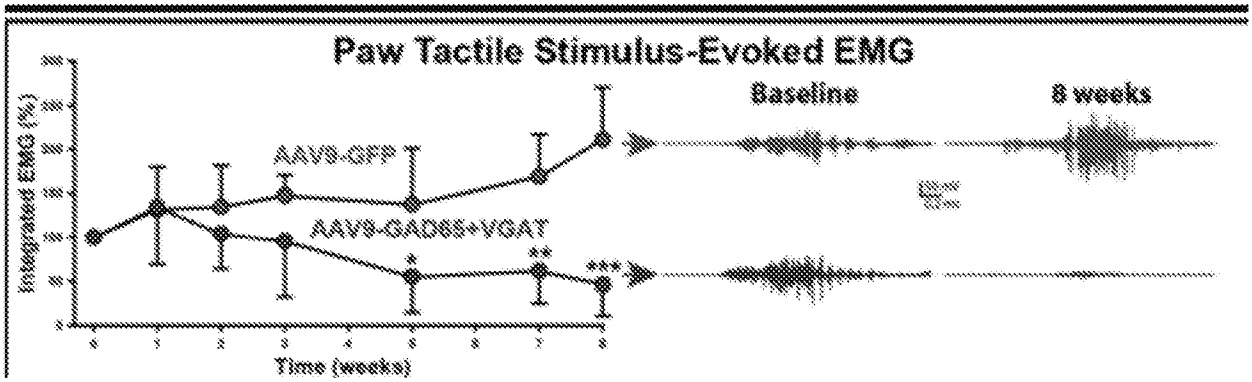


FIG. 3A

FIG. 3B

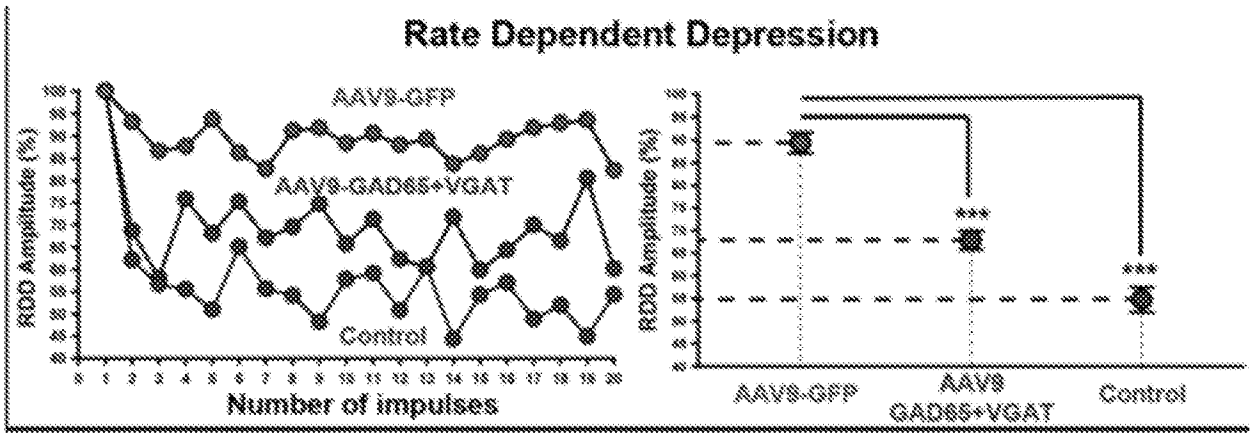


FIG. 3C

FIG. 3D

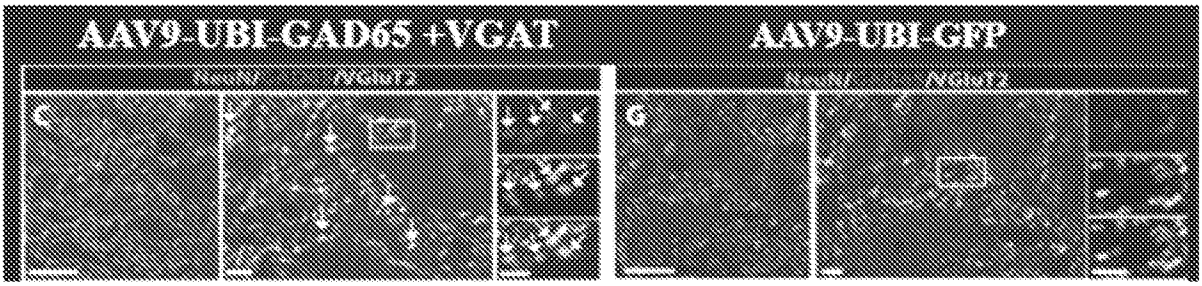


FIG. 4A

FIG. 4C

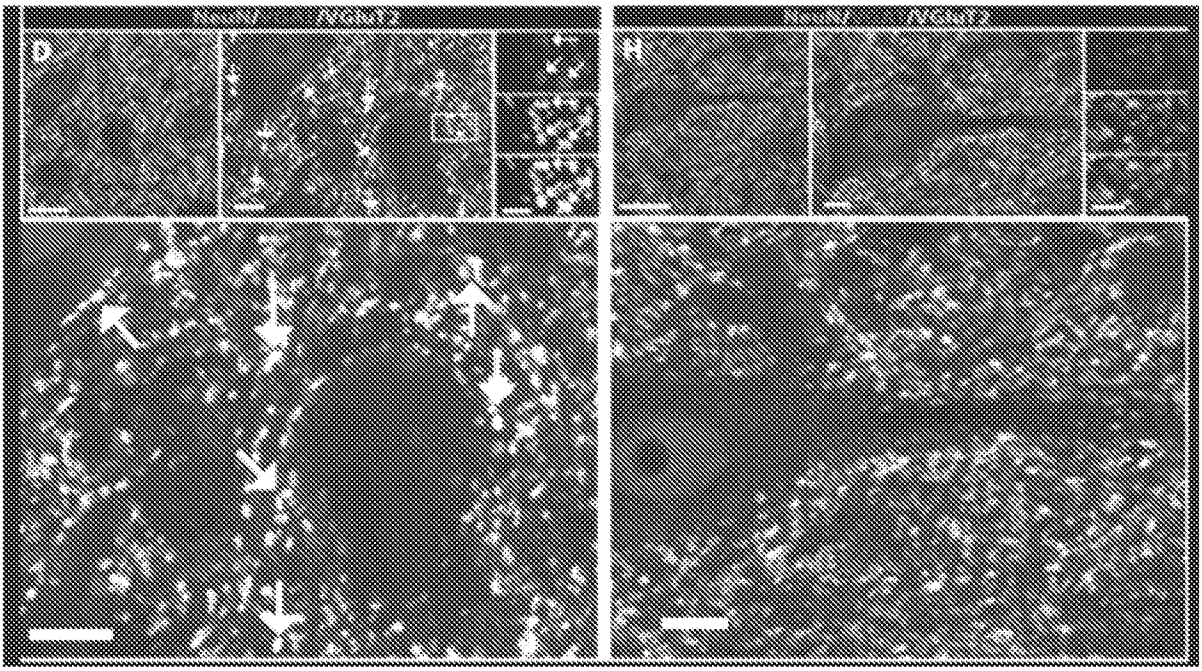
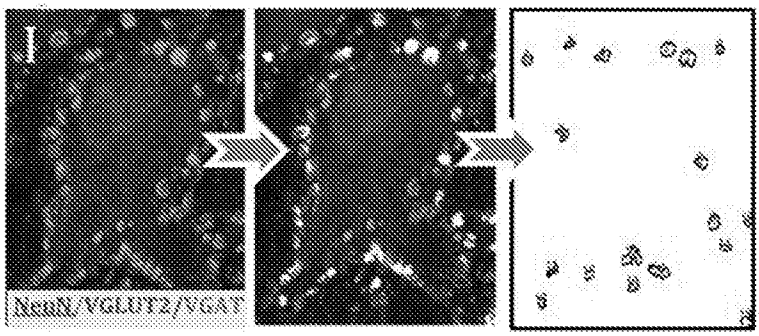


FIG. 4B

FIG. 4D



Quantitative analysis of GAD65 and VGAT expression.

Lumbar Subpial AAV9 delivery	GAD65	VGAT	VGLUT1 + GAD65	VGLUT1 + VGAT	VGLUT2+ GAD65	VGLUT2+ VGAT
Experimental Groups	% Normalized Signal (Integrated Density)		Co-expressing Puncta (* P<0.01)			
AAV9-GAD65/VGAT (n=4; 6 sections/animal)	208±19*	166±25*	14±8*	9±3.4*	245±97*	331±67*
AAV9-GFP (n=4; 6 sections/animal)	100±7	100±15	2±1	2±0.9	1±0.7	5±1

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/24285

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07C 233/69, C07C 235/34, C07C 235/42 (2017.01)
CPC - A61K 45/06, A61K 31/4535, C12N 9/88, A61K 38/51

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0343038 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 03 December 2015 (03.12.2015) Claim 8; Claim 11; Claim 12; Claim 16; Claim 20; para [0006]; para [0010]	1-21
Y	US 2002/0082390 A1 (FRIDDLE et al.) 27 June 2002 (27.06.2002) para [0004]; para [0011]; para [0040]; para [0059]	1-21
Y	JIN et al. "Demonstration of functional coupling between gamma-aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles." Proc Natl Acad Sci USA. 1 Apr 2003, Vol 100, No 7, pp 4293-8. Abstract; p4298, col 1, para 1 - col 2, para 1; p4293, col 1, last para	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 June 2017	10 AUG 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/24285

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-21, directed to a method of treating spasticity in a subject (having a spinal cord injury).

Group II, claims 22-25, directed to a vector comprising a polynucleotide encoding GAD65 and VGAT, and an isolated mammalian host cell containing the vector.

----------Please see continuation in first extra sheet----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-21

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/24285

Continuation of Box III Lack of Unity

The inventions listed as Groups I-II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features:

Group I has the special technical feature of administering to a subject in need thereof a therapeutically effective amount of a viral vector comprising a polynucleotide encoding GAD65 and VGAT, upregulating GAD65 and VGAT, thereby treating spasticity in the subject, that is not required by Group II.

Group II has the special technical feature of a vector comprising a polynucleotide encoding GAD65 and VGAT, and an isolated mammalian host cell containing the vector, that is not required by Group I.

Common technical features:

Groups I-II share the common technical feature of a viral vector comprising a polynucleotide encoding GAD65 and VGAT. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is made obvious by US 2015/0343038 A1 to THE REGENTS OF THE UNIVERSITY OF CALIFORNIA (hereinafter 'Univ California') in view of US 2002/0082390 A1 to Friddle et al., (hereinafter 'Friddle') and the article entitled 'Demonstration of functional coupling between gamma-aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles ' by Jin et al., (hereinafter 'Jin') [PNAS April 1, 2003, vol. 100, no. 7, 4293?4298].

Univ California teaches a method of treating spasticity in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a viral vector comprising a polynucleotide encoding GAD65, thereby treating spasticity in the subject (Claim 12 - 'A method of treating spasticity in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a gamma-aminobutryic acid (GABA) uptake inhibitor in combination with a viral vector encoding GAD65 gene, thereby treating spasticity in the subject.'). Univ California does not teach the viral vector also encodes VGAT. Friddle teaches a nucleic acid sequence encoding VGAT that can be used for gene therapy using a viral vector (para [0004] - 'The novel human protein (NHP) described for the first time herein shares structural similarity with mammalian GABA transporters.'; para [0011] - 'The present invention encompasses the nucleotides presented in the Sequence Listing...or gene therapy constructs comprising a sequence first disclosed in the Sequence Listing'; para [0040] - 'Nucleotide constructs encoding such NHP products can be used to genetically engineer host cells to express such products in vivo...Nucleotide constructs encoding functional NHPs...can also be used in "gene therapy" approaches for the modulation of NHP expression. Thus, the invention also encompasses pharmaceutical formulations and methods for treating biological disorders.'; para [0059] - 'In mammalian host cells, a number of viral-based expression systems may be utilized.'). Jin teaches a functional coupling between synaptic vesicle-associated GAD65 and VGAT for GABA processing (Abstract - 'Here, we propose that there is a functional and structural coupling between the synthesis of gamma-aminobutyric acid (GABA) by membrane-associated GAD and its packaging into synaptic vesicles (SVs) by vesicular GABA transporter (VGAT). This notion is supported by the following observations. First, newly synthesized [3H]GABA from [3H]L-glutamate by membrane-associated GAD is taken up preferentially over preexisting GABA by using immunoaffinity-purified GABAergic SVs. Second, the activity of SV-associated GAD and VGAT seems to be coupled because inhibition of GAD also decreases VGAT activity. Third, VGAT and SV-associated Ca2+/calmodulin-dependent kinase II have been found to form a protein complex with GAD. A model is also proposed to link the neuronal stimulation to enhanced synthesis and packaging of GABA into Svs.'; p4293, col 1, para 2 - 'The evidence presented here will demonstrate that GABA synthesized by SV-associated GAD is preferentially transported into the SV by vesicular GABA transporters (VGATs).'; p4298, col 1, para 1 - col 2, para 1, 'One can imagine that the active site of VGAT and the catalytic site of GAD65 are tightly coupled in a key and lock manner. This tight structural coupling would allow an efficient transfer of GABA from its site of synthesis to the site of transport.'). Since Univ California teaches that GABA signaling is a key mechanism underlying spasticity (para [0006] - 'Loss of gamma-aminobutryic acid (GABA)-mediated presynaptic, recurrent and reciprocal postsynaptic inhibition as well as the loss of its inhibitory effect in flexor afferent pathways has been shown to represent one of the key mechanisms.'), and Jin teaches functional coupling between GAD65 and VGAT for loading of newly synthesized GABA into synaptic vesicles (p4298, col 1, para 1 - col 2, para 1), it would have been obvious to one of ordinary skill in the art that the increased generation of GABA by GAD65 gene therapy according to Univ California could be complemented by also enabling increased packaging of GABA into synaptic vesicles via increased functional VGAT obtained by gene therapy using a VGAT construct of Friddle, and use of a viral vector comprising a polynucleotide encoding both GAD65 and VGAT for providing a more efficient therapy for spasticity.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.