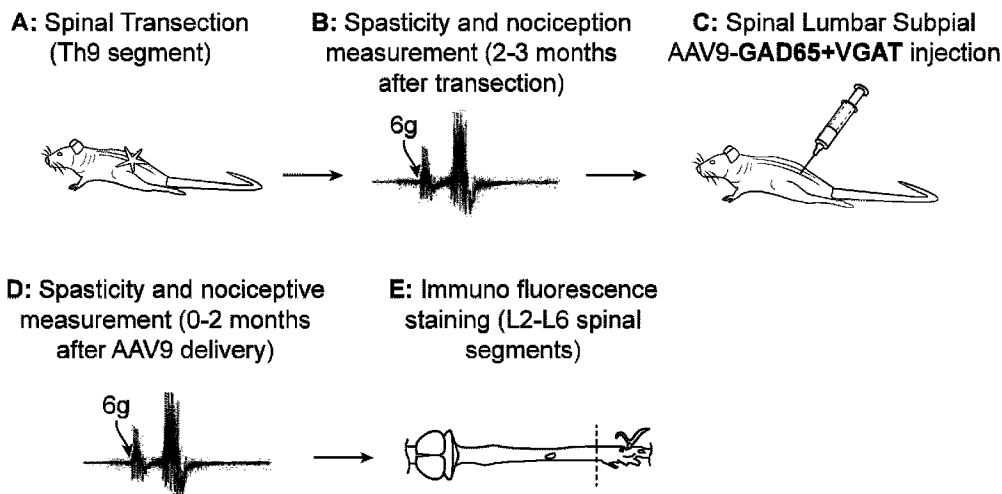




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The present invention provides a therapy for treating loss of GABA-mediated pre-synaptic inhibition after spinal injury. The therapeutic regimen includes spinal segment-specific upregulation of GAD65 (glutamate decarboxylase) and VGAT (vesicular GABA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

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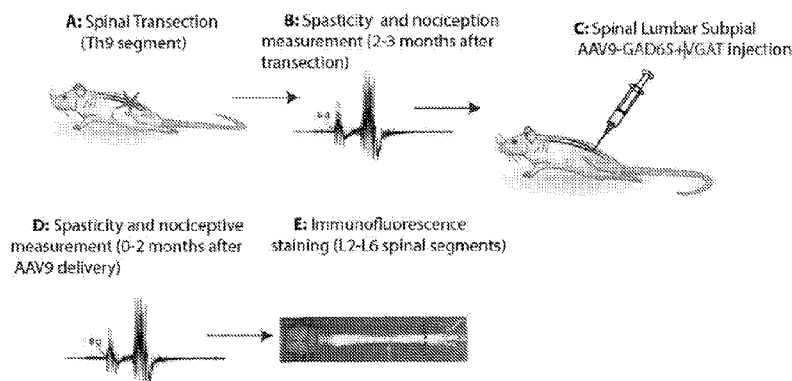


FIG. 1

(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**

[0001]

[0002]

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-aminobutyric 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/hyperreflexia. 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 (glutamate decarboxylase) 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 (glutamate-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 spasticity.

[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) of 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). See also, US Pub. No. 2016/0081956.

[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
MRSFGSGEWS FGSSEGGSGDS ENFGYARAWC QVAQKFTGGI GNKLCALLYG
      60      70      80      90     100
DARKPAEESGG SQFPRAAARK AACACDQKPC SCSEKVDNYA FLKATDLLEA
     110     120     130     140     150
CDGKRPILAF LQDVNNILLQ YVVKSPDRST KVIDPHYRNE LLQRYNWELA
     160     170     180     190     200
DQEQNLEERL NHCQTLKYA IRGHRFRYFN QLSTGLDQVG LAADNLTSTA
     210     220     230     240     250
NTNMPYIEIA FVFVLELYVT LKMKREIIGW FGGSGGSIPI FGGAISNNYA
     260     270     280     290     300
MMLAKFKKFP EVKEKGMAL PRLIAPTSEK SHPSLKKGAA ALGIGTDSVI
     310     320     330     340     350
LINCDEKGM IPSDLEKRIL EAEQKGFVFP LVSATAGTIV YGAFDILLAY
     360     370     380     390     400
ADICKYRIW NRVDAWEGE LLMSRKKKWK LSGVERANIV TWPNNMGGV
     410     420     430     440     450
ELQCSALLVR EEGLMQNCNQ MEASKLPQQD KHYDLSYDTG DEALQGRHV
     460     470     480     490     500
DVPKLMWNR ARGTTGFPAH VDKCLELAHY LYNIIKNREG YEMVFDGKPQ
     510     520     530     540     550
HTNVCTWIP PSLRTEEDNE ERMSRLSKVA FVIRARMMEY GTTNVSTQPL
     560     570     580
GKRVNFRNV ISNPAATHQD IDFLREIER LQQDL

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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 atgtccoccta tacatcacca tcaccatcac ctgggtccgc gtggatccga agcttcgaat
61 tctggcctttt ggtcttttcgg gtccggaagat ggctctgggg attccgagaa tcccgggcaca
121 ggcgcgagcct ggtgccaaggt ggctcaggaag tccaocgggg gcctccggaaa caaactgtgc
181 gcccctgctct acggagacgc cggagaagcgg gcggagagcgg gcgggagcga acccccgccg
241 gcccgcgcgcc ggaagggccc ctgcccctgc gaccaggaag cctgcccagctg ctccaaagtg
301 gatgtcaact acgctgttct ccatgcaaca gacctgctgc cggcgtgtga tggagaaagg
361 cccacccttgg cgtttcttgc agatgttatg aacatcttao ttcagtatgt ggtgaaaagt
421 ttcgatagat caaccaaaagt gattgatctc catttatccta atgagcttct ccaagaaatc
481 aattgggaat tggcagacca accacaaaat tgggaggaaa ttttgatgca tggccaaaca
541 actctaaaaat atgcaattca acacggggcat cctagatcct tcaactcaact tctactggt
601 tgggatagg ttggattagc agcagactgg ctgacatcaa cagcaaatca taaactgttc
661 accatagaaa ttgctccagt atttgtgctt tgggaatatg tccactaaa gaaaatgaga
721 gaaatcattg gctggccagg gggctctggc gatgggatat tttctcccg tggcccaata
781 tctaacatgt atgccatgat gatccgacgc tttaaagatgt tcccagaagt caaggagaaa
841 ggaatggctg ctcttcccg gctcattgct tccactctg aacatagctc tttttctctc
901 aagaaggggg ctgcagcctt agggatttga acagacggcg tgattctgat taaatgtgat
961 gagagagggg aaatgattcc atctgatctt gaaagaaggg tctctgagc caaacagaaa
1021 gggtttgttc cttctcctgt gagtccaca gctggaaaca ccgtgtaocg agcatctgac
1081 cccctcttag ctgtcgttga catttgcaaa aggtataaga tctggatgca tgtggtgca
1141 gcttgggggt ggggattact gatgtcccca aaacacaagt ggaactgag tggcgtggg
1201 agggcccaact ctgtgactg gaatccacac aagatgatgg gactccctt gcagtgctct
1261 gctctcctgg ttgagagaga gggattgatg cagaattgca accaaatgca tgcctctac
1321 ctcttccagc sagataaaca ttatgacctg tcttatgaca ctggagacaa ggccttacag
1381 tggcgagccc acgttgatgt ttttaactca tggctgatgt ggaaggcaaa ggggactacc
1441 gggtttgaag cgcattgtga taaatgtttg gagtggcag agtatctata caaacatcaba
1501 aaaaaccggg aaggabatga gatgggtctt gatgggagc ctcagcacac aaatgtctgc
1561 tctctgtaca ttcctccaaag cctgctgact ctggaaagaca atgaaagag agttagctgc
1621 ctctggaagg tggctccagt gattaaagcc agaattgatg agtatggaac cacaaatgctc
1681 agctaccaac ccttggggaga caaggctcaat tctctccgca tggctcctc aaaaaccagcg
1741 gcaactcacg aagacattga ctctctgatt gaagaaatag aacgcttgg acaagattca
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).

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):

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      10      20      30      40      50
MATLLRSLKLS NVATSVSNKS QAKMSCHFAR MGPQARTDEE AVGFARCDDE
      60      70      80      90     100
DFEKQQLQM DILKAEGERC GDEGAEAFVE GDHSYQNGSG APLPESGSKD
     110     120     130     140     150
QVGGGGEFEG HDKPKITANE AGNVTNAIQ GMFVLGLPYA ILGGVLEGLF
     160     170     180     190     200
LIIFAAVVCC YEGKILIAEL YEKNEDEGEVV SVRDSYVAIA NACCAPEEPT
     210     220     230     240     250
LGRVYVVAQ IIELVNTEIL YVVVSNLKY NSFFGLFVSQ KWSIIATAV
     260     270     280     290     300
LLPCAFLENL KAVSEPSLFC TLANFVINIL VIAVCLSRAR DWAKKVKFY
     310     320     330     340     350
IDVKKFISL GIIVFENTSQ IFLPSLEGDM QQPSEPHCMK HWIRIAACVL
     360     370     380     390     400
NGLEMLWAYL INADETKEVI TDGLPGSIRA VVNIETWAKA LLSYFLPFPA
     410     420     430     440     450
AVEYLEKSLF QEGSRAPFPA CYGGGGRLEK NGLTILCALV VPELLWAIYV
     460     470     480     490     500
PHTALMSILT GSLTGAGLCP ELPSLEWLRD LAKKLLNHQV PEGVAIFVIG
     510     520
GICSVSGFVH SLSGLIARIN TNARD

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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  gcttcggcggcc  cggcggccagct  ccgcaagtcgca  ctaggccaccca  ccgcccggccgc  cggccgctccg
61  ccagaccctggc  tggccaggcttg  ccggggtccag  cccctgaggaga  gcccctgcaacg  ccaggctggcga
121  ggggtcatgag  ccaggaggagcc  ccggggcggcc  gggccggagag  caagccggaga  tagcagacttc
181  gggcccccaca  gcccctggcct  ccttggcatcg  cgttcccggc  atcctcgggt  ccttctgtcc
241  tcttcggctgt  ccccaaccggcc  gcccctggccca  ccttgcctccg  cagcaagctg  tccaaccgtgg
301  ccaagctccg  gtcaccaaccag  tcccaggccca  agactgaggcgg  caagtccggcc  aggcatgggtt
361  ttcaggccggc  caagggatggc  gaggcggctgg  gcttcggccca  ttggcaccgac  ctcgaacttcg
421  agccaccggcca  gggccctggc  atggcaacctc  tgaaaagccca  gggagagagcc  tggcggggacc
481  agggccctga  agcccccctc  gaggggagaca  tccatctatca  gccggccagc  gggcctctcc
541  tggcccccctc  cggcctcccaag  gaccaccgctg  gaggctgctgg  cgaactccgg  ggcaccgaca
601  agcccaaaaat  caagggctgg  gaggccagctt  ggaaccctgac  caaccggcatc  cagggtcatgt
661  tggctgctgg  cctaccctca  gcccctctgg  accggcggcca  cctggggctg  tctctctaca
721  tcttcggccgc  cgttctgtgc  tggcaccctcc  gcaagctcct  cctccggctg  ctgtaccagg
781  agaactgaga  cggccggagtg  gtcggcctgg  gggcactcgt  cgtggccata  gccaaccgct
841  gctggccccc  gggcttccca  accgctgggg  gcccggctgg  gaaccgtagg  caggatcactg
901  agctggctgat  gaccctggctc  ctgtaccctg  tggctgagctg  caaccctcag  taccaccagct
961  tcccggggct  gcccctgtgg  caagaagctc  ggtcccctat  cggcaccggcc  gctgctggctc
1021  cttgcccctt  ccttaaggac  cccaaggccc  tgtcccaagt  cagctcctgg  tggactccgg
1081  cccactctct  cactcaactac  cctggctccag  cctaccctgt  accggcgggg  cggcactggg
1141  cctgggggaa  ggtcaagttc  tcaatcggag  tcaaggagct  ccccactccc  attggtctca
1201  tggctgctca  ccaaccgctc  cagactcctc  tggcctccgt  ggaaggcaat  atgctggctg
1261  caagcggctt  ccaactggatg  atgaactgga  cggcaactcgc  agcctggctg  ctcaaggccc
1321  tcttcggctt  cgtccggctc  ctcaccctgg  cggaccggag  caaggaggct  atcaaccgata
1381  accctggccg  ctccatccgc  gcccctggctc  accctctctt  gctggccagg  ggcctgtctg
1441  cctaccctct  gcccctctct  gcccctctct  aggtcctctg  gaagctcggc  tcccaaggag
1501  gcaagcggcc  cttcttccgc  gcccctctca  gggcggagcc  ggcctcggag  tctcggggg
1561  tcaaccctct  ctggccctcc  gtcctctctc  cgtctctctc  ggcctctctc  gctcctctct
1621  ctggctctct  cctggctctc  accggcggcc  tcaagggctc  cggcctctct  tctctctctc
1681  caagcctctt  tcaaccctct  ctggctctct  gcaagctctt  gctggcctct  gctctctctc
1741  accctggccg  ctctctctct  gggcggcctc  gcaagctctt  cggctctctg  cactcctctg
1801  agggcctctc  cgaagctctc  cgaaccaccg  cggaggagct  gggcctcagg  ggcagccccc
1861  gcccggctct  tggcctctct  cctctctctc  ctcaaccctc  ccccaccagg  ccaagctctc
1921  ctggccctct  gcttggggag  ccaagctctt  aaactctctt  gctcctctct  tctgattctt
1981  cggggatggg  ggggatgggg  ggggagcagg  atcaaccgct  cactcctctt  gctctctctt
2041  tgtctctctt  tttccaccac  accctggctt  tggggggggg  cggggctctt  ttgggggag
2101  ggttctctct  ccttcccaag  ggggcccagg  caactctctt  ccaagctctt  aggggggttg
2161  gaaggggagg  agaggggggc  cagctctctt  gctggcacc  ttgacctctt  gggaaatctt
2221  cacatccatc  caggagctct  aactctcagg  gtcaccagct  tcccaaggag  agcctctctc
2281  attcgggagg  cgttcccaac  ctggcggggg  aaggctctg  tgggaaactc  atttggggg
2341  ggcattctct  tccaaccgag  cgggaggggg  agaagcctct  ggggggctag  cttgctctct
2401  ggttctctct  aactctcaact  cctcctctct  gcaattctct  aggtctctct  ctgtctctct
2461  gctgctctct  tctgctctct  gctgactcag  tgaactctct  atcagaaaa  aatctctctc
2521  taattctagag  tggcgtctct  aactctctct  gcaactcagg  aaggagcaga  ggtcaaaaa
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). As has been shown *in vitro*, infection of primary astrocytes led to a Ca²⁺ 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).

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 Helveticae* 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).

[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 or 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 an 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. Immunofluorescence 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.

[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 alert spinal inhibition) show a significant recovery if compared to control 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.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A composition for use in the treatment of spasticity in a subject, wherein the composition comprises a viral vector comprising a polynucleotide encoding GAD65 (glutamate decarboxylase 65kDa) and VGAT (vesicular gamma-aminobutyric acid (GABA) transporter) proteins, wherein the viral vector is for administration directly into the subpial space of the subject, and wherein expression of the GAD65 and VGAT proteins generate spinal interneurons with a mixed inhibitory-excitatory neurotransmitter phenotype, thereby treating spasticity in the subject.
2. The composition for use of claim 1, wherein the expression of the GAD65 and VGAT proteins is spinal-specific expression.
3. The composition for use of claim 1, wherein the GAD65 and VGAT proteins are overexpressed.
4. The composition for use of claim 1, wherein the vector is a lentiviral vector, adenoviral vector (AV), or an adeno-associated vector (AAV).
5. The composition for use of claim 4, wherein the AAV is AAV9.
6. A viral vector for use in the treatment of a subject having a spinal cord injury, the viral vector comprising a polynucleotide encoding GAD65 (glutamate decarboxylase 65kDa) and VGAT (vesicular gamma-aminobutyric acid (GABA) transporter) proteins, wherein the viral vector is for administration directly into the subpial space of the subject, and expression of the GAD65 and VGAT proteins generates spinal interneurons with a mixed inhibitory-excitatory neurotransmitter phenotype, thereby treating the spinal cord injury.
7. The viral vector for use of claim 6, wherein the GAD65 and VGAT proteins are overexpressed.
8. The viral vector for use of claim 6, wherein the vector is a lentiviral vector, an AV, or an AAV.

9. The viral vector for use of claim 8, wherein the AAV is AAV9.
10. A vector for use in the treatment of spasticity in a subject, the vector comprising a promoter functionally linked to a polynucleotide encoding GAD65 (glutamate decarboxylase 65kDa) and VGAT (vesicular gamma-aminobutyric acid (GABA) transporter) proteins, wherein the vector is for administration directly into the subpial space of the subject and expression of the GAD65 and VGAT proteins generate spinal interneurons with a mixed inhibitory-excitatory neurotransmitter phenotype.
11. The vector for use of claim 10, wherein the vector is a viral vector selected from the group consisting of a lentiviral, adenoviral, and an adeno-associated vector (AAV) vector.
12. The vector for use of claim 11, wherein the vector is AAV9-UBI-GAD65+VGAT, wherein the AAV9-UBI-GAD65+VGAT consists of an AAV9 vector backbone, a human ubiquitin 1 (UB1) promoter upstream of and functionally linked to the polynucleotide encoding GAD65 (glutamate decarboxylase 65kDa) and VGAT (vesicular gamma-aminobutyric acid (GABA) transporter) proteins to facilitate over expression in the subpial space.
13. An isolated mammalian host cell containing the vector as defined in claim 10 for use in the treatment of spasticity in a subject.
14. The viral vector for use of claim 6, wherein spinal cord injury-induced spasticity is treated.

A: Spinal Transection (Th9 segment) **B: Spasticity and nociception measurement (2-3 months after transection)** **C: Spinal Lumbar Subspial AAV9-GAD65+VGAT injection**



D: Spasticity and nociceptive measurement (0-2 months after AAV9 delivery) **E: Immuno fluorescence staining (L2-L6 spinal segments)**

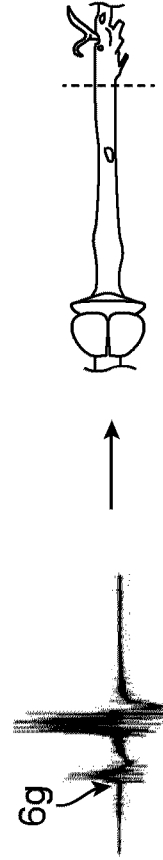


FIG. 1

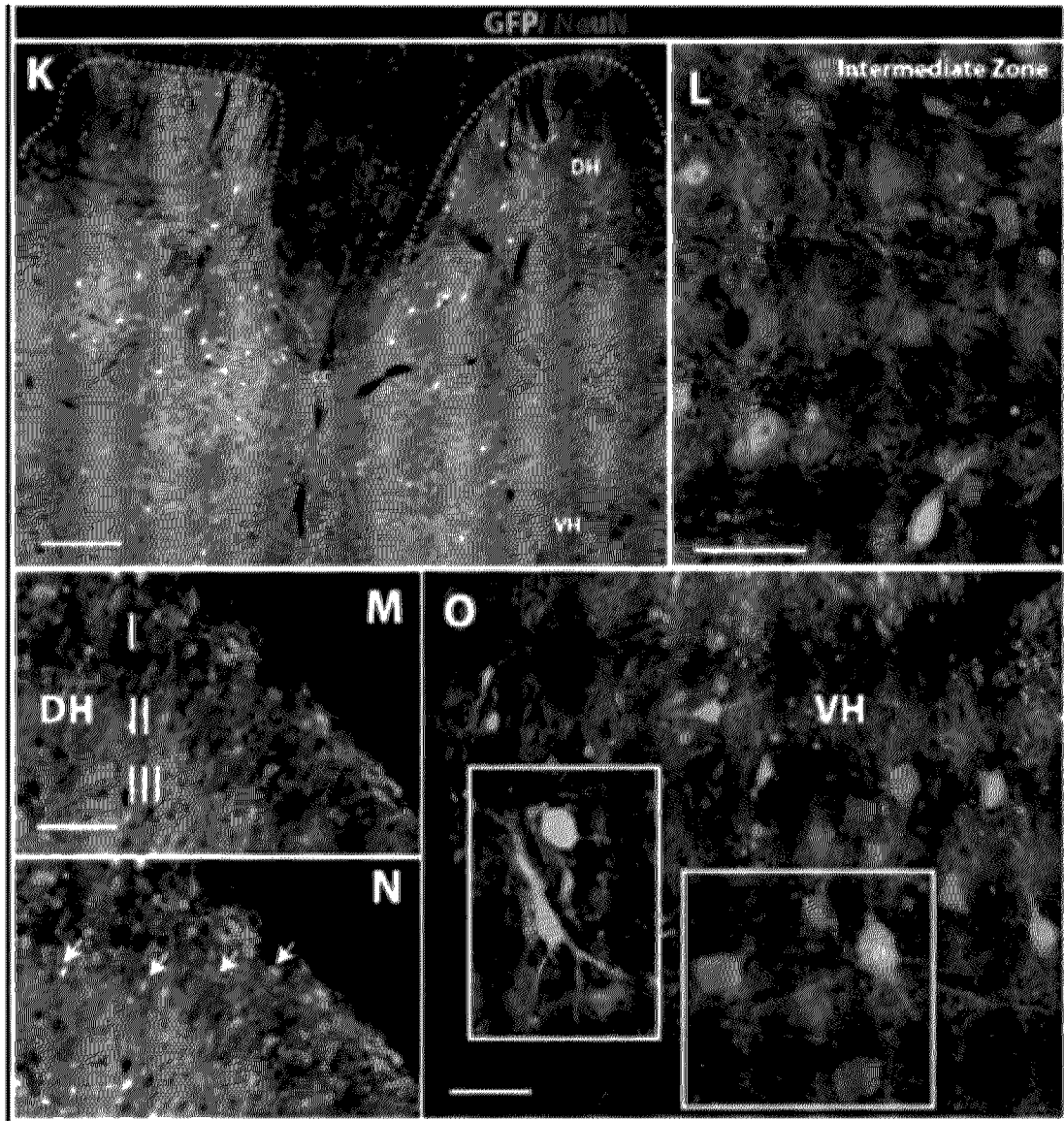
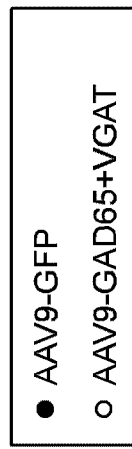


FIG. 2



Paw Tactile Stimulus-Evoked EMG

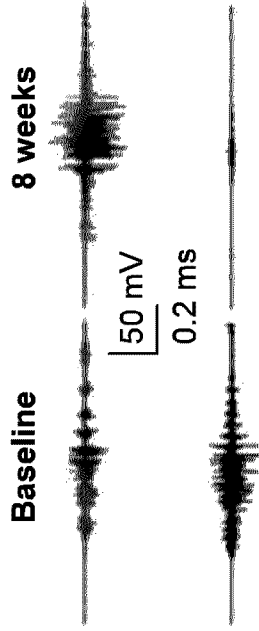
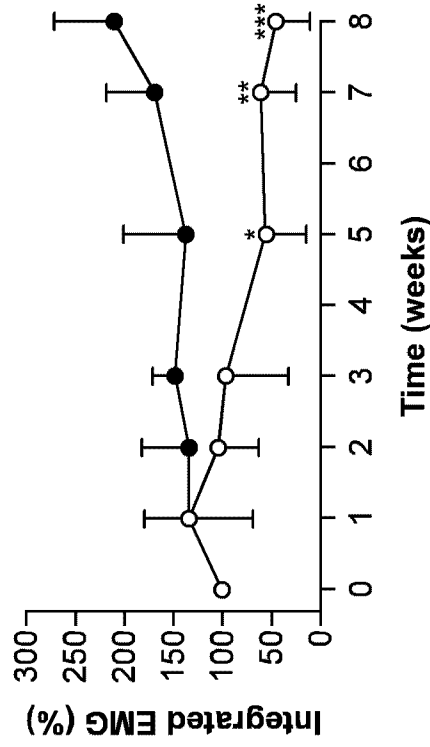


FIG. 3A

FIG. 3B

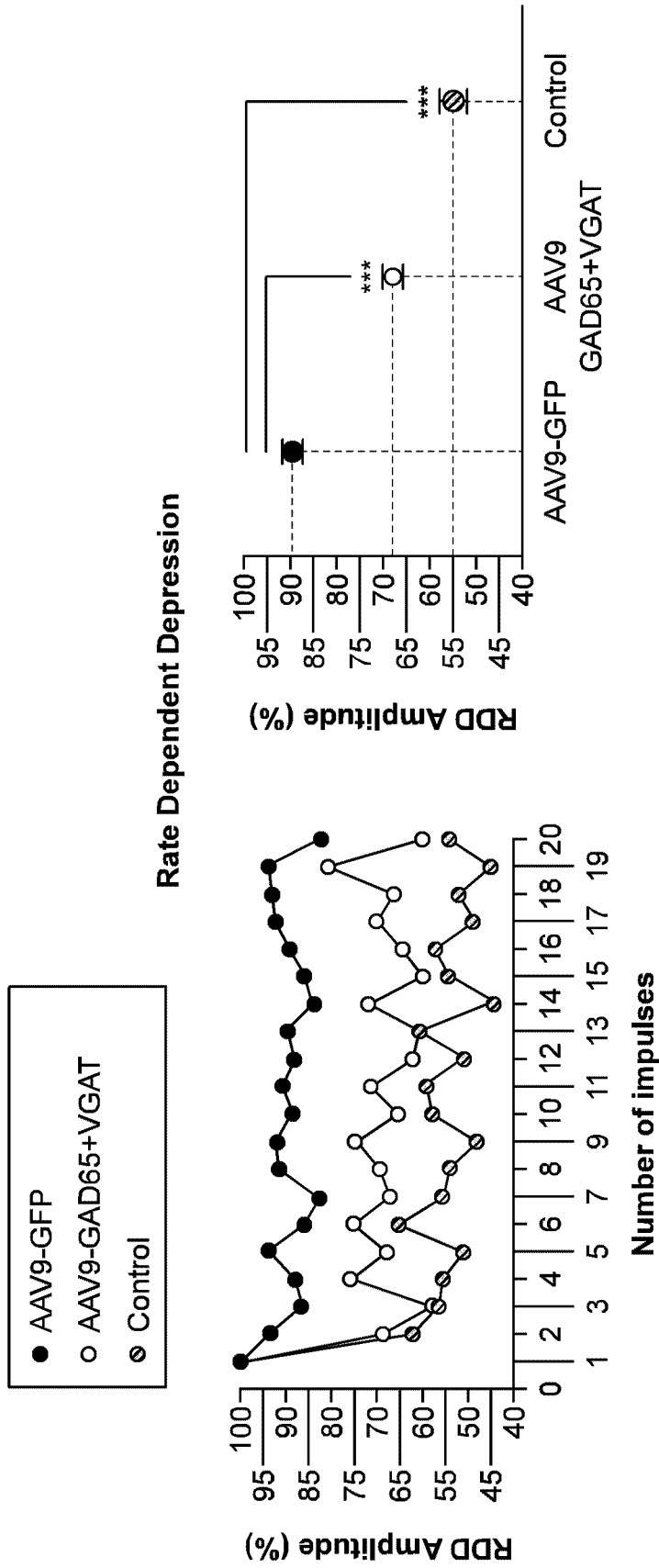


FIG. 3D

FIG. 3C

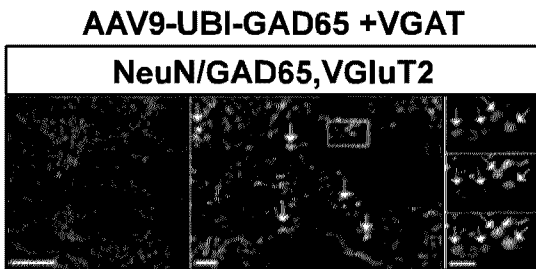


FIG. 4A

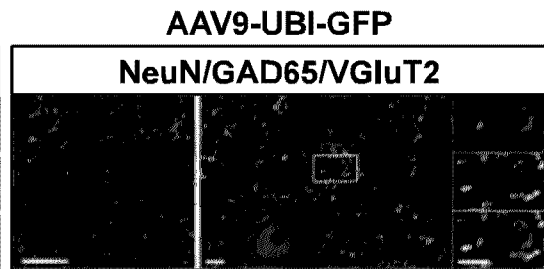


FIG. 4C

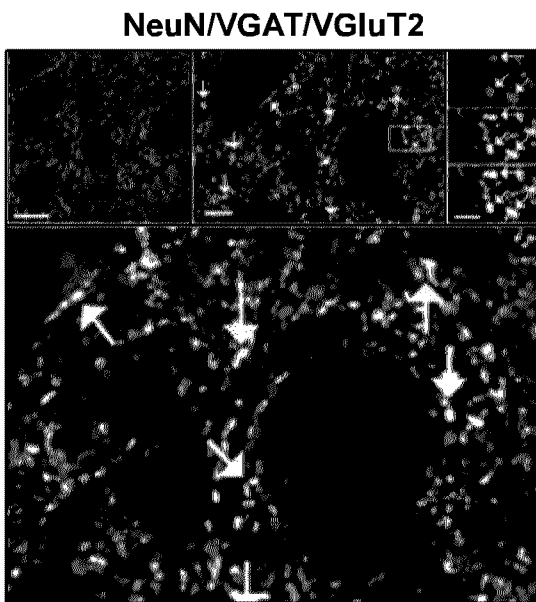


FIG. 4B

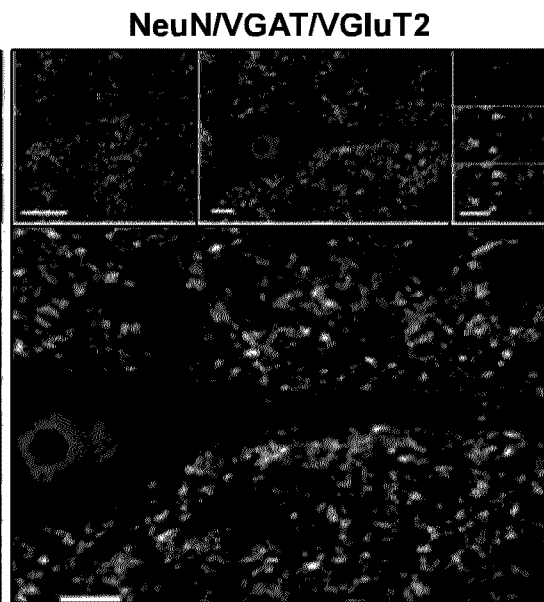
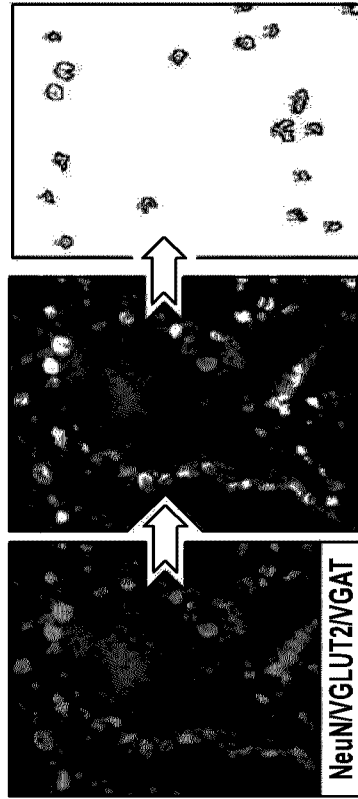


FIG. 4D



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)					
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
Co-expressing Puncta (* P<0.01)						

FIG. 5

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: Immuno fluorescence
staining (L2-L6 spinal
segments)

