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(54) Title: MODIFIED CHONDROITINASE POLYPEPTIDES

(57) Abstract: This invention relates to modified forms of bacterial glycosaminoglycan (GAG) polysaccharide lyase enzymes, such as chondroitinase ABC, that possess a reduced number of active N-glycosylation motifs. These modified enzymes can be synthesised and secreted by mammalian cells in an active form and may be useful, for example, in the treatment of nervous system damage, including human spinal cord injury.

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Modified Chondroitinase Polypeptides

This invention relates to modified bacterial chondroitinases which are modified for expression and secretion in an active form in mammalian  
5 cells. This may be useful, for example, in the treatment of nervous system damage, including human spinal cord injury.

Spinal cord injuries leave large numbers of people permanently paralysed every year. Most patients are young when injured and, in the  
10 face of the well-known regenerative failure of the mammalian CNS, can be expected to live for several more decades with little prospect of recovery. Spinal injury causes a glial 'scar' to form in the spinal cord, and cells in this scar zone express molecules that are inhibitory to axon regrowth [Fawcett et al., 2001; Fawcett & Asher,  
15 1999; Morgenstern et al. (2002); Silver & Miller, 2004; Yiu & He, 2006]. Together with growth-inhibitory proteins released by degenerating myelin, these are thought to provide a significant brake on regenerative growth by injured axons, as a result of which functional recovery fails to take place. Measures designed to  
20 stimulate recovery have had modest success in rodent models, but have not yet made the major improvements hoped for [Ramer et al., 2000; Silver & Miller, 2004; Ramer et al., 2005; Thuret et al., 2006; Bradbury & McMahon, 2006].

25 An important contribution to axon growth-inhibition in the glial scar is made by chondroitin sulphate proteoglycans (CSPGs) in the extracellular matrix, such as NG2 [Dou & Levine, 1994; Levine & Nishiyama, 1996; Fawcett & Asher, 1999; Bovolenta & Feraud-Espinosa, 2000; Silver & Miller, 2004; Tan et al., 2006; Yiu & He, 2006]. These  
30 molecules have long sulphated glycosaminoglycan (GAG) chains attached to their central protein component, and the GAG chains are responsible for much of the inhibitory activity [Grimpe & Silver, 2004].

Chondroitinase ABC is a bacterial enzyme that degrades these inhibitory carbohydrate chains. The use of chondroitinase to promote axon regeneration, using rats as an experimental model, was first demonstrated by Moon et al. (2001) in the nigrostriatal tract and by  
5 Bradbury et al. (2002) in spinal cord injury (SCI). Injection of bacterial chondroitinase ABC into mammalian CNS tissue in vivo was found to deplete GAG immunoreactivity surrounding an injury site [Pizzorusso et al., 2002] and concomitantly generate carbohydrate 'stub' products which are absent from normal tissue [Moon et al.,  
10 2001; Bradbury et al., 2002]. Repeated injection of the chondroitinase ABC at the site of spinal injury was shown to promote extensive regeneration of both corticospinal and sensory axons, accompanied by significant functional motor recovery (Bradbury et al., 2002). These results have been confirmed and extended by others (Yick et al., 2003;  
15 Caggiano et al., 2005; Steinmetz et al., 2005). Chondroitinase also enhances axon regeneration in combination with grafts of Schwann cells and/or olfactory ensheathing cells or neural stem/progenitor cells (Chau et al., 2004; Ikegami et al., 2005; Fouad et al., 2005; Houle et al., 2006).

20 The improvement seen after chondroitinase treatment is generally considered to be due to the enzyme removing the block to regeneration by CSPGs present in the glial scar. In addition, chondroitinase may also promote recovery by other mechanisms. One is to promote  
25 sprouting of spared axons at some distance from an injury site, which can then find their way through or round the CSPG-depleted injury region (Corvetti & Rossi, 2005; Barritt et al., 2006; Massey et al., 2006). Likewise, chondroitinase can increase the synaptic plasticity of undamaged neurons, possibly by removal of the CSPGs present in  
30 peri-neuronal nets (Pizzorusso et al., 2002, 2006; Rhodes & Fawcett, 2004). Such anatomical and synaptic plasticity can allow undamaged regions of CNS to take over the function of severed neurons and axons, and is particularly relevant to the case of SCI where two thirds of patients have incomplete lesions with axons surviving through the site

of injury. Finally, the degradation of CSPGs by chondroitinase results in the generation of disaccharides that support neural repair by inducing neurite outgrowth and preventing neuronal death and axonal collapse, both in vitro and in vivo (Rolls et al., 2004). Whatever  
5 the predominant mechanism, chondroitinase is clearly a promising treatment for spinal cord injury.

However, the use of chondroitinase in human CNS will require considerable modification of present techniques. Delivery of the  
10 enzyme by local injection into the spinal cord region is technically problematic, for several reasons. From the cerebrospinal fluid, the enzyme would be required to penetrate a considerable distance through the spinal cord parenchyma. Also, chronic infusion or repeated injections would be required to relieve axon growth-inhibition for the  
15 extended periods needed for functional recovery, especially in view of the limited stability of the enzyme (Chau et al., 2004). Chronic delivery carries risks of tissue damage, infection, and immunogenicity. Moreover, there could be deleterious effects of widespread CSPG degradation, as CSPG is present normally in  
20 perineuronal nets (Pizzorusso et al., 2002; Rhodes & Fawcett, 2004

The present inventors have discovered that bacterial chondroitinase ABC can be modified without reducing its activity so that mammalian cells are able synthesize and secrete active enzyme. This may be  
25 useful, for example, for the transfection of neurons and/or glia at an injury site, so the cells secrete the chondroitinase and facilitate repair of neural tissue without the need for chronic infusion or repeated injections.

30 An aspect of the invention provides a chondroitinase polypeptide comprising a modified bacterial glycosaminoglycan (GAG) polysaccharide lyase sequence, said modified sequence having a reduced number of active N-glycosylation motifs relative to the unmodified sequence, and

said chondroitinase polypeptide being secretable in an active form by mammalian cells.

Glycosaminoglycan (GAG) polysaccharide lyases are secreted bacterial lyase enzymes which degrade glycosaminoglycans, such as hyaluronan and chondroitin, in the extracellular matrix. GAG lyases recognize uronyl residues in polysaccharides and cleave their glycosidic bonds via a beta-elimination reaction to form a double bond between C-4 and C-5 of the non-reducing terminal uronyl residues of released products.

10 Glycosaminoglycan (GAG) polysaccharide lyases include chondroitin AC lyases, chondroitin ABC lyases, xanthan lyases, and hyalurate lyases.

Glycosaminoglycan (GAG) polysaccharide lyases are also known as polysaccharide lyase family 8 and are listed in the NCBI Conserved Domain Database under the reference number cd01083.1.

Examples of Glycosaminoglycan (GAG) polysaccharide lyases are provided in table 3.

20 A chondroitinase polypeptide as described herein may comprise an amino acid sequence having at least 50% sequence identity to the sequence of a bacterial glycosaminoglycan (GAG) polysaccharide lyase listed in Table 3 and having a reduced number of active N-glycosylation motifs relative to said sequence. Sequence identity is described in more detail below.

The number of N-glycosylation motifs may be reduced in the amino acid sequence by mutating a residue in one or more N-glycosylation motifs which are present in the bacterial GAG lyase sequence.

30

The mutation of N-glycosylation motifs is described in more detail below.

In preferred embodiments, the bacterial GAG lyase sequence is a chondroitinase ABC sequence or a chondroitinase AC sequence.

Chondroitinase ABC sequences include the *P. vulgaris* sequence shown in SEQ ID NO: 1 and the *Y. pestis* sequence of database entry

5 ZP\_00797056.1 GI: 77634965. Chondroitinase AC sequences include the *Flavobacterium columnare* sequence of database entry AY912281.1 GI: 59800135 and the *Pedobacter heparinus* sequence of database entry U27583.1 GI: 1002524.

10 In some preferred embodiments, a chondroitinase polypeptide may comprise an amino acid sequence having at least 20% sequence identity to the sequence of SEQ ID NO: 1 or a fragment thereof having chondroitinase activity,

the amino acid sequence having a reduced number of active N-glycosylation motifs relative to the sequence of SEQ ID NO: 1 or the  
15 fragment thereof, and

the chondroitinase polypeptide being secretable in an active form by mammalian cells.

20 A fragment of the chondroitinase having chondroitinase activity may comprise residues 282 to 960 of SEQ ID NO: 1. For example, a chondroitinase polypeptide may comprise an amino acid sequence having at least 20% sequence identity to the *P. vulgaris* Chondroitinase ABC sequence of residues 282 to 960 of SEQ ID NO: 1,

25 said amino acid sequence having a reduced number of active N-glycosylation motifs relative to the sequence of residues 282 to 960 of SEQ ID NO: 1, and

said chondroitinase polypeptide being secretable in an active form by mammalian cells.

30

Other suitable fragments may comprise residues 282 to 963 of SEQ ID NO: 1. A chondroitinase polypeptide may comprise an amino acid sequence having at least 20% sequence identity to the sequence of residues 282 to 963 of SEQ ID NO: 1 and having a reduced number of

active N-glycosylation motifs relative to the sequence of residues 282 to 963 of SEQ ID NO: 1.

Other suitable fragments may comprise residues 25 to 1021 of SEQ ID NO: 1. A chondroitinase polypeptide may comprise an amino acid sequence having at least 20% sequence identity to the sequence of residues 25 to 1021 of SEQ ID NO: 1 and having a reduced number of active N-glycosylation motifs relative to the sequence of residues 25 to 1021 of SEQ ID NO: 1.

Reducing the number of N-glycosylation motifs in the amino acid sequence reduces the amount of glycosylation which is undergone by the chondroitinase polypeptide when expressed in mammalian cells.

The number of N-glycosylation motifs may be reduced in the amino acid sequence by mutating a residue in one or more N-glycosylation motifs which are present in the corresponding sequence of SEQ ID NO: 1 and SEQ ID NO:3, for example, the sequence of residues 282 to 960, residues 282 to 963 or residues 25 to 1021 of SEQ ID NO: 1 or SEQ ID NO: 3. Mutation of a residue within a N-glycosylation motif as described herein prevents N-glycosylation of the motif during expression in a mammalian cell.

An N-glycosylation motif consists of the sequence N-X-S/T, where X is any amino acid.

An N-glycosylation motif may be mutated in the chondroitinase polypeptide by means of a conservative or semi-conservative substitution of the N or S/T residue of the sequence to produce a mutated N-glycosylation motif which is not glycosylated in mammalian cells.

A conservative substitution is an amino acid replacement that preserves the structure and functional properties of the native

protein. Similarity matrices such as the PAM 250 or Blosum 45 are commonly used in the art to determine conservative or semi-conservative variation.

- 5 In some embodiments, the N residue of the N-glycosylation motif may be mutated, for example by substitution for another amino acid, such as Q, K, D or A.

10 In some embodiments, the S or T residue of the N-glycosylation motif may be mutated, for example by substitution for another amino acid, such as A, G or L.

The *Proteus vulgaris* chondroitinase ABC amino acid sequence which is shown in residues 25 to 1021 of SEQ ID NO: 1 or SEQ ID NO: 3 has N-glycosylation motifs at positions N72, N171, N174, N202, N282, N338, N345, N515, N603, N675, N751, N773, N836, N856, N918, N963, N976.

20 The amino acid sequence between residues 282 and 960 of SEQ ID NO: 1 or SEQ ID NO: 3 has N-glycosylation motifs at positions N282, N338, N345, N515, N603, N675, N751, N773, N836, N856 and N918.

25 The sequence between residues 282 and 963 of SEQ ID NO: 1 or SEQ ID NO: 3 has N-glycosylation motifs at positions N282, N338, N345, N515, N603, N675, N751, N773, N836, N856, N918 and N963.

However, the N-glycosylation motif at position N751 has unfavourable flanking sequences and is shown herein to be unglycosylated in mammalian cells.

- 30 One or more of these N-glycosylation motifs may be mutated in the chondroitinase polypeptides described herein. For example, a chondroitinase polypeptide may comprise an amino acid sequence having at least 20% sequence identity to residues 282 to 960 of SEQ ID NO: 1 which has a mutation in one or more N-glycosylation motifs selected

from the group consisting of the N-glycosylation motifs at positions N282, N338, N345, N515, N675, N773 and N856 in SEQ ID NO: 1 or an amino acid sequence having at least 20% sequence identity to residues 282-963 or residues 25 to 1021 of SEQ ID NO: 1 which has a mutation in one or more N-glycosylation motifs selected from the group consisting of the N-glycosylation motifs at positions N282, N338, N345, N515, N675, N773, N856 and N963 in SEQ ID NO: 1.

More preferably, the amino acid sequence has a mutation at one, two or three N-glycosylation motifs selected from the group consisting of the N-glycosylation motifs at positions N282, N345, and N515 of SEQ ID NO: 1.

More preferably, the amino acid sequence has a mutation at one, two, three, four or all five N-glycosylation motifs selected from the group consisting of the N-glycosylation motifs at positions N282, N345, N336, N515 and N675 of SEQ ID NO: 1.

A chondroitinase polypeptide as described herein may comprise an amino acid sequence having a mutation in one or more, two or more, three or more, four or more or five or more N-glycosylation motifs of a bacterial GAG lyase sequence.

The mutation of the N-glycosylation motifs at N751 in SEQ ID NO: 1 are shown herein to have no effect on expression and secretion of active chondroitinase polypeptide. In some embodiments, this motif may be unmutated.

Examples of chondroitinase polypeptides include the B1, X12, X30, Y13, A10 and Y133 mutants listed in Table 1.

A chondroitinase polypeptide catalyses the depolymerization of chondroitin sulfate (EC 4.2.2.4) to unsaturated disaccharides. In particular, the chondroitinase polypeptide degrades the sulphated

glycosaminoglycan (GAG) chains of chondroitin sulfate proteoglycans (CSPGs), such as NG2.

The chondroitinase polypeptide described herein is active under physiological conditions and is able to be synthesised and secreted in an active form by mammalian host cells. Secretion of active chondroitinase polypeptide as described herein may be useful in neuronal regeneration or repair, for example at a site of neural injury or damage, such as a spinal cord lesion.

The *Proteus vulgaris* chondroitinase ABC sequence (Genbank Acc No; AAB43331.1 GI: 1828877) is shown in SEQ ID NO: 1. Residues 1 to 24 represent the signal peptide and residues 25 to 1021 represent the active enzyme. A modified *Proteus vulgaris* chondroitinase ABC sequence comprising a mammalian signal peptide is shown in SEQ ID NO 3. The first 29 residues (here numbered -4 to 24) represent the signal peptide and residues 25 to 1021 represent the active enzyme.

Chondroitinase polypeptides described herein may comprise at least 20% sequence identity or sequence similarity with the sequence of residues 282 to 960, residues 282 to 963 or residues 25 to 1021 of SEQ ID NO: 1 or SEQ ID NO:3 or other reference GAG lyase sequence. Preferably, the chondroitinase polypeptide comprises an amino acid sequence which shares greater than 30%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 85%, greater than 90%, greater than 95% or greater than 98% sequence identity or sequence similarity with the reference GAG lyase sequence.

Chondroitinase polypeptides described herein include mutations in one or more N-glycosylation motifs which are present in the sequence of a reference bacterial GAG lyase sequence, for example SEQ ID NO:1 or a fragment thereof, such as residues 282 to 960, residues 282 to 963 or residues 25 to 1021 of SEQ ID NO: 1 or SEQ ID NO: 3. The sequence of the chondroitinase polypeptide may also differ from the reference bacterial GAG lyase sequence by one or more of addition, insertion,

deletion or substitution of one or more amino acids at positions other than N-glycosylation motifs.

In some embodiments, up to about 5, 10, 15, 20, 30 or 40 amino acids may be altered in a chondroitinase polypeptide described herein. Such alterations may be caused by one or more of addition, insertion, deletion or substitution of one or more nucleotides in the encoding nucleic acid.

Preferably the sequence of the chondroitinase polypeptide does not differ from the sequence of residues 282 to 960 of SEQ ID NO: 1 or SEQ ID NO: 3 at residues H501, Y508, R560 and E653 as set out in SEQ ID NO: 1 or SEQ ID NO: 3.

Sequence similarity and identity are commonly defined with reference to the algorithm GAP (Wisconsin GCG package, Accelrys Inc, San Diego USA). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul et al. (1990) *J. Mol. Biol.* 215: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) *PNAS USA* 85: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) *J. Mol Biol.* 147: 195-197), or the TBLASTN program, of Altschul et al. (1990) supra, generally employing default parameters. In particular, the psi-Blast algorithm (Nucl. Acids Res. (1997) 25 3389-3402) may be used. Sequence identity and similarity may also be determined using Genomequest™ software (Gene-IT, Worcester MA USA).

Sequence comparisons are preferably made over the entire length of the relevant sequence described herein.

In some preferred embodiments, a chondroitinase polypeptide described herein may comprise an amino acid sequence which is the sequence of SEQ ID NO:1 or a fragment thereof with a mutation in an N-glycosylation motif thereof,

5       said mutation preventing N-glycosylation of the motif.

Suitable fragments of SEQ ID NO: 1 may comprise residues 282 to 960, residues 282 to 963 or residues 25 to 1021 of SEQ ID NO: 1.

10   One or more heterologous amino acids may be joined or fused to a chondroitinase polypeptide set out herein and a polypeptide may comprise a polypeptide sequence as described above linked or fused to one or more heterologous amino acids. One or more heterologous amino acids may include sequences from a source other than a bacterial  
15   chondroitinase protein.

In preferred embodiments, the chondroitinase polypeptide described above is coupled to a signal sequence which directs secretion of the chondroitinase polypeptide. Preferably, the signal sequence is a  
20   eukaryotic signal sequence, which directs secretion of the chondroitinase polypeptide in eukaryotic cells. Many suitable eukaryotic signal sequences are known in the art. In some preferred embodiments, a mammalian signal sequence may be employed, for example the mouse MMP2 signal sequence shown in the first 29 residues of SEQ  
25   ID NO: 3 (residues -4 to 25). Other suitable signal sequences include GDNF, prolactin, or immunoglobulin signal sequences.

In some embodiments, bacterial signal sequences, including the chondroitinase ABC signal sequence, may be employed.

30   Heterologous peptides might be attached at the N- or C-terminus of the chondroitinase polypeptide, for example to allow recognition by an antibody, or to direct secretion to a particular part of the cell such as the neuronal growth cone.

The invention also encompasses nucleic acids, vectors and cells which are suitable for use in methods of producing a chondroitinase polypeptide, as described above.

5

Accordingly, a nucleic acid may comprise a nucleic acid sequence which encodes a chondroitinase polypeptide as described above.

10 A suitable nucleic acid may comprise a nucleotide sequence having at least 40% sequence identity to a reference bacterial GAG lyase nucleotide sequence, for example SEQ ID NO:2, SEQ ID NO:4 or a fragment thereof, such as a sequence comprising nucleotides 191-3181 of SEQ ID NO:2 or nucleotides 297-3287 of SEQ ID NO: 4 ,

15 wherein the nucleotide sequence includes one or more mutations relative to the reference bacterial GAG lyase nucleotide sequence which reduce the number of N-glycosylation motifs in the encoded amino acid sequence relative to the amino acid sequence of the reference bacterial GAG lyase amino acid sequence, for example SEQ ID NO: 1 or SEQ ID NO: 3 or a fragment thereof, such as a sequence comprising  
20 residues 282 to 963 of SEQ ID NO: 1 or SEQ ID NO: 3.

A mutation which reduces the number of N-glycosylation motifs in the encoded amino acid sequence may be any mutation, for example the addition, insertion, deletion or substitution of one or more  
25 nucleotides, which leads to a substitution of the N or S/T residue of an N-glycosylation motif to produce a mutated N-glycosylation motif which is not glycosylated in mammalian cells. In some embodiments, the substitution may be a conservative substitution.

30 A suitable nucleic acid may share greater than 50% sequence identity, greater than 60% sequence identity, greater than 70%, greater than about 80%, greater than 90% or greater than 95% with the reference bacterial GAG lyase nucleotide sequence, for example all or part of

the nucleic acid sequence from SEQ ID NO: 2 or SEQ ID NO: 4 as described above.

A suitable nucleic acid may have one or more of addition, insertion, deletion or substitution of one or more nucleotides in the sequence of the reference bacterial GAG lyase nucleotide sequence. For example, up to 10, 20, 30, 50, 100, 500 or 1000 nucleotides may be added, inserted, deleted or substituted as described.

10 In some embodiments, a nucleic acid may comprise one or more codons optimized for eukaryotic expression, preferably mammalian cell expression. The optimisation of codons for expression in a particular host, for example by replacing preferred bacterial codons with codons preferred in mammalian cells, is well-known in the art. An optimised  
15 nucleic acid sequence may comprise the nucleic acid sequence from nucleotides 297-3287 of SEQ ID NO: 4 with one or more mutations which reduce the number of N-glycosylation motifs.

In some embodiments, the nucleic acid may comprise an optimized Kozak  
20 sequence for improved expression in a eukaryotic mammalian host cell. For example, the nucleic acid may comprise the kozak sequence which is shown in SEQ ID NO: 4.

The nucleotide sequence encoding the chondroitinase polypeptide may be  
25 operably linked to a heterologous regulatory sequence.

Suitable regulatory sequences to drive the expression of heterologous nucleic acid coding sequences in expression systems are well known in the art and include constitutive promoters, for example viral  
30 promoters such as CMV or SV40, and inducible promoters, such as Tet-on, ecdysone or tamoxifen controlled promoters.

In some preferred embodiments, promoters specific to neurons (such as neuron-specific enolase) or to astrocytes (such as GFAP) or to other glial cells may be employed.

5 The term "heterologous" indicates that the gene/sequence of nucleotides in question or a sequence regulating the gene/sequence in question, is a recombinant sequence which has been introduced into a construct, vector or cell, artificially, using genetic engineering or recombinant means, i.e. by human intervention. Heterologous  
10 nucleotide sequences are sequences which do not naturally occur together in nature. Nucleotide sequences which are heterologous to a cell may be non-naturally occurring in cells of that type, variety or species (i.e. exogenous or foreign) or may be sequences which are non-naturally occurring in that sub-cellular or genomic environment of the  
15 cells or may be sequences which are non-naturally regulated in the cells i.e. operably linked to a regulatory element other than the natural regulatory element for the sequence.

Nucleic acid sequences and constructs as described above may be  
20 comprised within a vector. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Preferably, the vector contains appropriate regulatory sequences to  
25 drive the expression of the nucleic acid in mammalian, in particular human, cells. A vector may also comprise sequences, such as origins of replication and selectable markers, which allow for its selection and replication in bacterial hosts such as *E. coli* and/or in eukaryotic cells.

30 Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 3rd edition, Russell et al., 2001, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for

manipulation of nucleic acid, for example in preparation of vectors and the introduction of DNA into cells are described in detail in *Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds. John Wiley & Sons, 1992.

5

In some embodiments, the vector is a viral vector suitable for expression in mammalian cells, in particular human cells. Suitable viral vectors include adenovirus, adeno-associated virus (AAV), for example AAV serotype 2 virus, retrovirus, lentivirus, recombinant  
10 adenovirus, 'gutless' adenovirus, herpes simplex virus, and poliovirus vectors.

A viral vector may be packaged into a viral particle comprising one or more capsid proteins prior to transfection of host cells.

15

In some embodiments, the viral vector may be packaged into a heterologous viral particle. For example, a lentiviral vector may be pseudotyped with a rabies glycoprotein, such as rabies-G envelope protein, which provides for transduction of neurons *in vivo* (Mazarakis  
20 et al Human Molecular Genetics (2001) 10 2109-2121)

A nucleic acid or vector as described herein may be introduced into a host cell. This may occur, for example, *in vitro* or *in vivo* or *ex vivo*.

25

Suitable host cells include neural cells, such as neurons and glial cells, preferably mammalian neurons and glial cells, including Schwann cells, oligodendrocytes, and astrocytes. Bacterial cells such as *E. coli* may also be useful as host cells for some purposes, for example  
30 in the production of nucleic acid for use as described herein.

Techniques for the introduction of nucleic acid into cells are well established in the art and any suitable technique may be employed, in accordance with the particular circumstances. For eukaryotic cells,

suitable techniques may include DEAE-dextran, polyethyleneimine, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. adenovirus, AAV, lentivirus or vaccinia. For bacterial cells, suitable techniques may include transformation of chemically competent cells, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art. In some embodiments, a marker such as green fluorescent protein may be used to identify cells expressing the introduced nucleic acid. Marker genes may be comprised in the same vector as the nucleic acid encoding the chondroitinase polypeptide, or on separate vectors, which may be co-transfected into a host cell.

The introduced nucleic acid may be on an extra-chromosomal vector within the cell or the nucleic acid may be integrated into the genome of the host cell. Integration may be promoted by inclusion of sequences within the nucleic acid or vector which promote recombination with the genome, in accordance with standard techniques.

The introduction may be followed by expression of the nucleic acid to produce the encoded chondroitinase polypeptide.

In some embodiments, host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) may be cultured *in vitro* under conditions for expression of the nucleic acid, so that the encoded chondroitinase polypeptide is produced. When an inducible promoter is used, expression may require the activation of the inducible promoter.

A host cell which expresses the nucleic acid and secretes the expressed chondroitinase polypeptide may be isolated and/or purified.

In some embodiments, host cells, in particular glial cells, may be obtained from an individual, preferably an individual requiring treatment for neural damage. Nucleic acid encoding the chondroitinase polypeptide may be then be introduced into the host cells *ex vivo* using standard transfection or transduction techniques and, optionally, cultured, isolated and/or purified, prior to implantation or administration to the individual, for example at a site of neural damage.

In some embodiments, nucleic acid may be introduced into a cell *in vivo*. For example, a nucleic acid or vector may be administered to the individual such that one or more cells of the individual incorporate the nucleic acid or vector. Preferably, the nucleic acid or vector is administered at or adjacent to the site of neural tissue damage to facilitate uptake of the nucleic acid or vector by neural cells e.g. neurons and glial cells, at the damage site. Neurons and/or glial cells, including astrocytes, oligodendrocytes and Schwann cells at the damage site express the nucleic acid and secrete the chondroitinase polypeptide. The secreted chondroitinase degrades extracellular CSPGs, for example in glial scars and this may be useful in the repair of neural damage and the improvement of nerve function.

A method of producing a chondroitinase polypeptide as described herein may comprise;

introducing a nucleic acid encoding a chondroitinase polypeptide as described above into a host cell, for example a mammalian neural cell, and;

expressing said nucleic acid in the cell to produce the chondroitinase polypeptide.

Nucleic acid may be expressed by allowing the host cells (which may include cells actually transformed, although more likely the cells will be descendants of the transformed cells) to grow under conditions under which they express the introduced nucleic acid to produce the

chondroitinase polypeptide. Following expression, the chondroitinase polypeptide is secreted by the host cells.

As described above, the nucleic acid may be introduced into the host cell in vitro, in vivo or ex vivo. Preferably the cell produces and secretes the chondroitinase polypeptide in vivo at the site of nervous tissue damage.

Further aspects of the invention provide a host cell comprising a vector or nucleic acid construct as described herein, and a method of producing a host cell as described herein may comprise introducing a nucleic acid as described herein into the host cell.

A host cell may contain a nucleic acid sequence encoding a chondroitinase polypeptide as a result of the introduction of the nucleic acid sequence into an ancestor cell.

A range of host cells suitable for the production of chondroitinase polypeptides are known in the art. Suitable host cells may include mammalian cells, in particular neural cells, for example neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cells, olfactory ensheathing cells, and Schwann cells. For therapeutic applications, the cells are preferably human cells. As described above, cells which express and secrete the chondroitinase polypeptide may be transplanted to a site of neural tissue damage or cells in situ at site of neural tissue damage may be transfected with a nucleic acid encoding the chondroitinase polypeptide.

Aspects of the invention provide a nucleic acid, viral particle, vector or cell as described herein for use in a method of treatment, for example, a method of treating neural tissue damage, in particular spinal cord injury, and a pharmaceutical composition comprising a nucleic acid, viral particle, vector or cell as described herein.

Other aspects of the invention provide the use of a nucleic acid, viral particle, vector or cell in the manufacture of a medicament for use in treating neural tissue damage comprising

administering a nucleic acid, viral particle, vector or cell as  
5 described herein to an individual in need thereof.

The methods described herein may be useful in promoting nerve repair at a site of neural damage, for example in the treatment of spinal cord injury.

10

In some embodiments, the damaged or injured nerve is within the central nervous system (CNS). The CNS includes the brain, the spinal cord, and the retina. It includes neurons whose cell bodies lie within, or have a primary synapse in, the brain or spinal cord,  
15 including neurons of origin of the corticospinal tract. The CNS also includes axons of sensory neurons whose cell bodies lie outside the CNS.

The neural injury or damage may be a spinal cord injury, for example  
20 an injury caused by assault, accident, tumour, intervertebral disc or bone abnormality, or surgery, e.g. surgery for spinal problems and/or surgery to remove tumours.

In preferred embodiments, the nucleic acid, vector or cell is  
25 administered directly to the site of neural tissue damage, for example by injection or infusion. Suitable infusion techniques are known in the art and commonly used in therapy (see, e.g., Rosenberg et al., New Eng. J. of Med., 319:1676, 1988).

30 The term "treatment", as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of a human or an animal (e.g. in veterinary applications), in which some desired therapeutic effect is achieved, for example, the inhibition of the progress of the condition, and includes a reduction in the rate of

progress, a halt in the rate of progress, amelioration of the condition, and cure of the condition.

Whether it is a nucleic acid, vector, viral particle or cell according  
5 to the present invention that is to be given to an individual, administration is preferably in a "therapeutically effective amount" this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated.  
10 Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of medical practitioners.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in  
15 addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.  
20 A pharmaceutical composition may thus comprise a nucleic acid encoding a chondroitinase polypeptide or a vector or cell comprising such a nucleic acid as described herein and a pharmaceutically acceptable excipient.

25 A pharmaceutical composition may be produced by admixing or formulating the nucleic acid, viral particle, vector, or cell with a pharmaceutically acceptable excipient.

Formulations suitable for administration (e.g. by infusion), include  
30 aqueous and non-aqueous isotonic, pyrogen-free, sterile injection solutions which may contain anti-oxidants, buffers, preservatives, stabilisers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents

and thickening agents. Examples of suitable isotonic vehicles for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Suitable vehicles can be found in standard pharmaceutical texts, for example, Remington's  
5 Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a  
10 parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles as described above. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be  
15 included, as required.

Pharmaceutical compositions comprising nucleic acids for use in gene therapy can be stored and administered in a sterile physiologically acceptable carrier, where the nucleic acid is dispersed in conjunction  
20 with any agents which aid in the introduction of the nucleic acid into cells.

The term "pharmaceutically acceptable" as used herein pertains to compounds, materials, compositions, and/or dosage forms which are,  
25 within the scope of sound medical judgement, suitable for use in contact with the tissues of a subject (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, excipient, etc. must also be "acceptable" in the sense of  
30 being compatible with the other ingredients of the formulation.

Administration of nucleic acid, vectors or cells in vivo can be effected in one dose, continuously or intermittently (e.g., in divided doses at appropriate intervals) throughout the course of treatment.

The determination of the most effective means and dosage of administration is within the remit of the medical practitioner and will vary with the formulation used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated.

5 Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician.

Neural cells expressing the chondroitinase polypeptide may be administered directly at the site of neural damage by cell-therapy

10 techniques. The administered cells at the site of neural damage produce and secrete the chondroitinase protein locally. In some embodiments, the neural cells may be contained within an implant or nerve guide which is positioned at a site of neural tissue damage. In some

15 embodiments, the implant may be fixed in position. Any convenient technique may be employed, for example, the implant may be sutured or glued in place. Suitable implants include, for example collagen gel implants.

For gene therapy to be practical, it is desirable to employ a nucleic acid transfer method that: (1) directs the therapeutic sequence into

20 specific target cell types (e.g. neurons, or glial cells), (2) is highly efficient in mediating uptake of the therapeutic polynucleotide into the target cell population, and (3) is suited for use *in vivo* for therapeutic application.

25 Nucleic acid encoding the chondroitinase polypeptide may be delivered using any of a variety of techniques. Current techniques may be divided into three major groups: transfection with a viral vector, such as a replication-defective retroviral vector (Cornetta et al, Hum. Gene. Ther. 2:215 (1991), adenoviral vector (Rosenfeld et al, Cell 68:143 (1992)), or adenoassociated virus; fusion with a lipid, for example in a liposome or immunoliposome (Litzinger and Huang, Biochem. Biophys. Acta 1113:201 (1992); Gao and Huang, Biochem. Biophys. Res. Commun. 179:280 (1991) Wang and Huang, Proc. Natl. Acad.

30

Sci. USA 84:7851 (1987); Trubetskoy et al, Biochem. Biophys. Acta 1131:311 (1992)); and DNA transfer with a cation such as polyethyleneimine or polylysine. The selection of which technique to use depends upon the particular circumstances and is within the remit  
5 of skilled persons in the field.

Any suitable delivery method may be used to deliver nucleic acid as described herein to cells at a site of neural tissue damage. In some embodiments, direct physical application of naked or liposome  
10 encapsulated nucleic acid to cells at the site of neural tissue damage may be preferred.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present  
15 disclosure. All documents mentioned in this specification are incorporated herein by reference in their entirety.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the  
20 other. For example "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of  
25 the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be  
30 illustrated by way of example and with reference to the figures and tables described below.

Figures 1 and 2 show recombinant chondroitinase ABC is active in vitro, after multi-site mutagenesis to correct mutations as described in the methods section below.

5 Figure 1 shows products of 35S-labelled in vitro transcription and translation (IVTT) on SDS-PAGE. Lane 1 shows a truncated product made by a plasmid with an internal stop codon. Lane 2 shows a full-length product after correction of the stop codon. L represents Luciferase (from control mRNA). The double band in each lane is apparently a  
10 result of initiation of protein synthesis at an internal AUG which is an artifact of in vitro translation.

Figure 2 shows in vitro activity assay using the Morgan-Elson reaction. Left panel, calibration curve for commercial Chondroitinase  
15 ABC (0 to 2 mU). Right panel, results of chondroitinase-ABC activity assay on products of IVTT reactions with chondroitinase clones. Clone 02 has a Leu745-Pro mutation and is inactive. Correction of the proline mutation, as in clones B5 and C4, generates enzyme activity. B5, C4, Chondroitinase ABC clones: C4 has the fully normalised  
20 sequence, and B5 additionally has one potential N-glycosylation site mutated.

Figure 3 shows an SDS-PAGE of IVTT reactions with microsomes to assess glycosylation. IVTT reactions were labeled with biotinylated lysine  
25 and incubated with canine microsomes. After SDS-PAGE and blotting, the products were detected with streptavidin-linked HRP. The lower arrow indicates unglycosylated chondroitinase-ABC; the upper arrow indicates the highest glycosylated band (from clone C4), which is not produced without microsomes. The glycosylated band migrates faster in products  
30 of clones with several mutations of N-glycosylation sites. The grey bar denotes a non-specific band, masked out for clarity, which obscures the glycosylated band from clones X12 and X30.

Figure 4 shows proteinase K protection after IVTT reaction with microsomes. The lower arrow indicates unglycosylated chondroitinase-ABC; the upper arrow, the glycosylated form. Only the glycosylated form is protected from Proteinase K, indicating that it has been internalised in the microsomes.

Figures 5 to 7 show western blots of medium from non-transfected and chondroitinase-transfected Neu7 cells, for the proteoglycan NG2. Each lane is labelled according to the clone used for transfection; those marked "+Ch'ase" were digested with commercial chondroitinase in vitro before SDS-PAGE.

Figure 5 shows, in lanes 1-4, positive and negative controls. In medium from untransfected or GFP-transfected cells, NG2 appears largely as a characteristic 'smear' as expected due to the GAG chains, and this is all converted to core protein by digestion with commercial chondroitinase (lanes 1 and 3). After transfection with modified chondroitinase A10, there is a reduction of the GAG smear and compensating intensification of the core protein band. After transfection with unmodified chondroitinase (clone C4\*, in this case with the original bacterial signal sequence), there is no such digestion of the GAG chains.

Figure 6 shows a similar experiment to Figure 5 in which the NG2 also appears to have been digested by protease in the medium, showing complete removal of the immunoreactive smear by commercial chondroitinase (lane 4) or by transfection with clone Y13 (lanes 1 and 2). (In this case the medium was collected from 16 to 22 h post-transfection, and the western blot was probed with Chemicon antibody against immuno-purified NG2.)

Figure 7 shows results from conditioned medium from untransfected Neu7 cells placed onto COS7 cells from 24 to 48 hrs after transfection with the indicated clones. Clones B5 and C4 show no secreted chondroitinase

activity, but clone Y13 shows complete digestion of the CSPG smear to core band. COS7 cells do not produce NG2, which may be why the digestion was complete in this experiment but incomplete in figure 5.

5 Figure 8 shows detachment of Neu7 cells following transfection with the modified chondroitinase clone Y13. All were photographed after 48h in culture. Cell rounding and detachment is not seen using a similar construct that lacks a signal sequence for secretion (d), nor with GFP (a,b), indicating that the effect is a consequence of chondroitinase  
10 secretion.

Figure 9 shows western blots of Neu7 conditioned medium (a source of CSPGs) placed on transfected COS7 cells, probed with antibody 1B5 against the carbohydrate 'stub' epitope that represents the residue of  
15 chondroitinase digestion. Figure 9(a) shows that medium incubated with GFP-transfected control cells shows little immunoreactivity (lane 2), but digestion with commercial chondroitinase in vitro generates extensive reactivity (lane 1). Lane 3 shows medium incubated with  
20 COS7 cells transfected with chondroitinase A10, and shows reactivity as great as that produced by chondroitinase in vitro. (\*) Saturated bands which have bleached before autoradiography. Figure 9(b) shows the same experiment repeated with additional clones. Neu7 medium for control lanes 1 and 2 was not incubated with COS7 cells.

25 Figure 10 shows western blots prepared as in Figure 9, showing that chondroitinase activity is routinely produced by several mutated genes with 3 or more selected glycosylation sites changed. The upper panels show 'stub' immunoreactivity as in Figure 9; the lower panels show NG2 immunoreactivity as in Figures 5-7.

30 Figure 11 shows western blots as in Figure 10, showing the activity of transfected chondroitinase mutants Y13 and Y133 relative to unmodified sequence C4 in glial cell lines SCTM and Neu7.

Figure 12 shows western blot lanes, probed for 'stub' immunoreactivity, showing the activity of transfected chondroitinase mutant A10 (with a signal sequence from human prolactin replacing the previous signal sequence; see Methods) in a primary astrocyte cell culture.

5

Figure 13 shows western blot lanes, probed for NG2, of Neu7 conditioned medium that was placed on COS7 cells after transfection with the chondroitinase mutant A10 coupled to different signal sequences (see Methods). Chondroitinase activity is revealed by degradation of NG2 glycan as in Figures 5-7. It is produced by A10 with three different signal sequences, but not by the negative control (C4-Csig).

10

### Experiments

#### 15 Materials and methods

The sequence of the gene for *Proteus vulgaris* chondroitinase ABC was reported by Ryan et al (1994) [Entrez accession number AAB43331, gil828877, SEQ ID NO: 2] and confirmed by Prabhakar et al. (2005a). The encoded sequence was also confirmed from the protein crystal structure by Huang et al (2003). These studies showed that an independent sequence reported by Sato et al. (1994) contained several errors.

20

A cDNA for *Proteus vulgaris* chondroitinase ABC, in a prokaryotic expression vector, was obtained from Glyko Inc. Sequencing revealed that this construct had the prokaryotic signal sequence removed, and contained two mutations, at nucleotide 1671 (C to T, glutamine to stop codon) and at nucleotide 2458 (T to C, leucine-745 to proline). Apart from these codons, the clone encoded the same sequence reported by Ryan et al (1994). We corrected the two mutations by site-directed mutagenesis, and added a eukaryotic signal sequence from mouse matrix metalloprotease 2 (GenBank accession no. NM008610 = gi47271505) (Reponen et al., 1992), to direct enzyme secretion from transfected cells. An optimized Kozak sequence was also inserted to allow

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recognition by eukaryotic ribosomes and to maximize protein yield. We further changed some of the codons that are unfavourable for translation by eukaryotic ribosomes, replacing them with those used more frequently by eukaryotic cells. The resulting coding sequence is set out in SEQ ID NO: 4 and the sequence of the encoded protein is set out in SEQ ID NO: 3.

The numbering of nucleotides and codons in the chondroitinase coding region (nucl. 297 onwards) is as in Sato et al. (1994); the section that was frame-shifted in Sato et al. (1994) is marked. The sequence up to nucl. 297 is replaced by a NotI site (underlined: used for cloning into pcDNA3.1) overlapping a Kozak initiation sequence (bold, purple), then the coding sequence for mouse MMP-2 signal sequence, which is joined to nucl. 297 of the chondroitinase ABC sequence.

(Nucls 297-300, and the encoded Ala-Ala sequence, comprise overlap of identical residues representing the cleavage site of the signal sequence in both MMP-2 and chondroitinase ABC.) From then on the sequence is identical to that of the *P. vulgaris* chondroitinase ABC gene, except for individual nucleotides in bold where synonymous substitutions have been made to alter restriction sites or to substitute codons more frequently used in mammalian genes. Underlined sections labelled G1 to G9 indicate matches to oligonucleotides used to introduce mutations in N-glycosylation sites.

This modified cDNA was subcloned into the eukaryotic expression vector pcDNA 3.1 (Invitrogen), in which transcription is directed by the viral CMV promoter, which directs high-level expression in a wide range of eukaryotic cells.

#### Site-directed mutagenesis

Mutagenesis was carried out using the QuikChange Multi-Site-Directed Mutagenesis kit (Stratagene). All mutagenesis was carried out using constructs inserted in pcDNA 3.1.

Primers containing the desired mutations are shown in SEQ.ID.NO.5. They were designed where possible to insert or delete a restriction site to allow easy identification of mutant clones. The primers were modified with a 5' phosphate and PAGE-purified to improve mutation efficiency. All clones were sequenced to confirm successful mutagenesis. Each construct was assayed using the TNT system to assess the effect of the mutation on enzyme activity. This allows us to distinguish the direct effect of the mutation on enzyme activity separately from effects due to glycosylation inside the cell.

Variants of clone A10 were made in which the MMP-2 signal sequence was replaced with signal sequences from the following genes:

1) original bacterial chondroitinase [Ryan et al, 1994 see above; Entrez ref.no. AAB43331, gi: 1828877]

2) rat GDNF [Lin, L.F. et al (1993) Science 260, 1130-1132 Entrez ref. no. NM\_019139, gi: 9506720]

3) human prolactin [Jungnickel, B. and Rapoport, TM (1995). Cell 71, 489-503; Entrez ref. no. BC088370; gi: 56971865]

4) mouse immunoglobulin kappa [Coloma, M. J. et al (1992). J. Imm. Methods 152, 89-104]

#### In vitro transcription-translation

In vitro transcription/translation (IVTT) reactions were carried out with rabbit reticulocyte lysate in a coupled reaction with T7 polymerase, using the TNT Quick Coupled Transcription/Translation kit (Promega). Each 25 µl reaction contained 1 µg of plasmid. Labelled reactions also included 1 µl biotinylated lysine (Transcend) or 1 µl 35S-methionine (Redivue L-methionine, 37 MBq/mmol, Amersham).

Reactions to assess glycosylation also contained 1 µl of canine microsomes (Promega). The samples were incubated at 30°C for 90mins.

The products of the IVTT reactions were then separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as follows:

35S-labeled samples: 1-2  $\mu$ l samples were run on Nupage 4-12% bis/tris gradient gels (Invitrogen), fixed in methanol/ acetic acid, and incubated in Amplify (Amersham) for 30 min prior to drying. The dried gels were then exposed to X-ray film for 2 h.

Biotin-labeled samples: 1-2  $\mu$ l samples of IVTT reactions labeled with biotinylated lysine were run on 10% Tris/glycine gels, transferred to nitrocellulose membrane using a semi-dry-blot (Invitrogen), and then probed with streptavidin-linked horse radish peroxidase (Promega), prior to development using chemiluminescence (Promega). All chemiluminescence products were detected using chemiluminescence film (Amersham).

Protease protection assay (Schmidt-Rose & Jentsch, 1997): The translation mixture was brought to 10 mM  $\text{CaCl}_2$  and chilled on ice. Aliquots of 10  $\mu$ l were incubated with proteinase K, 30  $\mu$ g/ml (Roche), in the presence or absence of 1% Triton X100. Controls remained without proteinase and detergent. Proteolysis proceeded on ice for 60min and was stopped by adding 5 mM phenylmethylsulfonyl fluoride. After 10 min on ice, 50  $\mu$ l of preheated sample buffer (95°C) was added and the sample was boiled for 15 min to inactivate the protease. The samples were then run on a gel.

#### Morgan-Elson reaction

This reaction measures chondroitinase activity by the N-acetylation of product disaccharides and subsequent reaction to give a coloured product [Morgan and Elson, 1934; Reissig et al., 1955]. The procedure was adapted from standard protocols [Yamagata, T. et al *J. Biol. Chem.*, 243, 1523 (19680)]. Initially the reaction resulted in unstable Colour intensity due to the formation of precipitate during the reaction, as previously noted by others [Takahashi et al., 2003].

Therefore, activity could only be estimated qualitatively. We then refined the procedure by centrifuging at two stages to minimise the presence of precipitate at the end, thereby allowing spectrophotometry to be used to quantify activity.

5

The reaction was made up of 100  $\mu$ l of 40 mM NaAc, 40 mM TrisCl pH 8.0, 10mg/ml chondroitin-6-sulphate (Sigma), mixed with 20  $\mu$ l enzyme sample (IVTT product or standard). *Proteus vulgaris* chondroitinase ABC (Sigma) was used as standard. The reaction was incubated at 37°C for 10 20 min, then stopped by boiling for 1 minute. Potassium borate solution (0.8 M, pH 9.1, 100  $\mu$ l) was added and the mixture was boiled for 7 mins. It was chilled on ice then centrifuged in a microfuge at 13000 rpm for 10 min. To the supernatant, 1ml glacial acetic acid was added and mixed before centrifugation for a further 20-30 min. To 15 1 ml of supernatant, 0.4 ml of Morgan-Elson Reagent (10g para-dimethylaminobenzaldehyde in 100 ml in glacial acetic acid with 12.5% concentrated HCl) was added and incubated at 37°C for 20 min. Product was measured by absorbance at 550 nm. (This wavelength gives higher and more consistent absorption values than the standard 585 nm.)

20

The Morgan-Elson reaction is not sensitive enough to detect enzyme secreted by transfected cells in unconcentrated medium, and does not work in concentrated conditioned medium. We determined this by mixing commercial chondroitinase ABC with Neu7 conditioned medium that had 25 been concentrated ten-fold with a Centricon-50 unit: activity in the Morgan-Elson reaction was abolished. Therefore, other methods had to be used to assay secretion of the enzyme.

#### Cell transfection

30 COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen) plus 10% fetal bovine serum plus standard concentrations of penicillin, streptomycin and fungazone (CDMEM). Neu7 cells [Smith-Thomas et al., 1994; Fok-Seang et al., 1995] were grown in CDMEM plus 10% horse serum.

Transfection was performed in 25-cm<sup>2</sup> flasks with the cells ~60-70% confluent, with polyethyleneimine (PEI) and pAdVantage vector to increase translation efficiency (Promega), as follows. For two  
5 flasks, 10 µl of chondroitinase or GFP plasmid at 1 µg/µl in Tris/EDTA, plus 1 µg of pAdVantage, was mixed with 50 µl of 0.15 M NaCl, then rapidly mixed with 53 µl of 0.1 M PEI in 0.15 M NaCl, and incubated at room temperature for 30 min. The cells were rinsed with DMEM with ITS3+ (Sigma). Each flask then received 0.5 ml of DMEM with  
10 ITS3+ and 55 µl of the DNA/PEI mixture, and was incubated at 37° for 4 hours. The mixture was then replaced with CDMEM and incubation continued. After 24 hr, the medium was replaced with DMEM with ITS3+, or (for COS7 cells) with medium of the same composition which had been incubated for 48 hours with just-confluent Neu7 cells (Neu7  
15 conditioned medium). This conditioned medium was collected after 48 hrs, centrifuged to remove detached cells, and concentrated 7- to -10-fold by centrifugation in a Centricon-50 unit (Millipore), mixed with protease inhibitor cocktail (Sigma P8340), and stored at -20° for subsequent electrophoresis.

20  
In every round of transfections, one transfection was performed with GFP to assess transfection efficiency by fluorescence microscopy of the live cells, and in some cases by fixation and counterstaining with bisbenzamide (Hoechst 33258). Transfection efficiencies were 17-30%  
25 for COS cells and 24-50% for Neu7 cells.

#### Western blots

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were done by standard techniques. For detection  
30 of proteoglycans, samples were 50 µl of concentrated conditioned medium. Controls were similar samples from non-transfected cells, one of which was digested with chondroitinase ABC (Sigma, 20 mU) at 37° for 3 hrs. Samples were mixed with 10 µl of 5x non-reducing Laemmli sample buffer, boiled for 2 min, separated by SDS-PAGE (5% acrylamide

gel), and electroblotted in a Transblot Semi-dry Transfer Cell blotter (Bio-Rad) to Hybond-ECL membrane.

IVTT product samples consisted of 5 µl IVTT product, 6 µl 5x reducing Laemmli sample buffer, 19 µl chondroitinase buffer (40 mM NaAc, 40 mM TrisCl pH 8.0). Proteins were separated by SDS-PAGE (6% acrylamide gel) and transferred to Hybond-ECL membrane.

Antibodies were: mouse anti-NG2 (Santa Cruz sc33666 = mcAb 132.38), diluted 1:1000 and mouse anti-'stub' (Seikagaku, mcAb 1B5, 1:250).

Membranes were incubated in 2% ECL Advanced Blocking Agent (Amersham) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at room temperature for 3-4 hours, then incubated with primary antibody in blocking solution, overnight at 4°C. Membranes were washed in TBS-T before incubation with secondary antibody (peroxidase-labelled anti-mouse), 1:10,000 in blocking solution) for 1 hour at room temperature. Membranes were washed in TBS-T before reaction with ECL chemiluminescence detection reagent and visualisation on Hyperfilm (Amersham).

## Results

### *1. Recombinant chondroitinase ABC is active in vitro.*

As described in Methods, we constructed a cDNA clone that encodes the natural sequence of chondroitinase ABC, with a Kozak sequence to initiate translation in mammalian cells, the mouse MMP2 signal sequence, and some codons modified to be more favourable for mammalian expression. This 'normalised' cDNA, subcloned into the expression vector pcDNA 3.1, was named clone C4. The sequence is set out in SEQ.ID.NOS 3 and 4.

These initial modifications were sufficient for active enzyme to be produced when testing the construct in an *in vitro* transcription/translation (IVTT) system using rabbit reticulocyte

lysate. A band of the expected size was synthesised (Fig.1) and active enzyme could be detected by colorimetric assay (Fig.2).

However, no secreted enzyme activity was detected 48 hours after transfection of COS 7 cells with the construct, even though RT-PCR confirmed that the cDNA was efficiently transcribed by the cells.

One explanation for the lack of detectable secreted product may be the presence of cryptic signals in the bacterial protein which are inappropriately recognized and processed by eukaryotic cells. Computer prediction programs enabled us to screen for such signals. We established the absence of the endoplasmic reticulum (ER) retention signal KDEL and signal sequences directing proteins to organelles, but we did find 17 predicted sites for N-glycosylation in the protein sequence. Such modifications could interfere with enzyme folding, impeding transit through the secretory pathway, and/or causing loss of activity by alteration of the active site or by sterically hindering substrate binding. Misfolding of a protein could also direct it to the proteosome for degradation.

The IVTT results are consistent with the possibility that abnormal N-glycosylation causes loss of chondroitinase activity. N-glycosylation does not occur with the rabbit reticulocyte lysate system, which produces active enzyme. However, when canine microsomes, which carry out N-glycosylation, were added to our IVTT, enzyme activity was completely suppressed.

We used a protein 3D-imaging program to identify the location of potential N-glycosylation sites in chondroitinase ABC, and found several in regions very likely to affect protein folding and/or substrate binding. We first performed multi-site-directed mutagenesis on the five sites marked (Table 1 and SEQ ID NOS: 5 to 13). The glycosylation recognition sequences were abolished by conservative

substitutions using either Asn->Gln if the residue was on the surface or Ser->Val, if it was buried or slightly buried.

2. *Mutations at most N-glycosylation sites do not impair enzyme activity in vitro.*

The different constructs were tested for activity by transcription and translation in the rabbit reticulocyte lysate system (IVTT), and the products assayed by the colorimetric assay (Morgan-Elson reaction). All constructs produced similar amounts of protein. This was demonstrated either by western blots using an antibody to the bacterial enzyme or by labeling the products with biotinylated lysine which was detected by strepavidin-linked HRP followed by chemiluminescence.

All the clones listed produced similar levels of activity in the Morgan-Elson reaction, within a factor of two, like clones B5 and C4 (Fig.2).

3. *Mutations at N-glycosylation sites reduce glycosylation in vitro.*

Our hypothesis is that these sites are being inappropriately glycosylated in a eukaryotic system. To test this, and identify which of the sites are actually glycosylated, we compared different constructs in the reticulocyte lysate system in the presence of canine microsomes (endoplasmic reticulum preparation), which incorporate and N-glycosylate the nascent polypeptide as in an animal cell.

Fig.3 shows that the unmodified enzyme (C4) is heavily glycosylated in the presence of microsomes. The unglycosylated form produced in the absence of microsomes is seen as a band of around 110 kDa. In the presence of microsomes, an additional band is seen at higher molecular weight, as expected for glycosylation. To confirm that it was internalised by the microsomes, a Proteinase K protection experiment was performed (Fig.4). N-linked glycosylation occurs only within intact microsomes, and this assay makes use of the protection afforded

the translocated protein domain by the microsomal membrane. Thus, translocated proteins are protected from exogenously added protease, and only the glycosylated form remains.

5 The glycosylated products of clones C4 and B5 comigrate (Fig.3), so the mutation of Asn-751 in B5 made no visible difference to the glycosylation. This indicates that Asn-751 is not detectably glycosylated, consistent with the sequence flanking Asn-751 which makes N-glycosylation unlikely [Petrescu et al., 2004]. Conversely,  
10 the glycosylated products of clones with mutations at other sites show increased mobility on the gel (Fig.3). Comparison of these products with the mutations they contain (Table 1) indicates that Asn-515, 345, 338 and 282 are all glycosylated as predicted. The glycosylation of Asn-515 is of particular significance because it lies in the cleft  
15 which constitutes the enzyme's active site.

Similar experiments with other constructs have shown that Asn-675, 856, and 963 are also glycosylated, but Asn-836 is not. Thus 7 out of 9 sites so far tested are glycosylated as predicted.

20

*4. Chondroitinase ABC with reduced N-glycosylation can degrade NG2 proteoglycan from transfected Neu7 cells.*

We have transfected these constructs, containing up to five mutations, into the astrocyte cell line Neu7. These cells were derived by  
25 transforming rat brain glia with the Neu oncogene [Fok-Seang et al., 1995], and secrete substantial amounts of the axon-inhibitory CSPG, NG2, into the medium and the matrix [Smith-Thomas et al., 1994; Fidler et al., 1999]. NG2 consists of a core protein of ~290 kDa, and a proportion of the molecules are glycanated and therefore run as a  
30 higher-Mr smear on a gel [Levine & Nishiyama, 1996]. Chondroitinase activity would be demonstrated by reduction or loss of the glycanated smear, which is converted to the core protein. To assess whether our clones would generate chondroitinase activity, we transfected Neu7 cells with each clone, incubated them with serum-free medium from 24

hr to 72 hr after transfection, and analysed the conditioned medium by western blotting for NG2.

The results are shown in Figs 5 to 7 and Table 2. There was no significant GAG degradation by cells transfected with GFP, or with clones C4, B5, B1, or X12, which have from 0 to 3 glycosylation sites mutated. Conversely, GAG degradation was clearly seen following transfection with clones A10 or Y13, which have additional glycosylation mutations.

In one experiment, a higher-resolution view of the GAG digestion was fortuitously obtained as the NG2 core protein was cleaved by endogenous protease activity in the medium (Fig.6). The antibody detected a broad smear in control (GFP-transfected) medium which was removed by treatment with commercial chondroitinase. Medium from Y13-transfected cells showed the same appearance as chondroitinase-treated control medium.

In the same series of transfections, the Neu7 cells reproducibly detached from the substratum by 48 hrs after transfection with clone Y13, but not with GFP, nor with a Y13 clone lacking a signal sequence (Fig.8). The same effect was produced by adding commercial chondroitinase ABC to the cultures. This effect may be due to degradation of the matrix, as a consequence of chondroitinase secretion, in a batch of Neu7 cells which were also secreting large amounts of proteases. However other batches of Neu7 cells did not show this effect, demonstrating that chondroitinase synthesis does not have a toxic effect on the cells.

#### *5. Chondroitinase ABC with reduced N-glycosylation is secreted from transfected COS7 cells.*

The preceding results showed that abolishing selected glycosylation sites resulted in chondroitinase activity in transfected astrocytes in culture. However, the sensitivity of the assays was limited because

the cells do not produce much CSPG for the first 48 hrs after transfection. Moreover, it did not exclude the possibility that the enzyme remained cell-associated and acted during the secretion of the CSPG rather than within the medium. We therefore transfected the same  
5 constructs into COS7 cells, which do not synthesise CSPGs, and added conditioned medium from newly confluent Neu7 cells as a reliable source of CSPGs. After 24-48 hrs, this medium was then analysed by western blotting for NG2 as before (Fig.9). In addition, western  
10 blots were probed with the antibody 1B5 which recognizes the carbohydrate 'stub' epitope remaining after chondroitinase has cleaved off the CSPG chains (Fig.9). This offers greater sensitivity because it detects the appearance of a novel product rather than the diminution of a substrate.

15 The western blots (Figs.9 & 10) show that several of the modified clones produce as much stub reactivity as in vitro treatment with commercial chondroitinase ABC. The results parallel those with antibody against NG2 (above and Fig.10) but as expected are more sensitive; thus clones B1 and X12 produced little or no visible  
20 degradation of NG2, but generated distinct stub immunoreactivity. Activity of the enzymes following transfection correlates with the number of glycosylation sites which have been ablated (Table 2).

A range of mutant chondroitinase constructs were also transfected into cell  
25 lines representing glial cells of the nervous system, viz. SCTM cells (Schwann cell line) and Neu7 cells (astrocyte cell line) and incubated in Neu7 conditioned medium as a source of CSPGs. Active chondroitinase was secreted from both SCTM cells and Neu7 cells (Fig.11). Active chondroitinase was also secreted following transfection of primary  
30 astrocytes from rat brain (Fig.12).

The level of secretion of active chondroitinase was found to be further improved by mutagenesis of site Asn-675, generating clone

Y133, which is the most active mutant chondroitinase identified so far (Figs. 10 and 11).

All these constructs used the signal sequence from the mouse MMP2 gene to direct secretion, but there is evidence that signal sequences show differential activity in different cell types. Therefore, for one of the more active mutants (A10), alternative signal sequences were substituted, from the MMP2, GDNF, immunoglobulin kappa, and prolactin genes. The resulting constructs were transfected into COS cells and incubated in Neu7 conditioned medium for 24-48 hrs. The medium was then analysed by western blotting for NG2. Several of the various signal sequences were shown to direct secretion of active chondroitinase from cells transfected with our mutagenised genes (Fig. 13).

Little is known about how a bacterial protein is trafficked in a eukaryotic cell. The data herein shows that bacterial chondroitinase is inappropriately glycosylated by eukaryotic cells and that this has a detrimental effect on secretion of active enzyme.

Mutations of many N-glycosylation sites are permissible without impairing enzyme activity. However, a construct equivalent to X12 plus a Thr-Ala substitution at Thr-338 was inactive in the in vitro assay, whereas a mutation at Asn-336, eliminating the same N-glycosylation site in clone A10, was well tolerated.

An *in vitro* assay was also useful to show whether the enzyme could enter the secretory pathway. In the presence of microsomes, a protein destined for secretion is internalised and N-glycosylated, and this was observed to happen with our chondroitinase constructs. However, a positive result in this assay does not guarantee that the enzyme will be adequately secreted by transfected cells. All clones, including the native sequence C4, showed internalisation and glycosylation of a proportion of their products in microsomes *in vitro*, whereas some of

them failed to produce detectable activity in medium from transfected cells.

We have shown that ablation of strategic glycosylation sites results  
5 in secretion of active enzyme from transfected mammalian cells.  
First, transfection of an astrocyte cell line, Neu7, with two of our  
modified constructs led to the degradation of the matrix, accompanied  
by the removal of the GAG chains from NG2, the main inhibitory  
component of the matrix. These results were consistent with active  
10 chondroitinase being produced and secreted. This conclusion was  
further supported by experiments on COS7 cells (which do not produce  
NG2 nor other CSPGs). COS7 cells were transfected with several of the  
modified cDNAs, and conditioned medium containing CSPGs was incubated  
with transfected cells. Western blots showed that GAG chains were  
15 cleaved from NG2 molecules, and that the stub epitope was generated.  
The latter assay was more sensitive because it depended on detection  
of a new product rather than diminution of a substrate. It also  
conclusively showed that the chondroitinase was secreted, as the only  
source of CSPG substrate was the conditioned medium added to the  
20 transfected cells.

Recently, Cafferty et al. (2007) have reported production of Proteus  
chondroitinase ABC in transgenic mice, using the GFAP promoter and the  
original bacterial signal sequence to express it in astrocytes, and  
25 demonstrating activity in the injured CNS by staining with a stub  
antibody. However the stub antibody can detect small amounts of  
chondroitinase product, and indeed it could have detected products of  
intracellular chondroitinase activity. Moreover, the astrocytic  
expression was not sufficient to allow corticospinal axons to  
30 regenerate beyond the lesion, unlike the injection experiments of  
Bradbury et al. (2002), and neuronal expression was not attempted; it  
remains to be seen whether the bacterial sequence will be adequate for  
secretion from axons. As we have shown that mutations of  
glycosylation sites can greatly enhance secretability, it is clearly

important to use these mutants in future transgenic experiments for optimal expression, to improve the efficacy of chondroitinase as a treatment for spinal cord injury.

- 5 Curinga et al. (2007) have recently reported expression of a different bacterial enzyme, chondroitinase AC, in mammalian cells, using an immunoglobulin signal sequence and adenovirus vector. However the specific activity of the secreted product was very low and it was ineffective in vivo.

10

Bacterial chondroitinase ABC has shown considerable promise in animal models as a treatment for spinal cord injury. It not only promotes regeneration of injured axons and functional recovery following spinal injury, but also increases plasticity so new axons can grow and take  
15 over the function of damaged axons. The strategy of making cells at the site of injury secrete the enzyme will have two major advantages: it delivers the enzyme to the precise place it is needed, and it circumvents the requirement of repeated injections.

- 20 The above data shows that ablation of strategic glycosylation sites results in secretion of active enzyme from transfected mammalian cells. Therefore, it is now possible to transfect it into cells such as Schwann cells which could be grafted into the CNS to form a bridge across the injury site. We have previously produced Schwann cell lines  
25 secreting GDNF and shown that bridge grafts of these promoted axon regeneration in a rat model of Parkinson's disease (Wilby et al., 1999). The modified chondroitinase gene could also be expressed from viral vectors to target it to neurons and/or glia in the injured CNS (Murray & Fischer, 2001).

| <u>Clone no:</u>          | <u>C4</u> | <u>B5</u> | <u>B1</u> | <u>X12</u> | <u>X30</u> | <u>Y13</u> | <u>A10</u> | <u>Y133</u> |
|---------------------------|-----------|-----------|-----------|------------|------------|------------|------------|-------------|
| <u>Positions changed:</u> |           |           |           |            |            |            |            |             |
| Asn-751 (N-Q)             | -         | +         | +         | +          | +          | +          | +          | +           |
| Asn-515 (S-A)             | -         | -         | +         | +          | +          | +          | +          | +           |
| Asn-345 (N-Q)             | -         | -         | -         | +          | -          | +          | +          | +           |
| Asn-336 (N-Q)             | -         | -         | -         | -          | -          | -          | +          | -           |
| Asn-282 (N-K, or N-Q)     | -         | -         | -         | -          | N-K        | N-K        | N-Q        | N-K         |
| Asn-675                   | -         | -         | -         | -          | -          | -          | -          | +           |

The table indicates whether each clone had a mutation at each of 5 Asn-X-(Ser/Thr) sites. Mutations were Asn-Gln (N-Q), Asn-Lys (N-K), or Ser-Ala (S-A; at position +2 relative to the Asn), as indicated.

5

Table 1: Mutations of N-glycosylation sites

| <u>Clone no:</u>                    | <u>C4</u> | <u>B5</u> | <u>B1</u> | <u>X12</u> | <u>X30</u> | <u>Y13</u> | <u>A10</u> | <u>Y133</u> |
|-------------------------------------|-----------|-----------|-----------|------------|------------|------------|------------|-------------|
| <u>No. of positions changed:</u>    | 0         | 1         | 2         | 3          | 3          | 4          | 5          | 5           |
| <u>Activity after transfection:</u> |           |           |           |            |            |            |            |             |
| (Neu7 cells)                        | -         | -         | -         | (+/-)      | nd         | +          | +          | +           |
| (COS7 cells)                        | -         | (+/-)     | +         | ++         | ++         | ++         | ++         | +++         |

The table summarises the level of chondroitinase activity observed in media from transfected cells, either Neu7 cells generating endogenous CSPGs, or COS7 cells provided with Neu7 conditioned medium as a source of CSPGs. They were scored using both NG2 glycan degradation, and appearance of stub immunoreactivity. (nd, not determined).

Table 2: Results of transfections

| Protein                                  | Source                       | Version       | Identifier   |
|------------------------------------------|------------------------------|---------------|--------------|
| chondroitinase AC                        | Flavobacterium columnare     | AAX07421.1    | GI:59800136  |
| Polysaccharide lyase family 8            | Pseudomonas syringae         | AAZ36518.1    | GI:71557307  |
| chondroitinase ABC                       | Yersinia pestis Angola       | ZP_00797056.1 | GI:77634965  |
| chondroitinase AC                        | Pedobacter heparinus         | U27583.1      | GI:1002524   |
| chondroitin lyase                        | Bacteroides thetaiotaomicron | NP_812262.1   | GI:29348759  |
| chondroitinase                           | Photobacterium profundum     | CAG22026.1    | GI:46915253  |
| XalA                                     | Paenibacillus alginolyticus  | AAG24953.1    | GI:10952528  |
| Polysaccharide lyase family 8            | Clostridium phytofermentans  | ZP_01352004.1 | GI:106884635 |
| chondroitinase AC precursor              | Bacteroides fragilis         | BAD48335.1    | GI:52215742  |
| pyruvated mannose-specific xanthan lyase | Bacillus cereus              | AAV60495.1    | GI:66970519  |
| hyaluronate lyase                        | Streptococcus pyogenes       | AAF29533.1    | GI:6851375   |
| hyaluronate lyase precursor              | Vibrio fischeri ES114        | AAW88064.1    | GI:59482425  |

Table 3: Examples of bacterial GAG lyases

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Sequences

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5     121 idgyltidfg eklistseaq agfkvkl dft gwrvvgvsln ndlenremtl natntssdgt
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SEQ ID NO: 2 (I29953.1 GI: 1820744)

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|-----|--|-----|-----|-----|-----|-----|-----|-----|------------|------------|------------|-----|-----|-----|-----|-----|------------|
|     |  |     |     |     |     |     |     |     |            |            |            | M   | E   | A   | R   | V   | NotI, NcoI |
|     |  | gcc | tgg | gga | gcg | ctg | gcc | gga | cct        | ctg        | cgg        | gtt | ctc | tgc | gtc | ctg |            |
|     |  | A   | W   | G   | A   | L   | A   | G   | P          | L          | R          | V   | L   | C   | V   | L   |            |
| 270 |  | tgc | tgc | ctg | ttg | ggc | cgc | gcc | atc        | gcc        | <u>gcc</u> | act | agt | aat | cct | gca | NT 297     |
| 16  |  | C   | C   | L   | L   | G   | R   | A   | I          | A          | A          | T   | S   | N   | P   | A   |            |
| 315 |  | ttt | gat | cct | aaa | aat | ctg | atg | cag        | tca        | gaa        | att | tac | cat | ttt | gca |            |
| 31  |  | F   | D   | P   | K   | N   | L   | M   | Q          | S          | E          | I   | Y   | H   | F   | A   |            |
| 360 |  | caa | aat | aac | cca | tta | gca | gac | ttc        | tca        | tca        | gat | aaa | aac | tca | ata |            |
| 46  |  | Q   | N   | N   | P   | L   | A   | D   | F          | S          | S          | D   | K   | N   | S   | I   |            |
| 405 |  | cta | acg | tta | tct | gat | aaa | cgt | agc        | att        | atg        | gga | aac | caa | tct | ctt |            |
| 61  |  | L   | T   | L   | S   | D   | K   | R   | S          | I          | M          | G   | N   | Q   | S   | L   |            |
| 450 |  | tta | tgg | aaa | tgg | aaa | ggg | ggg | agt        | agc        | ttt        | act | tta | cat | aaa | aaa |            |
| 76  |  | L   | W   | K   | W   | K   | G   | G   | S          | S          | F          | T   | L   | H   | K   | K   |            |
| 495 |  | ctg | att | gtc | ccc | acc | gat | aaa | gaa        | gca        | tct        | aaa | gca | tgg | gga | cgc |            |
| 91  |  | L   | I   | V   | P   | T   | D   | K   | E          | A          | S          | K   | A   | W   | G   | R   |            |
| 540 |  | tca | tct | acc | ccc | gtt | ttc | tca | ttt        | tgg        | ctt        | tac | aat | gaa | aaa | ccg |            |
| 106 |  | S   | S   | T   | P   | V   | F   | S   | F          | W          | L          | Y   | N   | E   | K   | P   |            |
| 585 |  | att | gat | ggg | tat | ctt | act | atc | gat        | ttc        | gga        | gaa | aaa | ctc | att | tca |            |
| 121 |  | I   | D   | G   | Y   | L   | T   | I   | D          | F          | G          | E   | K   | L   | I   | S   |            |
| 630 |  | acc | agt | gag | gct | cag | gca | ggc | ttt        | aaa        | gta        | aaa | tta | gat | ttc | act |            |
| 136 |  | T   | S   | E   | A   | Q   | A   | G   | F          | K          | V          | K   | L   | D   | F   | T   |            |
| 675 |  | ggc | tgg | cgt | gct | gtg | gga | gtc | tct        | tta        | aat        | aac | gat | ctt | gaa | aat |            |
| 151 |  | G   | W   | R   | A   | V   | G   | V   | S          | L          | N          | N   | D   | L   | E   | N   |            |
| 720 |  | cga | gag | atg | acc | tta | aat | gca | acc        | aat        | acc        | tcc | tct | gat | ggg | act |            |
| 166 |  | R   | E   | M   | T   | L   | N   | A   | T          | N          | T          | S   | S   | D   | G   | T   |            |
| 765 |  | caa | gac | agc | att | ggg | cgt | tct | tta        | ggg        | gct        | aaa | gtc | gat | agt | att |            |
| 181 |  | Q   | D   | S   | I   | G   | R   | S   | L          | G          | A          | K   | V   | D   | S   | I   |            |
| 810 |  | cgt | ttt | aaa | gcg | cct | tct | aat | gtg        | agt        | cag        | ggg | gaa | atc | tat | atc |            |
| 196 |  | R   | F   | K   | A   | P   | S   | N   | V          | S          | Q          | G   | E   | I   | Y   | I   |            |
| 855 |  | gac | cgt | att | atg | ttt | tct | gtc | gat        | gat        | gct        | cgc | tac | caa | tgg | tct |            |
| 211 |  | D   | R   | I   | M   | F   | S   | V   | D          | D          | A          | R   | Y   | Q   | W   | S   |            |
| 900 |  | gat | tat | caa | gta | aaa | act | cgc | tta        | tca        | gaa        | cct | gaa | att | caa | ttt |            |
| 226 |  | D   | Y   | Q   | V   | K   | T   | R   | L          | S          | E          | P   | E   | I   | Q   | F   |            |
| 945 |  | cac | aac | gta | aag | cca | caa | cta | cct        | gta        | aca        | cct | gaa | aat | tta | gcg |            |
| 241 |  | H   | N   | V   | K   | P   | Q   | L   | P          | V          | T          | P   | E   | N   | L   | A   |            |
| 990 |  | gcc | att | gat | ctt | att | cgc | caa | cgt        | cta        | att        | aat | gaa | ttt | gtc | gga |            |

|      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |             |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------------|
| 256  | A   | I   | D   | L   | I   | R   | Q   | R   | L   | I   | N   | E   | F   | V   | G   |             |
| 1035 | ggt | gaa | aaa | gag | aca | aac | ctc | gca | tta | gaa | gag | aat | atc | agc | aaa | G1b         |
| 271  | G   | E   | K   | E   | T   | N   | L   | A   | L   | E   | E   | N   | I   | S   | K   |             |
| 1080 | tta | aaa | agt | gat | ttc | gat | gct | ctt | aat | att | cac | act | tta | gca | aat |             |
| 286  | L   | K   | S   | D   | F   | D   | A   | L   | N   | I   | H   | T   | L   | A   | N   |             |
| 1125 | ggt | gga | acg | caa | ggc | aga | cat | ctg | atc | act | gat | aaa | caa | atc | att |             |
| 301  | G   | G   | T   | Q   | G   | R   | H   | L   | I   | T   | D   | K   | Q   | I   | I   |             |
| 1170 | att | tat | caa | cca | gag | aat | ctt | aac | tcc | caa | gat | aaa | caa | cta | ttt |             |
| 316  | I   | Y   | Q   | P   | E   | N   | L   | N   | S   | Q   | D   | K   | Q   | L   | F   |             |
| 1215 | gat | aat | tat | gtt | att | tta | ggt | aat | tac | acg | aca | tta | atg | ttt | aat | G2E         |
| 331  | D   | N   | Y   | V   | I   | L   | G   | N   | Y   | T   | T   | L   | M   | F   | N   |             |
| 1260 | att | agc | cgt | gct | tat | gtg | ctg | gaa | aaa | gat | ccc | aca | caa | aag | gcg |             |
| 346  | I   | S   | R   | A   | Y   | V   | L   | E   | K   | D   | P   | T   | Q   | K   | A   |             |
| 1305 | caa | cta | aag | cag | atg | tac | tta | tta | atg | aca | aag | cat | tta | tta | gat |             |
| 361  | Q   | L   | K   | Q   | M   | Y   | L   | L   | M   | T   | K   | H   | L   | L   | D   |             |
| 1350 | caa | ggc | ttt | gtt | aaa | ggg | agt | gct | tta | gtg | aca | acc | cat | cac | tgg |             |
| 376  | Q   | G   | F   | V   | K   | G   | S   | A   | L   | V   | T   | T   | H   | H   | W   |             |
| 1395 | gga | tac | agt | tct | cgt | tgg | tgg | tat | att | tcc | acg | tta | tta | atg | tct |             |
| 391  | G   | Y   | S   | S   | R   | W   | W   | Y   | I   | S   | T   | L   | L   | M   | S   |             |
| 1440 | gat | gca | cta | aaa | gaa | gcg | aac | cta | caa | act | caa | gtt | tat | gat | tca |             |
| 406  | D   | A   | L   | K   | E   | A   | N   | L   | Q   | T   | Q   | V   | Y   | D   | S   |             |
| 1485 | tta | ctg | tgg | tat | tca | cgt | gag | ttt | aaa | agt | agt | ttt | gat | atg | aaa |             |
| 421  | L   | L   | W   | Y   | S   | R   | E   | F   | K   | S   | S   | F   | D   | M   | K   |             |
| 1530 | gta | agt | gct | gat | agc | tct | gat | cta | gat | tat | ttc | aat | acc | tta | tct | P2          |
| 436  | V   | S   | A   | D   | S   | S   | D   | L   | D   | Y   | F   | N   | T   | L   | S   |             |
| 1575 | cgc | caa | cat | tta | gcc | ttg | ctg | tta | ctg | gag | cct | gat | gat | caa | aag |             |
| 451  | R   | Q   | H   | L   | A   | L   | L   | L   | L   | E   | P   | D   | D   | Q   | K   |             |
| 1620 | cgt | atc | aac | tta | gtt | aat | act | ttc | agc | cat | tat | atc | act | ggc | gca | P1          |
| 466  | R   | I   | N   | L   | V   | N   | T   | F   | S   | H   | Y   | I   | T   | G   | A   |             |
| 1665 | ttg | acg | caa | gtg | cca | ccg | ggt | ggt | aaa | gat | ggt | tta | cgc | cct | gat |             |
| 481  | L   | T   | Q   | V   | P   | P   | G   | G   | K   | D   | G   | L   | R   | P   | D   | Frame Shift |
| 1710 | ggt | aca | gca | tgg | cga | cat | gaa | ggc | aac | tat | ccg | ggc | tac | tct | ttc | start       |
| 495  | G   | T   | A   | W   | R   | H   | E   | G   | N   | Y   | P   | G   | Y   | S   | F   |             |
| 1755 | cca | gcc | ttt | aaa | aat | gcc | tct | cag | ctt | att | tat | tta | tta | cgc | gat | Frame Shift |
| 511  | P   | A   | F   | K   | N   | A   | S   | Q   | L   | I   | Y   | L   | L   | R   | D   | G3          |
| 1800 | aca | cca | ttt | tca | gtg | ggt | gaa | agt | ggt | tgg | aat | aac | ctg | aaa | aaa | Frame Shift |
| 526  | T   | P   | F   | S   | V   | G   | E   | S   | G   | W   | N   | N   | L   | K   | K   | end: remove |
| 1845 | gcg | atg | gtt | tca | gcg | tgg | atc | tac | agt | aat | cca | gaa | gtt | gga | tta | t @ NT 1812 |
| 541  | A   | M   | V   | S   | A   | W   | I   | Y   | S   | N   | P   | E   | V   | G   | L   |             |

|      |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |
|------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| 1890 | ccg        | ctt        | gca        | gga        | aga        | cac        | cct        | ttt        | aac        | tca        | cct        | tcg        | tta        | aaa        | tca        |
| 556  | P          | L          | A          | G          | R          | H          | P          | F          | N          | S          | P          | S          | L          | K          | S          |
| 1935 | gtc        | gct        | caa        | ggc        | tat        | tac        | tgg        | ctt        | gcc        | atg        | tct        | gca        | aaa        | tca        | tcg        |
| 571  | V          | A          | Q          | G          | Y          | Y          | W          | L          | A          | M          | S          | A          | K          | S          | S          |
| 1980 | cct        | gat        | aaa        | aca        | ctt        | gca        | tct        | att        | tat        | ctt        | gcg        | att        | agt        | gat        | aaa        |
| 586  | P          | D          | K          | T          | L          | A          | S          | I          | Y          | L          | A          | I          | S          | D          | K          |
| 2025 | aca        | caa        | aat        | gaa        | tca        | act        | gct        | att        | ttt        | gga        | gaa        | act        | att        | aca        | cca        |
| 601  | T          | Q          | N          | E          | S          | T          | A          | I          | F          | G          | E          | T          | I          | T          | P          |
| 2070 | gcg        | tct        | tta        | cct        | caa        | ggg        | ttc        | tat        | gcc        | ttt        | aat        | ggc        | ggg        | gct        | ttt        |
| 616  | A          | S          | L          | P          | Q          | G          | F          | Y          | A          | F          | N          | G          | G          | A          | F          |
| 2115 | ggg        | att        | cat        | cgt        | tgg        | caa        | gat        | aaa        | atg        | gtg        | aca        | ctg        | aaa        | gct        | tat        |
| 631  | G          | I          | H          | R          | W          | Q          | D          | K          | M          | V          | T          | L          | K          | A          | Y          |
| 2160 | aac        | acc        | aat        | gtt        | tgg        | tca        | tct        | gaa        | att        | tat        | aac        | aaa        | gat        | aac        | cgt        |
| 646  | N          | T          | N          | V          | W          | S          | S          | E          | I          | Y          | N          | K          | D          | N          | R          |
| 2205 | tat        | ggc        | cgt        | tac        | caa        | agt        | cat        | ggg        | gtc        | gct        | caa        | ata        | gtg        | agt        | aat        |
| 661  | Y          | G          | R          | Y          | Q          | S          | H          | G          | V          | A          | Q          | I          | V          | S          | N          |
| 2250 | ggc        | tcg        | cag        | ctt        | tca        | cag        | ggc        | tat        | cag        | caa        | gaa        | ggg        | tgg        | gat        | tgg        |
| 676  | G          | S          | Q          | L          | S          | Q          | G          | Y          | Q          | Q          | E          | G          | W          | D          | W          |
| 2295 | aat        | aga        | atg        | caa        | ggg        | gca        | acc        | act        | att        | cac        | ctt        | cct        | ctt        | aaa        | gac        |
| 690  | N          | R          | M          | Q          | G          | A          | T          | T          | I          | H          | L          | P          | L          | K          | D          |
| 2340 | tta        | gac        | agt        | cct        | aaa        | cct        | cat        | acc        | tta        | atg        | caa        | cgt        | gga        | gag        | cgt        |
| 706  | L          | D          | S          | P          | K          | P          | H          | T          | L          | M          | Q          | R          | G          | E          | R          |
| 2385 | gga        | ttt        | agc        | gga        | aca        | tca        | tcc        | ctt        | gaa        | ggg        | caa        | tat        | ggc        | atg        | atg        |
| 721  | G          | F          | S          | G          | T          | S          | S          | L          | E          | G          | Q          | Y          | G          | M          | M          |
| 2430 | gca        | ttc        | gat        | ctt        | att        | tat        | ccc        | gcc        | aat        | ctg        | gag        | cgt        | ttt        | gat        | cct        |
| 736  | A          | F          | D          | L          | I          | Y          | P          | A          | N          | L          | E          | R          | F          | D          | P          |
| 2475 | <u>aac</u> | <u>ttc</u> | <u>act</u> | <u>gcg</u> | aaa        | aag        | agt        | gta        | tta        | gcc        | gct        | gat        | aat        | cac        | tta        |
| 751  | N          | F          | T          | A          | K          | K          | S          | V          | L          | A          | A          | D          | N          | H          | L          |
| 2520 | att        | ttt        | att        | <u>ggg</u> | <u>agc</u> | <u>aat</u> | <u>ata</u> | <u>aat</u> | <u>agt</u> | <u>agt</u> | <u>gat</u> | <u>aaa</u> | <u>aat</u> | <u>aaa</u> | <u>aat</u> |
| 766  | I          | F          | I          | G          | S          | N          | I          | N          | S          | S          | D          | K          | N          | K          | N          |
| 2565 | <u>gtt</u> | <u>gaa</u> | <u>acg</u> | acc        | tta        | ttc        | caa        | cat        | gcc        | att        | act        | cca        | aca        | tta        | aat        |
| 781  | V          | E          | T          | T          | L          | F          | Q          | H          | A          | I          | T          | P          | T          | L          | N          |
| 2610 | acc        | ctt        | tgg        | att        | aat        | gga        | caa        | aag        | ata        | gaa        | aac        | atg        | cct        | tat        | caa        |
| 796  | T          | L          | W          | I          | N          | G          | Q          | K          | I          | E          | N          | M          | P          | Y          | Q          |
| 2655 | aca        | aca        | ctt        | caa        | caa        | ggg        | gat        | tgg        | tta        | att        | gat        | agc        | aat        | ggc        | aat        |
| 811  | T          | T          | L          | Q          | Q          | G          | D          | W          | L          | I          | D          | S          | N          | G          | N          |
| 2700 | ggg        | tac        | tta        | att        | act        | caa        | <u>gca</u> | <u>gaa</u> | <u>aaa</u> | <u>gta</u> | <u>aat</u> | <u>gta</u> | <u>agt</u> | <u>cgc</u> | <u>caa</u> |
| 826  | G          | Y          | L          | I          | T          | Q          | A          | E          | K          | V          | N          | V          | S          | R          | Q          |
| 2745 | <u>cat</u> | <u>cag</u> | <u>gtt</u> | <u>tca</u> | <u>gcg</u> | gaa        | aat        | aaa        | aat        | cgc        | caa        | <u>ccg</u> | <u>aca</u> | <u>gaa</u> | <u>gga</u> |

G5b

G4 &amp; G4b

G9

G6

G7

```

841  H   Q   V   S   A   E   N   K   N   R   Q   P   T   E   G
2790  aac ttt agc tcg gca tgg atc gat cac agc act cgc ccc aaa gat
856  N   F   S   S   A   W   I   D   H   S   T   R   P   K   D
2835  gcc agt tat gag tat atg gtc ttt tta gat gcg aca cct gaa aaa
871  A   S   Y   E   Y   M   V   F   L   D   A   T   P   E   K
2880  atg gga gag atg gca caa aaa ttc cgt gaa aat aat ggg tta tat
886  M   G   E   M   A   Q   K   F   R   E   N   N   G   L   Y
2925  cag gtt ctt cgt aag gat aaa gac gtt cat att att ctc gat aaa
901  Q   V   L   R   K   D   K   D   V   H   I   I   L   D   K
2970  ctc agc aat gta acg gga tat gcc ttt tat cag cca gca tca att
916  L   S   N   V   T   G   Y   A   F   Y   Q   P   A   S   I
3015  gaa gac aaa tgg atc aaa aag gtt aat aaa cct gca att gtg atg
931  E   D   K   W   I   K   K   V   N   K   P   A   I   V   M
3060  act cat cga caa aaa gac act ctt att gtc agt gca gtt aca cct G8
946  T   H   R   Q   K   D   T   L   I   V   S   A   V   T   P
3105  gat tta aat atg act cgc caa aaa gca gca act cct gtc acc atc
961  D   L   N   M   T   R   Q   K   A   A   T   P   V   T   I
3150  aat gtc acg att aat ggc aaa tgg caa tct gct gat aaa aat agt
976  N   V   T   I   N   G   K   W   Q   S   A   D   K   N   S
3195  gaa gtg aaa tat cag gtt tct ggt gat aac act gaa ctg acg ttt
991  E   V   K   Y   Q   V   S   G   D   N   T   E   L   T   F
3240  acg agt tac ttt ggt att cca caa gaa atc aaa ctc tcg cca ctc
1006  T   S   Y   F   G   I   P   Q   E   I   K   L   S   P   L
3285  cct tga
1021  P   *

```

SEQ ID NO;4

SEQ ID NO;3

SEQ ID NOS: 3 and 4

G1b

5 Start NT 1052, End NT 1099

 $N_{282} \rightarrow Q$ 

5'

3'

```

c ctc gca tta gaa gag CaG atc agc aaa tta aaa agt gat ttc gat gc
L   A   L   E   E   Q   I   S   K   L   K   S   D   F   D

```

SEQ ID NO: 5

G2E

Start NT 1224, End NT 1280

N<sub>338</sub> → Q, N<sub>345</sub> → Q, L<sub>342</sub> improved codon.

5' 3'

ggt att tta ggt CaG tac acg aca Cta atg ttt CaG att agc cgt gct tat gtg ctg

V I L G Q Y T T L M F Q I S R A Y V L

SEQ ID NO: 6

5 G3

Start NT 1756, End NT 1804

(N<sub>515</sub>) S<sub>517</sub> → A, L<sub>519</sub> and L<sub>522</sub> codons improved.

5' PvuII 3'

ca gcc Ttt aaa aat gcc Gct cag ctG att tat CtG tta cgc gat aca cc

A F K N A A Q L I Y L L R D T

SEQ ID NO: 7

10

G4

Starts NT 2446 Ends NT 2486

P<sub>745</sub> → L, N<sub>751</sub> → Q

5' XhoI 3'

at ccc gcc aat cTC gag cgt ttt gat cct CaG ttc act gcg

P A N L E R F D P Q F T A

SEQ ID NO: 8

15

54

G5b

Start NT 2237 End NT 2277

N<sub>675</sub> → Q

5' Pvu 3'  
a ata gtg agt CaG ggc gcg Cag ctG tca cag ggc tat cag c

SEQ ID NO: 9

5 G6

Start NT 2718 End NT 2757

N<sub>836</sub> → D (Aspartic acid)

5' SexAI / 3'  
gca gaa aaa gta Gat gta agt cgc caa caC cag gtt tca g

SEQ ID NO: 10

10

G7b

Start NT 2761 End NT 2800

N<sub>856</sub> → Q

5' 3'  
g aca gaa gga CaG ttt agc tcg gca tgg atc gat cac agg

SEQ ID NO: 11

15

55

G8b

Start NT 3081 End NT 3123

N<sub>963</sub> → Q

5' SfcI 3'  
gtc agt gca gtt aca cct gat Cta CaG atg act cgc c

SEQ ID NO: 12

5 G9

Start NT 2529 End NT 2573

N<sub>773</sub> → Q

5' SspI 3'  
ggt agc aat atT CaG agt agt gat aaa aat aaa aat gtt gaa acg

10 SEQ ID NO: 13

## Claims:

1. A chondroitinase polypeptide comprising a modified bacterial glycosaminoglycan (GAG) polysaccharide lyase sequence, said sequence  
5 having a reduced number of active N-glycosylation motifs relative to the unmodified sequence, and;  
said chondroitinase polypeptide being secretable in an active form by mammalian cells.
- 10 2. A chondroitinase polypeptide according to claim 1 comprising an amino acid sequence having at least 20% sequence identity to the sequence of SEQ ID NO: 1 or a fragment thereof having chondroitinase activity,  
the amino acid sequence having a reduced number of active N-  
15 glycosylation motifs relative to the sequence of SEQ ID NO: 1 or the fragment thereof, and  
the chondroitinase polypeptide being secretable in an active form by mammalian cells.
- 20 3. A chondroitinase polypeptide according to claim 2 wherein said fragment comprises residues 282 to 963 of SEQ ID NO: 1.
4. A chondroitinase polypeptide according to claim 2 or claim 3 wherein said amino acid sequence comprises a mutation in one or more  
25 N-glycosylation motifs which are present in the sequence of SEQ ID NO: 1 or the fragment thereof,  
said mutation preventing N-glycosylation of the motif.
5. A chondroitinase polypeptide according to claim 4 wherein the  
30 amino acid sequence comprises a conservative substitution of the N or S/T residue of an N-glycosylation motif.

6. A chondroitinase polypeptide according to claim 5 wherein the amino acid sequence comprises an N-Q or N-K or N-D substitution of the N residue of a N-glycosylation motif.

5 7. A chondroitinase polypeptide according to claim 5 wherein the mutation is an S/T to A substitution of the S/T residue of the N-glycosylation motif.

10 8. A chondroitinase polypeptide according to any one of claims 4 to 7 wherein said amino acid sequence comprises a mutation in one or more N-glycosylation motifs selected from the group consisting of the N-glycosylation motifs positioned at N282, N345, N336, N515, N675, N773, N856 and N963 of SEQ ID NO:1.

15 9. A chondroitinase polypeptide according to any one of claims 4 to 8 wherein said amino acid sequence comprises a mutation in two or more N-glycosylation motifs.

20 10. A chondroitinase polypeptide according to claim 9 wherein said amino acid sequence comprises mutations in the N-glycosylation motifs positioned at N282, N336, N345 and N515 of SEQ ID NO: 1.

25 11. A chondroitinase polypeptide according to claim 10 wherein said amino acid sequence further comprises a mutation in the N-glycosylation motif positioned at N675 of SEQ ID NO: 1.

30 12. A chondroitinase polypeptide according to any one of claims 2 to 11 wherein the amino acid sequence comprises the sequence of SEQ ID NO: 1 or a fragment thereof with mutations in one or more N-glycosylation motifs thereof,

said mutations preventing N-glycosylation of the one or more motifs.

13. A chondroitinase polypeptide according to claim 12 comprising mutations in one or more N-glycosylation motifs selected from the group consisting of the N-glycosylation motifs positioned at N282, N345, N336, N515, N675, N773, N856 and N963 of SEQ ID NO:1.

5

14. A polypeptide comprising a chondroitinase polypeptide according to any one of the preceding claims coupled to a signal sequence.

15. A polypeptide according to claim 14 wherein said signal sequence  
10 is a mammalian signal sequence.

16. A nucleic acid encoding a chondroitinase polypeptide according to any one of claims 1 to 13 or a polypeptide according to claim 14 or claim 15.

15

17. A nucleic acid according to claim 16 having a sequence which is optimised for eukaryotic codon usage.

18. A nucleic acid according to claim 16 or claim 17 comprising a  
20 kozak sequence optimised for eukaryotic expression.

19. A nucleic acid according to any one of claims 16 to 18 operably linked to a heterologous regulatory element.

25 20. A nucleic acid according to any one of claims 16 to 19 wherein said heterologous regulatory element is suitable for expression in mammalian neural cells.

21. A nucleic acid according to claim 20 wherein said heterologous  
30 regulatory element is suitable for expression in mammalian glial cells.

22. A vector comprising a nucleic acid according to any one of claims 16 to 21.

23. A vector according to claim 22 which is a viral vector.

24. A vector according to claim 23 which is a adenovirus, adeno-  
5 associated virus (AAV), for example AAV serotype 2 virus, retrovirus,  
lentivirus, recombinant adenovirus, 'gutless' adenovirus, herpes  
simplex virus, or poliovirus vector.

25. A host cell comprising a nucleic acid according to any one of  
10 claims 16 to 21 or a vector according to any one of claims 22 to 24.

26. A host cell according to claim 25 which is a mammalian neural  
cell.

15 27. A host cell according to claim 26 which is a human neural cell.

28. A host cell according to claim 27 which is a human glial cell.

29. A nucleic acid according to any one of claims 16 to 21, a vector  
20 according to any one of claims 22 to 24 or a host cell according to  
any one of claims 25 to 28 for use in a method of treatment of the  
human or animal body.

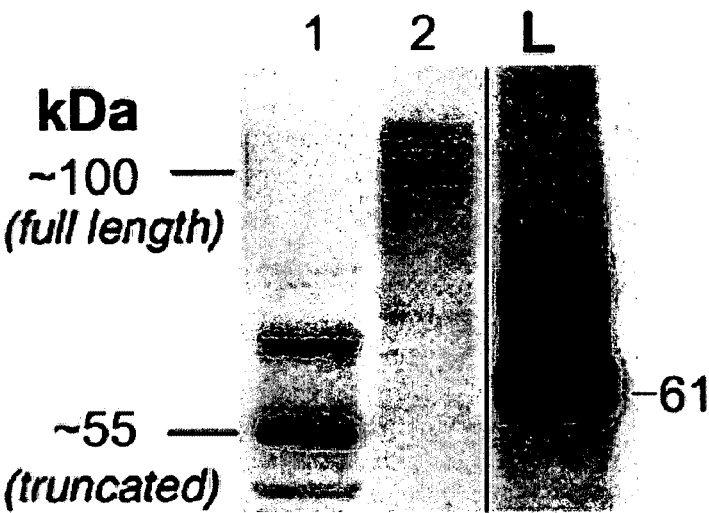
30. A nucleic acid according to any one of claims 16 to 21, a vector  
25 according to any one of claims 22 to 24 or a host cell according to  
any one of claims 25 to 28 for use in a method of treatment of neural  
damage.

31. Use of a nucleic acid according to any one of claims 16 to 21, a  
30 vector according to any one of claims 22 to 24 or a host cell  
according to any one of claims 25 to 28 in the manufacture of a  
medicament for use in the treatment of neural damage.

32. A method of treating neural damage comprising

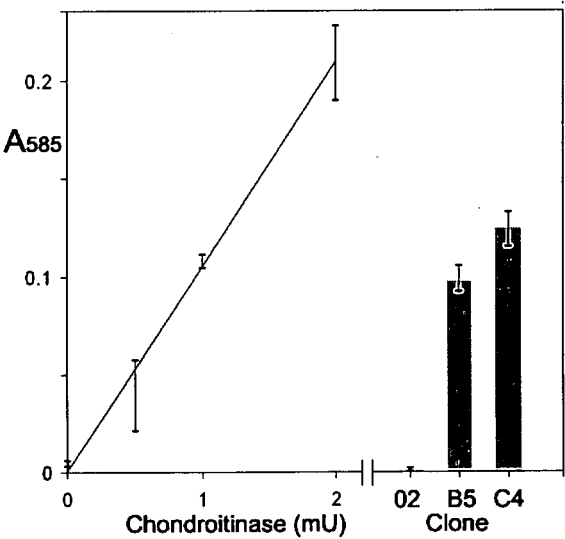
administering a nucleic acid according to any one of claims 16 to 21, a vector according to any one of claims 22 to 24 or a host cell according to any one of claims 25 to 28 to an individual in need

5 thereof.



5

Figure 1



10

Figure 2

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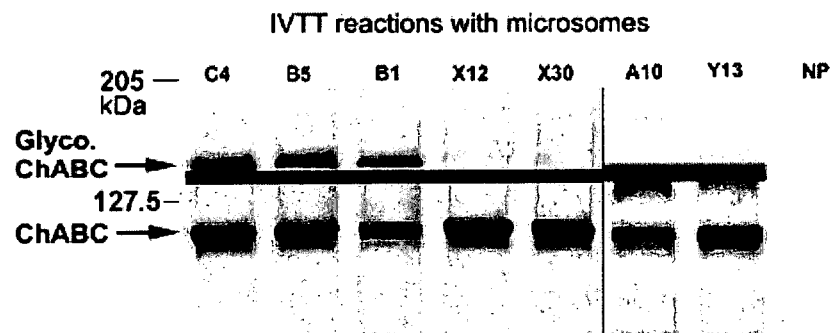


Figure 3

5

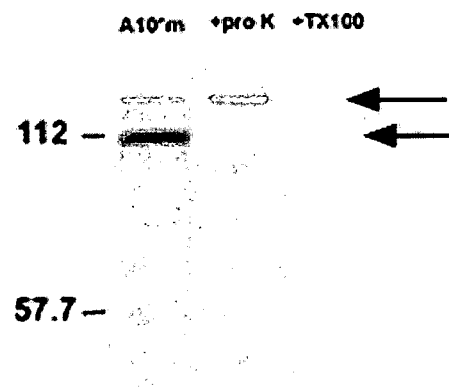
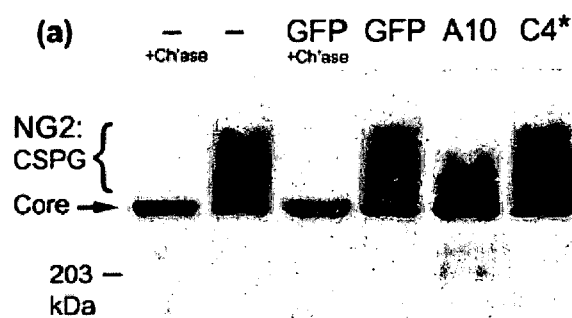


Figure 4



10

Figure 5

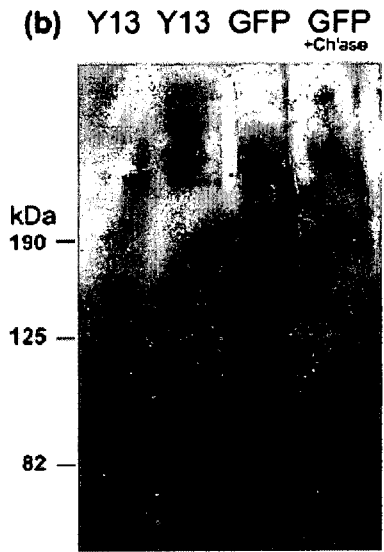


Figure 6

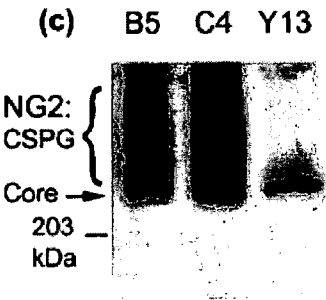


Figure 7

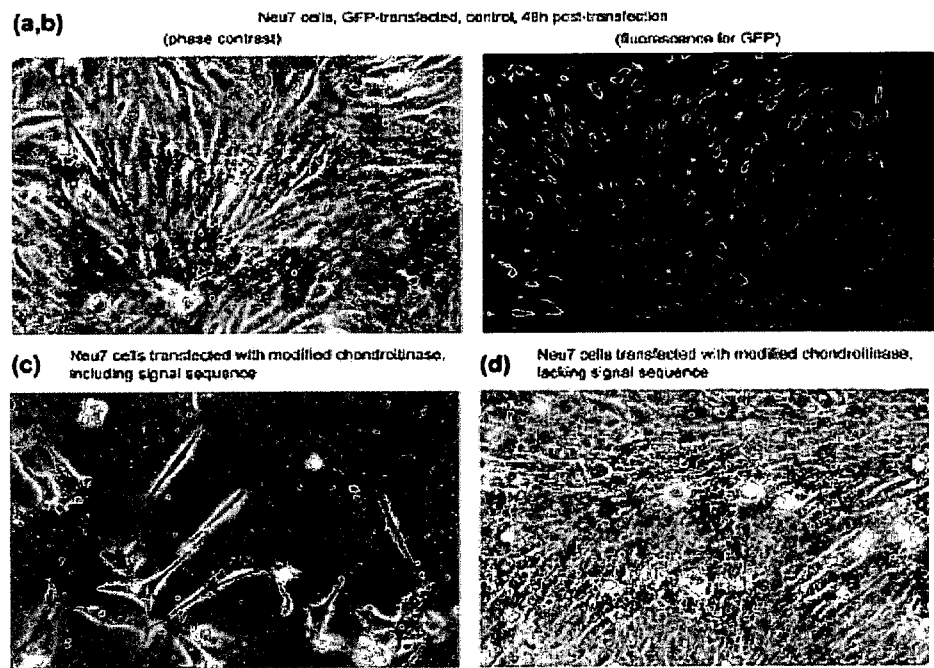


Figure 8

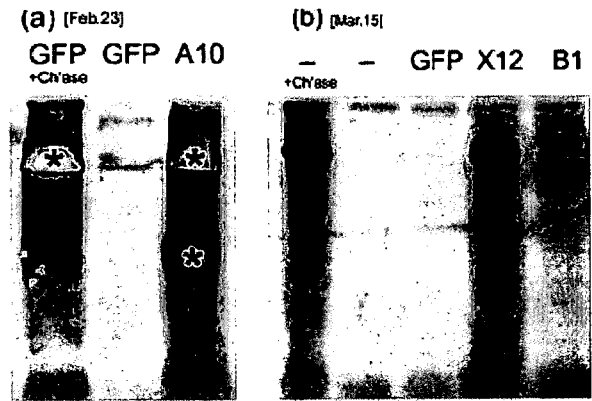


Figure 9

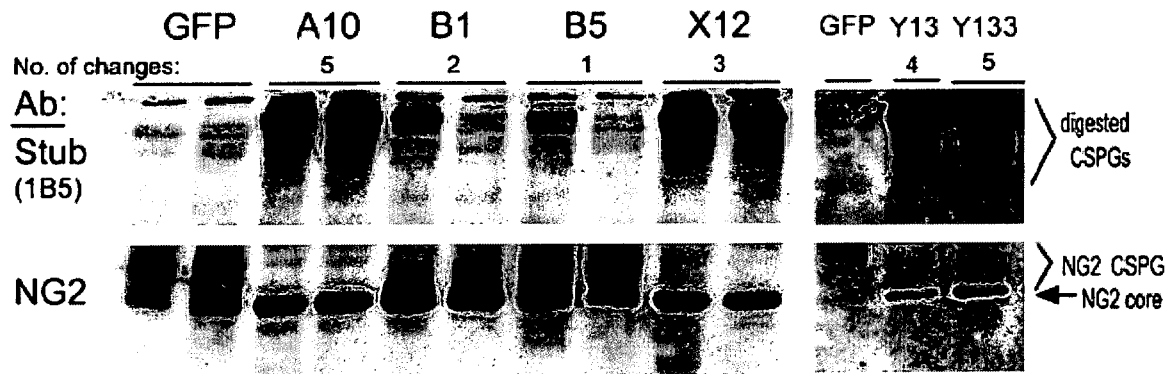


Figure 10

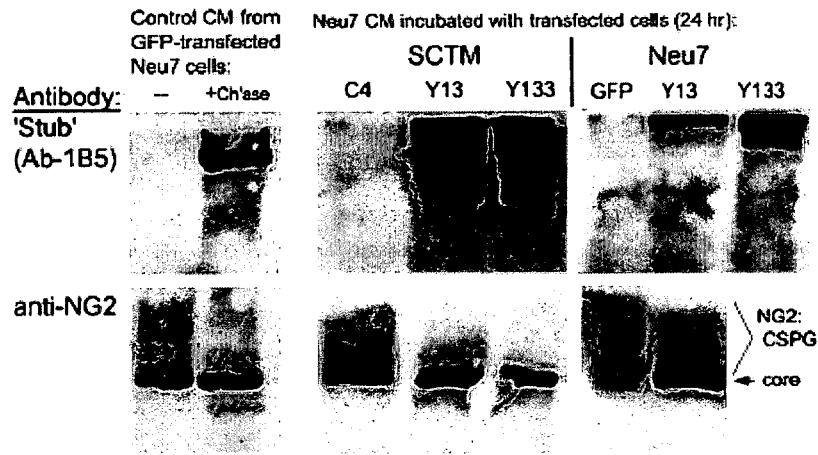


Figure 11

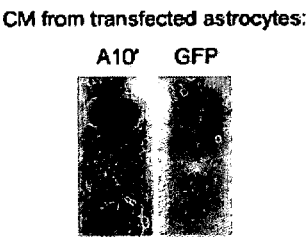
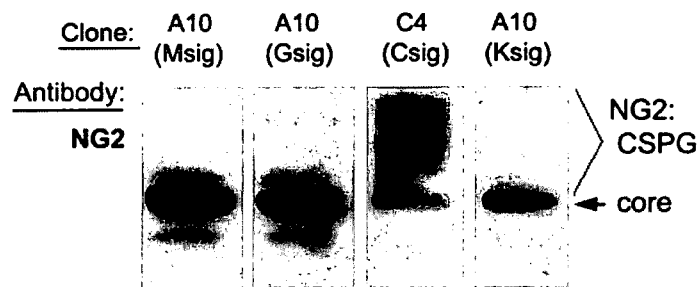


Figure 12

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Signal sequences from the following genes:  
Msig, MMP-2; Gsig, GDNF;  
Csig, bacterial chondroitinase (neg. control);  
Ksig, immunoglobulin K

Figure 13

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB2008/002143

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/88 C12N15/60 A61K38/46 A61K38/51 C12N9/14

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)

C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                   | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | WO 2005/087920 A (MASSACHUSETTS INST TECHNOLOGY [US]; PRABHAKAR VIKAS [US]; CAPILA ISHAN)<br>22 September 2005 (2005-09-22)<br>Whole document, in particular p.4, 43 |                       |
| A         | EP 0 613 949 A (MARUHA CORP [JP] MARUHA CORP [GB]; MARUHA CORP [JP])<br>7 September 1994 (1994-09-07)<br>Whole document, in particular p.3                           |                       |
| A         | US 2004/091472 A1 (POJASEK KEVIN [US] ET AL) 13 May 2004 (2004-05-13)<br>Whole document, in particular p.8-9                                                         |                       |

☒ 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 document 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.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

10 October 2008

Date of mailing of the international search report

24/10/2008

Name and mailing address of the ISA/

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Authorized officer

Roscoe, Richard

## INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2008/002143

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                  | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | US 2006/154337 A1 (SATO NOBUYUKI [JP] ET AL) 13 July 2006 (2006-07-13)<br>Whole document, in particular [0019], [0028]<br>the whole document                                                                                                                                                                        |                       |
| A         | -----<br>FETHIERE J ET AL: "Crystal structure of chondroitin AC lyase, a representative of a family of glycosaminoglycan degrading enzymes"<br>JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 288, no. 4, 14 May 1999 (1999-05-14), pages 635-647, XP004462090<br>ISSN: 0022-2836<br>the whole document             |                       |
| A         | -----<br>HUANG W ET AL: "Crystal structure of chondroitinase B from Flavobacterium heparinum and its complex with a disaccharide product at 1.7 Å resolution"<br>JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 294, no. 5, 17 December 1999 (1999-12-17), pages 1257-1269, XP004461802<br>ISSN: 0022-2836<br>----- |                       |

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB2008/002143

## 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:  
Although claims 32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
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:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

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

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB2008/002143

| Patent document<br>cited in search report |    | Publication<br>date | Patent family<br>member(s)                                        | Publication<br>date                                  |
|-------------------------------------------|----|---------------------|-------------------------------------------------------------------|------------------------------------------------------|
| WO 2005087920                             | A  | 22-09-2005          | CA 2558984 A1<br>EP 1737954 A2<br>US 2006078959 A1                | 22-09-2005<br>03-01-2007<br>13-04-2006               |
| EP 0613949                                | A  | 07-09-1994          | DE 69434206 D1<br>DE 69434206 T2<br>JP 3419811 B2<br>JP 6245774 A | 03-02-2005<br>08-12-2005<br>23-06-2003<br>06-09-1994 |
| US 2004091472                             | A1 | 13-05-2004          | US 2005227320 A1<br>US 2005233419 A1                              | 13-10-2005<br>20-10-2005                             |
| US 2006154337                             | A1 | 13-07-2006          | NONE                                                              |                                                      |