

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 December 2007 (06.12.2007)

PCT

(10) International Publication Number
WO 2007/138236 A1

(51) International Patent Classification:

A61K 38/18 (2006.01) A61P 25/28 (2006.01)
A61K 38/48 (2006.01)

(21) International Application Number:

PCT/GB2006/001904

(22) International Filing Date: 24 May 2006 (24.05.2006)

(25) Filing Language: English

(26) Publication Language: English

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SHEDDASE ENZYMES FOR NEURON GROWTH

(57) Abstract: The invention provides a method of promoting axon and/or neurite branching and/or outgrowth and/or for inhibiting cell apoptosis in a subject, the method comprising administering to a subject in need of such treatment a composition comprising a sheddase enzyme, or analogue, or derivative, or activator thereof. The invention also provides a delivery system for use in a gene therapy technique, and compositions for use in treating disease conditions characterised by damaged or impaired nerves.

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SHEDDASE ENZYMES FOR NEURON GROWTH

The present invention relates to neuron growth, and particularly to the modulation of neural growth and regeneration in the central nervous system. The invention also provides medicaments, and *in vivo* and *ex vivo* methods of using such medicaments to modulate neuron and neurite growth.

Nerve cells or neurons can be generally divided into three main functional parts, i.e. the cell body, the axon, and the dendrites. The cell body is where cellular metabolism occurs, and the dendrites are the part of the cell that receive signals and conducts these signals to the rest of the neuron. The axon is responsible for passing these signals between neighbouring neurons. The term neurites is used to define either axons or dendrites when the neuron is in cell culture, i.e. *in vitro* or *ex vivo*.

Axons and dendrites elongate profusely during development both in the central nervous system (CNS) and peripheral nervous systems (PNS) of all animal species. However, in adults, axonal and dendritic regrowth in the CNS is increasingly lost with evolutionary progression. In the PNS, after infliction of a lesion, axons of all vertebrate species are able to regrow to some extent. However, in the CNS of mammals, axon regrowth following damage is limited to axon sprouting. Regrowth of neuronal processes is, however, possible in the CNS of lower vertebrate species.

Glia are the decisive determinants for controlling axon regrowth. Mammalian glia are generally permissive for axon outgrowth in the CNS during development and in the adult PNS. Thus, upon infliction of a lesion, glia of the adult mammalian PNS can re-express their earlier axon outgrowth-promoting potential and foster regeneration. The CNS glia of some lower vertebrates remain permissive for axon regrowth in adulthood. However, in contrast, CNS glia of adult mammals do not re-express their developmental growth properties following lesions. Hence, repair and regrowth of neurons in the CNS following a lesion is at best very limited.

The extent of growth of axons (or neurites in culture) may be measured by essentially two parameters, i.e. the level of branching, and the level of outgrowth. Axon/neurite

branching relates to the number of axons/neurites that are associated with a cell body of the neuron. Hence, branching is a measure of axon density, whether an axon either grows out from the cell body itself, or forms a sub-branch off another axon. Axon/neurite outgrowth relates to the lengths of the axons/neurites associated with the cell body. It will be appreciated that if it were possible to increase the number (branching) and length (outgrowth) of axons/neurites in a patient, then it would in turn increase the likelihood of achieving a functional connection between adjacent neurons in the patient. Hence, damaged or disconnected axons could be re-connected.

Neurotrophic factors (NTF) are present during the normal development of the nervous system. During such development, neuronal target structures produce limited amounts of specific NTFs necessary for the survival, differentiation and growth of the neurons projecting into target structures. NTFs promote the survival and/or maintenance of mature neurons and are primary determinants of neuronal regeneration after CNS injury. Furthermore, peripheral nerve glia (i.e. Schwann Cells) produce neurotrophic factors (NTF) which are presumed to be responsible for axon regeneration after injury in the adult PNS. However, long term experiments demonstrate that peripheral nerve implants grafted into the CNS do not maintain CNS axon regeneration beyond 30 days post lesion (dpl) and by 100 dpl most axons degenerate, presumably because Schwann cells in the peripheral nerve implants stop producing NTFs at about 20 days post-implantation, possibly due to a lack of axonal contact.

Despite numerous efforts spanning many years, neural regeneration in the CNS has never been achieved therapeutically. For instance, Logan *et. al.* (Eur. J. Neurosci. 1994 16(3) 355-63) investigated the effects of modulating levels of Transforming Growth Factor β_1 (TGF β_1) in the injured CNS to see if neuron regeneration could be induced. TGF β_1 is a neurotrophic factor in some circumstances and is also a potent fibrogenic factor, stimulating the production of matrix molecules including CSPG (an axon growth inhibitory ligand), thereby potentially modulating the axon growth inhibitory pathway. They demonstrated that TGF β_1 participates in scar formation, which in turn restricts the growth of axonal projections in the injured CNS around the scarred region, probably by active axon growth inhibition via the inhibitory ligands like CSPG contained therein. They showed that blocking TGF β_1 activity did suppress scarring. However, there was little or no associated axon growth,

suggesting that blocking scar formation and the production of some categories of inhibitory molecules does not lead to enhanced axon regeneration. Therefore, there is a distinct need to provide regenerative therapies that can promote neural growth in the CNS. Such therapies may be used to enable damaged or diseased nerves to survive, re-grow and function again following injury or lesion.

The inventors of the present invention have previously focussed their research on the Rho-A inhibitory pathway in attempt to see if modulation of this pathway could enhance NTF-stimulated neuron survival and axon outgrowth. CNS myelin is a rich source of axon growth inhibitors, including myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), Nogo and, chondroitin sulphate proteoglycans (CSPG), which arrest axon growth by binding to the Nogo-66 receptor (NgR). The Nogo receptor associates with LINGO-1 (LRR and Ig domain-containing Nogo receptor interacting protein) and either the low affinity neurotrophin receptor (p75^{NTR}), or TROY, to initiate growth cone collapse via the Rho-A inhibitory signalling pathway and/or Ca²⁺-dependent activation of epidermal growth factor receptor (EGFR) through an unknown signalling pathway. Hence, inhibitory signals are transduced by activating downstream Rho-A, leading to sequential ROCK/LIM kinase/cofilin-mediated actin filament depolymerisation and growth cone collapse. Accordingly, NgR, p75^{NTR}, TROY, EGFR, and the RhoA inhibitory molecule collectively form key elements of the Rho-A axon growth inhibitory pathway. It will be appreciated that numerous other molecules, such as ROCK/LIM, cofilin and LINGO-1, are also involved in the pathway.

In their previous experiments, the inventors successfully demonstrated that it is possible to cause disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75^{NTR} and Rho-A. Hence, they have demonstrated the efficacy of using siRNA knockdown of p75^{NTR}, NgR and Rho-A as a potential therapeutic strategy for enhancing CNS axon regeneration *in vivo* (Ahmed, 2005, Mol.Cell Neurosci. 28, 509-523).

Following on from these experiments, the inventors decided to see if it is possible to find alternative therapeutic strategies for enhancing axon growth *in vivo* (or neurite growth *ex vivo*) based on their investigations of the Rho-A growth inhibitory pathway. In particular, the

inventors investigated the use of enzymes known as sheddases, which proteolytically cleave membrane-bound proteins to release their Extracellular Domain (ECD), as they thought that these could have an effect on neuron growth, and therefore may have a therapeutic use.

5 One example of a sheddase, which the inventors thought may have potential therapeutic activity includes the enzyme, tumor necrosis factor (TNF)- α converting enzyme (TACE). TACE is a member of the metalloprotease-disintegrin family (i.e. ADAMs) causing the release of TNF- α from cells by liberating the ECD of membrane-bound pro-TNF. TACE is known to be produced by the glia in brain tissue in minute amounts but, up until now, the
10 specific role and function of TACE in the brain has not been understood. Furthermore, it is known that following a lesion, CNS glia of adult mammals do not re-express their developmental growth properties, including production of TACE. Hence, up until now, any potential therapeutic effect of TACE has not been realised.

15 The inventors therefore investigated disinhibition of axon outgrowth using an *in vitro* model in which addition of a pre-determined inhibitory concentration of CNS myelin ligands to dorsal root ganglion cell (DRG) cultures blocked FGF2-stimulated neurite outgrowth from DRG neurons (DRGN). Upon addition of the sheddase enzyme, TACE, to these DRG cultures, the inventors were surprised to find:- (i) cleavage of the ectodomain of NgR and
20 p75^{NTR}, and (ii) blockade of Rho-A activation. Furthermore, the inventors were very surprised to find that the addition of the sheddase to the neurites resulted in the significant promotion of FGF2-stimulated neurite outgrowth and also branching, in the presence of the inhibitory CNS myelin ligands. Thus, while the inventors do not wish to be bound by any hypothesis, they believe that sheddase-induced cleavage of NgR and p75^{NTR} abrogates axon
25 growth inhibitory signalling, and results in effective disinhibition of CNS axon/neurite growth. Hence, the inventors believe that they have found the first use of a sheddase enzyme as a medicament.

Therefore, according to a first aspect of the present invention, there is provided a
30 sheddase enzyme, or analogue, or derivative, or activator thereof, for use as a medicament.

The inventors also believe that they are the first to demonstrate that the medicament of the first aspect, which cleaves the ectodomain of NgR and p75^{NTR} and TROY to thereby

block Rho-A activation of the Rho-A inhibitory pathway, may be used for promoting axon and/or neurite branching and/or outgrowth.

Hence, in a second aspect, there is provided use of a sheddase enzyme, or analogue, or derivative, or activator thereof, for the manufacture of a medicament for promoting axon and/or neurite branching and/or outgrowth.

It will be appreciated that axons are responsible for passing the nerve signals between neighbouring neurons *in vivo*, and that the term “neurites” is used to define either axons or dendrites when in cell culture (i.e. *ex vivo* or *in vitro*). It should be appreciated therefore that a significant advantage of the invention is that the number, the length, and also branches of axons or neurites increases when exposed to the medicament comprising the sheddase or analogue, derivative or activator thereof. Accordingly, as the number, length and extent of branching of axons/neurites increase upon exposure to the sheddase, so too does the likelihood of achieving a functional connection with adjacent axons/neurites either in the body or in culture. Hence, damaged or dis-connected circuits of axons or neurites may be effectively re-connected upon treatment with the medicament of the first or second aspect aspect, which is a significant surprising advantage.

By the expression “Rho-A inhibitory pathway”, we mean the axon growth inhibitory pathway, which includes the NgR, p75^{NTR}, TROY, EGFR, and RhoA inhibitory molecules, amongst others, which will be known to the skilled technician.

By the term “promoting axon and/or neurite branching”, we mean the number of branches (or ‘off-shoots’) from axons or neurites (*ex vivo* or *in vitro*) increases when in the presence of the medicament comprising a sheddase, or analogue, or derivative, or activator thereof, as compared to the number of axons or neurites when the medicament is absent. As described in the Examples section, an axon/neurite must be at least 20µm long for it to be counted as an axon/neurite (Bouquet *et al.*, J.Neurosci. 24, 7204-7213, 2004).

By the term “promoting axon and/or neurite outgrowth”, we mean the length of axons (*in vivo*), or neurites (*ex vivo* or *in vitro*) increases when in the presence of the medicament comprising a sheddase, or analogue, or derivative, or activator thereof, as compared to the

length of axons or neurites when the medicament is absent. The skilled technician will know how to measure whether axon/neurite outgrowth has increased, and an example is given in the Examples section.

5 Preferably, the medicament is used to promote axon and/or neurite branching and/or outgrowth in the Central Nervous System (CNS) and/or the Peripheral Nervous System (PNS) in a subject being treated. Preferably, the medicament is for the treatment of diseases resulting from neural injury, which may have resulted from surgery, trauma, compression, contusion, transection, neurotoxicity, or other physical injury, from vasculature
10 pharmacologic or other insults including hemorrhagic or ischemic damage or from neurodegenerative or other neurological diseases. Examples of such diseases, which may be treated include spinal cord injury (SCI), glaucoma, and neurodegenerative disorders, such as MS, ALS, Alzheimer's, Parkinson's disease, diabetic neuropathy, and spinal muscular atrophy (SMA). Furthermore, ailments, characterised by impaired or failing axon growth,
15 which may be treated by the medicament, are preferably characterised by neuronal injury. For instance, the ailment may be chronic or acute brain trauma, spinal cord injury, neurotoxicity, stroke, glaucoma, optic nerve damage, blindness, haemorrhage, facial nerve injury, caused by elective surgery, nerve compression, concussion, ischaemia, burns and the like.

20 The inventors were also surprised to establish that use of a sheddase according to the invention may be used to treat conditions characterised by an increase in cell death.

Therefore, according to a third aspect of the present invention, there is provided use
25 of a sheddase enzyme, or analogue, or derivative, or activator thereof, for the manufacture of a medicament for treating or inhibiting cell apoptosis.

By the term "apoptosis", we mean programmed cell death. Hence, the medicament comprising the sheddase enzyme, or analogue, or derivative, or activator thereof may be used
30 to treat conditions in subjects where neuron (axon or neurite) survival is a significant issue. For instance, the medicament may be used to treat neuro-degenerative diseases such as dementia, Parkinson's Disease, Huntingdon's Disease, Alzheimers Disease, Motor Neuron Disease, CJD, diabetic neuropathy, and the like.

Hence, it will be appreciated from the foregoing that the invention has many uses in both *in vitro* and *in vivo* applications. For example, the sheddase, or analogue, derivative or activator thereof, may be used to stimulate neurite outgrowth and/or branching in cell culture, following which the neuronal culture may then be transferred to a subject in need of treatment to repair damaged areas. Alternatively, or additionally, the sheddase, or analogue, derivative or activator thereof, may be used to stimulate axon outgrowth and/or branching *in vivo* in a subject being treated with the medicament of the invention to treat any of the conditions described herein.

By the term “shedase”, we mean any one of the known specialized proteases, which are capable of cleaving an extracellular domain (ECD) of a membrane-bound protein. Hence, this post-translational proteolysis step carried out by a sheddase releases a fragment of the membrane protein with a biologically active domain. Proteolysis may also control the surface expression of multiple integral membrane proteins, and may be used to down-regulate the protein at the cell surface.

Sheddases are also referred to in the art as secretases, or membrane-protein-solubilizing proteases (MPSP). Examples of secretases include α -secretases. Accordingly, the sheddase used in the medicament of the invention may comprise a secretase, and preferably an α -secretase; or a membrane protein solubilising protease.

Sheddases may be sub-classified into a number of different sub-categories, such as metalloproteases, cysteine proteases, aspartic proteases, and serine proteases (Blobel C. Nat Rev Mol Cell Biol 2005, 32-43). Hence, the sheddase used in the medicament of the invention may comprise an enzyme independently selected from a group consisting of a metalloprotease; a cysteine protease; an aspartic protease; or a serine protease, or any combination thereof.

The skilled technician will appreciate that each of these categories of sheddase will comprise many members, and each of the inventors believe that any of these would make effective examples of sheddase for use in the medicament according to the invention. For example, cysteine proteases require a cysteine residue for activity, examples of which include

caspases and cathepsins. Hence, the sheddase used in the medicament may comprise a caspase or cathepsin.

5 In addition, aspartic proteases require an aspartate residue for activity, examples of which include pepsins. Hence, the sheddase used in the medicament may comprise a pepsin.

Furthermore, serine proteases require a serine residue for activity, examples of which include kallikreins. Hence, the sheddase used in the medicament may comprise a kallikrein.

10 In a preferred embodiment, it is preferred that the sheddase used comprises a metalloprotease. Metalloproteases, which require a metal ion, include ACEs, MMPs, NEPs and ADAMs. Hence, the sheddase used in the medicament preferably comprises a metalloprotease independently selected from a group consisting of angiotensin converting enzyme (ACE), matrix metalloproteinase (MMP), NEP, and a disintegrin and
15 metalloproteinase (ADAM). The metal ion is zinc in most cases and so the enzymes are referred to as zinc metalloproteinases. Therefore, it is preferred that the sheddase used in the medicament comprises a metalloproteinase, and more preferably, a zinc metalloproteinase. The skilled technician will appreciate the various types of metalloproteases. However, an example of a zinc metalloproteinase, are those, which are characterised by a disintegrin and a
20 metalloproteinase domain, which are known in the art as ADAMs. There are at least 30 ADAMs (ADAM 1 to ADAM 30), which are typically 700-800 amino acids in length and are composed of, from the N-terminus, a pro-peptide region, a reprolysin-type Zn-metalloproteinase domain, a disintegrin domain, a cysteine-rich region, a transmembrane region and a cytoplasmic tail. The removal of the pro-peptide domain is required for
25 activation of the metalloproteinase. Hence, it is preferred that the sheddase used in the medicament comprises an ADAM enzyme.

It is preferred that the sheddase used in accordance with the invention comprises at least one enzyme selected from the group consisting of ADAM1 to ADAM 30, i.e. ADAM 1, ADAM 2, ADAM 3, and so on to ADAM 30. A preferred sheddase comprises ADAM 10. However, a more preferred ADAM, which may be used in accordance with the invention, comprises ADAM 17. ADAM 17 is also known in the art as Tumor Necrosis Factor- α Converting Enzyme (i.e. TACE), or CD156q. When used herein, the terms TACE,
30

ADAM17, and CD156q are all interchangeable, and are intended to refer to Tumor Necrosis Factor- α Converting Enzyme (Black, 2002, Int. J. Biochem & Cell Biol, 34, 1-5). TACE releases TNF- α from cells by liberating the Extracellular Domain of membrane-bound pro-TNF.

5

It will be appreciated that full characterising details of the sheddase, TACE, may be found in the publicly available database, <http://www.ncbi.nlm.nih.gov>. For example, the DNA sequence for TACE is publicly available under accession code, AC073195, and is incorporated herein by reference, and is referred to as SEQ ID No.1.

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The mRNA transcript for TACE has accession code NM_003183, and comprises substantially the following sequence:-

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1      acctgcactt ctggggggcgt cgagcctggc ggtagaatct tcccagtagg cggcgcgggga
15  61      gggaaaagag gattgagggg ctaggccggg cggatcccggt cctccccga tgtgagcagt
    121      tttccgaaac cccgtcaggc gaaggctgcc cagagaggtg gagtcggtag cggggccggg
    181      aacatgaggg agtctctcct attcctgacc agcgtggttc ctttcgtgct ggcgcgcgga
    241      cctccggatg acccgggctt cggcccccac cagagactcg agaagcttga ttctttgctc
    301      tcagactacg atattctctc tttatctaataccagcagc attcggtaag aaaaagagat
20  361      ctacagactt caacacatgt agaaacacta ctaacttttt cagctttgaa aaggcatttt
    421      aaattatacc tgacatcaag tactgaacgt ttttcacaaa atttcaagggt cgtggtggtg
    481      gatggtaaaa acgaaagcga gtacactgta aaatggcagg acttcttcac tggacacgtg
    541      gttggtgagc ctgactctag ggttctagcc cacataagag atgatgatgt tataatcaga
    601      atcaacacag atggggccga atataacata gagccacttt ggagatttgt taatgatacc
25  661      aaagacaaaa gaatgttagt ttataaatct gaagatatca agaatgtttc acgtttgcag
    721      tctccaaaag tgtgtggtta tttaaaagtg gataatgaag agttgctccc aaaagggtta
    781      gtagacagag aaccacctga agagcttggt catcgagtga aaagaagagc tgaccagat
    841      cccatgaaga acacgtgtaa attattggtg gtagcagatc atcgcttcta cagatacatg
    901      ggcagagggg aagagagtac aactacaaat tacttaatag agctaattga cagagttgat
30  961      gacatctatc ggaacacttc atgggctaata gcaggtttta aaggctatgg aatacagata
    1021     gacagatttc gcattctcaa gtctccacaa gaggtaaaaa ctggtgaaaa gcactacaac
    1081     atggcaaaaa gttaccacaa tgaagaaaag gatgcttggg atgtgaagat gttgctagag
    1141     caatttagct ttgatatagc tgaggaaagca tctaaagttt gcttggcaca ccttttcaca
    1201     taccaagatt ttgatatggg aactcttgga ttagcttatg ttggctctcc cagagcaaac
35  1261     agccatggag gtgtttgtcc aaaggcttat tatagcccag ttgggaagaa aaatatctat
    1321     ttgaatagtg gtttgacgag cacaaagaat tatggtaaaa ccatccttac aaaggaagct
    1381     gacctggtta caactcatga attgggacat aattttggag cagaacatga tccggatggt
    1441     ctagcagaat gtgccccgaa tgaggaccag ggagggaaat atgtcatgta tcccatagct
    1501     gtgagtggcg atcacgagaa caataagatg ttttcaaact gcagtaaaaca atcaatctat
40  1561     aagaccattg aaagtaaggc ccaggagtgt tttcaagaac gcagcaataa agtttgtggg
    1621     aactcgaggg tggatgaagg agaagagtgt gatcctggca tcatgtatct gaacaacgac
    1681     acctgctgca acagcgactg cacgttgaag gaagggtgtcc agtgcagtga caggaacagt
    1741     ccttgctgta aaaactgtca gtttgagact gccagaaga agtgccagga ggcgattaat
    1801     gctacttgca aaggcgtgtc ctactgcaca ggtaatagca gtgagtgcc gcctccagga
45  1861     aatgctgaag atgacactgt ttgcttggat cttggcaagt gtaaggatgg gaaatgcata
    1921     cctttctgcg agagggaaca gcagctggag tcctgtgcat gtaatgaaac tgacaactcc
    1981     tgcaagggtg gctgcaggga cctttctggc cgctgtgtgc cctatgtcga tgctgaacaa
    2041     aagaacttat ttttgaggaa aggaaagccc tgtacagtag gattttgtga catgaatggc
    2101     aaatgtgaga aacgagtaca ggatgtaatt gaacgatttt gggatttcat tgaccagctg
50  2161     agcatcaata cttttggaaa gtttttagca gacaacatcg ttgggtctgt cctgggtttc

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2221    tccttgatat tttggattcc tttcagcatt cttgtccatt gtgtggataa gaaattggat
2281    aaacagtatg aatctctgtc tctgtttcac cccagtaacg tcgaaatgct gaggcagcatg
2341    gattctgcat cggttcgcac tatcaaacc cttcctgcgc cccagactcc aggcgcgctg
2401    cagcctgccc ctgtgatccc ttcgggccca gcagctccaa aactggacca ccagagaatg
5 2461    gacaccatcc aggaagaccc cagcacagac tcacatatgg acgaggatgg gtttgagaag
2521    gaccccttcc caaatagcag cacagctgcc aagtcatttg aggatctcac ggaccatccg
2581    gtcaccagaa gtgaaaaggc tgccctcctt aaactgcagc gtcagaatcg tgttgacagc
2641    aaagaaacag agtgctaatt tagttctcag ctcttctgac ttaagtgtgc aaaatatttt
2701    tatagatttg acctacaaat caatcacagc ttgtattttg tgaagactgg gaagtgactt
10 2761    agcagatgct ggtcatgtgt ttgaacttcc tgcaggtaaa cagttcttgt gtggtttggc
2821    ccttctcctt ttgaaaaggc aagggtgaagg tgaatctagc ttattttgag gctttcaggt
2881    ttttagttttt aaaatatctt ttgacctgtg gtgcaaaagc agaaaataca gctggattgg
2941    gttatgaata tttacgtttt tgtaaattaa tcttttata tgaataacagc actgactagg
3001    gaaatgatca gttttttttt atacactgta atgaaccgct gaatatgagg catttggcat
15 3061    ttattttgtg tgacaactgg aatagttttt tttttttttt tttttttttg ccttcaacta
3121    aaaacaaagg agataaatct agtatacatt gtctctaaat tgtgggtcta tttctagtta
3181    ttaccagag tttttatgta gcagggaaaa tatatatcta aatttagaaa tcatttgggt
3241    taatatggct cttcataatt ctaagactaa tgctctctag aaacctaac acctaccta
20 3301    cagtggggc tatacatggt agccagttga atttatggaa tctaccaact gtttagggcc
3361    ctgatttgct gggcagtttt tctgtatttt ataagtatct tcatgtatcc ctgttactga
3421    tagggatata tgctcttaga aaattcacta ttggctggga gtggtggctc atgcctgtaa
3481    tcccagcact tggagaggct gaggttgccg cactacactc cagcctgggt gacagagtga
3541    gactctgcct caaaaaaaaaa aaaaaaaaaa aa

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[SEQ ID No.2]

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In addition, the protein sequence of human TACE has accession code NP_003174, and comprises substantially the following sequence:-

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30 MRQSLFLFLTSVVPFVLAPRPDDPGFGPHQRLEKLDLSLDYDILSLSNIQHRSVRKRDLQSTHVEITLLTFSA
LKRHFKLYLTSSSTERFSQNFKVVVVVDGKNESEYTVKWQDFFTGHVVGEPDSRVLAHIRDDVIRINTDGAEYN
IEPLWRFVNDTKDKRMLVYKSEDIKNVSRLQSPKVCGYLKVDNEELLPKGLVDREPPPELVHRVKRRADPDPMK
NTCKLLVVADHRFYRYMGRGEESTTTNYLIELIDRVDDIYRNTSWDNAGFKGYGIQIEQIRILKSPQEVKPGKEK
HYNMAKSYPNEEKDAWDVKMLLEQFSFDIAEEASKVCLAHLFYQDFDMGTLGLAYVGSPRANSHGGVCCKAYY
35 SPVGKKNIYLNGLTSTKNYKTLTKEADLVTTHELGHNFGEHDPDGLAECAPNEDQGGKYVMYPIAVSGDH
ENNKMFSNCSKQSIYKTIESKAQECFQERSNKCNSRVDEGEEDPGIMYLNNDTCCNSDCTLKEGVQCSNRN
SPCKKNCQFETAQKKCQEAINATCKGVSCTGNSSECPPPGNAEDDTVCLDLGKCKDGKCIFFCEREQQLESCE
CNETDNSCKVCCRDLSGRCPYPYVDAEQKNLFLRKGPCTVGFCDMNGKCEKRVQDVIERFWDFFIDQLSINTFGK
FLADNIVGSVLVFLIFWIPFSILVHCVDKLDKQYESLSLFHPSNVEMLSMDSASVRIKPPFAPQTPGRLQ
PAPVIPSAPAAPKLDHQRMDTIQEDPSTDSHMDEDFEKPDPFNSSTAASFEGLTDHPVTRSEKAASFQKLQRQ
40 NRVDSKETEC

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[SEQ ID No.3]

Examples 1 and 2 describe a test paradigm delivering the sheddase, TACE, against p75^{NTR} mRNA in cultured Dorsal Root Ganglion Cells (DRGC), which have been treated with FGF-2 in the presence of inhibitory CNS myelin extract. As shown in Figures 3B, 4A and 4B, the inventors found to their surprise that the addition of exogenous TACE on its own induced neurite outgrowth *and* branching in the DRGC neurons in the presence of inhibitory CNS myelin extract, both in the presence and also the absence of a neurotrophic factor, such as FGF-2. The inventors believe that the addition of TACE cleaved p75^{NTR}, and thereby

caused a significant increase in (a) the number of DRGCs with neurites, (b) the number of branches/neurite, and (b) the mean neurite length. The inventors therefore realized that an important feature of the present invention is that a sheddase enzyme or analogue, derivative or activator thereof, may be used in combination with a molecule, which stimulates growth of axons/neuritis, such as a Neurotrophic Factor (NTF).

The inventors have shown that such a combination has a surprising synergistic effect on axon/neurite outgrowth and branching, and neuron survival. When used separately, these agents have either modest or minimal effects, whereas in combination the agents can dramatically improve neural regeneration, and this could not have been predicted. Examples of suitable growth stimulators may include any Neurotrophic Factor (NTF).

Hence, it is especially preferred that the medicament of the first aspect or the use of the second or third aspect comprises combining the sheddase, or analogue, derivative or activator thereof, with a neurotrophic factor (NTF), for the manufacture of the medicament.

The inventors believe that use of these two components (i.e. the sheddase and an NTF) results in the manufacture of a very effective medicament for treating disease conditions characterised by damaged or impaired nerves. The NTF may be either TRK-dependent or TRK-independent. An example of a preferred NTF includes Ciliary Neurotrophic Factor (CNTF) or Fibroblast Growth Factor 2 (FGF2). It will be appreciated that CNTF is an effective growth stimulator of neurites in Retinal Ganglion Cells (RGC), and FGF2 is an effective growth stimulator of neurites in Dorsal Root Ganglia Neurons (DRGN). Other NTFs that may be used in conjunction with a sheddase include but are not limited to:- NGF, NT-3, NT-4, BDNF, GDNF, FGF-1, FGF-5, CT-1, CDF, insulin, IGF-1, IGF-2, IL-6, LIF, NPF, PDGF, PN-1, S-100, TGF- β , and VIP (Oppenheim, 1996, Neuron 17:195-197).

Furthermore, as shown in Figures 3B, 4A and 4B, even more surprisingly was the observation that not only did the TACE cause dis-inhibition of the Rho-A inhibitory pathway, it was also capable of inducing much greater neural growth (i.e. both outgrowth and branching) than would be stimulated in the absence of inhibitory myelin derived ligands. This was particularly surprising, as due to some unknown mechanism, TACE was able to stimulate growth over and above that seen in the absence of myelin extract. While the

inventors do not wish to be bound by any theory, they believe that this may be because the enzyme was disinhibiting inhibitors other than those that are myelin derived, for example, ephrins, semaphorins, and chondroitin sulphate proteoglycans (CSPG).

5 Hence, it is especially preferred that the medicament of the invention comprises a sheddase, or analogue, derivative or activator thereof, and an axon/neurite growth stimulating molecule.

10 The inventors believe that use of these two components (i.e. the sheddase and an axon/neurite growth stimulatory molecule) results in the manufacture of a surprisingly effective medicament for treating disease conditions characterised by damaged or impaired nerves, and that this effect was totally unexpected. Examples of axon/neurite growth inhibitor molecules include myelin-related molecules, chondroitin sulphate proteoglycans (CSPG), ephrins and semaphorins. In this embodiment, the use of the sheddase, not only reverses
15 inhibition of the NTF-stimulated axonal growth caused by myelin-derived neurite growth inhibitor molecules such as the myelin-related molecules indicated, but also surprisingly stimulates axon outgrowth and branching to a greater extent than seen in the absence of the myelin-derived inhibitor molecules. For instance, the effect is greater than found in control experiments in which neurites are stimulated with an NTF in the absence of myelin-derived
20 neurite growth inhibitor molecules. Again, the inventors believe that this effect was totally unexpected.

 Hence, it is especially preferred that the medicament of the invention comprises a sheddase, or analogue, derivative or activator thereof, and a neurotrophic factor (NTF). The
25 inventors believe that use of these two components (ie. the sheddase, and the NTF) results in the manufacture of a surprisingly effective medicament for treating disease conditions characterised by damaged or impaired nerves.

 By the term "activator of a sheddase enzyme", we mean a positive modulator, or an
30 agonist of a sheddase enzyme, which may be present in a subject to be treated. Suitable activators may comprise:- (i) compounds, which increase the expression, transcription, or translation and therefore concentration of sheddase; and (ii) compounds, which decrease the rate of degradation of the sheddase, or analogue or derivative thereof.

Alternatively, in another embodiment, the sheddase activator may comprise a sheddase expression system adapted to increase the expression level and, hence, concentration of the sheddase or analogue or derivative thereof in a target cell, preferably in a subject to be treated. For example, if the medicament of the invention is to be administered to the CNS of a subject, then the expression system may be administered to target cells in the CNS. It is preferred that the expression system may be administered directly to the site requiring promotion of axon/neurite outgrowth and branching. Preferably, the expression system comprises a nucleic acid sequence encoding a sheddase enzyme or analogue or derivative thereof, and preferably comprises elements capable of controlling and/or enhancing expression of the nucleic acid. The nucleic acid sequence may be a DNA sequence, which is preferably derived from the same source as the subject, which is to be treated, for example, human.

Sequences for suitable sheddase enzymes, which may be used according to the invention will be known to the skilled technician, and may be readily obtainable from publicly available databases. The DNA sequence for human TACE comprises SEQ ID NO.1, which is publicly available, and the mRNA and protein sequences for human TACE are given as SEQ ID No.2 and SEQ ID No.3, respectively, which are also publicly available. Therefore, by way of example, the sequence of the nucleic acid in the expression system may be a nucleotide sequence such as SEQ ID No.1, which may be transcribed into the mRNA of SEQ ID No.2, which may be translated into the sheddase enzyme, TACE, for example, as identified as SEQ ID No.3.

The nucleic acid may be contained within a suitable vector to form a recombinant vector. For example, the vector may be derived from a plasmid, cosmid, phage, or virus. Examples of a suitable virus include a retrovirus, herpes virus, pox virus, vaccina virus, adenovirus, lentivirus and the like). Such recombinant vectors are highly useful for transforming target cells (e.g. cells of the CNS) with the nucleic acid molecule encoding the sheddase. Recombinant vectors may also comprise other functional elements. For instance, recombinant vectors may be designed such that the vector may autonomously replicate in the target cell. In this case, elements that induce nucleic acid replication may be required in the recombinant vector.

Alternatively, the recombinant vector may be designed such that the vector and recombinant nucleic acid molecule integrates into the genome of a target cell. In this case, it is preferred that the vector comprises nucleic acid sequences, which favour targeted integration (e.g. by homologous recombination). Recombinant vectors may also comprise DNA coding for genes that may be used as selectable markers in the cloning process, which will be known to the skilled technician.

The recombinant vector may comprise a promoter or regulator for controlling expression of the gene, as required. The nucleic acid molecule may (but not necessarily) be one, which becomes incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed leading to the production of genetically modified daughter cells (in which case regulation of expression in the subject may be required e.g. with specific transcription factors or gene activators). Alternatively, the expression system may be designed to favour unstable or transient transformation of differentiated cells in the subject being treated. When this is the case, regulation of expression may be less important because expression of the DNA molecule will stop when the transformed cells die or stop expressing the protein (ideally when the required therapeutic effect has been achieved).

In another embodiment, the expression system may provide the nucleic acid molecule encoding the sheddase, or analogue or derivative thereof to the subject without it being incorporated in a vector. For instance, the nucleic acid molecule may be incorporated within a liposome or a virus particle. Alternatively, a "naked" nucleic acid molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The nucleic acid molecule may be transferred to the cells of a subject to be treated by transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the nucleic acid molecule, viral vectors (e.g. adenovirus) and means of providing direct nucleic acid uptake (e.g. endocytosis) by application of the nucleic acid molecule directly.

It is envisaged that the expression system defined herein may be used in a gene therapy technique for the treatment of diseases characterised by damaged or impaired nerves.

Hence, in a fourth aspect, there is provided a delivery system for use in a gene therapy technique, the delivery system comprising a nucleic acid molecule encoding a protein which directly or indirectly modulates activity of a sheddase, or analogue or derivative thereof, wherein said nucleic acid molecule is capable of being transcribed to allow the expression of the protein, which is effective for treating a disease condition characterised by a damaged or impaired nerves.

Advantageously, the delivery system of the fourth aspect is highly suitable for achieving sustained levels of a protein, which is therapeutically active for combating diseases as described herein over a longer period of time than is possible for most conventional therapeutic regimes. The delivery system may be used to induce continuous protein expression in cells in a target tissue to be treated, for example, the CNS or PNS, that have been transformed with the DNA molecule. Therefore, even if the protein has a very short half-life as agent *in vivo*, therapeutically effective amounts of the protein may be continuously expressed in the treated tissue.

The delivery system of the fourth aspect preferably comprises the expression system as described herein. The nucleic acid molecule may be a DNA molecule, which may be derived from human, and may encode a sheddase, or analogue or derivative thereof. For example, the nucleic acid molecule may comprise SEQ ID No.1, which may encode the sheddase shown as SEQ ID No.3.

Furthermore, advantageously, the delivery system of the invention may be used to provide the nucleic acid molecule (and thereby the protein which is the active therapeutic agent) without the need to use conventional pharmaceutical vehicles such as those required in tablets, capsules or liquids. The delivery system of the invention is such that the nucleic acid molecule is capable of being expressed (when the delivery system is administered to a patient) to produce a protein that directly or indirectly has activity for modulating activity of a sheddase. By “directly”, we mean that the product of gene expression *per se* has the required activity. Hence, the product of gene expression is itself a sheddase enzyme, or analogue, or derivative thereof. By “indirectly”, we mean that the product of gene expression

undergoes or mediates (e.g. as an enzyme) at least one further reaction to provide an agent effective for modulating the sheddase activity.

The delivery system may further comprise a nucleic acid sequence (which is preferably DNA), which encodes a neurotrophic factor (NTF). The skilled technician will appreciate that there are many types of NTF, which may be used to increase the effect of the sheddase. Examples include FGF-2 (NM_002006) and CNTF (NM_000614), the DNA and amino acid sequences of which are readily available to the skilled technician.

For example, the mRNA sequence (spliced transcript) for FGF-2 is set out as:-

```

1      cggccccaga aaacccgagc gagtaggggg cggcgcgag gagggaggag aactgggggc
61     gcgggaggct ggtgggtgtc gggggtggag atgtagaaga tgtgacgccg cggcccggcg
121    ggtgccagat tagcggagcg gctgcccgcg gttgcaacgg gatcccgggc gctgcagctt
181    gggaggcggc tctccccagg cggcgctccg ggagacaccc atccgtgaac ccaggtccc
15 241    gggccgccgg ctgcgccgcg accaggggcc ggcggacaga agagcggccg agcggctcga
301    ggctggggga ccgcggggcg ggccgcgcg tgcggggcgg gaggtgggg ggccggggcc
361    ggggcccgtgc cccggagcgg gtcggaggcc ggggcccggg ccgggggacg gcggtcccc
421    gcgcggctcc agcggctcgg ggatcccgcg cgggccccgc agggaccatg gcagccggga
481    gcatcaccac gctgcccggc ttgcccggag atggcggcag cggcgcttc ccgcccggcc
20 541    acttcaagga cccaagcgg ctgtactgca aaaacggggg cttcttctct cgcatccacc
601    ccgacggccg agttgacggg gtccgggaga agagcgaccc tcacatcaag ctacaacttc
661    aagcagaaga gagaggagtt gtgtctatca aaggagtgtg tgctaaccgt tacctggcta
721    tgaaggaaga tggaagatta ctggcttcta aatgtgttac ggatgagtgt ttcttttttg
781    aacgattgga atctaataac tacaatactt accggtcaag gaaatacacc agttggtatg
25 841    tggcactgaa acgaactggg cagtataaac ttggatccaa aacaggacct gggcagaaag
901    ctatactttt tcttccaatg tctgctaaga gctgatttta atggccacat ctaatctcat
961    ttcacatgaa agaagaagta tatttttagaa atttgttaat gagagtaaaa gaaaataaat
1021   gtgtatagct cagtttggat aattggtcaa acaatttttt atccagtagt aaaatatgta
1081   accattgtcc cagtaaagaa aaataacaaa agtttgaataa tgtatatctt cctttttata
30 1141   ttgcatctgc tgttaccagg tgaagcttac ctagagcaat gatctttttt acgcatttgc
1201   tttattcgaa aagaggcttt taaatgtgc atgttttaga acaaaatttt ttcattgaaa
1261   tcatatacat tagaaaatca cagtcagatg tttaatcaat ccaaaatgtc cactatttct
1321   tatgtcatte gttagtctac atgtttctaa acatataaat gtgaatttaa tcaattcctt
1381   tcatagtttt ataattctct ggcagttcct tatgatagag tttataaaac agtctgtgt
35 1441   aaactgctgg aagtctctcc acagtcaggc caattttgtc aaaccttct ctgtacccat
1501   acagcagcag cctagcaact ctgctggtga tgggagttgt attttcagtc ttcgccaggt
1561   cattgagatc catccactca catcttaagc attcttctct gcaaaaatth atggtgaatg
1621   aatatggctt taggcggcag atgatataca tatctgactt cccaaaagct ccaggatttg
1681   tgtgtctgtg ccgaatactc aggacggacc tgaattctga ttttatacca gtctcttcaa
40 1741   aaacttctcg aaccgctgtg tctcctacgt aaaaaagag atgtacaaat caataataat
1801   tacactttta gaaactgtat catcaaagat tttcagttaa agtagcatta tgtaaaggct
1861   caaaacatta ccctaacaaa gtaaagtttt caatacaaat tctttgcctt gtggatatca
1921   agaaatccca aaataatttt ttaccactgt aaattcaaga agcttttgaa atgctgaata
1981   tttcttttgg tgctacttgg aggccttact acctgtacat ttttgggggc agctcttttt
45 2041   aacttcttgc tgctcttttt cccaaaaggc aaaaatatag attgaaaagt taaaacattt
2101   tgcattggctg cagttccttt gtttcttgag ataagattcc aaagaactta gattcatttc
2161   ttcaacaccg aaatgctgga ggtgtttgat cagttttcaa gaaacttggg atataaataa
2221   ttttataatt caacaaaggc tttcacattt tataagggtg atttttcaat taaatgcaaa
2281   tttgtgtggc aggattttta ttgccattaa catatttttg tggctgcttt ttctacacat
50 2341   ccagatgggc cctctaactg ggctttctct aattttgtga tgttctgtca ttgtctccca
2401   aagtatttag gagaagccct ttaaaaagct gccttctct accactttgc tggaaagctt
2461   cacaattgtc acagacaaag atttttgttc caatactcgt tttgcctcta tttttcttgt

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2521	ttgtcaaata	gtaaatagata	tttgcccttg	cagtaattct	actggtgaaa	aacatgcaaa
2581	gaagaggaag	tcacagaaac	atgtctcaat	tcccatgtgc	tgtgactgta	gactgtctta
2641	ccatagactg	tcttaccat	cccctggata	tgctcttggt	ttttccctct	aatagctatg
2701	gaaagatgca	tagaaagagt	ataatgtttt	aaaacataag	gcattcatct	gccatttttc
5 2761	aattacatgc	tgacttccct	tacaattgag	atttgcccat	agggttaaaca	tggttagaaa
2821	caactgaaag	cataaaagaa	aaatctaggc	cgggtgcagt	ggctcatgcc	tatattccct
2881	gcactttggg	aggccaaagc	aggaggatcg	cttgagccca	ggagttcaag	accaacctgg
2941	tgaaaccccc	tctctacaaa	aaaacacaaa	aaatagccag	gcatggtggc	gtgtacatgt
3001	ggtctcagat	acttggggagg	ctgagggtggg	agggttgatc	acttgaggct	gagagggtcaa
10 3061	ggttgagctg	agccataatc	gtgccactgc	agtccagcct	aggcaacaga	gtgagacttt
3121	gtctcaaaaa	aagagaaatt	ttccttaata	agaaaagtaa	tttttactct	gatgtgcaat
3181	acatttggtta	ttaaatttat	tatttaagat	ggtagcacta	gtcttaaaatt	gtataaaata
3241	tcccctaaca	tgtttaaatg	tccattttta	ttcattatgc	tttgaaaaat	aattatgggg
3301	aaatacatgt	ttgttattaa	atltattatt	aaagatagta	gcactagtct	taaatattgat
15 3361	ataacatctc	ctaacttggt	taaatgtcca	tttttattct	ttatgcttga	aaataaatta
3421	tggggatcct	atltagctct	tagtaccact	aatcaaaagt	tcggcatgta	gctcatgatc
3481	tatgctgttt	ctatgtcgtg	gaagcaccgg	atgggggtag	tgagcaaatc	tgccctgctc
3541	agcagtcacc	atagcagctg	actgaaaatc	agcactgcct	gagtagtttt	gatcagttta
3601	acttgaatca	ctaactgact	gaaaattgaa	tgggcaaata	agtgcctttg	tctccagagt
20 3661	atgcgggaga	cccttccacc	tcaagatgga	tatttcttcc	ccaaggattt	caagatgaat
3721	tgaaattttt	aatcaagata	gtgtgcttta	ttctgttgta	ttttttatta	ttttaatata
3781	ctgtaagcca	aactgaaata	acatttgctg	ttttataggt	ttgaagaaca	taggaaaaac
3841	taagagggtt	tgtttttatt	tttgctgatg	aagagatatg	tttaaataatg	ttgtattggt
3901	ttgttttagtt	acaggacaat	aatgaaatgg	agtttatatt	tgttattttct	atltttgttat
25 3961	atlttaataat	agaattagat	tgaaataaaa	tataatggga	aataatctgc	agaatgtggg
4021	tttctctggtg	tttctctctga	ctctagtgc	ctgatgatct	ctgataaggc	tcagctgctt
4081	tatagttctc	tggctaattgc	agcagatact	cttctctgcca	gtggtaatac	gattttttta
4141	gaaggcagtt	tgtcaatttt	aatccttggtg	atacctttat	actcttaggg	tattattttta
4201	tacaaaagcc	ttgaggattg	cattctatlt	tctatatgac	cctcttgata	tttaaaaaac
30 4261	actatggata	acaattcttc	atltacctag	tattatgaaa	gaatgaagga	gttcaaacaa
4321	atgtgtttcc	cagtttaacta	gggtttactg	tttgagccaa	tataaatggt	taactgtttg
4381	tgatggcagt	atltcctaaag	tacattgc	gttttcttaa	atacagagtt	aaataatttt
4441	cagtaattct	tagatgatc	agcttcac	ttaaagaat	cttttgtttt	atgttgagtt
4501	agaaatgcct	tcataatagac	atagcttttc	agacctctac	tgctcagtttt	catttctagc
35 4561	tgttttcagg	gttttatgaa	ttttcaggca	aagctttaat	ttataactaag	cttaggaagt
4621	atggctaattg	ccaacggcag	tttttttctt	cttaattcca	catgactgag	gcataatga
4681	tctctgggta	gggtgagttgt	tgtgacaacc	acaagcactt	tttttttttt	taaagaaaaa
4741	aaggtagtga	atlttttaatc	atctggactt	taagaaggat	tctggagtat	acttaggcct
4801	gaaattatat	atatttggct	tggaaatgtg	tttttcttca	attacatcta	caagtaagta
40 4861	cagctgaaat	tcagaggacc	cataagagtt	cacatgaaaa	aaatcaattc	atlttgaaaag
4921	gcaagatgca	ggagagagga	agccttgcaa	acctgcagac	tgcttttttg	ccaatataga
4981	ttgggtaagg	ctgcaaaaaca	taagcttaat	tagctcacat	gctctgctct	cacgtggcac
5041	cagtggatag	tgtgagagaa	ttaggctgta	gaacaaatgg	ccttctcttt	cagcattcac
5101	accactacaa	aatcatcttt	tatatcaaca	gaagaataag	cataaactaa	gcaaaaggtc
45 5161	aataagtacc	tgaaaccaag	attggctaga	gatatatctt	aatgcaatcc	atlttctgat
5221	ggattgttac	gagttggcta	tataatgtat	gtatgggtatt	ttgatttggtg	taaaagtttt
5281	aaaaatcaag	ctttaagtac	atggacattt	ttaaataaaa	tatttaagaa	caatttagaa
5341	aattgcctta	atatcattgt	tggctaataa	gaatagggga	catgcataat	aaggaaaagg
5401	tcattggagaa	ataatatttg	tatcaaaca	atacattgat	ttgtcatgat	acacattgaa
50 5461	tttgatccaa	tagtttaagg	aataggtagg	aaaatttggt	ttctattttt	cgatttctctg
5521	taaatcagtg	acataaataa	ttcttagctt	atltttatatt	tccttgtctt	aaatactgag
5581	ctcagtaagt	tgtgttaggg	gattattttct	cagttgagac	tttcttatat	gacattttac
5641	tatgttttga	cttctgact	attaaaaata	aatagtagaa	acaattttca	taaagtgaag
5701	aattatataa	tcactgcttt	ataactgact	ttattatatt	tatttcaaag	ttcattttaa
55 5761	ggctaactat	catctctgt	gatggaatgg	tcagggaattt	gttttctcat	agtttaattc
5821	caacaacaat	attagtcgta	tccaaaataa	cctttaatgc	taaactttac	tgatgtatat
5881	ccaaagcttc	tccttttcag	acagattaat	ccagaagcag	tcataaacag	aagaatagggt
5941	ggtatgttcc	taatgatatt	atlttctacta	atggaataaa	ctgtaataat	agaaattatg
6001	ctgctaatta	tatcagctct	gaggtaattt	ctgaaatggt	cagactcagt	cggaacaaat
60 6061	tggaaaattt	aaatttttat	tcttagctat	aaagcaagaa	agtaaacaca	ttaatttcct
6121	caacattttt	aagccaatta	aaaatataaa	agatacacac	caatatcttc	ttcaggctct

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6181   gacaggcctc ctggaaactt ccacatattt ttcaactgca gtataaagtc agaaaataaa
6241   gttaacataa ctttcaactaa cacacacata tgtagatttc acaaaatcca cctataattg
6301   gtcaaagtgg ttgagaatat attttttagt aattgcatgc aaaatttttc tagcttccat
6361   ccttttctcc tctgttcttc ttttttggg ggagctggta actgatgaaa tcttttccca
5   6421   ccttttctct tcaggaaata taagtggttt tgtttgggta acgtgatata ttctgtatga
6481   atgaaacatt ggaggggaaac atctactgaa tttctgtaat ttaaaatatt ttgctgctag
6541   ttaactatga acagatagaa gaatcttaca gatgctgcta taaataagta gaaaatataa
6601   atttcatcac taaaatatgc tattttaaaa tctatttcct atattgtatt tctaatacaga
6661   tgtattactc ttattatttc tattgtatgt gttaatgatt ttatgtaaaa atgtaattgc
10  6721   ttttcatgag tagtatgaat aaaattgatt agtttgtgtt ttcttgtctc ccgaaaaaaa
6781   aaaaaaaaaa aaaaaaaaaa aaa

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[SEQ ID No.4]

For example, the mRNA sequence (spliced transcript) for CNTF is set out as:-

```

15  1      gagagtcaca tctcttattt ggaccagtat agacagaagt aaaccagct gacttgtttc
61      ctgggacagt tgagttaagg gatggctttc acagagcatt caccgctgac cctcaccgt
121     cgggacctct gtagccgctc tatctggcta gcaaggaga ttcgttcaga cctgactgct
181     cttacggaat cctatgtgaa gcatcagggc ctgaacaaga acatcaacct ggactctgcg
20  241     gatgggatgc cagtggcaag cactgatcag tggagtgagc tgaccgaggc agagcgactc
301     caagagaacc ttcaagctta tcgtaccttc catgttttgt tggccaggct cttagaagac
361     cagcaggtgc attttaccct aaccgaaggt gacttccatc aagctataca tacccttctt
421     ctccaagtcg ctgcctttgc ataccagata gaggagttaa tgatactcct ggaatacaag
481     atcccccgca atgaggctga tgggatgcct attaatgttg gagatggtgg tctctttgag
25  541     aagaagctgt ggggcctaaa ggtgctgcag gagctttcac agtggacagt aaggctccatc
601     catgaccttc gtttcatttc ttctcatcag actgggatcc cagcacgtgg gagccattat
661     attgctaaca acaagaaaat gtagcagtta gtcccttctc tcttccctgc tttctcttct
721     aatggaatat gcgtagttcc ctggggcctc gctttcccat cttaaatttc taaaaacagt
781     taagacaaca ggcattttct ttcttttttc tctgaccacc tgcagcctgt tgaaggacta
30  841     caggtatttt catcaagtag cggtggagac atacacaaat gggcatacaa gtttagcctg
901     gggggtgtga tttgtgtgcg tgcttgcagtg tgctgcaggt gtaagagagt gggagcaggg
961     acaacgtcct tccacttcag ggttctaacc tttctaacc actaagtaac ctctacaggc
1021    atttaactgc cttacagaca gaatatacat atgttaattc tagtctgga tgactcggtc
1081    tgagaagatt caatttaaaa tcagactctt tagttgattt aaactcttag agaataagaa
35  1141    taataatggc taacttttat tatcttctat attaaggcag tatgccaagg gtctttatgt
1201    atattatgta cagcgtttac aaccttgtga gcaagggtgt gttactccca ttaggtagat
1261    gagaaaacag gctcacagag atttggttaa gctcacacag ctaacaagta gcacactgag
1321    tttgaacaca gatcattctc cttgtaaaag cctatgtgcc tttcacttta gaggcttgat
1381    catgaatcac tgcacctctt tgtcacaggg tgttggaga tgcattcatg taatctattc
40  1441    ccacgctggt aaaacagctg ctgttagatg tcctcagaag tcagttgcaa attttagcgt
1501    taaagtcagg atttattgtt cactactggc ggtgaggagg gcagctggag atcttaagat
1561    tccatttttg aaaatgatta ggcccgccta acttctgaac tttggaagct ggggatgttt
1621    agtaatacag cctggttttc aagtactcac taaaagttct caaatattgg gttgggcacg
1681    gcttatacca ggttacctca cttttaatta gtgatgcagg cagtgttaac caagcatttg
45  1741    tggacaatga gtggaatact aaagttaaaa agtcaaactt tcacctcaga ttttctggac
1801    ttagtcatga ggagaggggt aggcccaact tgttccctact ggagatacca gagactctga
1861    aactatagaa taaagcctct gtgctgcaca a

```

[SEQ ID No.5]

50

It is envisaged that the gene therapy technique comprises administering the delivery system to the subject being treated such that the nucleic acid molecule (and where applicable the further nucleic acid sequence(s)) is expressed in the subject. Expression of the protein(s)

causes direct or indirect modulation of the activity of the sheddase enzyme, or analogue or derivative thereof, thereby promoting axon and/or neurite outgrowth and branching in the subject, thereby treating the disease characterised by damaged or impaired nerves.

5 Derivatives or analogues of the sheddase enzyme used in the medicament according to the invention may include derivatives or analogues that increase or decrease the enzyme's half-life *in vivo* or *ex vivo*. It is preferred that the derivative or analogue exhibits enhanced resistance to hydrolysis, by for example, peptidases. It will also be appreciated that the invention extends to the amino acid sequence of a sheddase enzyme, or a nucleic acid
10 encoding a sheddase enzyme, or a derivative, or an analogue thereof, which comprises substantially the amino acid or nucleic acid sequences of any of the sequences referred to herein, including functional variants or functional fragments thereof.

Hence, by the terms "analogue of a sheddase enzyme" or "derivative of a sheddase enzyme", or "substantially the amino acid/polynucleotide/polypeptide sequence", "functional
15 variant" and "functional fragment", we mean that the sequence has at least 30% sequence identity with the amino acid/polynucleotide/polypeptide sequences of any one of the sequences referred to herein. For example, a derivative or analogue of sheddase may comprise 30% identity with the sheddase gene identified as SEQ ID No.1, or 30% identity with the sheddase polypeptide identified as SEQ ID No.3. Amino
20 acid/polynucleotide/polypeptide sequences with a sequence identity which is greater than 65%, more preferably, greater than 70%, even more preferably, greater than 75%, and still more preferably, greater than 80% sequence identity to any of the sequences referred to is also envisaged. Preferably, the amino acid/polynucleotide/polypeptide sequence has 85% identity with any of the sequences referred to, more preferably 90% identity, even more
25 preferably 92% identity, even more preferably 95% identity, even more preferably 97% identity, even more preferably 98% identity and, most preferably, 99% identity with any of the referred to sequences.

The skilled technician will appreciate how to calculate the percentage identity
30 between two amino acid/polynucleotide/polypeptide sequences, for example, as described in http://wikiomics.org/wiki/Percentage_identity. In order to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences, an alignment of the two sequences must first be prepared, followed by calculation of the sequence identity value.

The percentage identity for two sequences may take different values depending on:-

- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs
5 global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of
10 sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

Hence, it will be appreciated that the accurate alignment of protein or DNA sequences
15 is a complex process. The popular multiple alignment program ClustalW (Thompson et al., 1994, Nucleic Acids Research, 22, 4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW may be as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and
20 Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

Preferably, calculation of percentage identities between two amino
25 acid/polynucleotide/polypeptide sequences is then calculated from such an alignment as $(N/T)*100$, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating percentage identity between two sequences defined herein comprises (i) preparing a sequence alignment using the ClustalW
30 program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:- Sequence Identity = $(N/T)*100$.

Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to the sequences shown in SEQ ID No.1 or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to
5 filter-bound DNA or RNA in 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x SSC/0.1% SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequences shown in SEQ ID No.3.

Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence
10 could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence,
15 which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids
20 include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids may be replaced with an amino acid having similar biophysical properties, and the skilled technician will know the nucleotide sequences encoding these amino acids.

It will be appreciated that use of the sheddase enzyme or analogue, derivative or activator thereof according to the present invention may be used in a monotherapy (e.g. use of a sheddases *per se*, or analogue or derivative or activator thereof (e.g. an expression or delivery system according to the invention to promote axon and/or neurite outgrowth and/or branching). However, it is preferred that the sheddase is used as an adjunct, or in
30 combination with, any other therapies, which may be used to promote axon/neurite branching/outgrowth (for example, the addition of an NTF). It is therefore particularly

preferred that combination therapy comprises a sheddase, or analogue or derivative thereof, or an expression system therefor, and a Neurotrophic Factor (e.g FGF2 or CNTF etc).

The sheddase or analogue, derivative or activator thereof, may be combined in a composition having a number of different forms depending, in particular on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, transdermal patch, liposome or any other suitable form that may be administered to a person or animal suffering from a disease state characterised by impaired neuron (axon) growth. It will be appreciated that the vehicle of the composition of the invention should be one which is well tolerated by the subject to whom it is given, and preferably enables delivery of the molecule to the target site requiring axon growth and/or improved neuron survival.

Compositions comprising a sheddase or analogue, derivative or activator thereof may be used in a number of ways. For instance, systemic administration may be required in which case the compound may be contained within a composition that may, for example, be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion), subcutaneous (bolus or infusion), intraventricular or subarachnoidal. Preferably, the injection is intraneural. Alternatively, the composition may be administered by inhalation (e.g. intranasally or via the mouth).

The sheddase or analogue, derivative or activator thereof may also be incorporated within a slow or delayed release device. Such devices may, for example, be inserted at the site of a CNS or PNS lesion, and the sheddase may be released over weeks or months. Such devices may be particularly advantageous when long-term treatment with a sheddase according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

It will be appreciated that the amount of sheddase or analogue, derivative or activator thereof that is required is determined by the type of sheddase itself, its biological activity and its bioavailability, which in turn depends on the mode of administration, the physicochemical properties of the sheddase employed, and whether it is being used as a monotherapy or in a combined therapy, for example; with an NTF. The frequency of administration will also be

influenced by the above-mentioned factors and particularly the half-life of the sheddase within the subject being treated.

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular sheddase or analogue, derivative or activator thereof in use, the strength of the preparation, the mode of administration, and the advancement or severity of the disease condition, and the urgency of the requirement for axon growth. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to establish specific formulations of the medicament for use according to the invention and precise therapeutic regimes (such as daily doses of the sheddase and the frequency of administration).

Generally, a daily dose of between 0.01 µg/kg of body weight and 0.5 g/kg of body weight of a sheddase according to the invention may be used for the stimulation of axon growth (i.e. promoting axon outgrowth/branching), depending upon which specific sheddase enzyme is used. More preferably, the daily dose is between 0.01 mg/kg of body weight and 200 mg/kg of body weight, and more preferably, between approximately 0.1mg/kg and 100 mg/kg, and even more preferably, between about 1mg/kg and 10mg/kg sheddase.

When the sheddase or analogue, derivative or activator thereof is delivered to a cell (and *de novo* synthesis is not required in the target cell), daily doses may be given as a single administration (e.g. a single daily injection). Typically, a therapeutically effective dosage should provide about 1ng to 100µg/kg of the sheddase per single dose, and preferably, 2ng to 50ng per dose.

Alternatively, the sheddase may require administration twice or more times during a day. As an example, sheddases according to the invention may be administered as two (or more depending upon the severity of the condition) daily doses of between 0.1mg/kg and 10mg/kg (i.e. assuming a body weight of 70kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at

3 or 4 hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses to a patient without the need to administer repeated doses.

According to a fifth aspect, there is provided a composition comprising a therapeutically effective amount of a sheddase or analogue, derivative or activator thereof, and a pharmaceutically acceptable excipient.

In one embodiment, the composition according to the fifth aspect of the invention may comprise about 0.01 μ g and 0.5 g of the sheddase or analogue, derivative or activator thereof. More preferably, the composition comprises between about 0.01 mg and 200 mg, and more preferably, between approximately 0.1mg and 100 mg, and even more preferably, between about 1mg and 10mg of the sheddase or analogue, derivative or activator thereof. Most preferably, the composition comprises between approximately 2mg and 5mg of the sheddase. It will also be appreciated that the invention is not limited to using just one type of sheddase enzyme, and it is envisaged that more than one sheddase may be used in the medicament according to the invention, and the composition of the fifth aspect.

Preferably, the composition comprises approximately 0.1% (w/w) to 90% (w/w) of the sheddase, and more preferably, 1% (w/w) to 10% (w/w) of the sheddase or analogue, derivative or activator thereof. The rest of the composition may comprise the excipient.

This invention further provides in a sixth aspect, a process for making a pharmaceutical composition comprising combining a therapeutically effective amount of a sheddase or analogue, derivative or activator thereof, and a pharmaceutically acceptable excipient.

A "therapeutically effective amount" is any amount of a sheddase enzyme or analogue, derivative or activator thereof which, when administered to a subject promotes axon or neurite outgrowth or branching.

30

A "subject" may be a vertebrate, mammal, domestic animal or human being.

A "pharmaceutically acceptable excipient" as referred to herein is any physiological carrier vehicle known to those of ordinary skill in the art useful in formulating pharmaceutical compositions.

5 In a preferred embodiment, the excipient comprises liquid and the pharmaceutical composition is in the form of a solution. In another embodiment, the pharmaceutical vehicle is a gel and the composition is in the form of a cream or the like. Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by for example, intramuscular, intraneural, intrathecal, epidural, intraperitoneal, intravenous and particularly
10 subcutaneous, intracerebral or intracerebroventricular injection. The sheddase may be a sterile composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Vehicles are intended to include necessary and inert binders, suspending agents, lubricants, flavourants, sweeteners, preservatives, dyes, and coatings.

15 The combination of a sheddase or analogue, derivative or activator thereof, and a neurotrophic factor (NTF) represents an important feature of the present invention. The inventors have therefore found that the simultaneous combination of these two active ingredients is particularly beneficial for promoting axon/neurite outgrowth/branching
20 (inducing neuron growth and promoting cell survival) in culture and in injured tissues in a subject.

Therefore, it is preferred that the composition of the fifth aspect comprises a therapeutically effective amount of an NTF. Examples of suitable NTF include FGF-2 and
25 CNTF.

It will be appreciated that the composition of the fifth aspect may be used as a medicament. Hence, according to a seventh aspect, there is provided the composition according to the fifth aspect, for use as a medicament.

30

The medicament may be used to treat individuals suffering from various diseases characterised by damaged or inappropriate nerve growth, such as CNS or PNS injury.

Hence, according to an eighth aspect, there is provided a method of promoting axon outgrowth and/or branching and/or for inhibiting cell apoptosis in a subject, the method comprising administering to a subject in need of such treatment a composition comprising a sheddase enzyme, or analogue, or derivative, or activator thereof.

5

Preferably, the method according to the eighth aspect comprises administering to the subject in need of treatment the composition according to the fifth aspect.

It is preferred that the composition used in the method according to the eighth aspect further comprises a neurotrophic factor (NTF) in addition to the sheddase, or analogue, derivative or activator thereof. It is most preferred that the composition used in the method of the eighth aspect comprises (i) a sheddase, or analogue, or derivative, or activator thereof; and (ii) a neurotrophic factor (NTF).

It will be appreciated that the invention has many uses in both *in vitro* and *in vivo* applications. For example, the sheddase, or analogue, derivative or activator thereof, may be used to stimulate axon outgrowth and/or branching *in vivo* in a subject being treated. Alternatively, or additionally, the sheddase, or analogue, derivative or activator thereof, may be used to stimulate neurite outgrowth and/or branching in cell culture, following which the neuronal culture may then be transferred to a subject in need of treatment to repair damaged areas.

Hence, in a ninth aspect there is provided a method of promoting neurite outgrowth and/or branching in culture, the method comprising administering a composition comprising a sheddase, or analogue or derivative or activator thereof, to a culture of neurons.

Preferably, the composition used in the method of the ninth aspect comprises a neurotrophic factor. Examples of suitable neurotrophic growth factors (NTF) may include CNTF, FGF2, NGF, NT-3, NT-4, BDNF, GDNF, FGF-1, FGF-5, CT-1, CDF, insulin, IGF-1, IGF-2, IL-6, LIF, NPF, PDGF, PN-1, S-100, TGF- β , and VIP and the like (Oppenheim, 1996, Neuron 17:195-197). The amount of NTF may be between approximately 0.01 $\mu\text{g/kg}$ of body weight and 0.5 g/kg of body weight, more preferably, about 0.1mg/kg and 10mg/kg.

Neuron cultures prepared using the method of the ninth aspect may be used either for *in vitro* experiments, or may be introduced into a subject into a target cell to repair damaged or impaired nerves.

5 All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

10 For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

Figure 1 shows TACE-induced RIP of p75^{NTR} and suppression of Rho activation in the presence of CNS myelin ligands. (A) Mean number of DRGN and glia with and without treatment with the mitotic inhibitor 5-fluoro-2'-deoxyuridine (5-FDU). (B) Representative Western blot/Rho immunoprecipitation in cell lysates and media of p75^{NTR}, p75^{CTF}, p75^{ICD}, p75^{ECD}, Rho-GTP, total Rho and β -actin in DRG cultures after FGF2, TACE, PMA and TIMP3 treatments singly, and in combination. Control = DRG cultures without NTF and CNS myelin. (C) Mean integrated optical density of protein bands from lysates and media in A (mean integrated density) and % reduction in Rho-GTP compared to control for treatments of DRG cultures with TACE, TACE + FGF2, PMA + FGF2 and TACE + FGF2 + TIMP3. The figure demonstrates that p75^{NTR} fragmentation into p75^{ECD} and p75^{ICD} suppressed Rho activation after the addition of TACE. TIMP3 blocked fragmentation of p75^{NTR} by TACE but had no affect on Rho activation. β -actin was used as a loading control. *** $P < 0.0001$;

Figure 2 shows TACE-induced fragmentation of NgR. (A) and (B) Addition of TACE induces fragmentation of NgR and fragmented NgR is present in the media. (C) and (D) TROY did not appear to be fragmented by TACE. β -actin was used as a loading control. *** $P < 0.0001$;

Figure 3 shows TACE-induced RIP of p75^{NTR} enhanced DRGN neurite outgrowth in the presence of CNS myelin ligands. (A) Representative β III-tubulin immunocytochemistry in

control DRG cultures, and after DRGN exposure to FGF2, FGF2 + CNS myelin, TACE + CNS myelin, and FGF2 + TACE + CNS myelin. (B) Quantification of mean longest neurite lengths to demonstrate significantly enhanced FGF2-stimulated DRGN neurite outgrowth after addition of TACE + FGF + CNS myelin. Once again, addition of TIMP3 blocked TACE-mediated DRGN neurite outgrowth. *** $P < 0.0001$;

Figure 4 shows TACE-induced RIP of p75^{NTR} enhanced neuritic branching in DRGN in the presence of CNS myelin ligands. (A) The number of primary DRGN neurites and (B) branches, after 3 days in culture in controls, and after the exposure to FGF2, FGF2 + myelin, TACE + CNS myelin, and FGF2 + TACE + CNS myelin, increased significantly. Treatment with TIMP3 restored the inhibitory potential of CNS myelin ligands and blocked DRGN neurite outgrowth. (C-E) Representative β III-tubulin immunocytochemistry of DRGN in 3 day-old cultures to demonstrate branching after exposure to FGF2 with no myelin, TACE + FGF2 + CNS myelin, and PMA + FGF2 + CNS myelin. DRGN have >5 primary neurites with >10 branches. After exposure to TACE + FGF2 in the presence of CNS myelin. *** $P < 0.0001$;

Figure 5 shows TACE-induced RIP of p75^{NTR} correlated with enhanced MAP1B levels in the presence of CNS myelin. Representative Western blots and subsequent densitometry of MAP1B levels in lysates of cells from control cultures and cultures exposed to FGF2 and TACE alone, and to combinations of TACE + FGF2, PMA + FGF2, and TACE + FGF2 + TIMP3. Exposure to TACE + FGF2 in the presence of CNS myelin enhanced MAP1B levels significantly compared to other treatments, while addition of TIMP3 restored MAP1B levels to those observed in controls. β -actin was used as a loading control. *** $P < 0.0001$;

Figure 6 shows upregulation of TACE activity and presenilin-1 (PS1) levels in retinae and ON in the RM. *A*, Reprobed representative Western blot (n=3) of the same retinal samples shown in Figure 2 shows inactive (*) and active (arrow) TACE, PS1 and β -actin taken from intact 0d controls, and from RM and NRM at 6, 8 and 20dpi. *B*, Representative double immunolocalisation (n=3) of TACE (green) with β III-tubulin (red) in the ganglion cell layer of the retinae of intact 0d controls, and of retinae from RM and NRM at 20dpi (scale bar=10 μ m). *C*, Western blot of inactive (*) and active (arrow) TACE, PS1 and β -actin in ON

samples from intact 0d controls, and from RM and NRM at 6, 8, and 20dpi, taken from retinal samples;

Figure 7 shows NTF activated TACE, while addition of TACE to RGC cultures fragments p75^{NTR} and blocks Rho-A activation. *A*, Representative Western blot and corresponding densitometry (n= 3) of inactive (*) and active (arrow) TACE in control cultures grown in the presence of myelin extract without NTF and from cultures grown in the presence of the TACE activator, PMA, NTF, and NTF plus the TACE inhibitor, TIMP3 (***= $P < 0.0001$). β -actin acted as a loading control in the same Western blot. *B*, Representative Western blot/immunoprecipitation of Rho-GTP and corresponding densitometry (n=3) of p75^{NTR}, p75_{ICD} from cell lysates and p75_{ECD} from supernatant of dissociated retinal cultures grown with CNS myelin extracts in the presence of NTF, NTF plus TIMP3, active TACE, NTF plus active TACE or NTF plus PMA. β -actin acted as a loading control;

Figure 8 shows addition of TACE promotes RGC neurite outgrowth in the presence of inhibitory CNS myelin extract. *A*, Representative immunocytochemistry for β III-tubulin in retinal cultures to demonstrate RGC neurite outgrowth in the presence and absence of NTF, TACE, CNS myelin extract and the TACE activator, PMA. *B*, Quantification of the number of RGC with neurites after treatment with NTF no CNS myelin extract, NTF plus CNS myelin extract, TACE no CNS myelin extract, TACE plus CNS myelin extract, NTF plus TACE plus CNS myelin extract, NTF plus TACE plus TIMP3 plus CNS myelin extract, NTF plus γ -secretase inhibitor, and NTF plus TACE plus γ -secretase inhibitor. *** $P < 0.0001$. *C*, Quantification of mean neurite length after various treatments with/without NTF in the presence/absence of CNS myelin extracts.

Figure 9 shows a proposed mechanism of NTF-stimulated disinhibition of RGC axon growth by TACE-mediated p75^{NTR} shedding. Intravitreally-delivered NTF stimulate RGC to release TACE at the somata and at the growth cone, which cleaves p75_{ECD} from p75^{NTR} while γ -secretase cleaves p75_{ICD} by RIP. Accordingly, full length p75^{NTR} is not available at the growth cone to associate with NgR so that there is no transduction of inhibitory signalling after ligand/NgR binding, and downstream Rho-A activation is suppressed, favouring actin polymerisation in the growth cone. Growth cone integrity and mobility are preserved and,

hence, unimpeded regeneration through the putative inhibitory environment of the distal ON stump is promoted.

Examples

5 The inventors conducted a series of experiments (summarised in Examples 1 and 2 below) to investigate the efficacy of exogenously added sheddases on the growth of neurons. As a model, they used the sheddase, TACE.

Example 1

Materials and Methods

Adult DRG cultures

15 Dissociated adult rat (6-8 week-old) DRGN were cultured in 4-well plates at a density of 1500/well, as described elsewhere (Ahmed, 2005, Mol.Cell Neurosci. 28, 509-523.), on glass coverslips pre-coated with 100 µg/ml poly-D-lysine followed by 20 µg/ml Laminin-I (both from Sigma, Dorset, UK) in Neurobasal-A containing B27 supplement (Invitrogen, Paisley, UK). To limit glial proliferation, the mixed DRG cultures were treated with 30 µM 5-fluoro-2-deoxyuridine (Sigma) for 3 d (Russell, 2002, FASEB J. 16, 1738-1748). DRGN
20 were cultured either in the presence, or absence of adult rat CNS myelin extracts for a further 3 d at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of CNS myelin

25 CNS myelin was prepared according to our earlier published method (Ahmed *supra*, 2005). Briefly, adult Sprague-Dawley rat brains were homogenised in 0.32M sucrose, 1mM EDTA, pH 7.0 at 4°C and centrifuged. The supernatant was resuspended in 0.9M sucrose, overlaid with 1-2 ml of 0.32 M sucrose, and centrifuged at 20,000g for 60 min. The CNS myelin at the interface of the two sucrose layers was collected, dispersed in 20 volumes of 0.32 M sucrose, and centrifuged at 13,000g for 25 min. CNS myelin extract was then diluted
30 in 25 volumes of water and centrifuged at 20,000g for 25 min. The final white pellet was resuspended in a small volume of water, freeze dried overnight and the protein content determined using the Pierce BCA assay (BioRad, Hercules, CA, USA). Western blotting of the CNS myelin extracts confirmed the presence of Nogo-A, MAG, OMgp and chondroitin

sulphate proteoglycan (CSPG) (Ahmed *supra*, 2005). The myelin extract was added to DRG cultures at a protein concentration of 200 µg/ml, previously determined to be optimally inhibitory to DRGN neurite outgrowth.

5 Treatment of DRGN with FGF2 and TACE

FGF2 (Peprotech, London, UK) was added at 10 ng/ml (pre-determined to cause optimal DRGN neurite outgrowth) and active TACE (R & D Systems, Abingdon, UK) was added at 10 ng/ml (pre-determined to cause optimal p75^{NTR} peptide cleavage) to DRGN culture plates in triplicates. DRGN were also treated with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) to upregulate TACE production (Weskamp et al., J.Biol.Chem. 279, 4241-4249, 2004; Kanning et al., J.Neurosci. 23, 5425-5436, 2003), or TIMP3 (50 nM, Chemicon, Hampshire, UK), a specific inhibitor of TACE activity (Black, Nat.Genet. 36, 934-935, 2004; Karan et al., Int.J.Oncol. 23, 1365-1371, 2003; Lee et al., Biochem.J. 364, 227-234, 2002). Cells were treated for 72 hr before harvesting for Western blotting and subsequent quantitative assessment of proteins by densitometry as described below.

Antibodies

Monoclonal β-III tubulin antibody (Sigma) was used at 1:100 to label DRGN neurites by immunocytochemistry (ICC). Polyclonal anti-p75^{NTR} antibody was used to identify and localise p75^{NTR}, p75^{CTF}, p75^{ICD} and p75^{ECD} (Promega, 1:500 dilution for both Western blots and ICC). Microtubule associated protein-1B (MAP1B) (Abcam Ltd, Cambridge, UK) was used at 1:500 dilution for Western blots. Anti-mouse NgR (Autogen Bioclear, Wiltshire, UK) was used at 1:500 dilution for Western blots. A second NgR antibody, goat anti-human NgR (Santa Cruz Biotechnology, San Diego, USA) was used at 1:500 dilution to confirm the results obtained with anti-mouse NgR. A polyclonal anti-human TROY antibody was used to detect TROY in Western blots (1:500, Santa Cruz Biotechnology).

Immunocytochemistry

DRG cultures were fixed in 4% paraformaldehyde for 10 min (TAAB Laboratories, Berkshire, UK) before washing ×3 in phosphate buffered saline (PBS), blocked in PBS containing 0.5% bovine serum albumin (BSA, Sigma) and 1% Triton X-100 (Sigma), and incubated with the relevant primary antibody diluted 1:100 in PBS containing 0.5% BSA and

0.5% Tween-20 (Sigma) for 1 hr at room temperature in a humidified chamber. Cells were then washed X3 in PBS and incubated with either AlexaFluor 488 (Green), or Texas Red (Red) (both from Invitrogen), diluted 1:100 in PBS-T-BSA for 1 hr at room temperature. Following further washes in PBS, coverslips were mounted in FluorSave (Calbiochem, San Diego, USA) and viewed under a fluorescent microscope (Carl Zeiss, Welwyn-Garden City, UK).

Measurement of DRGN neurite outgrowth (neurite length)

Photomicrographs of β III-tubulin⁺ immunostained DRGN neurites were captured using Axiovision Software (Carl Zeiss, Hertfordshire, UK) from 30 randomly selected DRGN/coverslip and neurite lengths measured using Axiovision (Carl Zeiss) and represented as means $\pm$ SD.

DRGN branching analysis (neurite number/density)

The numbers of primary β III tubulin⁺ DRGN neurites emerging directly from the DRGN somata were counted and from each neuritic tree, the total number of branches exceeding 20 μ m in length (Bouquet et al., J.Neurosci. 24, 7204-7213, 2004) recorded using Axiovision software (Carl Zeiss).

Counting of DRGN and glia

Each coverslip (n=3, 3 independent experiments) was partitioned into 9 equal quadrants and, in each, random photomicrographs of merged images from β III-tubulin⁺ DRGN and DAPI stained nuclei were photographed in Axiovision, as described above, and the relative proportions of β III-tubulin⁺ DRGN and glia (absent of β III-tubulin⁺ staining) determined.

Protein extraction and Western blotting

To determine fragmentation of NgR and p75^{NTR} into p75^{ECD}, p75^{CTF} and p75^{ICD} after addition of TACE, DRG cultures were washed X2 with PBS and incubated for 15 min at 37°C with 0.25 % trypsin/EDTA (Invitrogen), followed by trituration and centrifugation at 1300 rpm for 5 min. The cell pellets were re-suspended in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1 % NP-40 (Sigma) and PI cocktail (Sigma) and incubated on ice for 30 min, centrifuged at 13,000 rpm at 4°C

and cell lysates normalised for protein concentration using a colorimetric protein assay (Bio-Rad). To determine the levels of shed NgR and p75^{ECD}, cell culture media were collected and concentrated using microconcentrators (Millipore, Bedford, MA, USA). Each sample (40 µg total protein) was incubated with ×2 Laemmli loading buffer at 90 °C for 4 min and
5 separated on a 12% SDS-polyacrylamide gel to probe for NgR and p75^{NTR} (Invitrogen) and 6% SDS gels for MAP1B. Proteins were transferred to PVDF membranes (Millipore UK, Gloucestershire, UK), blocked for 1 hr at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat milk. Membranes were blotted overnight with the relevant
10 antibody. For detection of protein bands, an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) and HRP-conjugated secondary antibody (1:1,000; Amersham) were used. Each blot was stripped and re-probed with additional relevant antibodies thereafter. Each experiment was performed 6 times.

Rho activation assay

15 GTP-bound Rho was assayed from DRG cell culture lysates using a commercially available Rho activation assay kit, following the manufacturers instructions (Upstate Biotechnology, Milton Keynes, UK).

Densitometry

20 Western blots were scanned into Adobe Photoshop, keeping all scanning parameters the same for each blot. Captured images were used to quantify the integrated optical density of each band using the built-in gel plotting macros in ScionImage software (Scion Corporation/NIH Image, Maryland, CA, USA).

Statistical analysis

25 Sample means were calculated and differences analysed for significance using GraphPad Prism (GraphPad Software Inc., Version 4.0, San Diego, USA) by one-way analysis of variance (ANOVA) followed by *post-hoc* testing with Dunnett's method to identify statistically significant groups.

Results

1.1) TACE-induced RIP of p75^{NTR} in DRG cultures

Since glia produce minute amounts of TACE in the brain, the inventors first determined the relative proportions of DRGN and glia (satellite and Schwann cells) in FGF2-stimulated cultures. The number of β III-tubulin⁺ DRGN was determined as 1,500 at 0 d and their number remained constant over the 6 d culture period (Fig. 1A). The initial number of glia (~7,500) increased significantly to 20,000 at 6 d. Treatment of FGF2-stimulated cultures with the mitotic inhibitor, 5-fluoro-2'-deoxyuridine (5-FDU), halted glia proliferation to a level equivalent to that at 0 d. When cells from both TACE- and FGF2-treated cultures were lysed, only 7% more p75^{NTR} fragments were observed in the presence/absence of mitotic inhibitor (not shown) (Fig. 1A). Hence, in all subsequent experiments, the mitotic inhibitor was added to normalise the contribution of glia-derived TACE.

Addition of FGF2 to DRG cultures in the presence of inhibitory CNS myelin extracts induced limited fragmentation of p75^{NTR} compared to untreated controls (Fig. 1B and C), while addition of either active TACE alone, or active TACE plus FGF2, cleaved most of the p75^{NTR} present, eliminating full length p75^{NTR} protein (Fig. 1B and C). PMA, which activates TACE, also cleaved p75^{NTR} in FGF2-stimulated cultures, but fragmentation was less than that observed with the addition of active TACE. After p75^{NTR} fragmentation, p75^{ECD} accumulated in the culture medium and p75^{CTF} and p75^{ICD}, which appeared in the cell lysates (Fig. 1B and C). TACE-induced RIP of p75^{NTR} coincided with a significant reduction in the levels of Rho-GTP within DRG cultures in the presence of CNS myelin, while total Rho levels did not change (Fig. 1B and C). The further addition of TIMP3 blocked TACE-mediated fragmentation of p75^{NTR} in the presence of FGF2, and restored the levels of Rho-GTP to those seen in FGF-2 treated cultures with CNS myelin extracts (Fig. 1B and C). Similar levels of fragmentation were observed in the absence of CNS myelin. However, Rho-GTP was not detected, confirming that there was no activation of Rho (Fig. 1B and C).

1.2) TACE induced fragmentation of NgR but not of TROY

Addition of TACE to DRG cultures either in the presence or absence, of CNS myelin, also caused fragmentation of NgR, yielding a shed 48kDa band in the cell culture medium (Fig. 2). However, when the blots were reprobbed, no fragmentation of TROY was detected (Fig. 2).

1.3) TACE-induced RIP of p75^{NTR} promoted DRGN neurite outgrowth in the presence of inhibitory CNS myelin ligands

The effect of TACE on the length of neurites was investigated.

In the absence of CNS myelin, FGF2 promoted significant DRGN neurite outgrowth while addition of either FGF2 plus PMA, or FGF2 plus TACE plus TIMP3 did not affect neurite outgrowth. The addition of either TACE alone, or PMA alone did not promote neurite outgrowth (Fig. 3A and B).

After the addition of a pre-determined concentration of CNS myelin protein extract, neurite outgrowth was completely blocked in the presence of FGF2 (Fig. 3A and B). With the addition of CNS myelin ligands to cultures, TACE significantly disinhibited FGF2-stimulated DRGN neurite outgrowth compared to that seen: (1), without CNS myelin; (2), with TACE alone; and (3), with PMA ($P < 0.0001$, Fig. 3A and B). Addition of TIMP3 blocked TACE activity in FGF-2-stimulated DRG cultures and restored the inhibition of DRGN neurite outgrowth mediated by CNS myelin (Fig. 3B).

1.4) Addition of TACE enhanced DRGN branching

The effect of TACE on the number/density of neurites was investigated.

TACE added to FGF2-stimulated DRGN, cultured in the presence of CNS myelin, increased the number of DRGN primary neurites and their branching frequency (Fig. 4A and B). After treatment with FGF2 without CNS myelin, the majority of DRGN had 3-4 primary neurites, each with 1-4 branches (Fig. 4A, B and C). After the addition of TACE and FGF2 in the presence of CNS myelin, the majority of DRGN grew >5 primary neurites, each with >10 branches (Fig. 4A, B and D). Treatment of DRGN with PMA plus FGF2 similarly increased the numbers of primary neurites and their branches, compared to DRGN treated with PMA alone (Fig. 4A, B and E). TIMP3 restored the inhibitory potential of CNS myelin extracts in the presence of TACE and blocked neurite outgrowth and branching in FGF2-stimulated cultured DRGN (Fig. 4A and B).

1.5) TACE-mediated branching of DRGN was correlated with upregulation of MAP1B

The effect of TACE on MAP1B was then investigated.

5 Microtubule associated protein-1B (MAP1B), a member of the MAP family is expressed in regenerating axons and their growth cones, and promotes branching of DRGN neurites. When lysates of DRG cultures were probed for MAP1B, there was a significant upregulation of MAP1B protein after treatment with TACE plus FGF2 in both the presence and absence of CNS myelin (Fig. 5). MAP1B was also upregulated in DRGN treated with
10 PMA plus FGF2 in the presence of CNS myelin compared to PMA alone, but MAP1B levels were significantly less than those observed with TACE plus FGF2 ($P < 0.001$ TACE + FGF2 vs PMA + FGF2, Fig. 5). In TIMP3 treated cultures, the levels of MAP1B were similar to those in control DRGN (Fig. 5).

15 Discussion

In this study, the inventors have shown that addition of TACE to DRG cultures induced fragmentation of both NgR and p75^{NTR}, leading to blocked Rho-A activation and disinhibition of FGF2-stimulated DRGN neurite outgrowth *and* branching in the presence of inhibitory CNS myelin extracts. NgR and p75^{NTR} fragmentation was blocked by the addition
20 of TIMP3, a specific inhibitor of TACE activation, re-instating inhibition of DRGN neurites in the presence of CNS myelin. The results suggest that TACE-induced RIP of NgR and p75^{NTR} suppresses Rho activation, paralyses the signalling cascade mediating growth cone collapse and disinhibits NTF-stimulated neurite outgrowth and branching in the presence of inhibitory myelin ligands. The inventors therefore suggest that TACE-mediated receptor
25 shedding may be used a therapeutic strategy to block the function of receptor in the axon growth inhibitory cascade.

Example 230 Materials and Methods

Animals and treatment groups

All animal procedures were licensed by the UK Home Office. Adult, female 200-250g Fischer rats were anaesthetised with Hypnorm/Hypnovel anaesthetic (Janssen Pharmaceuticals, Oxford, UK). The experimental groups comprised a regenerating ON model (RM) in which the ON was crushed (ONC) intraorbitally and a freshly teased sciatic nerve (PN, sciatic nerve) segment immediately transplanted intravitreally and held in place with Sterispon gelatine sponge (Sterispon, Allen and Hanbury, London, UK), and a non-regenerating ON model (NRM) in which the ON was crushed (ONC) without intravitreal PN implantation. Five additional groups separately controlled for: (1) base line parameters, using untreated intact rats, i.e. 0d intact controls; (2), effects of PN alone, by intravitreal PN implantation without ONC; (3), intravitreal inflammation and effects of scleral/vitreous injury without ONC, by intravitreal Sterispon implantation alone; (4), intravitreal inflammation and effects of scleral/vitreous injury with ONC, by Sterispon transplantation and ONC; and (5), presence of non-proteinaceous PN-derived growth factors, by intravitreal implantation of freeze/thawed PN to denature all proteins and kill all Schwann and other PN cells. The ON was exposed through a supraorbital approach and crushed using forceps as described previously (Berry et al., J Neurocytol 25: 147-170, 1996; Berry et al., J Neurocytol 28: 721-741, 1999). ON and retinae were processed for RNase protection assay, Western blotting, and immunohistochemistry at 6, 8, and 20 days post injury (dpi), as described below.

Preparation of CNS myelin extract

CNS myelin was prepared according to (Cuzner et al., J Neurochem 12: 469-481, 1965). Briefly, adult rat brains were homogenised in 0.32 M sucrose, 1 mM EDTA, pH 7.0 at 4°C, centrifuged at 800 g for 10 min and the supernatant collected. The cell pellet was diluted to the original volume in 0.32 M sucrose, 1 mM EDTA, pH 7.0 and centrifuged, supernatant collected and the combined supernatants centrifuged at 13,000 g for 20 min. After removal of the supernatant, the pellet was resuspended in 0.9 M sucrose, distributed equally amongst a set of tubes, carefully overlaid with 1-2 ml of 0.32 M sucrose and centrifuged at 20,000 g for 60 min. The white material at the interface of the two sucrose layers was collected in the minimum volume possible, dispersed in 20 volumes of 0.32 M sucrose, and centrifuged at 13,000 g for 25 min. The white pellet containing the myelin, was collected, diluted in 25 volumes of pure water, left on ice for 30 min before centrifuging at 20,000 g for 25 min. The final myelin pellet was resuspended in a small volume of water and freeze dried overnight.

Protein content of the CNS myelin was determined using the Pierce BCA assay (BioRad, Hercules, CA, USA) by dissolving a small aliquot of myelin with 10% SDS which was diluted to <1% SDS for the assay. We have shown previously that the myelin extract contains various inhibitory molecules, including Nogo-A, OMgp, MAG and CSPG (Ahmed *supra*, 2005).

Ribonuclease protection assay

A 457-bp fragment (coding region) of the rat p75^{NTR} cDNA (from Dr M. Knipper, Tuebingen Hearing Research Centre, Tuebingen, Germany) cloned into the *Pst* I/*Sac* II site of pBluescript KS⁺ was used to detect p75^{NTR} mRNA. Cyclophilin (peptidyl-propyl *cis-trans*-isomerase) cDNA, a housekeeping gene, was used as a reference DNA (from Dr J. Douglass, Oregon Health Sciences University, Portland, Oregon, USA), utilising a 295-bp fragment (coding region) of the rat cyclophilin cDNA cloned into the *Pst* I/*Bam*H I sites of pSP65.

Total RNA was prepared from groups of three animals per time-point using a commercial reagent RNA-B (Biogenesis, Southampton, UK) and RNase protection assay was performed according to the method described previously (Smith et al., 2001). Autoradiographs were digitally scanned and intensities of bands corresponding to p75^{NTR} were quantified using NIH Image software (NIH, USA). For control animals, the mean intensity was given a value of 1.0 and the relative fold-change in lesioned animals was then calculated. mRNA levels were internally standardized by quantifying cyclophilin mRNA levels. Data were examined statistically by one-way analysis of variance (ANOVA) with results judged as significant at $P < 0.05$.

Tissue preparation and immunohistochemistry

Groups of at least 3 rats were killed by anaesthetic overdose and perfusion fixed with 4% paraformaldehyde and their retinae and ON excised, cryoprotected, immersed in OCT (Miles Inc, CA, USA), and frozen in liquid nitrogen. Longitudinal and parasagittal cryostat sections, 10 μ m thick, were cut through ON and retinae (Bright Instrument Co. Ltd., Cambridgeshire, UK) at 4°C, collected onto Vectabond coated slides (Vector Laboratories, Cambridgeshire, UK), air dried and processed for immunohistochemistry as previously described (Lorber et al., 2002). Cultured retinal cells on glass coverslips were fixed in 4% paraformaldehyde and also processed as previously described (Lorber et al., 2002).

In situ hybridisation (ISH)

Parasagittal cryostat sections, 10 µm thick, were cut through retinae and collected onto charged slides (Vector Laboratories), air dried and processed for ISH using a TSA Biotin System (NEN Life Sciences Inc., Boston, MA) as previously described (Lagord et al., 2002). Oligonucleotide probes used for p75^{NTR} (Radeke et al., 1987; Suzuki et al., 1998) and TACE (Black et al., 1997) were designed from published sequences.

Protein extraction and Western blotting

At 0, 6, 8 and 20dpi, 3 rats in each treatment group (3 independent experiments) were killed and both retinae and ON dissected, proteins extracted and processed for Western blotting as previously described (Winton et al., 2002). To determine levels of p75^{NTR} in siRNA treated RGC cultures by Western blotting, 375 x 10³ cells/siRNA treatment (n=6) were lysed and blotted as described previously (Winton et al., 2002).

Affinity precipitation of Rho-GTP

GTP-bound Rho was assayed from tissues and cell lysates using a Rho activation assay kit (Upstate Biotechnology, Milton Keynes, UK) following the manufacturer's instructions, and as described earlier (Dubreuil et al., 2003).

In situ Rho-GTP pull-down assay

In situ localisation of Rho-GTP was determined according to a modified earlier published method (Dubreuil et al., 2003). Briefly, 10 µm thick post-fixed sections of retinae were incubated with either GST-RBD, or GST alone (both from Upstate Biotechnology) overnight at 4°C. Sections were then washed X3 in PBS and blocked in 3% bovine serum albumin for 1 hr at room temperature and incubated with an anti-GST antibody (New England Biolabs, Hertfordshire, UK) and βIII-tubulin antibody (Sigma), overnight at 4°C. Sections were then washed in PBS, incubated with FITC or Texas red secondary antibodies (Molecular Probes), mounted in Fluorsave (Calbiochem) and viewed under an epi-fluorescent microscope (Zeiss, Hertfordshire, UK).

Adult retinal cultures

Adult rats (6-8 wk-old) were killed by cervical dislocation and retinae removed by dissection and dissociated using a papain system according to the manufacturer's protocol (Worthington Biochem, New Jersey, USA). Dissociated retinal cells, 125×10^3 , containing RGC were cultured on glass coverslips precoated with 100 $\mu\text{g/ml}$ poly-D-lysine (Sigma, Dorset, UK) and 20 $\mu\text{g/ml}$ merosin (Chemicon, Harrow, UK) in 4-well tissue culture plates (Nunc, UK) in supplemented Neurobasal-A (Invitrogen) medium for 4 d at 37°C in a humidified 5% CO₂ atmosphere.

Treatment of retinal cultures with NTF

Experimental adult retinal cultures were grown in triplicates on glass coverslips, as described above, and subjected to treatment with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) to upregulate TACE production (Weskamp et al., 2004; Kanning et al., 2003), or a combined NTF cocktail consisting of: CNTF (10 ng/ml); NT-3 (50 ng/ml); FGF2 (10 ng/ml); and BDNF (50 ng/ml) (all purchased from Peprotech, London, UK) to stimulate TACE production. Retinal cultures were also treated with either recombinant TACE enzyme (10 ng/ml) (R&D Systems, UK), or active human TIMP3 enzyme (5 $\mu\text{g/ml}$, Chemicon) to inhibit TACE production, or γ -secretase inhibitor (30 μM) (S2188, Sigma) to block p75_{CTF} fragmentation and the production of p75_{ICD}. Cells were treated for 48 hr before harvesting for Western blotting and subsequent quantitative assessment of proteins by densitometry as described below. Cultures were prepared as above and comprised RGC which were either: (1), untreated; (2), treated with combined NTF; (3), treated with TACE and combined NTF; or (4), treated with TACE, combined NTF and TACE and γ -secretase inhibitors either in the presence, or absence of CNS myelin.

Antibodies

Monoclonal β -III tubulin (1:100) was from Sigma, Poole, UK. Polyclonal anti-p75^{NTR} (1:500 for Western blots and immunohisto/cyto-chemistry) which recognises intact p75^{NTR}, p75_{ECD} and p25_{ICD} was from Promega, Southampton, UK. Goat anti-human NgR (1:100 for Western blots) and TROY (1:500 for Western blots) (both from Santa Cruz, C.A., USA) were used to detect NgR and TROY levels in Western blots. Rabbit polyclonal TACE (ADAM-17) from Neomarkers, CA, USA, was used at 1:500 dilution to detect TACE and in Western blots and retinal sections. Chicken anti-presenilin-1 (PS1, Cambridge Bioscience, Cambridge, USA) was used at 1:200 in Western blots.

Densitometry

Western blots were scanned digitally in Adobe Photoshop keeping scanning parameters the same for all blots to provide a densitometric value for bands of interest. TIFF files were then analysed in ScionImage (version 4.0.2, Scion Corp, Maryland, USA) using the built-in gel plotting macros. The integrated density of each band in each lane was calculated for 3 separate blots from 3 independent experiments.

Quantification of neurite outgrowth and RGC survival

The mean number of RGC with neurites and mean neurite length were quantified by splitting each coverslip into 9 quadrants and randomly capturing images of β III-tubulin immunostained RGC from each quadrant using a Zeiss Axioplan epi-fluorescent microscope. Axiovision image analysis software (Zeiss, Hertfordshire, UK) was then used to measure neurite length and count the number of RGC in each coverslip using the built in macros (n = 9 coverslips/ condition), as previously described (Lorber et al., 2002).

Statistical analysis

Sample means were calculated and analysed for significance using GraphPad Prism (GraphPad Software Inc., Version 4.0, San Diego, USA) by one-way analysis of variance (ANOVA) followed by *post-hoc* testing with Dunnett's method.

Results

2.1) p75^{ECD}, p75^{CTF} and p75^{ICD} products were produced by TACE-induced RIP of p75^{NTR}.

Referring to Figure 6, at all dpi, there was an upregulation and activation of both pro- and active-TACE levels in retinae of RM but not NRM. In addition, the levels of presenilin-1 (PS1), which a major protein of the multi-component γ -secretase enzyme was significantly higher in the RM compared to NRM retinae at all time points (Fig. 6A). At 20dpi, TACE was predominantly co-localised to β III-tubulin⁺ RGC, with higher levels of immunoreactivity in RM than NRM RGC (Fig. 6B). Whilst levels of TACE rose post-injury in both RM and NRM on, in RM ON, TACE was predominantly in the active-, rather than the pro-form, while in NRM ON, most TACE was in the pro-form (Fig. 6C). PS1 levels also increased with

time post-injury and were significantly higher in RM compared to NRM ON (Fig. 6C). TACE was similarly localised within RM and NRM ON and, in RM ON, TACE and GAP-43 were seen co-localised to occasional axons (not shown). There was a small increase in TACE and GAP-43 in RGC of controls with intravitreal PN implantation (no ONC), accompanied by a modest proportional enhancement of p75^{NTR} fragmentation, but there were no changes in levels of downstream Rho-GTP (not shown). All other controls showed no modulation of any of these proteins, confirming that inflammatory/macrophage cell-derived factors did not contribute to receptor shedding in RM (not shown).

2.2) TACE was upregulated by NTF stimulation of retinal cultures, while exogenous addition of TACE induced RIP of p75^{NTR} and disinhibited NTF-stimulated RGC neurite outgrowth in the presence of CNS myelin

Levels of both active and pro-forms of TACE were significantly increased after addition of a combination of pre-optimised concentrations of CNTF, BDNF, NT-3 and FGF2 to adult rat retinal cultures. NTF increased TACE activation by 43% over that observed with PMA (phorbol 12-myristate 13-acetate, a known activator of TACE) treatment ($P < 0.0001$). Addition of TIMP3, a specific inhibitor of TACE activation, abolished active TACE from NTF-stimulated retinal cultures (Fig. 7A). Densitometry of Western blots confirmed the presence of significantly higher amounts of active TACE in response to NTF when compared with those produced in the presence of PMA (Fig. 7A).

The inventors then tested whether the addition of exogenous TACE to adult rat RGC in culture disinhibited RGC neurite outgrowth by initiating RIP of p75^{NTR} in the presence of a pre-determined inhibitory concentration of CNS myelin extract. The extent of RIP of p75^{NTR} (induced by NTF-stimulated activation of TACE) was further increased when NTF-treated retinal cultures were coincidentally treated with PMA (Fig. 7B). This RIP was correlated with the suppression of Rho activation. Exogenous TACE completely cleaved p75^{NTR} into p75^{ECD}, p75^{CTF} and p75^{ICD}, alone or with NTF, blocked Rho activation (Fig. 7B) and enhanced NTF-stimulated RGC neurite outgrowth in the presence of CNS myelin extract (Fig. 8A-C); increasing both the number of RGC with neurites (Fig. 8B) and mean neurite length (Fig. 8C). TIMP3 (a TACE inhibitor) blockade of NTF-induced RIP of p75^{NTR} (Fig. 7C) re-established the levels of active Rho and inhibition of RGC neurite outgrowth in the presence of CNS myelin extracts (Fig. 8A-C). The treatment of cultures with NTF + a γ -

secretase inhibitor in the presence of CNS myelin induced limited fragmentation of p75^{NTR} to produce p75^{ECD} and p75^{CTF} fragments but p75^{ICD} genesis was prevented. This treatment did not affect Rho activation, allowing CNS myelin to inhibit RGC neurite outgrowth (Fig. 7B, Fig 8B and C). Similarly, the addition of NTF + TACE + γ -secretase inhibitor, in the presence of CNS myelin, promoted fragmentation of most of the full length p75^{NTR} into p75^{ECD} and p75^{CTF}, but the generation of p75^{ICD} was prevented, with an accompanying reduction in levels of activated Rho, leading to a level of RGC neurite outgrowth in the presence of CNS myelin that was equivalent to that seen in the absence of CNS myelin (Fig. 7B, Fig 8B and C). It was noted that this treatment did not attenuate the levels of active Rho to the same degree as NTF + TACE treatment, and the levels of active Rho correlated with the extent of neurite outgrowth (see Fig. 8A-C). Whenever fragmentation of p75^{NTR} was observed in cell lysates, some p75^{ECD} was retained in the cell fraction, probably reflecting binding of the fragment to element of the plasmalemma, but most was shed into the medium

Discussion

Interestingly, enhanced levels of TACE-induced RIP of p75^{NTR} blocked the conversion of downstream Rho-GDP to Rho-GTP, consequently arresting inhibitory downstream signalling. However, TACE-mediated fragmentation of p75^{NTR} in the presence of γ -secretase inhibitor, which generates only p75^{ECD} and p75^{CTF} but not p75^{ICD}, did not significantly block Rho activation, suggesting that RIP of p75^{NTR} is required to effectively block Rho activation. This may explain why NTF plus TACE, which induced RIP of p75^{NTR} promoted significantly greater neurite outgrowth than NTF + TACE + γ -secretase inhibitor, in the presence of inhibitory CNS myelin. The release of Rho from Rho-GDP dissociation inhibitor (Rho-GDI) allows the GDP-bound form to be activated by guanine nucleotide exchange factors (GEF), probably by direct interaction of Rho-GDI with p75^{NTR}, an event that liberates Rho-GDP for conversion into its active form. It is possible that RIP of p75^{NTR} perturbs the interaction of Rho-GDI with p75^{NTR}, thereby preventing Rho activation by the failure of Rho-GDP dissociation from Rho-GDI. Accordingly, NTF-stimulated ON axon regeneration after intravitreal PN implantation may promote uninhibited polymerisation of actin in RGC growth cones after paralysis of p75^{NTR} inhibitory signalling.

In summary, the inventors have proposed a model (as shown in Figure 9) in which RIP of p75^{NTR} activated by TACE is a key factor in NTF-stimulated RGC axon/neurite

outgrowth through inhibitory CNS myelin ligand-rich environments. RIP of p75^{NTR} blocks Rho-A activation and inhibitory signalling, thereby stabilising growth cone morphology and allowing NTF-stimulated axons to regenerate in the presence of inhibitory ligands. Hence, examples 1 and 2 both illustrate that TACE-mediated receptor shedding may be used a
5 therapeutic strategy to block the function of receptor in the axon growth inhibitory cascade.

CLAIMS

1. A method of promoting axon and/or neurite branching and/or outgrowth and/or for inhibiting cell apoptosis in a subject, the method comprising administering to a subject in need of such treatment a composition comprising a sheddase enzyme, or analogue, or derivative, or activator thereof.
5
2. A method according to claim 1, wherein the composition is for the treatment of a disease condition characterised by damaged or impaired nerves.
- 10 3. A method according to claim 1, wherein the composition is for the treatment of spinal cord injury (SCI), glaucoma, and neurodegenerative disorders, such as MS, ALS, dementia, Huntingdon's Disease, Motor Neuron Disease, CJD, Alzheimer's disease, Parkinson's disease, diabetic neuropathy, and spinal muscular atrophy (SMA), chronic or acute brain trauma, spinal cord injury, neurotoxicity, stroke, glaucoma, optic nerve damage, blindness,
15 haemorrhage, facial nerve injury, caused by elective surgery, nerve compression, concussion, ischaemia, and burns.
4. A method according to claim 1, wherein the sheddase comprises an enzyme independently selected from a group consisting of a cysteine protease; an aspartic protease;
20 or a serine protease, or any combination thereof.
5. A method according to claim 1, wherein the sheddase comprises a metalloprotease.
6. A method according to claim 1, wherein the sheddase comprises a metalloprotease
25 independently selected from a group consisting of angiotensin converting enzyme (ACE), matrix metalloproteinase (MMP), and a NEP.
7. A method according to claim 1, wherein the sheddase comprises a zinc metalloprotease.
30
8. A method according to claim 1, wherein the sheddase comprises an ADAM.

9. A method according to claim 1, wherein the sheddase comprises ADAM 10 or ADAM 17 (TACE).

10. A method according to claim 1, wherein the sheddase comprises substantially the
5 sequence identified as SEQ ID No.3.

11. A method according to claim 1, wherein the sheddase, or analogue, derivative or activator thereof is used in combination with an axon/neurite growth-stimulating molecule.

10 12. A method according to claim 11, wherein the axon/neurite growth-stimulating molecule comprises a Neurotrophic Factor (NTF).

13. A method according to claim 12, wherein the NTF comprises NGF, NT-3, NT-4, BDNF, GDNF, FGF-1, FGF-5, CT-1, CDF, insulin, IGF-1, IGF-2, IL-6, LIF, NPF, PDGF,
15 PN-1, S-100, TGF- β , or VIP.

14. A method according to claim 12, wherein the NTF comprises Ciliary Neurotrophic Factor (CNTF) or Fibroblast Growth Factor 2 (FGF2).

20 15. A delivery system for use in a gene therapy technique, the delivery system comprising a nucleic acid molecule encoding a protein which directly or indirectly modulates activity of a sheddase, or analogue or derivative thereof, wherein said nucleic acid molecule is capable of being transcribed to allow the expression of the protein, which is effective for treating a disease condition characterised by a damaged or impaired nerves.

25 16. A delivery system according to claim 15, wherein the delivery system comprises a sheddase expression system adapted to increase the expression level of a sheddase or analogue or derivative thereof in a target cell.

30 17. A delivery system according to claim 16, wherein the expression system comprises a nucleic acid sequence encoding a sheddase enzyme or analogue or derivative thereof.

18. A delivery system according to claim 16, wherein the expression system comprises a nucleic acid sequence, which encodes a neurotrophic factor (NTF).

19. A composition for promoting axon and/or neurite branching and/or outgrowth, the composition comprising a sheddase or analogue, derivative or activator thereof, and a therapeutically effective amount of an NTF.

20. A composition according claim 19, wherein the NTF is FGF-2 or CNTF.

21. A composition according to claim 19, for use as a medicament.

22. A method of promoting neurite outgrowth and/or branching in culture, the method comprising administering a composition comprising a sheddase, or analogue or derivative or activator thereof, to a culture of neurons.

23. A method according to claim 22, wherein the composition comprises an NTF.

24. A method according to claim 23, wherein the NTF is FGF-2 or CNTF.

25. A sheddase enzyme, or analogue, or derivative, or activator thereof, for use as a medicament.

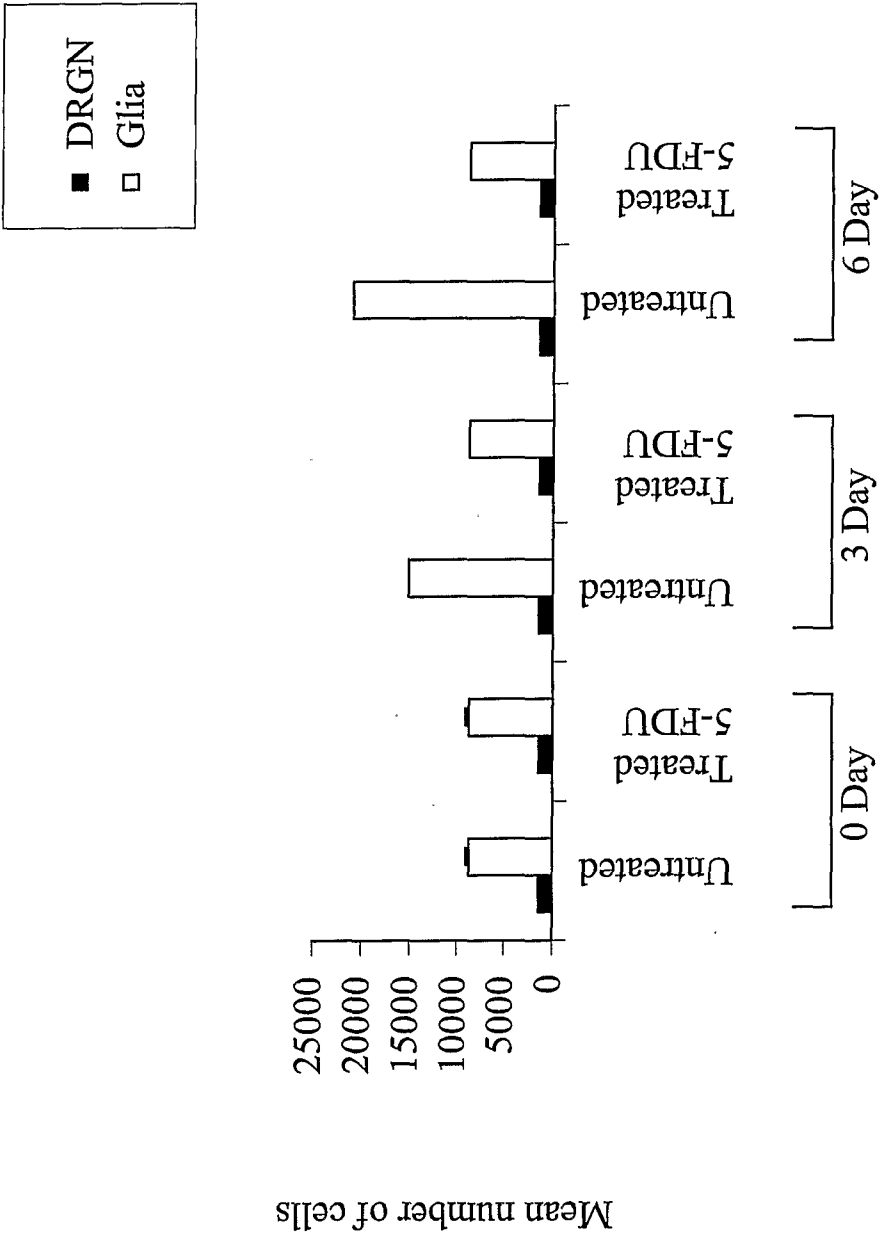


FIG 1^a

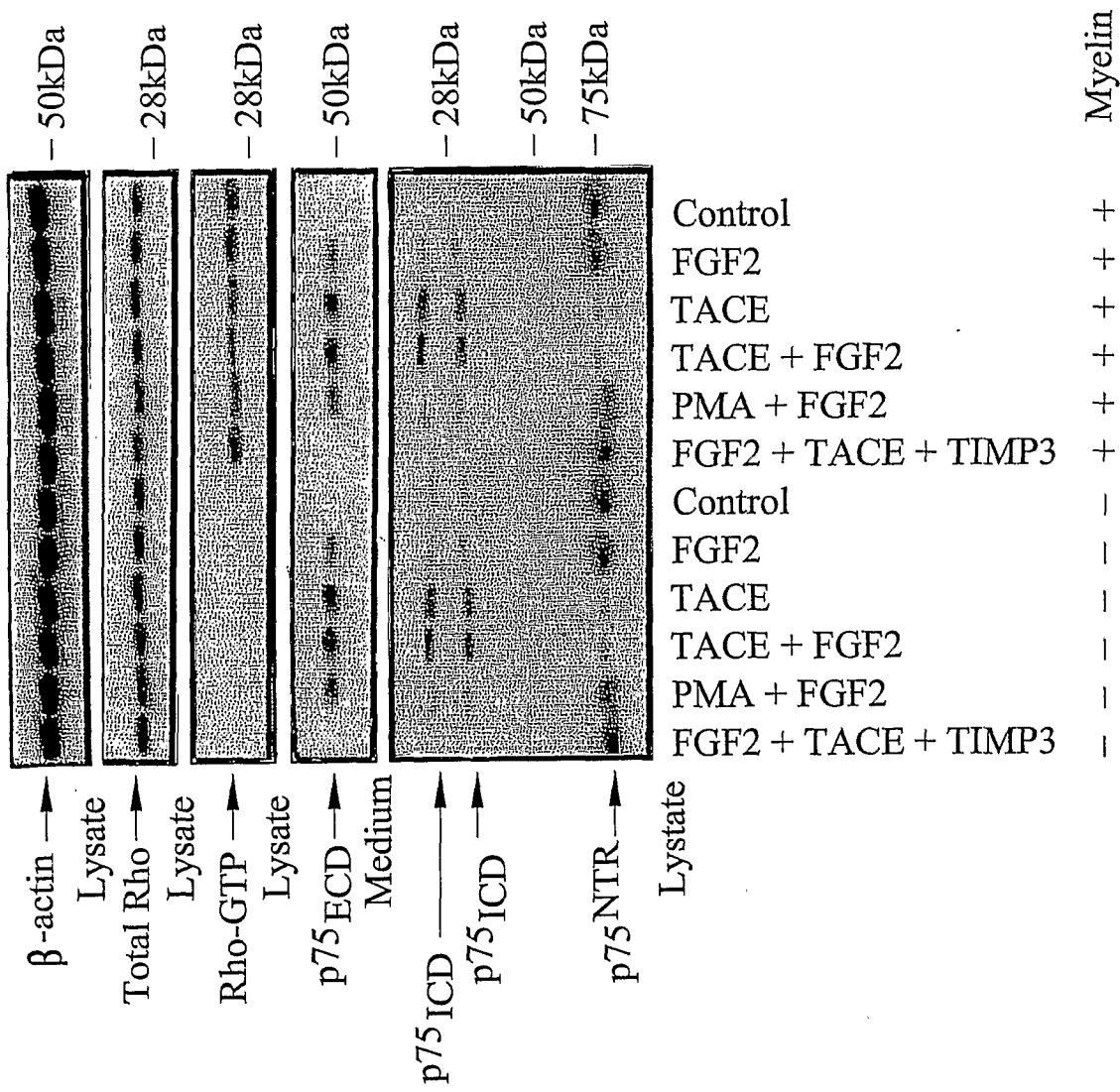


FIG 1^b

3 / 12

Myelin + + + + + - - - - -

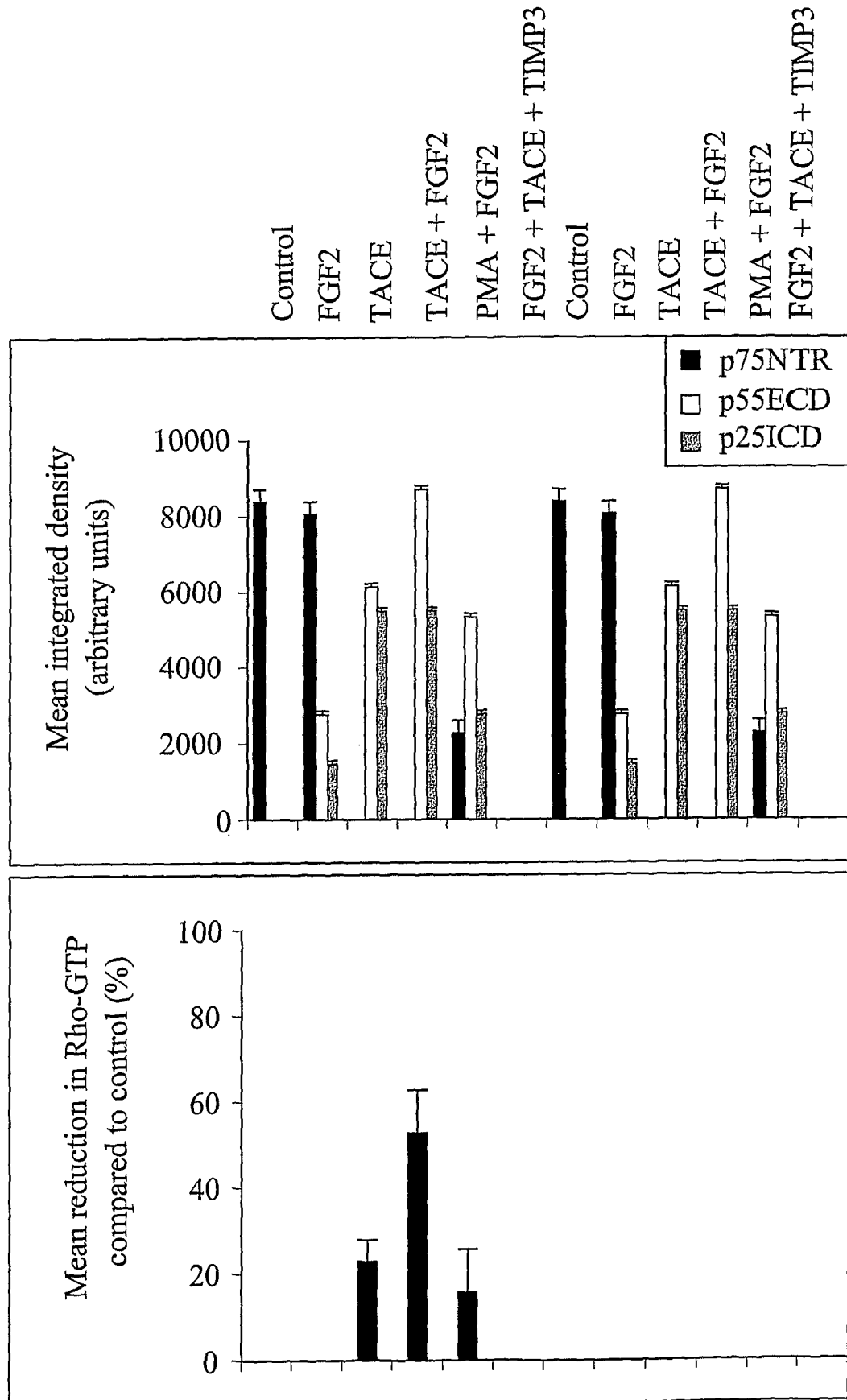
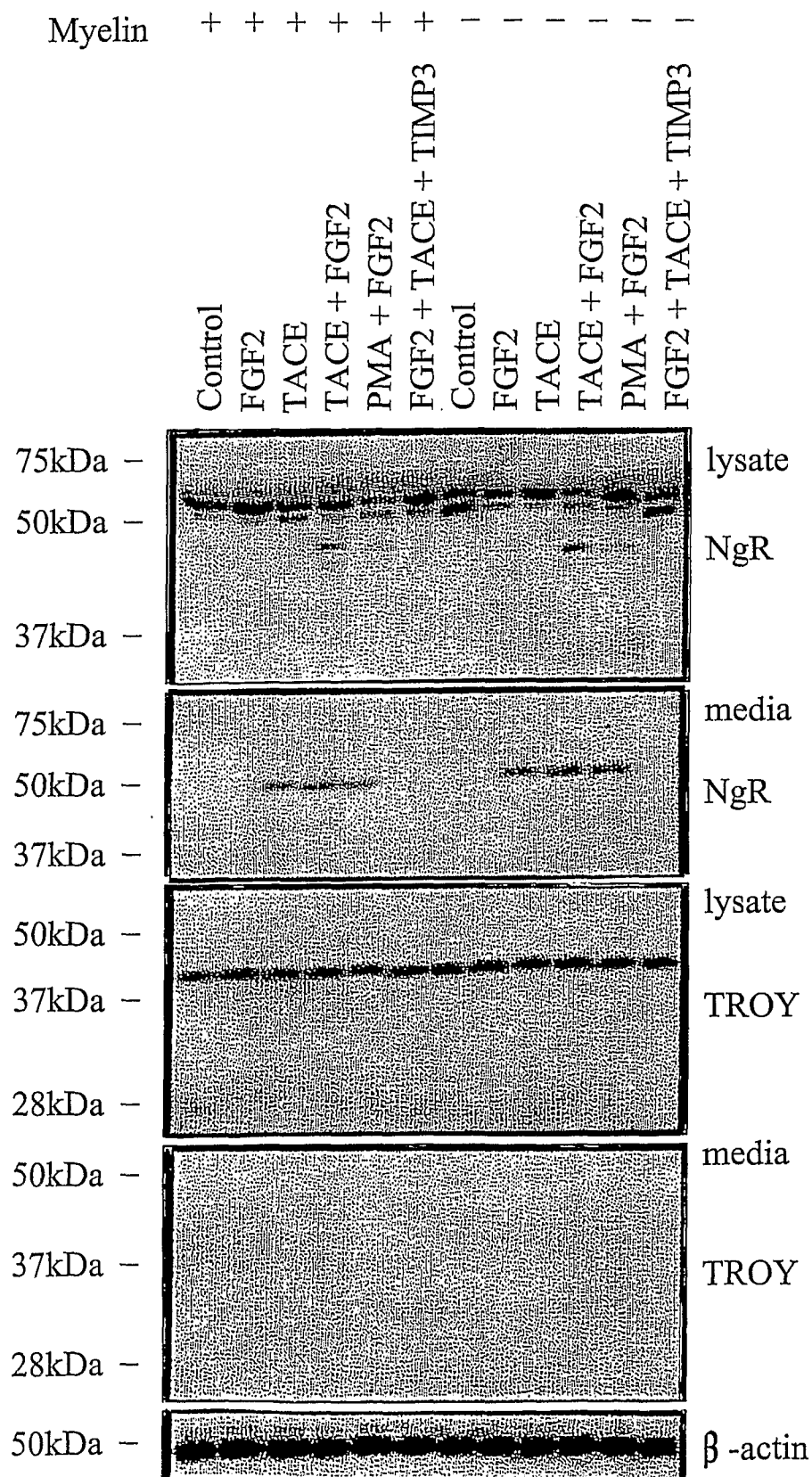


FIG 1^c

FIG 2

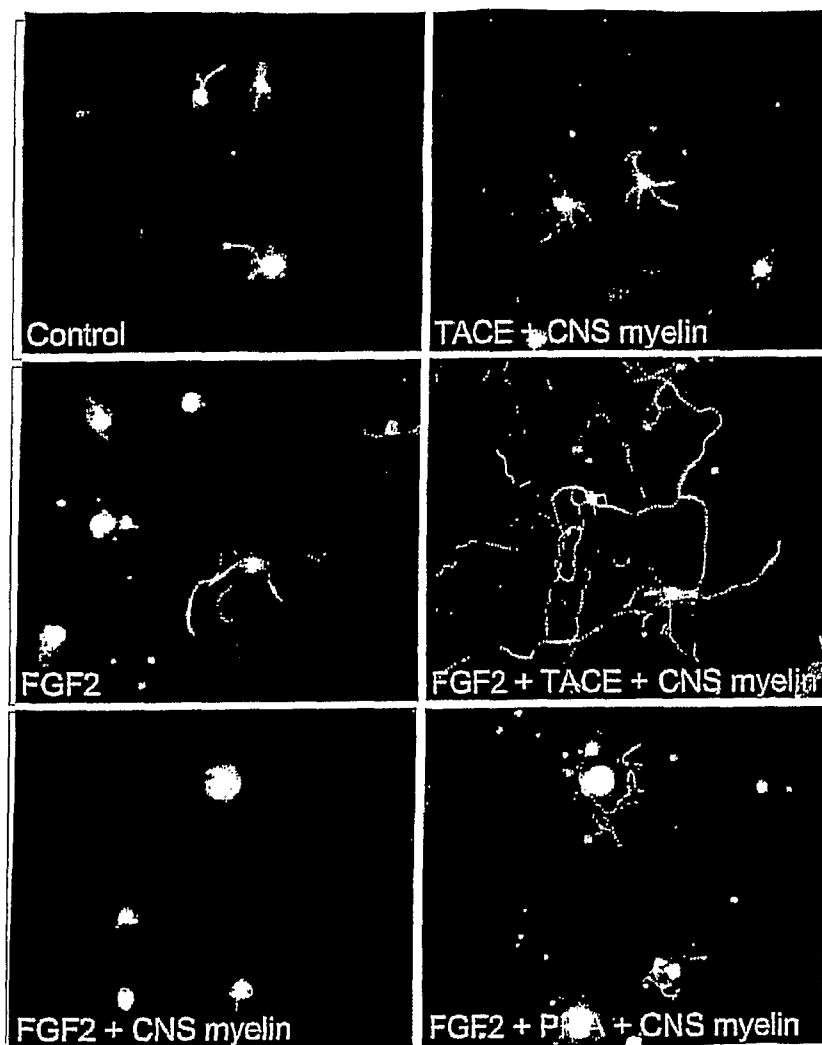


FIG 3^a

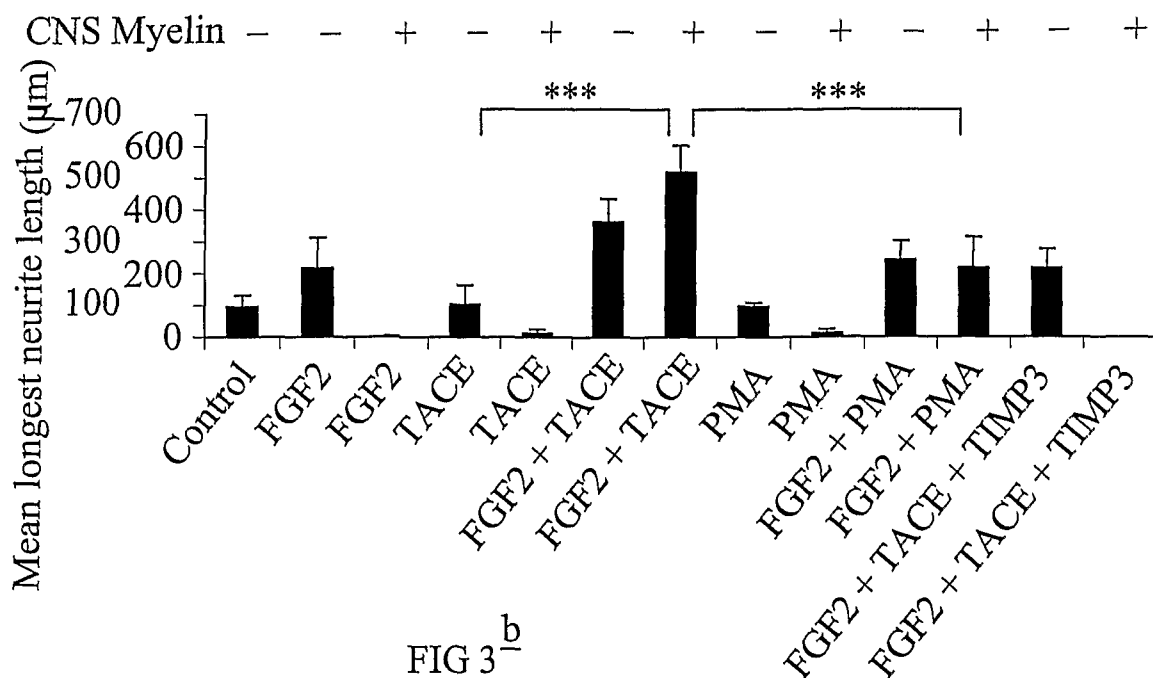


FIG 3^b

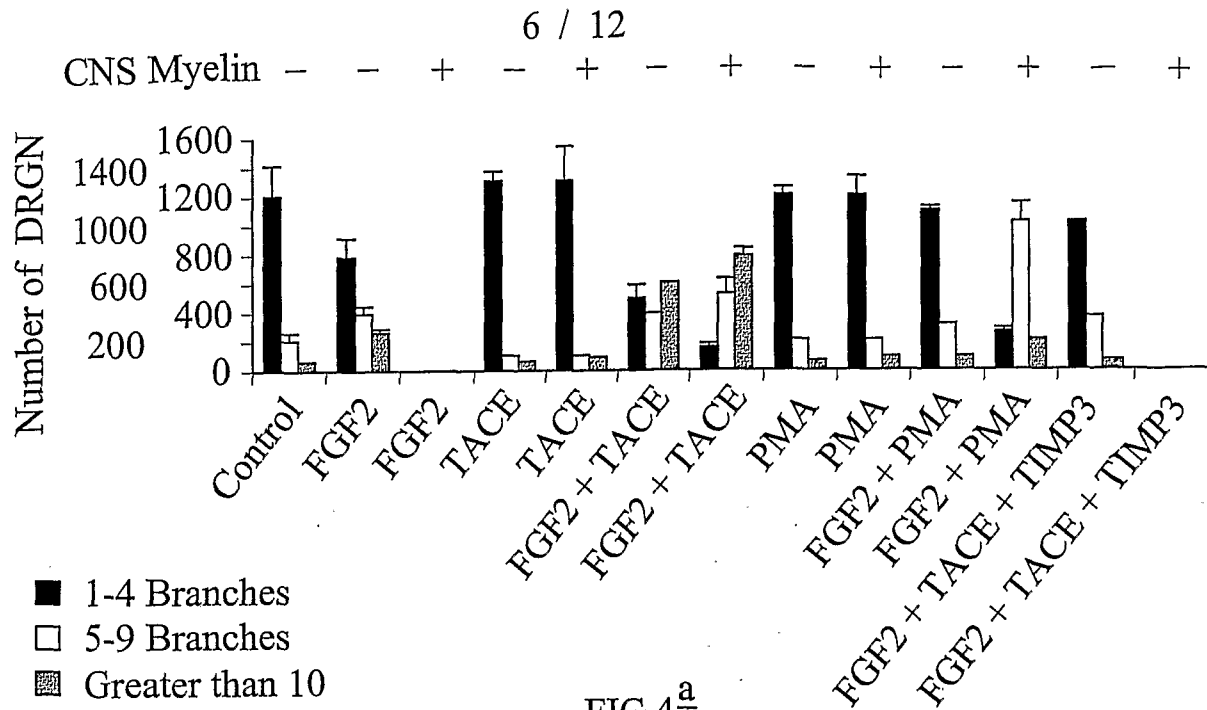


FIG 4^a

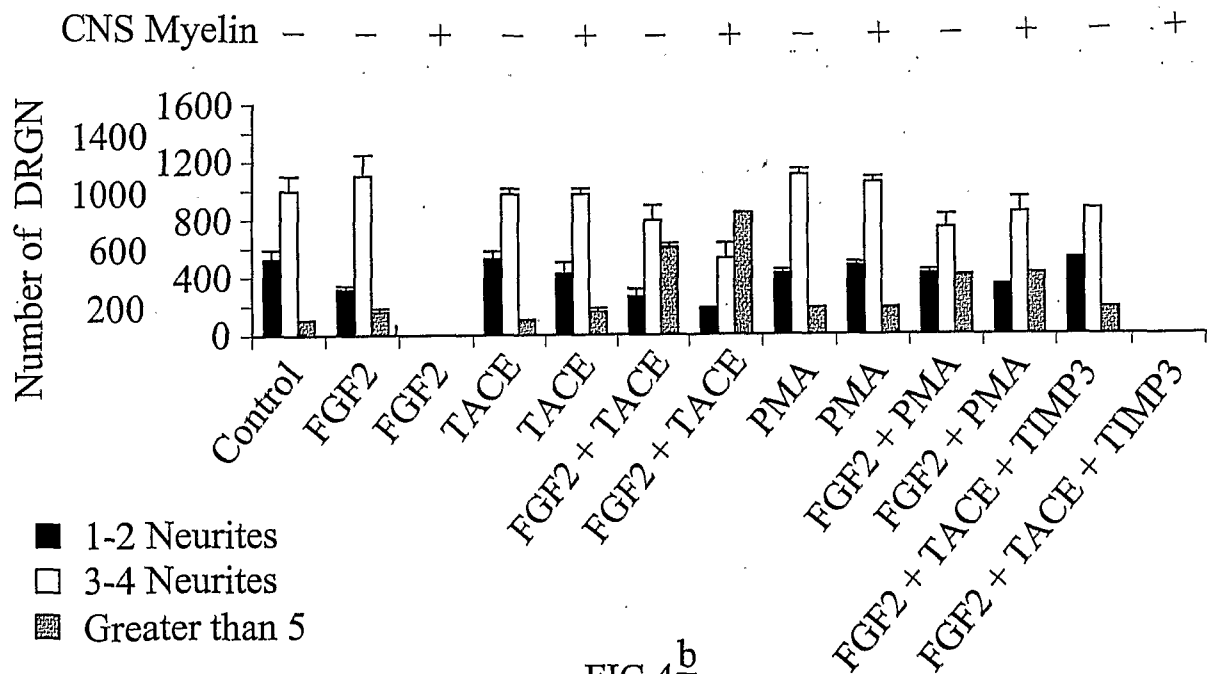


FIG 4^b

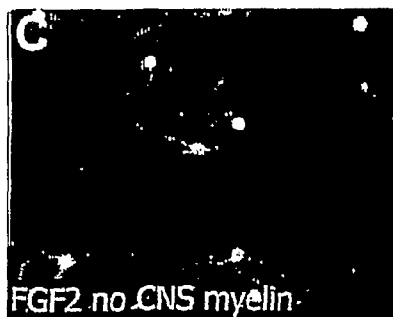


FIG 4^c

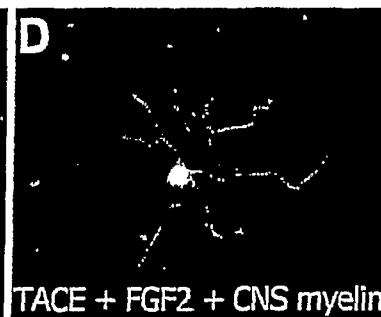


FIG 4^d

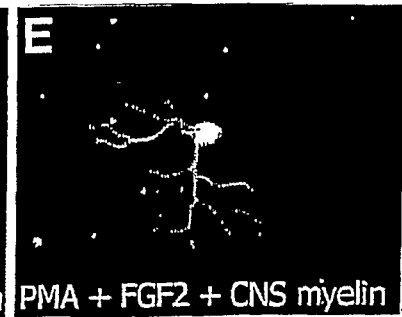


FIG 4^e

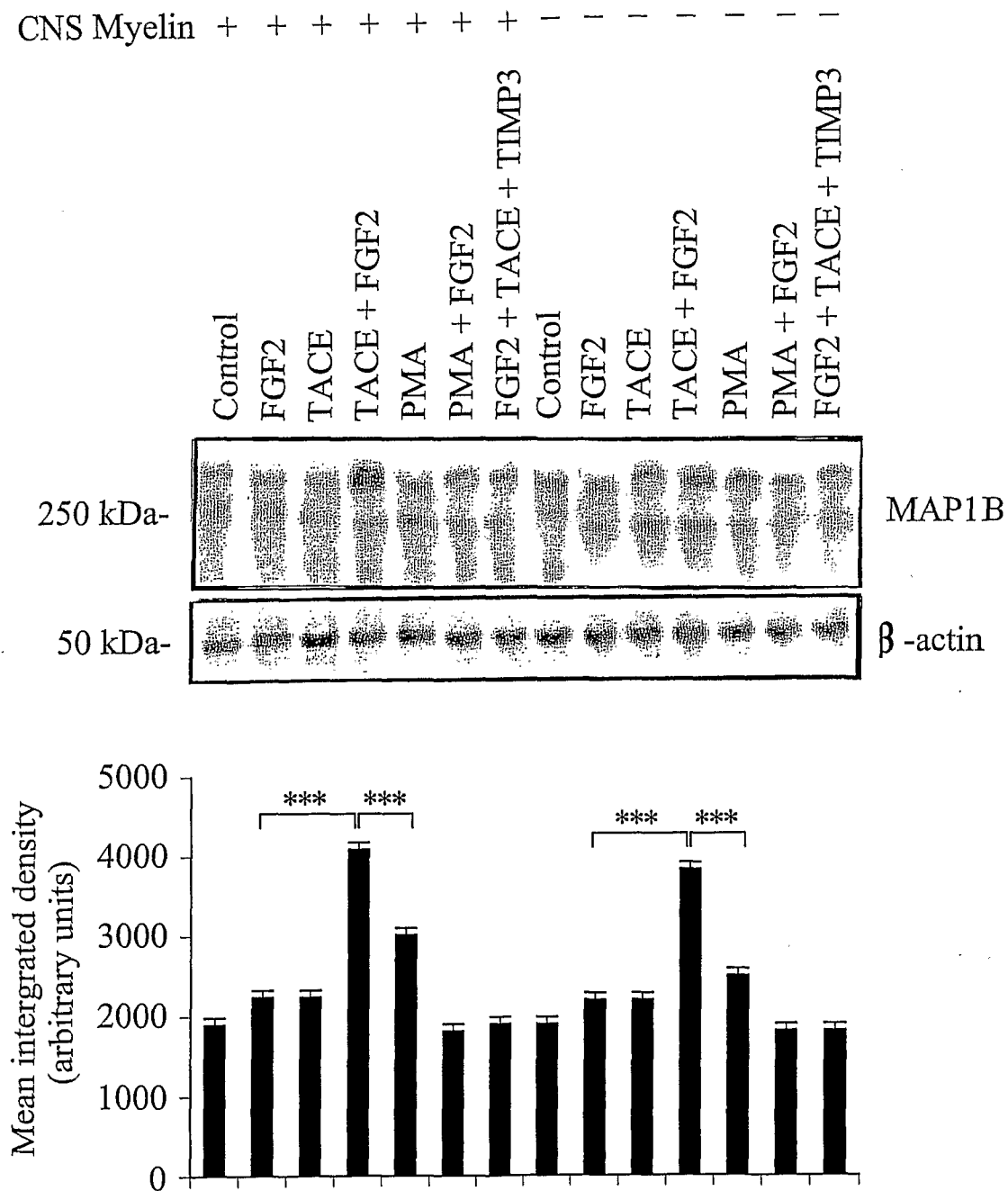
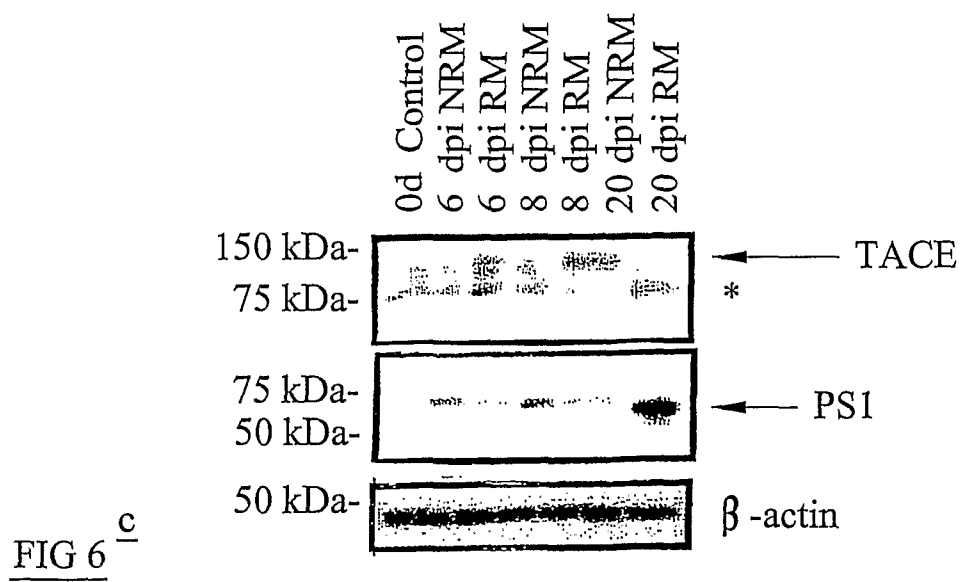
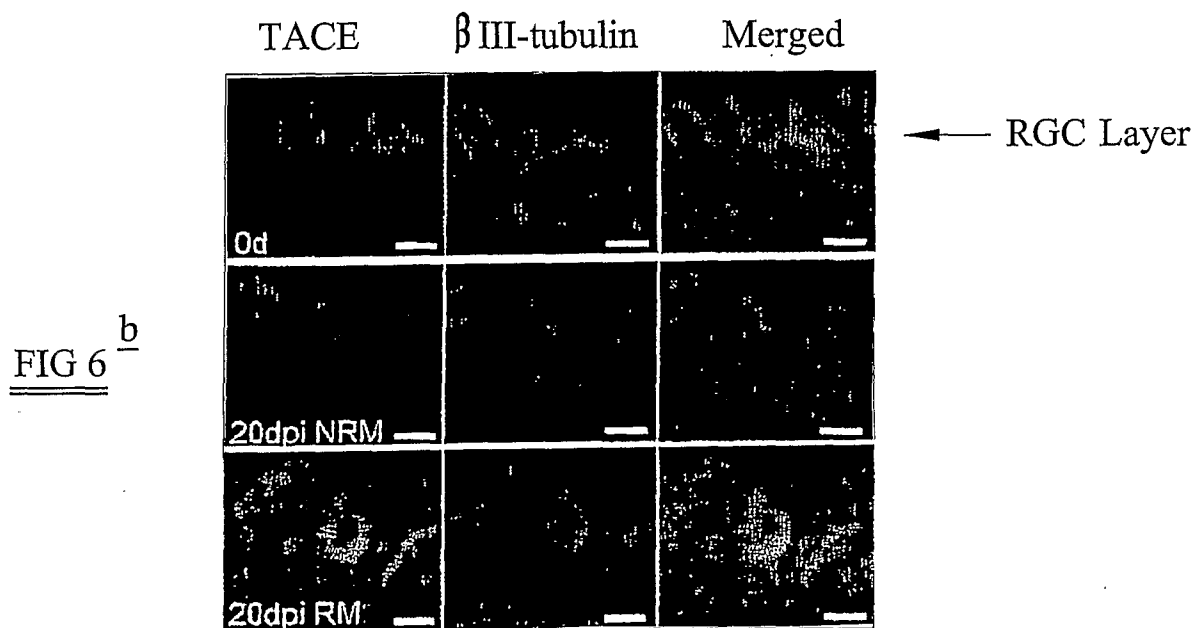
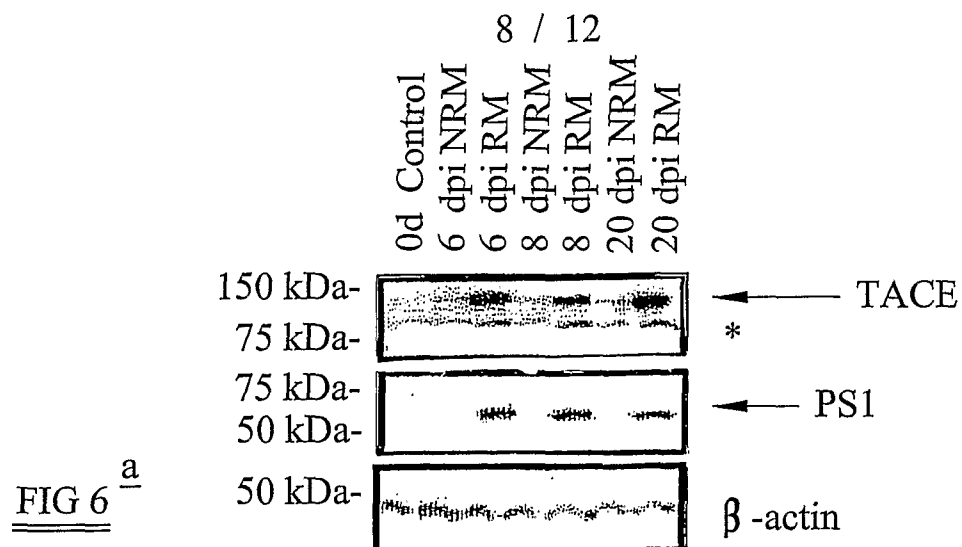


FIG 5



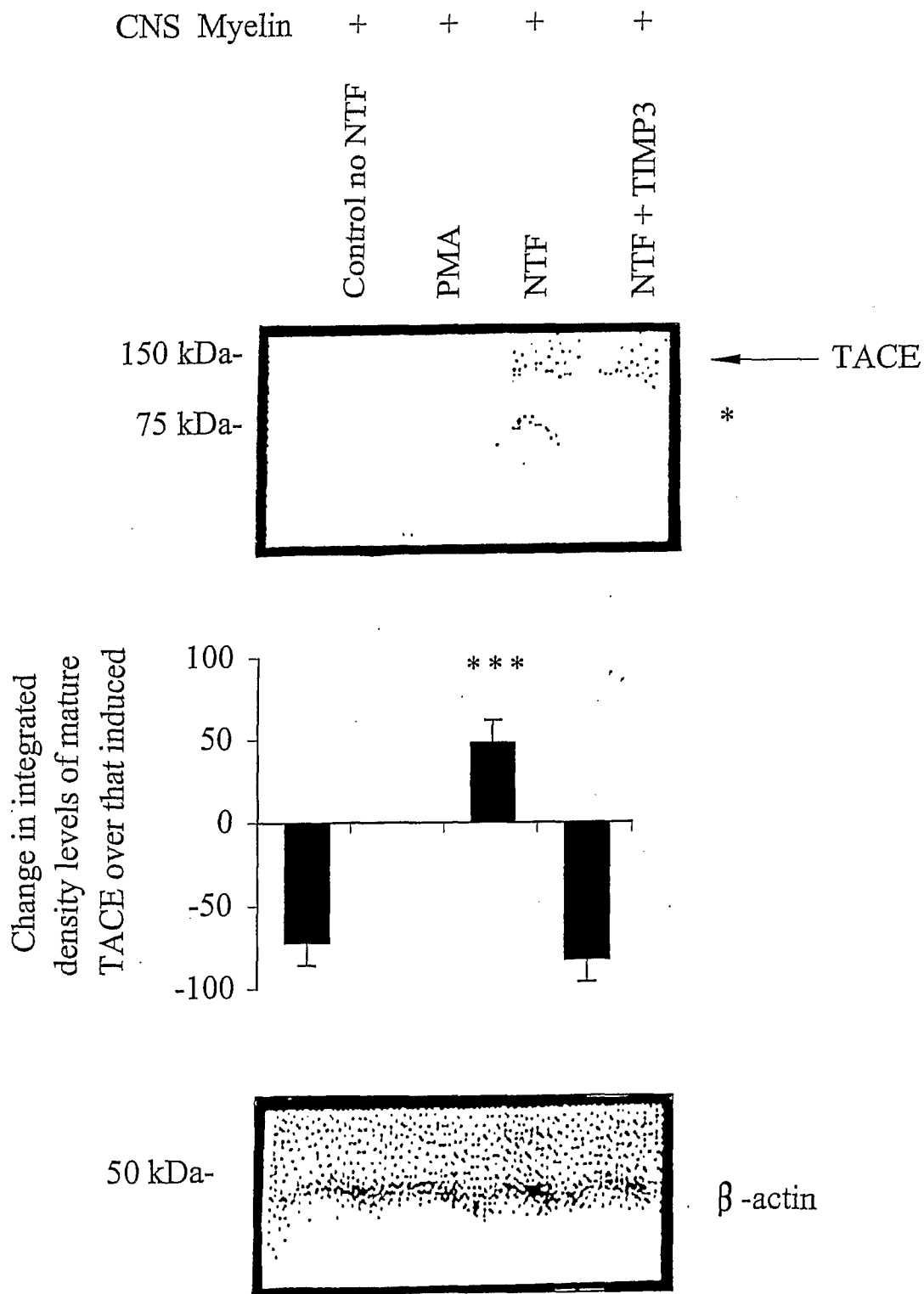


FIG 7^a

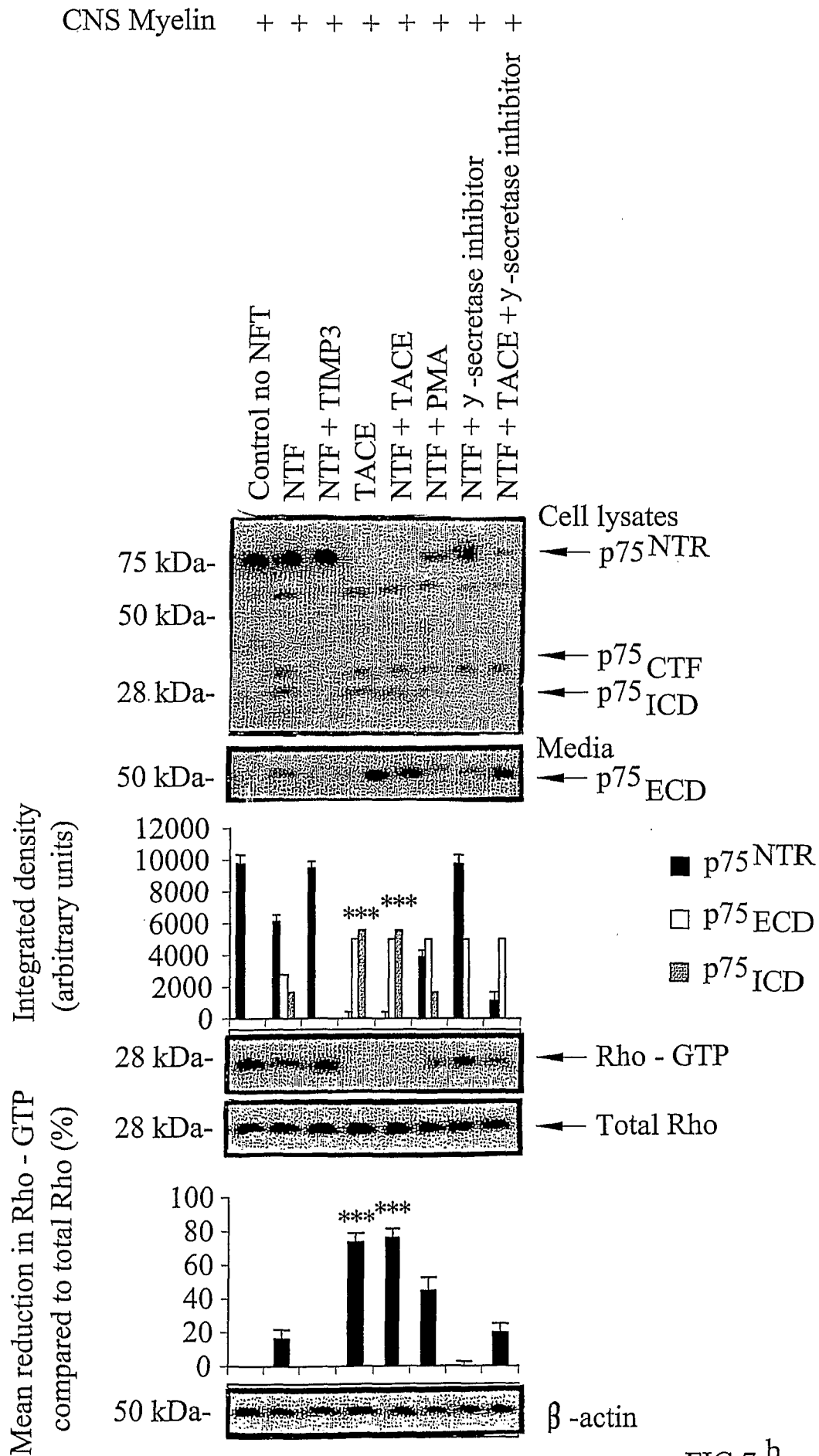


FIG 7 ^b

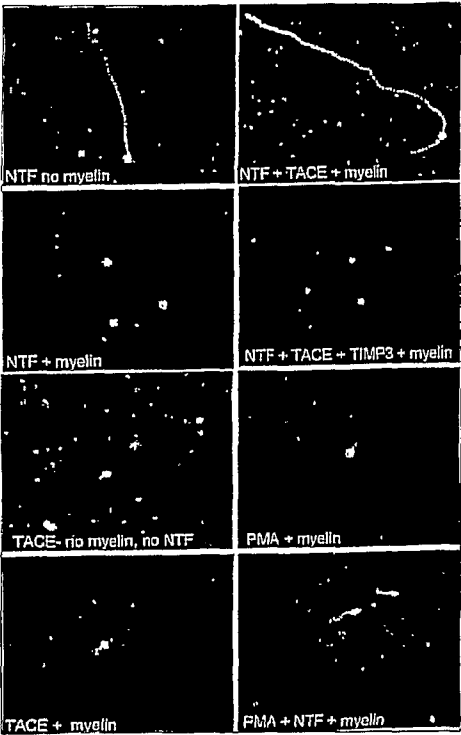


FIG 8^a

Mean number of RGC with neurites

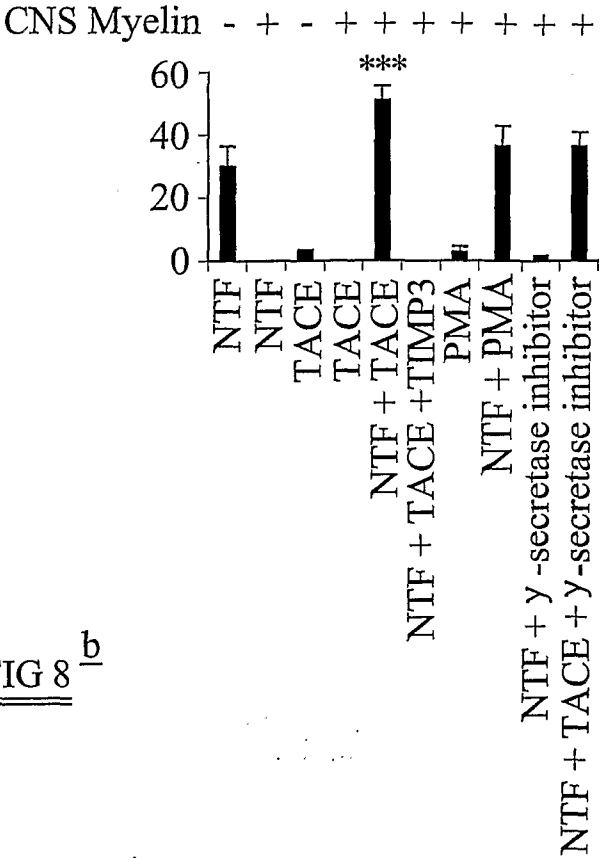


FIG 8^b

Mean neurite length (μm)

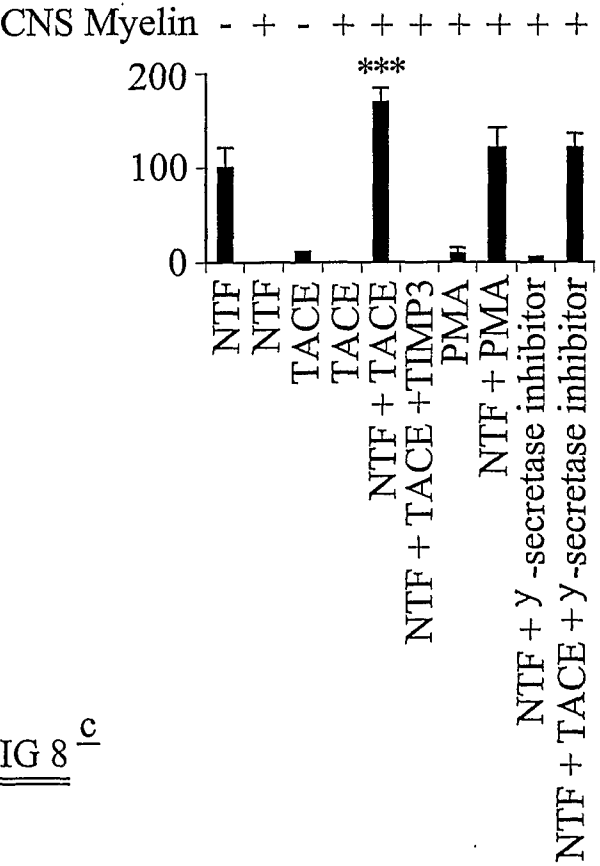
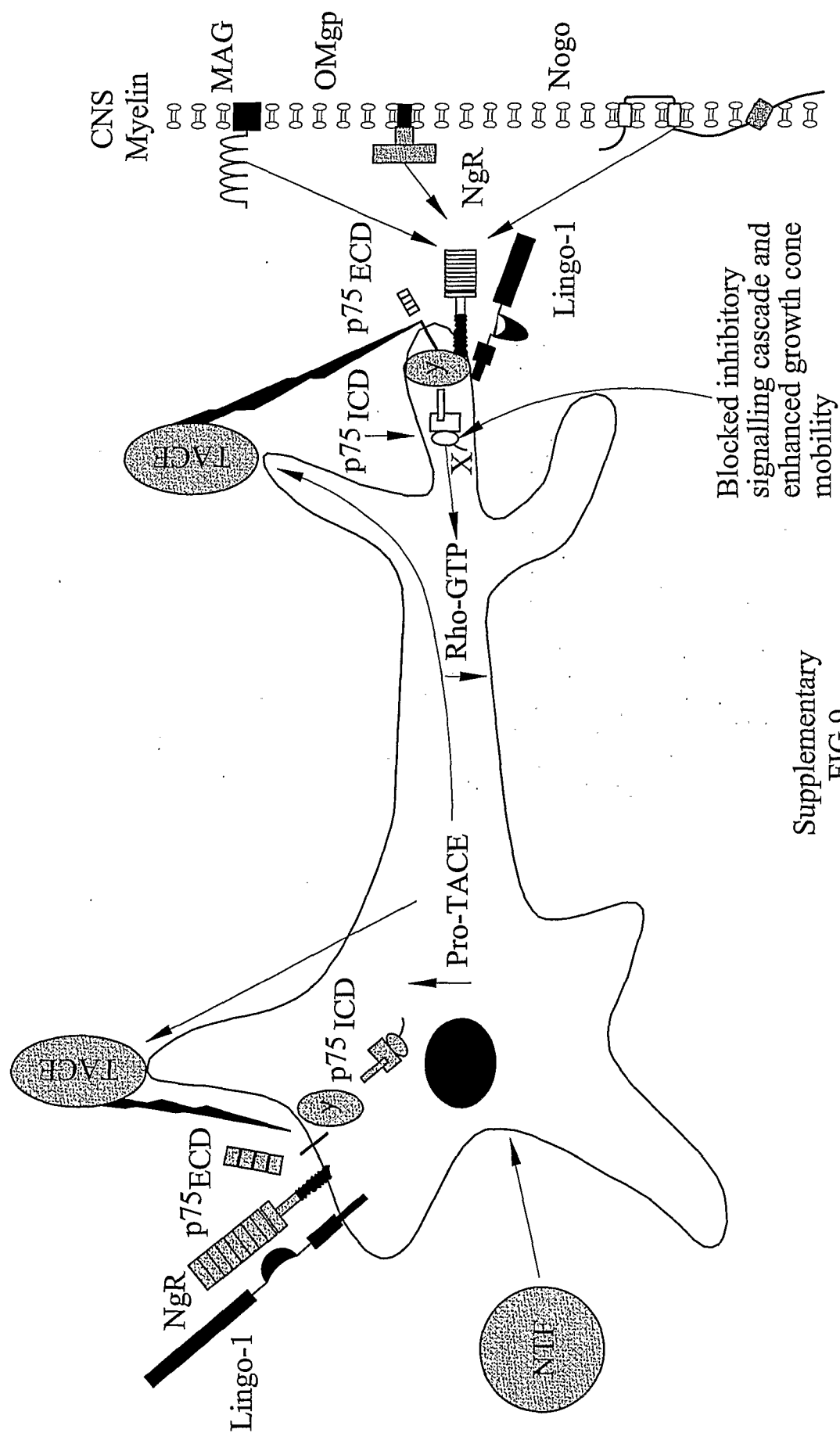


FIG 8^c

Supplementary
FIG 9

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2006/001904

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/18 A61K38/48 A61P25/28

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)

A61K A61P

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, WPI Data, EMBASE, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AHMED ZUBAIR ET AL: "Schwann cell-derived factor-induced modulation of the NgR/p75(NTR)/EGFR axis disinhibits axon growth through CNS myelin in vivo and in vitro" BRAIN, vol. 129, no. Part 6, 13 April 2006 (2006-04-13), pages 1517-1533, XP009077910 ISSN: 0006-8950 page 1519, right-hand column, paragraph 3 page 1522, right-hand column - page 1524 ----- -/-	1-25

☒ 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.
- * & * document member of the same patent family

Date of the actual completion of the international search

7 February 2007

Date of mailing of the international search report

16/02/2007

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Winger, Rudolf

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2006/001904

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOGAN ANN ET AL: "Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury" BRAIN, vol. 129, no. Part 2, February 2006 (2006-02), pages 490-502, XP009077917 ISSN: 0006-8950 abstract	1-25
X	US 2003/121064 A1 (LOGAN ANN [GB] ET AL) 26 June 2003 (2003-06-26) claims; examples	1-25
Y	CLARKE W E ET AL: "The role of matrix metalloproteinases (MMPs) in the regenerating optic nerve" JOURNAL OF ENDOCRINOLOGY, vol. 164, no. Suppl., March 2000 (2000-03), page P158, XP009077936 & 19TH JOINT MEETING OF THE BRITISH ENDOCRINE SOCIETIES, WITH THE EUROPEAN FEDERATION OF ENDOCRINE SO; BIRMINGHAM, ENGLAND, UK; MARCH 13-16, 2000 ISSN: 0022-0795 abstract	1-25
Y	WO 00/69822 A (DARWIN DISCOVERY LTD [GB]; BAXTER ANDREW DOUGLAS [GB]; OWEN DAVID ALAN) 23 November 2000 (2000-11-23) page 1, line 25 - line 30; claim 15	1-25
X	WO 97/35538 A (GLAXO GROUP LTD [GB]; MCGEEHAN GERARD M [US]; BECHERER JAMES DAVID [US]) 2 October 1997 (1997-10-02) claim 14	15-18,25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2006/001904

Box 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 1-14 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 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 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 all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
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.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2006/001904

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003121064	A1	26-06-2003	NONE
WO 0069822	A	23-11-2000	AU 4595800 A 05-12-2000 EP 1177174 A1 06-02-2002 US 6506764 B1 14-01-2003
WO 9735538	A	02-10-1997	AU 2291397 A 17-10-1997 CA 2249985 A1 02-10-1997 EP 0900272 A2 10-03-1999 JP 2000507943 T 27-06-2000