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(54) **Title**: TREATMENT OF NEURONAL INJURIES

(57) **Abstract**: The present invention relates to treatment of neuronal injuries. Specifically, the present invention relates to novel mechanisms and means for promoting neurite outgrowth, neural regeneration and/or maintenance of neurites (axons and dendrites) in pathologies where chondroitin sulphate proteoglycans (CSPGs) display adverse effects, such as in CNS injuries.

TREATMENT OF NEURONAL INJURIES

FIELD OF THE INVENTION

The present invention relates to treatment of neuronal injuries. Specifically, the present invention relates to novel mechanisms and means for promoting neurite outgrowth, neural regeneration and/or maintenance of neurites (axons and dendrites) in pathologies where chondroitin sulphate proteoglycans (CSPGs) display adverse effects, such as in CNS injuries.

BACKGROUND OF THE INVENTION

Nervous system injuries affect millions of people every year. As a result of this high incidence of neurological injuries, nerve regeneration and repair are becoming a rapidly growing field dedicated to the discovery of new ways to recover nerve functionality after injury. The nervous system is divided into two parts: the central nervous system (CNS), which consists of the brain and spinal cord, and the peripheral nervous system (PNS), which consists of cranial and spinal nerves along with their associated ganglia. A brain injury or brain damage is the destruction or degeneration of brain cells in the brain of a living organism. Brain injuries can be classified along several dimensions. Primary and secondary brain injuries are ways to classify the injury processes that occur in brain injury.

Post traumatic regeneration of the brain and spinal cord is a major unsolved medical problem because the brain and spinal cord are not able to regenerate like the peripheral nervous system. While peripheral axons regenerate in patients after nerve injury, brain and spinal cord axons fail to regenerate due to glial scar formation and the inhibitory action of chondroitin sulphate proteoglycans, CSPGs, in the scar. In addition, those factors that promote peripheral nerve regeneration, for instance nerve growth factor, NGF fail to improve regeneration in the brain and spinal cord. The central nervous system and peripheral nervous system are very different in their reactions to drug treatment and regeneration ability.

Traumatic brain injury (TBI) can result from direct impacts to the head, such as from a fall, or from acceleration/deceleration injuries, such as those encountered in motor vehicle accidents. TBI is the leading cause of death and disability in the most active population (<45 years of age). Individuals under the age of 5 and over the age of 75 are also particularly at risk.

Each year, in the US alone, it has been estimated that more than 1.7 million people sustain TBI. In addition, there are >5 million people coping with TBI related disabilities at an estimated cost of more than $60 billion per year. In Europe, there are an estimated ~775,500 new cases per year and a case fatality rate of 11%. TBI is also an epigenetic risk factor for Alzheimer's and Parkinson's disease. Thus, TBI is a major health concern and a significant socioeconomic burden.

For survivors of TBI, the most prevalent and debilitating features are personality disorders, cognitive defects and motor dysfunctions. Memory alterations, characterised by loss of both specific ones and the inability to form/store new ones, is the most common cognitive impairment, for which there is currently no effective treatment. Improvements in motor function can be attained through sustained physical therapy, but outcomes are varied and full return to capability cannot be assured. Natural recovery, from TBI, is greatest during the first 6 months, after which observed improvements are typically more gradual and frequently incomplete.

Neuroprotective treatments have been historically pursued for the treatment of TBI, but have failed to demonstrate clear efficacy in Phase III trials thus far. Instead, neurorestorative processes such as promotion of angiogenesis, neurogenesis and axonal remodelling are now being developed, in order to enhance endogenous brain plasticity processes and to improve functional recovery after TBI (Xiong *et al.*, 2010).

Bone marrow stromal cells (MSCs) are a promising source of cell-based therapy for TBI. The safety and feasibility of treatment with autologous MSCs has been assessed in patients with TBI, during which no toxicity related to the cell therapy was observed. Neurological function was significantly improved 6 months after cell therapy, but is potentially difficult to interpret in the absence of control data. Increased expression of erythropoietin (EPO), and its receptors, is found in neurons, neural progenitor cells and glial cells in response to injury. Clinical trials to investigate the safety of EPO treatment in patients with severe TBI and, separately, to investigate the early administration of EPO to TBI patients are both ongoing. However, in the Phase II trials, the high doses of EPO used led to an increase in hematocrit, which may cause adverse vascular effects, such as thrombosis. Other clinical trials are ongoing with statins, such as atorvastatin, which induce angiogenesis, neurogenesis and synaptogenesis, whilst enhancing functional recovery (Xiong *et al.*, 2010).

Spinal cord injury (SCI) is classified as damage to the spinal cord caused by trauma, instead of disease, with symptoms ranging from pain to paralysis to incontinence. Any injury that involves the head, pelvic fractures, penetrating injuries in the area of the spine or injuries that result due to a fall from height, may result in spinal cord damage. The most common causes of SCI are motor vehicle accidents, falls and violence.

In the US, there are an estimated 12,000 new cases of SCI each year, with approximately 260,000 individuals afflicted by SCI. In Europe, there are estimated to be roughly 9,000 new SCI cases per year. Most incidences of SCI occur in people between the ages of 16–30 and hence healthcare expenses can be considerable, varying depending upon the severity of injury. Estimated lifetime costs for a tetraplegic patient are greater than a $1,000,000. These figures do not include any indirect costs, such as losses in salary, which are estimated to be approximately $64,000 per year.

Treatment options for SCI are extremely limited, with physical therapy a major treatment modality. Methylprednisolone, which helps to reduce swelling in the spinal cord, is widely prescribed as an off-label drug, but does not serve most patients' needs. There are currently no therapies to alleviate, or repair, the incurred damage to the spinal cord. Very few compounds are in late stage development with the limited examples including Lyrica (a calcium channel modulator, targets neuropathic pain), umbilical cord blood mononuclear cell transplants (aimed at improving functional recovery) and Procord (autologous activated macrophage therapy, aimed at facilitating neuroprotection and wound healing). However, none of these molecules are expected to reach the market before 2017.

For nervous system injuries, especially for both TBI and SCI there are substantial patient populations with significant unmet needs, for which novel treatment options are desperately required. There is currently no treatment for recovering human nerve function after injury to the central nervous system.

Neuronal regeneration is important for neuronal injuries and neurodegenerative diseases and identifying molecular mechanisms guiding neuronal development has been a great challenge. Inhibition of chondroitin sulphate proteoglycans (CSPGs) as a mechanism to enhance neuronal growth has been of considerable interest. CSPGs have been implicated in inhibiting regeneration of axons and dendrites following CNS trauma (Silver and Miller,

2004). CSPGs are also known to be part of the glial scar that forms post-injury, acting as a barrier to prevent axon extension and regrowth. Levels of versican, neurocan, brevican and phosphacan (those CSPGs measured) have all been found to be upregulated after spinal cord injury (Jones *et al.,* 2003).

Some approaches to CSPG inhibition have focused on the use of molecules/agents that inhibit the interaction of CSPGs with its receptor RPTPσ. WO2011/022462 discloses the use of soluble fragments of RPTPs that bind CSPGs, thus acting as competitive inhibitors to prevent the CSPGs from binding RPTPs on the neuron. The neural cell can be associated with an injury or neurodegenerative condition. WO2012/112953 discloses methods for contacting a neuron with an agent that binds RPTPσ, to thereby induce neuronal outgrowth of the neuron. The agent may induce clustering of RPTPσ and/or inhibit binding of CSPGs to RPTPσ. Examples of suitable agents are heparan sulfate proteoglycan, heparan sulfate, heparan sulfate oligosaccharides, or heparin oligosaccharides.

Secondary injury mechanisms have, so far, been predominantly targeted through the use of neuroprotective treatments. For example, in TBI, calcium channel blockers, corticosteroids and NMDA receptor antagonists, amongst others, have all demonstrated pre-clinical efficacy in animal models. However, the compounds and approaches, which have been tested in clinical trials thus far, have disappointingly failed to demonstrate clear efficacy. Consequently, the use of neuroprotective strategies, as the primary treatment option for TBI and SCI, remains in doubt and hence novel approaches are required. Finding out mechanisms and means to promote nerve regeneration is important also clinically, as it is part of the pathogenesis of many diseases. In the hunt for neurostimulatory agents that promote nerve regeneration, well-defined models and analysis methods are required.

BRIEF DESCRIPTION OF THE INVENTION

An object of the invention is thus to provide novel means and mechanisms for promoting neurite outgrowth and/or neural regeneration e.g. in nervous system injuries. Specifically the nervous system injury is selected from a group consisting of neurodegenerative diseases, traumatic brain injury, spinal cord injury, multiple sclerosis (MS), Alzheimer's disease, amyotropic lateral sclerosis (ALS), stroke, Parkinson's disease, eye injury and skin burn.

A further object of the invention is to provide a method of promoting neurite outgrowth and/or neural regeneration.

The objects of the invention are achieved by an agent and methods which are characterized by what is stated in the independent claims. The preferred embodiments of the invention are disclosed in the dependent claims.

The invention is based on the surprising realization and unexpected finding that heparin-binding growth associated molecule, HB-GAM, (pleiotrophin) changes the CSPG matrix from regeneration inhibiting to regeneration activating structure. HB-GAM can be therefore used to reverse the CSPG effects in various diseases where the CSPGs have adverse effects in terms of neuronal regeneration or maintenance. Moreover, HB-GAM promotes neurite outgrowth and/or neural regeneration by increasing the amount of chondroitin sulphate proteoglycan, CSPG, binding to its receptor RPTPσ and clustering of this receptor. In view of the prior art it may have been expected that HB-GAM would sequester the CSPGs from RPTPσ, through interaction of its basic residues with the negatively charged sulphate side chains, but the present inventors found the reverse to be true. Through this novel mechanism, it is stated that HB-GAM can promote neurite outgrowth by modulating CSPG matrix that leads to its enhanced chondroitin suphate epitope binding to PTPsigma. It is also demonstrated here that HB-GAM can promote dendrite regeneration in the cortex. It is known that the regenerative ability of dendrites is also very different from that of axons (Le Roux and Reh, 1996). Hence, HB-GAM and the novel mechanism of action are advantageous and useful for the treatment of neuronal injuries.

An advantage of the invention is that HB-GAM promotes CNS regeneration by converting CSPG-enriched glial scar into permissive milieu for axon and dendrite growth in adult CNS. Moreover, dendrite regeneration has been an unexplored strategy for surviving dendrite damage. The present invention provides now an agent, HB-GAM that promotes dendrite regeneration.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention will be described in greater detail by means of preferred embodiments with reference to the attached drawings, in which

Figure 1A. HB-GAM promotes neurite growth on CSPG-coated substrate in rat cortical neurons *in vitro*. Chondroitin sulphate proteoglycan aggre-

can prevents attachment and neurite growth in embryonic rat cortical neurons. HB-GAM overcomes the inhibitory action of aggrecan, promoting neuronal attachment and neurite growth on the aggrecan-coated substrate *in vitro*.

Figure 1B. HB-GAM overcomes the inhibitory effect of neurocan and promotes neuronal attachment and neurite growth. Neurocan was precoated on plastic wells alone or co-precoated with HB-GAM under the same experimental conditions as in Figure 1. Neurocan precoated at 5 μg/ml inhibits neurite growth and cell attachment in dissociated culture of rat cortical neurons. HB-GAM precoated at 25 μg/ml together with neurocan (5 μg/ml) overcomes the inhibitory effect of neurocan and promotes neuronal attachment and neurite growth.

Figure 2. HB-GAM increases the amount of aggrecan binding to RPTPσ. Mutant RPTPσ with a defective CSPG-binding site was used as a negative control.

Figure 3. HB-GAM promotes dendrite regeneration after traumatic brain injury (TBI) in the adult mouse cortex. TBI was applied to adult mice as a needle prick to the parietal lobe of the brain cortex. Transgenic mice expressed YFP under Thy1 promoter in dendrites of neurons from cortical layers 4 and 5. Chronic cranial window was used to follow dendrite regeneration using two photon microscopy on live animals over 20 days after injury. Immunoglobulin G was used in parallel as a negative control.

Figure 4. HB-GAM turns CSPG into a potent activator of neurite growth. Embryonic cortical neurons were cultured for 3.5 days on unprecoated plastic (A, C, E) or on aggrecan (precoated at 10 μg/ml) (B, D, E). HB-GAM (10 μg/ml) was added to culture medium when plating cells in (C, D).

Figure 5. HB-GAM but not NGF overcomes the CSPG-dependent inhibition of neurite growth in PC12 cells. Cells were plated on the substrate precoated with aggrecan (AGG) or with AGG + or with AGG + HB-GAM (HB). Immunoglobulin G (IgG) was used for the unspecific control precoating. All samples were treated with NGF (100 ng/ml). In (B) neurite length was measured in live cell images obtained 1 day after plating. The scale bar in (A) is 20 μm.

Figure 6. HB-GAM enhances recovery of locomotor functions after spinal cord injury. Motor function was assessed in mice under control (vehicle) or HB-GAM treatment following cervical spinal cord hemisection. Starting from week 6 the animals treated with HB-GAM demonstrated significant improvement in frontlimb use as compared to the vehicle-treated controls (**A**). * $p<0.05$,

Mann-Whitney test. During the weeks 7–9 after trauma the vehicle-treated animals needed significantly more time to climb up on vertical screen as compared to the sham-operated and the HB-GAM-treated mice (**J**). *p<0.05 in Newman-Keuls post-hoc test for comparison of the vehicle-treated group with the sham group; # p<0.05 in Newman-Keuls post-hoc test for comparison of the vehicle-treated group with HB-GAM-treated mice. HB-GAM was injected within 30 min after injury at 1mg/ml in 7 μl injection volume. In sham animals laminectomy was performed but the spinal cord remained intact (**B**).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a use of HB-GAM or a fragment thereof for treating injuries of the nervous system in a subject by increasing the interaction of chondroitin sulphate proteoglycan (CSPG) to the receptor protein tyrosine phosphatase sigma (RPTPσ). Specifically, the present invention relates to a use of HB-GAM as anti-CSPG therapy in diseases in which the CSPGs restrict neuronal regeneration or maintenance. According to one embodiment of the invention HB-GAM promotes neurite outgrowth and neuronal attachment on GSPG-coated substrate by overcoming the inhibitory action of CSPG. In another embodiment regeneration of the dendritic tree is shown in vivo and by in vitro assays upon CSPG matrices modified by HB-GAM. As disclosed in the invention, HB-GAM actually changes the CSPG matrix from regeneration inhibiting to regeneration activating structure (Figure 4). By contrast, nerve growth factor (NGF) that is an extremely potent neurite-outgrowth promoting factor does not change the matrix from inhibitory to activating structure (Figure 5).

As used herein, heparin-binding growth-associated molecule (HB-GAM, also known as pleiotrophin) is an 18 kDa protein, which was first isolated from rat brain based on its neurite outgrowth promoting activity. Its expression in nervous tissue is developmentally regulated with the highest expression during the perinatal period (Rauvala, 1989). Neurite outgrowth induced by HB-GAM is dependent on the neuronal cell surface heparan sulphate proteoglycan N-syndecan, whilst the chondroitin sulfate proteoglycan RPTP beta/zeta (receptor-type tyrosine phosphatase beta/zeta) has also been implicated in the receptor mechanism of HB-GAM (Rauvala *et al.*, 2000).

HB-GAM contains two thrombospondin type 1 repeat (TSR) domains, which fold independently and do not interact with one another in solu-

tion (Raulo *et al.*, 2005). This TSR is found in a larger family of extracellular matrix-associated and cell surface proteins, such as thrombospondins 1 and 2, F-sponding, mindin and semaphorins F and G. A common feature for this superfamily is that their function in cell surface and matrix binding is dependent on heparan-type polysaccharides.

The individual domain structures from HB-GAM have been found to bind weakly to heparin/heparin sulphate and failed to influence neurite outgrowth and plasticity. However, when both TSR domains were present, heparin/heparin sulphate binding was observed together with an interaction with hippocampal neurons (Raulo *et al.*, 2005). HB-GAM contains a high proportion of basic amino acid residues, consisting of lysine clusters, at its N- and C- terminals. These lysine-rich tails have not been implicated in the binding interactions.

In addition to binding heparan sulphate proteoglycans, the chondroitin sulphate proteoglycans, neurocan and phosphacan, have also been demonstrated to bind to HB-GAM with high affinity (K_d = 0.3–8nM). Almost all of this binding (>80%) is abolished by chondroitinase treatment to remove the glycosylaminoglycan chains, suggesting that chondroitin sulphate is the major mediator of these binding interactions (Milev *et al.*,1998).

As used herein a “fragment” refers to any part of HB-GAM that is long enough to have the desired activity to reverse the inhibitory effects of the CSPGs. In a preferred embodiment of the invention, HB-GAM or a fragment thereof is native, i.e. a protein in its natural state, unaltered by heat, chemicals, enzyme action, or the exigencies of extraction. Preferably HB-GAM of the present invention is a recombinant protein. Recombinant proteins of the invention may be produced in a generally known manner. A polynucleotide fragment comprising the HB-GAM gene is isolated, the gene is inserted under a strong promoter into an expression vector, the vector is transformed into suitable host cells and the host cells are cultivated under conditions provoking production of the enzyme. Methods for protein production by recombinant technology in different host systems are well known in the art (Sambrook *et al.*, 2001; Coen, 2001; Gellissen, 2005). Preferably the recombinant proteins are produced in a baculovirus expression system.

As used herein, the term “neurite growth” or “neurite outgrowth includes the process by which axons or dendrites extend from a neuron. The outgrowth can result in a new neuritic projection or in the extension of a previ-

ously existing cellular process. Neurite outgrowth may include linear extension of an axonal process by five cell-diameters or more. "Central nervous system (CNS) neurons" include the neurons of the brain, the cranial nerves and the spinal cord. The invention relates not only to CNS neurons but also to peripheral neurons that make projections (axons) in CNS, for instance dorsal root ganglion neurons.

According to the present invention HB-GAM is used in treating neuronal disorders, which include disease, disorder, or condition directly or indirectly affecting the normal functioning or anatomy of a subject's nervous system. The disorder may be a neuronal injury, which can be acute or chronic. Examples of acute injury are those that results from surgery, trauma, compression, contusion, transection or other physical injury, vascular pharmacologic or other insults including hemorrhagic or ischemic damage. Chronic neuronal injury may result from repetitive stress, inflammation/oxidative stress within a neural tissue caused by disease, neurodegenerative or other neurological diseases.

According to the present invention HB-GAM is beneficial in all diseases where the CSPG matrix is inhibitory for regeneration or maintenance of axons and dendrites. In one embodiment of the present invention the disease is a neuronal injury selected from a group consisting of neurodegenerative diseases, traumatic brain injury, spinal cord injury, multiple sclerosis (MS), amyotropic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, stroke, peripheral nerve injury, eye injury and skin burn Preferably the neuronal injury is TBI or SCI.

"Traumatic brain injury, TBI" as used herein includes the condition in which a traumatic blow to the head causes damage to the brain or connecting spinal cord, with or without penetrating the skull. It relates more specifically to the actual mechanical damage that occurs at the type of trauma, such as shearing, tearing and stretching of axons, neurons and blood vessels. Usually, the initial trauma can result in expanding hematoma, subarachnoid hemorrhage, cerebral edema, raised intracranial pressure, and cerebral hypoxia, which can, in turn, lead to severe secondary events due to low cerebral blood flow.

"A spinal cord injury, SCI" as used herein is damage to any part of the spinal cord or nerves at the end of the spinal canal. It often causes permanent changes in strength, sensation and other body functions below the site of

the injury. The spinal cord injury may be a complete severing of the spinal cord, a partial severing of the spinal cord, or a crushing or compression injury of the spinal cord. Spinal cord injury SCI proceeds over minutes, hours, days and even months after the initial traumatic insult and can lead to significant expansion of the original damage. These secondary events are a consequence of delayed biochemical, metabolic and cellular changes, which are initiated by the primary injury, and includes inflammation, free radical induced cell death and glutamate excitotoxicity.

Axonal sprouting, from surviving neurons, is associated with spontaneous motor and sensory recovery following TBI and SCI. Although the CNS has a limited capacity to regenerate, spontaneous pericontusional axon sprouting does take place approximately 1–2 weeks after trauma. However, this process typically fails due to an inhibitory axonal environment promoted by chondroitin sulphate proteoglycans (CSPGs). Astrocytes, at the site of injury, produce CSPGs, beyond which the axons cannot regenerate (Silver and Miller, 2004). Inhibition of CSPG activity represents one potential approach to neuroregeneration, following either TBI or SCI. Evidence in support of this theory has been provided through the use of chondroitinase ABC (ChABC, an enzyme that degrades CSPGs) at the site of trauma in rodent models of TBI and SCI. ChABC treatment resulted in an enhanced and prolonged sprouting response with an increase in sensory, motor and autonomic function (Harris *et al.*, 2010, Starkey *et al.*, 2012).

Multiple sclerosis (MS) is a chronic immune-mediated disease that is characterized by demyelinating and degenerative processes within the central nervous system. MS potentially requires symptomatic and disease-modifying therapies. Numerous symptoms such as fatigue, spasticity, depression, bowel and bladder dysfunction, pain, and impaired mobility are associated with the neurologic damage that results from MS. Several therapies e.g. modafinil, dalfampridine, baclofen, diazepam, gabapentin, opioids are used for symptomatic treatment of disability and symptoms, but these do not improve disease outcome. Intravenous corticosteroids are used in the management of MS exacerbations, but do not appear to affect the degree of improvement from acute exacerbations. A more definitive therapy for MS should reduce relapse rate, prolong remission, limit the onset of new MS lesions, and postpone the development of long-term disability. There are currently available MS disease-

modifying therapies, but thus far no beneficial agent has been established in primary-progressive MS.

Amyotrophic lateral sclerosis (ALS), also referred to as motor neuron disease and Lou Gehrig's disease, is the most common form of the motor neuron diseases. The disorder is characterized by rapidly progressive weakness, muscle atrophy, twitching and spasticity, difficulty with speaking and swallowing and a decline in breathing ability. The defining feature of ALS is the death of both upper and lower motor neurons in the motor cortex of the brain, the brain stem, and the spinal cord. The disease has its onset usually in midlife and leads to death within 3–5 years from diagnosis, usually due to respiratory failure. Once diagnosed, only 10% of patients survive for longer than 10 years. In the US, there are approximately 30,000 ALS sufferers, with 5,000 new cases each year. Although there are currently several ongoing clinical trials for novel ALS treatments, there is no curative therapy for ALS and palliative care remains the most important means of treatment.

"Chondroitin sulphate proteoglycans, CSPGs" as used herein, represent a varied class of complex extracellular matrix macromolecules. They share a general molecular structure comprising a central core protein with heavily sulphated sugar side chains, usually glycosaminoglycans (GAGs), attached through covalent bonds. The GAG side chains are of different lengths, which partially define the different CSPGs e.g. aggrecan (CSPG1), versican (CSPG2), neurocan (CSPG3), brevican (CSPG7) and phosphacan. Some of these aggrecans share similar N-terminal and C-terminal domains.

CSPGs play an active role in the neural development of postnatal babies, acting as guidance cues for developing growth cones. Growing axons are found to avoid CSPG dense areas. Similarly, CSPGs found near and around embryonic roof plates inhibit axon elongation through the spinal cord and direct the axons in an alternative direction. CSPGs absent on roof plates were found to attract axonal elongation (Snow *et al.*, 1990).

The present invention discloses that HB-GAM changes the CSPG matrix from regeneration inhibiting to regeneration activating structure. As used herein the CSPG matrix means the type of extracellular matrix that expresses chondroitin sulphate proteoglycans. The extracellular matrix (ECM) provides a number of critical functions in the CNS, contributing both to the overall structural organization of the CNS and to control of individual cells. At the cellular level, the ECM affects its functions by a wide range of mechanisms, including

providing structural support to cells, regulating the activity of second messenger systems, and controlling the distribution and local concentration of growth and differentiation factors. The brain extracellular matrix has trophic effects on neuronal cells and affects neurite outgrowth. The GSPG-coated substrate is a simplified experimental model used in experimental studies. It can be made for example by coating a CSPG, such as e.g. aggregan or neurocan, on a tissue culture well.

Only recently has a receptor, protein tyrosine phosphatase sigma (RPTPσ) been identified for the CSPGs (Shen *et al.*, 2009). It was previously demonstrated that disruption of the RPTPσ gene enhanced regeneration in sciatic nerves, but the mechanism by which this was achieved was unclear. An interaction was demonstrated between both neurocan and aggrecan with RPTPσ, which was dependent upon the chondroitin sulphate side chains. RPTPσ has a conserved, positively charged, region in the first immunoglobulin domain that is known to interact with heparin sulphate (Arisescu *et al.*, 2002). Mutation of a cluster of four lysine residues in this area to alanines, reduced binding of CSPG to RPTPσ to background levels.

A functional effect of the RPTPσ and CSPG interaction, has been demonstrated by using dorsal root ganglion (DRG) neurons that constitutively express high levels of RPTPσ. Wild-type DRG neurons were cultured, in parallel with those from mice with a targeted gene disruption of RPTPσ, in the presence of a CSPG mixture. Control DRG neuron outgrowth was reduced by approximately 50% in the presence of the CSPG mixture, but had far less effect on those neurons from RPTPσ$^{-/-}$ mice. When the DRG neurons were challenged with purified neurocan, similar results were observed. The role of RPTPσ in inhibiting regeneration of axons and dendrites, through CSPG interaction, following injury has also been demonstrated using appropriate *in vivo* models. In PTPσ$^{-/-}$ mice, following a dorsal column crush injury, axonal extension into the lesion penumbra was significantly improved, compared to controls (Shen *et al.*, 2009). The ability of corticospinal tract (CST) axons to regenerate after spinal hemisection and contusion injury in PTPσ$^{-/-}$ mice has also been assessed. Damaged CST fibers, in PTPσ$^{-/-}$ mice, were found to regenerate and extend for long distances after injury to the thoracic spinal cord. In contrast, no long distance axon regeneration of CST fibers was seen after similar lesions in wild-type mice (Fry *et al.*, 2010).

RPTPσ is also known to bind heparan sulphate proteoglycans (HSPGs), which are similar to the CSPGs, in that they contain a protein core with heavily sulphated, negatively charged, sugar side chains. Although CSPGs, through RPTPσ, have been shown to have an inhibitory effect on neuronal outgrowth, HSPGs, also acting through RPTPσ, have been shown to strongly promote neuronal growth (Coles *et al.*, 2011). Both CSPGs and HSPGs bind to a common site on RPTPσ and these differential effects were rationalised based on RPTPσ oligomerisation status. Heparan sulphate GAGs were found to induce oligomerisation of RPTPσ fragments, but chondroitin sulphate GAGs did not support clustering. HSPGs and CSPGs differ in the composition of their GAG chains; sulphate groups are more evenly distributed in CSPGs, whereas HSPGs contain areas of high sulphation. Receptor oligomerisation may cause microdomains with high phosphotyrosine levels and support neuronal extension, which CSPGs are able to disrupt and hence inhibit axon growth (Coles *et al.*, 2011).

In the present invention the HB-GAM is used for treating neuronal injuries. Moreover, HB-GAM may be used for promoting dendrite regeneration. The present invention discloses that HB-GAM increases dendritic density (Figure 3) thus promoting dendritic regeneration. Treating and treatment refers to increasing, enhancing and promoting neuron regeneration and/or nerve growth in the presence of a neuronal injury. Treating and treatment encompass both therapeutic and prophylactic treatment regimens.

In a preferred embodiment of the present invention a subject is a human or and animal.

The present invention also relates to a method of promoting neuronal outgrowth and/or neural regeneration. This is achieved by contacting the neuron with a therapeutically effective amount of HB-GAM that increases CSPG binding to RPTPσ, to thereby induce neuronal outgrowth. The contacting can occur *in vitro* or *in vivo* to the neuronal cell. *In vitro*, HB-GAM can be added to a cell culture containing the neuron. *In vivo* HB-GAM can be administered to a subject, such that an effective amount of it comes in contact with the neuron to thereby induce the neuronal outgrowth. In one embodiment, the neuron is a central nervous system neuron.

The term "administered" or "administering" to a subject includes dispensing, delivering or applying the agent to the subject by any suitable route for delivery of the agent to a site in the body where neuronal outgrowth is

desired. HB-GAM may be delivered according to any known method in the art. These include, without limitation, subcutaneous, intramuscular, transdermal, intravenous, oral, sublingual, nasal, rectal and topical administrations. PEGylation of HB-GAM may be used to modify pharmacokinetics of a drug and its interaction with living tissues. PEGylation of HB-GAM may enhance its ability to promote neurite growth on CSPG-substrate in cortical neurons. HB-GAM can be administered directly to the nervous system (particularly to the site of injury), intracranially, intraspinally, intracerebroventricularly, intravenously, topically or intrathecally, e.g. into a chronic lesion of a neurodegenerative disease or at the site(s) of traumatic injury.

One preferred method of administration is to introduce HB-GAM to the site of injury in a surgical operation. Another preferred method of administration is to introduce HB-GAM to the site of injury without open surgery, e.g. by injection. Preferably HB-GAM is administered via direct injection or HB-GAM-bound carrier (scaffold) material implantation or viral vector expressing HB-GAM. The scaffolds may include natural components (aggrecan, neurocan, hyaluronic acid) or artificial materials. The route of administration and the dosage regimen will be determined by skilled clinicians, based on factors such as the exact nature of the condition being treated, the severity of the condition, and the age and general physical condition of the patient.

In one embodiment, administration is to thereby contact injured and/or non-injured neurons proximal to the injury site. In one embodiment, administration is such as to deliver the agent across the blood brain barrier. The agent of the present invention may be formulated as part of pharmaceutical compositions comprising one or more of the specific agents.

The term "effective amount" or "therapeutically effective amount" refers to the amount of an active agent sufficient to induce a desired biological result e.g. promotion and/or restoration of neuronal regeneration and/or neurite growth. That result may be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system.

Neuronal outgrowth induced by the methods described herein can be determined by a variety of methods, such as by determination of the formation of axons (e.g., detecting the formation of neuronal branching microscopically or by showing cytoplasmic transport of dyes). Neuronal outgrowth can also be detected by determination of the formation of neural connectivity. Outgrowth can also be determined by an increase or a restoration of function

of the neuron. Neuronal function can be measured by standard assays such as detection of action potential or nerve impulse conduction by standard assays.

EXAMPLES

Materials and Methods

Rat (Rattus norvegicus) HB-GAM sequence was used for baculovirus expression of recombinant HB-GAM protein. Bovine aggrecan was from Sigma. The DNA sequence (the template cDNA clone MGC:63375 IMAGE:6834684) of the CSPG-binding domain of mouse RPTPσ was used for Fc-tagged RPTPσ protein production.

Neurite outgrowth assay

Cortical or hippocampal neurons from E17 rat embryos were plated at 50,000 cells/cm^2 on plastic cell culture plates. Plates were pre-coated with aggrecan or aggrecan+IgG (both proteins at 10 μg/ml) or with neurocan (at 5 μg/ml). HB-GAM (10μg/ml) was pre-coated together with aggrecan or added to culture medium when plating neurons. HB-GAM was precoated at 25 μg/ml together with neurocan (5 μg/ml). Cells were cultured for 1.5–3.5 days and after that images of cell cultures were taken using phase contrast microscope with x20 objective. Neurite length was quantified using ImagePro software. Mouse neurons were immunostained with anti-tubulin βIII antibodies and imaged using fluorescent microscope.

RPTPσ binding assay

The Fc-tagged extracellular domain of RPTPσ was immobilized on protein G-coated plates. Binding of biotinylated aggrecan to RPTPσ was quantified via colorimetric assay using streptavidin-conjugated horse radish peroxidise. Experiments were done by using protein G coated 96 well plates (Pierce, prod. #15133). Wells were first washed briefly with PBS, 0.05%Tween-20 solution. RPTPσ wild type and mutant FC-fusion proteins were diluted into 2 μg/ml solution in buffer containing PBS, 1%BSA, 0.05% Tween-20. 150 μl of protein solution was added into the wells and plates were left for shaking at +RT for 1 hour. Wells were washed 3 times 5 minutes with 200 μl of PBS, 0.05% Tween-20. During the coating and washing steps biotinylated aggregan was diluted to 5 μg/ml with different amounts of HB-GAM (0-20 μg/ml) in solution containing PBS, 1%BSA, 0.05% Tween-20. Aggregan and HB-GAM was left together with

shaking for 30 minutes before applying 150 μl of this solution into the washed RPTPσ coated wells. Wells were left for shaking at +RT for 1 hour. Wells were washed 3 times 5 minutes with 200 μl of PBS, 0.05% Tween-20. Streptavidin−Peroxidase Polymer (SIGMA, S2438) was diluted 1/10 000 into PBS, 1%BSA, 0.05% Tween-20 solution and 150 μ was applied into the washed wells which were left for shaking +RT for 30 minutes. Wells were washed 3 times 5 minutes with 250 μl of PBS, 0.05% Tween-20. Detection of bound Streptavidin-Peroxidase Polymer was done by adding 200 μl of OPD (*o*-Phenylenediamine dihydrochloride, SIGMA P9187) substrate into the wells and was measured by reading the absorbance at 450 nm (A450).

Cortical prick trauma model (TBI model)

TBI was applied in adult mice as a 2 mm deep needle prick to the parietal lobe of the brain cortex. Transgenic mice expressed YFP under Thy1 promoter in dendrites of neurons from cortical layers 4 and 5. Chronic cranial window was used to follow dendrite regeneration using two photon microscopy on live animals over 40 days after injury. HB-GAM (1.5 μl, 1 mg/ml) was injected within 30 min after injury directly to the injury site. Immunoglobulin G was used in parallel as a negative control.

Example 1 – HB-GAM promotes neurite outgrowth on aggrecan or on neurocan substrate *in vitro*

Chondroitin sulphate proteoglycan aggrecan prevents attachment and neurite growth in embryonic rat cortical neurons in culture. HB-GAM overcame this inhibitory action of aggrecan, promoting neuronal attachment and neurite growth on the aggrecan-coated substrate *in vitro* (Figure 1A).

Neurocan precoated at 5 μg/ml inhibits neurite growth and cell attachment in dissociated culture of rat cortical neurons. HB-GAM precoated at 25 μg/ml together with neurocan (5 μg/ml) overcomes the inhibitory effect of neurocan and promotes neuronal attachment and neurite growth (Figure 1B).

Example 2 – HB-GAM increases aggrecan binding to RPTPσ

Aggrecan binding to RPTPσ was assessed via ELISA using Fc-tagged RPTPσ and biotinylated aggrecan. Binding was quantified using streptavidin-bound horse radish peroxidase. Mutant RPTPσ, with a defective CSPG-binding site, was used as a negative control. HB-GAM was found to increase aggrecan binding to wild type RPTPσ, by two fold, in a concentration-

dependent manner. By contrast, aggrecan binding to mutant RPTPσ was not affected by HB-GAM (Figure 2).

Example 3 – cortical prick trauma model (TBI model) with HB-GAM treatment

TBI was applied in adult mice as a 2 mm deep needle prick to the parietal lobe of the brain cortex. Transgenic mice expressed YFP under Thy1 promoter in dendrites of neurons from cortical layers 4 and 5. Chronic cranial window was used to follow dendrite regeneration using two photon microscopy on live animals over 40 days after injury. HB-GAM (1.5 μl, 1 mg/ml) was injected within 30 min after injury directly to the injury site. Immunoglobulin G was used in parallel as a negative control. HB-GAM increased dendritic density (i.e. dendritic regeneration) at the injury site at 20 days after injury, as compared to IgG control (Figure 3).

Example 4 – HB-GAM turns CSPG into a potent activator of neurite growth

Combination of soluble HB-GAM with precoated aggrecan becomes potent activator of neurite growth (Fig.4). Embrionic cortical neurons were cultured for 3.5 days on unprecoated plastic (A, C, E) or on aggrecan (precoated at 10 μg/ml) (B, D, E). HB-GAM (10 μg/ml) was added to culture medium when plating cells in (C, D). Example 4 shows that in the presence of HB-GAM in the medium, CSPG is even required for neurite outgrowth. So, depending on the conditions, CSPG can be either a negative signal or a positive signal.

Example 5 – HB-GAM but not NGF overcomes the CSPG-dependent inhibition of neurite growth in PC12 cells

Cells were plated on the substrate precoated with aggrecan (AGG) or with AGG + or with AGG + HB-GAM (HB). Immunoglobulin G (IgG) was used for the unspecific control precoating. All samples were treated with NGF (100 ng/ml). In (B) neurite length was measured in live cell images obtained 1 day after plating. The scale bar in (A) is 20 μm. NGF clearly fails to overcome the inhibitory effect of aggrecan on neurite growth (Figure 5).

Example 6 – HB-GAM enhances recovery of locomotor functions after spinal cord injury

Motor function was assessed in mice under control (vehicle) or HB-GAM treatment following cervical spinal cord hemisection (Figure 6). Starting from week 6 the animals treated with HB-GAM demonstrated significant improvement in frontlimb use as compared to the vehicle-treated controls (**A**). * $p<0.05$, Mann-Whitney test. During the weeks 7-9 after trauma the vehicle-treated animals needed significantly more time to climb up on vertical screen as compared to the sham-operated and the HB-GAM-treated mice (**J**). *$p<0.05$ in Newman-Keuls post-hoc test for comparison of the vehicle-treated group with the sham group; # $p<0.05$ in Newman-Keuls post-hoc test for comparison of the vehicle-treated group with HB-GAM-treated mice. HB-GAM was injected within 30 min after injury at 1 mg/ml in 7 μl injection volume. In sham animals laminectomy was performed but the spinal cord remained intact (**B**).

It will be obvious to a person skilled in the art that, as the technology advances, the inventive concept can be implemented in various ways. The invention and its embodiments are not limited to the examples described above but may vary within the scope of the claims.

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CLAIMS

1. HB-GAM or a fragment thereof for use in treating neuronal injuries in a subject by increasing the interaction of chondroitin sulphate proteoglycan (CSPG) to receptor protein tyrosine phosphatase sigma (RPTPσ).

2. HB-GAM for use according to claim 1, wherein a neuronal injury is selected from a group consisting of neurodegenerative diseases, traumatic brain injury, spinal cord injury, multiple sclerosis (MS), amyotropic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, stroke, eye injury and skin burn.

3. HB-GAM for use according to claims 1 or 2, wherein a subject is a human or an animal.

4. HB-GAM for use according to anyone of the previous claims, wherein treating neuronal injuries comprises administering to the subject an effective amount of HB-GAM.

5. HB-GAM for use according to claim 4, wherein said administration is intracranial or intracerebrospinal or intravenous or topical.

6. HB-GAM for use according to claim 4, wherein HB-GAM is administered via direct injection or HB-GAM-bound carrier material implantation or viral vector expressing HB-GAM.

7. A method of promoting outgrowth of a neuron and/or neuronal regeneration comprising contacting the neuron with HB-GAM or a fragment thereof that increases the interaction of chondroitin sulphate proteoglycan (CSPG) to receptor protein tyrosine phosphatase sigma (RPTPσ).

8. A method of claim 7, wherein the neuron is *in vivo*.

9. A method of claim 7, wherein the neuron is *in vitro*.

10. A method of any one of claims 7 to 9, wherein said HB-GAM is native.

11. A method of any one of claims 7 to 9, wherein said HB-GAM is recombinantly produced.

12. A method of claim 8, wherein contacting the neuron in vivo comprises administering to the subject an effective amount of HB-GAM.

13. The method of claim 12, wherein said administration is intracranial or intracerebrospinal or intravenous or topical.

14. The method of claim 12 or 13, wherein HB-GAM is administered via direct injection or HB-GAM-bound carrier material implantation or viral vector expressing HB-GAM.

15. The method of any one of claims 7 to 14, wherein the subject has experienced a brain injury selected from a group consisting of neurodegenerative diseases, traumatic brain injury, spinal cord injury, multiple sclerosis (MS), Alzheimer's disease, Parkinson's disease, stroke, eye injury and skin burn.

16. The method of claim 12, wherein the subject has experienced a spinal cord injury or traumatic brain injury.

Figure 1A

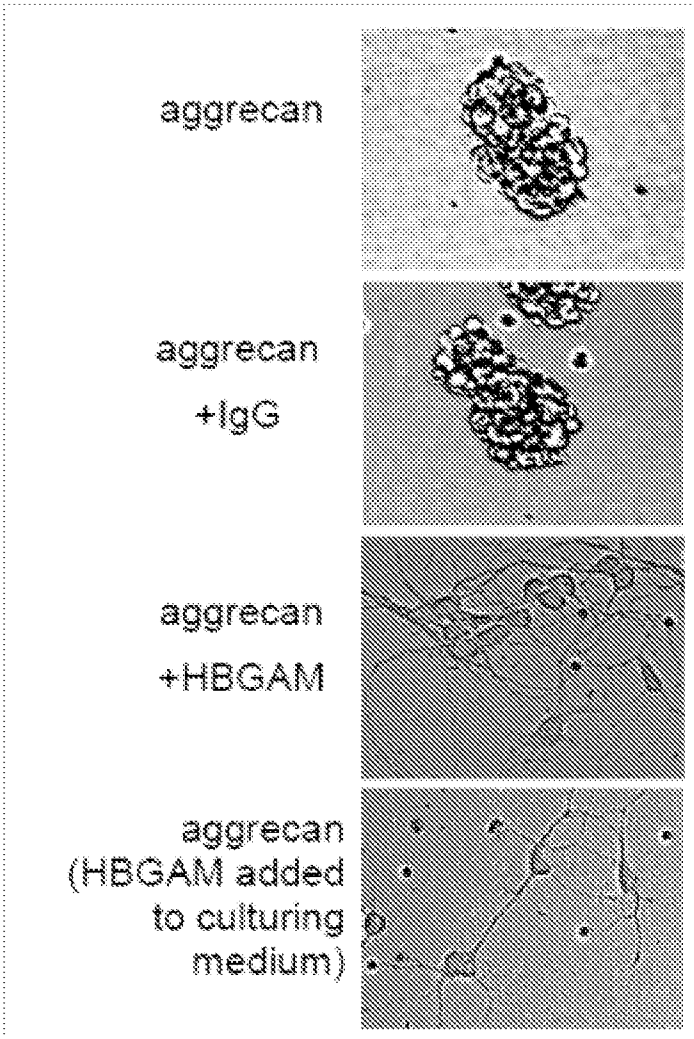


Figure 1B

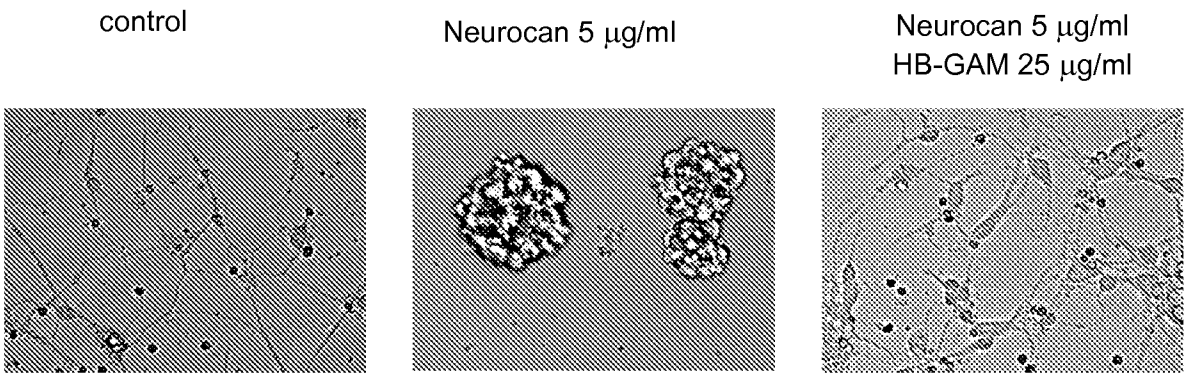


Figure 2

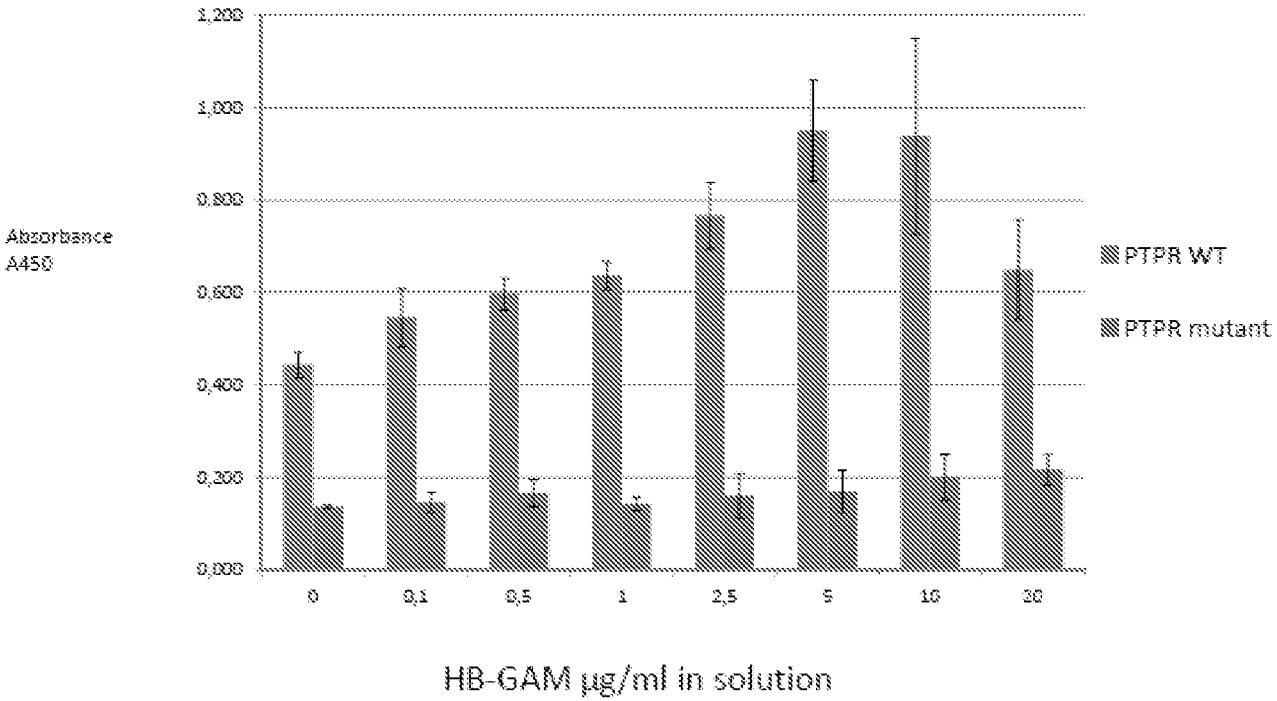
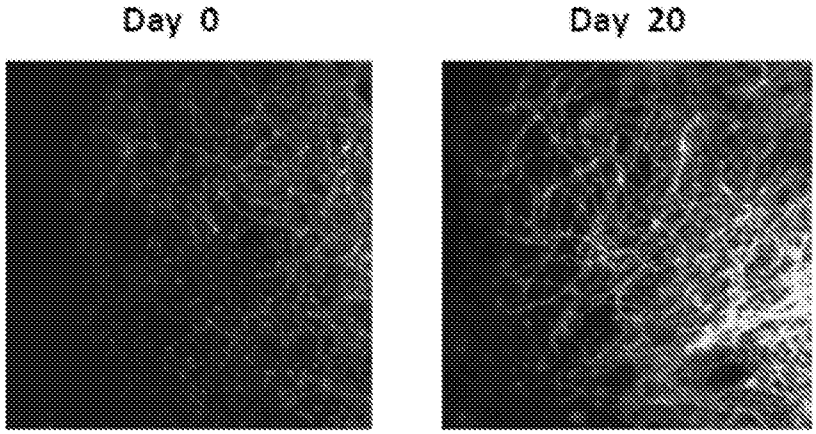

1,200
1,000
0,800
0,600
0,400
0,200
0,000
Absorbance
A450
0
0,1
0,5
1
2,5
5
10
30
HB-GAM µg/ml in solution
PTPR WT
PTPR mutant

Figure 3


Day 0
Day 20

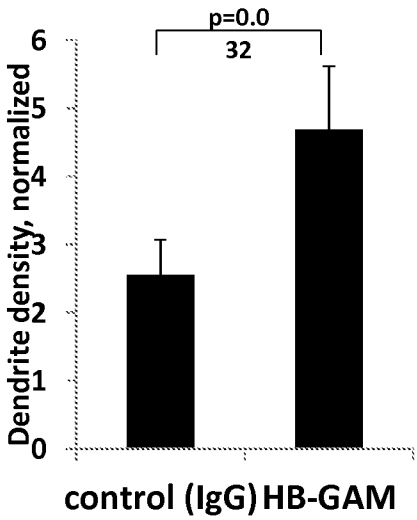

p=0.0
32
Dendrite density, normalized
0
1
2
3
4
5
6
control (IgG)
HB-GAM

Figure 4

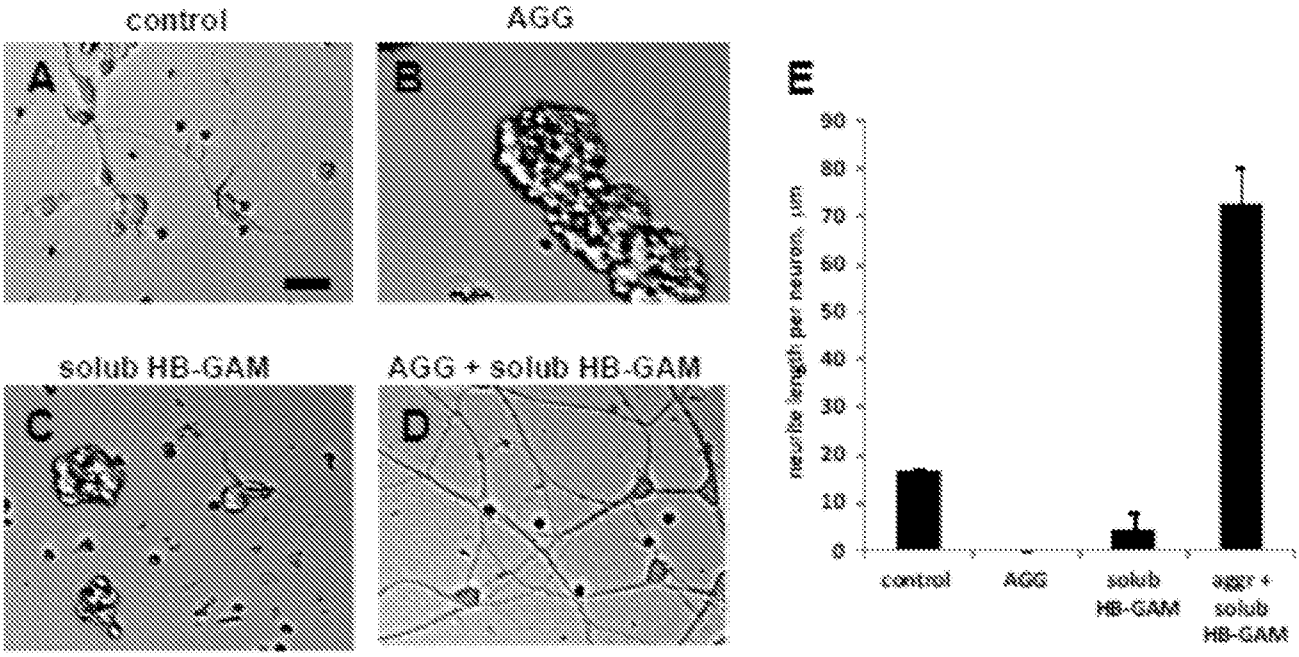

control
AGG
A
B
solub HB-GAM
AGG + solub HB-GAM
C
D
E
control
AGG
solub HB-GAM
aggr + solub HB-GAM

Figure 5

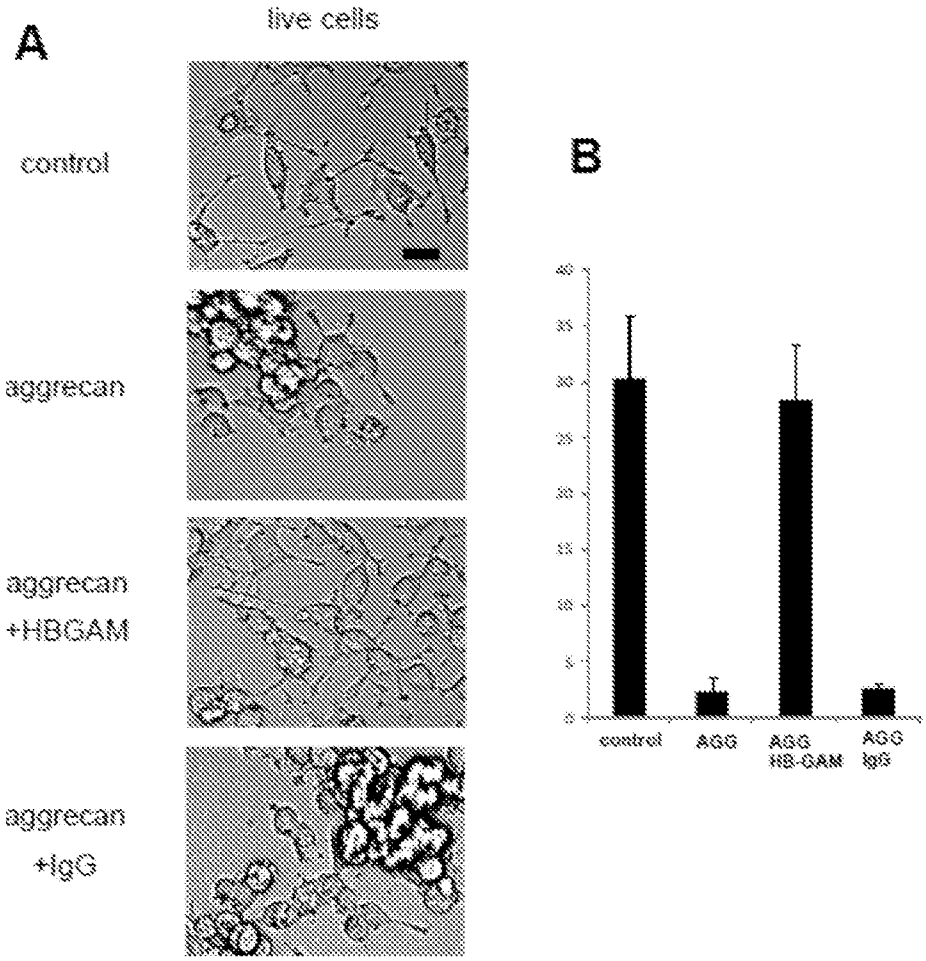
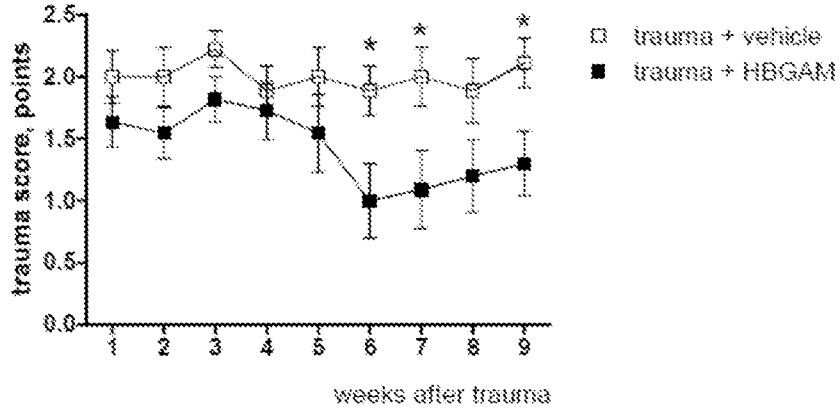
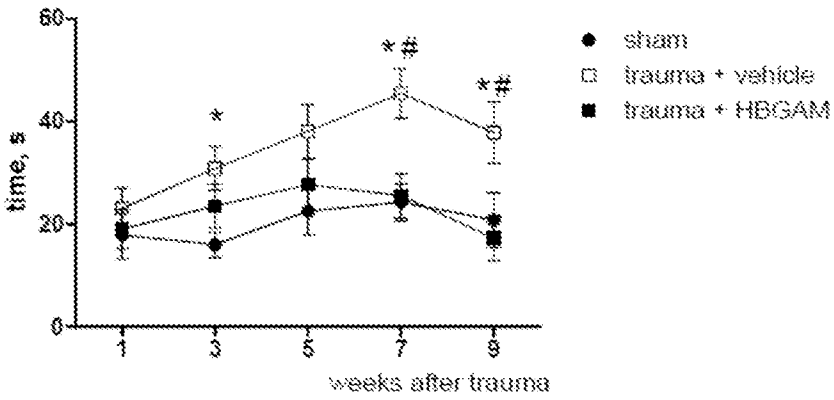

A
live cells
control
aggrecan
aggrecan
+HBGAM
aggrecan
+IgG
B
control
AGG
AGG
HB-GAM
AGG
IgG

Figure 6

A


trauma score, points
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
weeks after trauma
trauma + vehicle
trauma + HBGAM

B


time, s
60
40
20
0
1
3
5
7
9
weeks after trauma
sham
trauma + vehicle
trauma + HBGAM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/050490

A. **CLASSIFICATION OF SUBJECT MATTER**

See extra sheet

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)

IPC: A61K, C07K, A61P

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

FI, SE, NO, DK

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

EPO-Internal, WPI, XPESP, XPESP2, XPOAC, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006122116 A1 (HOKE AHMET [US] et al.) 08 June 2006 (08.06.2006) paragraphs [0005], [0017]-[0018]; [0021], [0031]; claims 1, 9, 13, 19, 20	1-6
X	EP 0474979 A1 (AMERICAN CYANAMID CO [US]) 18 March 1992 (18.03.1992) page 5, lines 20-25; page 6, line 41 - page 7, line 12; claims 8 and 10	7, 9-11
A	US 2006193831 A1 (MARCHIONINI DEANNA [US] et al.) 31 August 2006 (31.08.2006) abstract	1-7, 9-11

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2014 (30.09.2014)	03 October 2014 (03.10.2014)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/050490

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG, YT. et al. Upregulation of heparin-binding growth-associated molecule after spinal cord injury in adult rats. Acta Pharmacologica Sinica, 2004, Vol. 25, No. 5, pages 611-616 the whole document, esp. page 614, right column, last paragraph - page 615, left column, first paragraph	1-7, 9-11
A	US 2007254842 A1 (BANKIEWICZ KRYSTOF S [US]) 01 November 2007 (01.11.2007) abstract; claims 1, 19, 21	1-7, 9-11
A	WO 2012004291 A1 (NANOLOGICA AB [SE]) 12 January 2012 (12.01.2012) abstract; claims 1, 10, 12-17	1-7, 9-11
A	EP 1057489 A1 (MEIJI MILK PROD CO LTD [JP]) 06 December 2000 (06.12.2000) abstract; paragraphs [0020]-[0022], [0044]-[0045]	1-7, 9-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/050490

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

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

1. ☒ Claims Nos.: 7 (partly), 8, 10 (partly), 11 (partly), 12-16
because they relate to subject matter not required to be searched by this Authority, namely:
Methods for treatment of the human or animal body by therapy (Rule 39.1 (iv) PCT).

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/050490

CLASSIFICATION OF SUBJECT MATTER

IPC
A61K 38/18 (2006.01)
C07K 14/475 (2006.01)
A61P 25/00 (2006.01)

INTERNATIONAL SEARCH REPORT
Information on Patent Family Members

International application No.
PCT/FI2014/050490

Patent document cited in search report	Publication date	Patent family members(s)	Publication date
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		CA 2304956 C	03/12/2013

INTERNATIONAL SEARCH REPORT
Information on Patent Family Members

International application No.
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