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(54) Title: TREATMENT OF SENSORINEURAL HEARING LOSS

(57) Abstract: The present disclosure relates to agents which act to stimulate auditory neuron differentiation. The present disclosure also relates to the stimulation of neurite production of existing neurons using the agents. Included in the present disclosure are methods of treating neurosensory deafness comprising administering agents which are capable of stimulating auditory neuronal differentiation and/or neurite production. Exemplary agents include, for example, Protease Activated Receptor (PAR) agonists.

TREATMENT OF SENSORINEURAL HEARING LOSS

The present disclosure relates to agents which act to stimulate auditory neuron differentiation. The present disclosure also relates to the stimulation of neurite production of existing neurons using the agents. Included in the present disclosure are methods of treating neurosensory deafness which comprise administering agents which are capable of stimulating auditory neuronal differentiation and/or neurite production. In embodiments of the invention, the agents include for example proteases and receptor agonists e.g. PAR agonists. Also included in the present disclosure are devices and compositions for the treatment of neurosensory deafness, as well as other subject matter.

BACKGROUND

In the United States and European Community alone, there are an estimated 21 million people with significant conductive hearing loss, whilst there are over 90 million suffering from moderate to severe sensorineural hearing loss. Of these, more than 65 million hearing-impaired persons are without treatment.

Neurosensory deafness (also known as sensorineural deafness) involves damage of the sensory hair cells and their associated neurons. Sensory hair cells are the sensory receptors of the mammalian auditory system and are located in the cochlea of the inner ear. The hair cells are innervated by the neurons of the cochlear ganglion. There are various different causes of sensorineural deafness or hearing loss, including for example, old age, genetic (including hereditary) conditions, congenital conditions and disorders caused by infectious agents e.g. meningitis, mumps, measles, as well as ototoxic drugs.

Currently, there are only very limited therapeutic interventions for patients suffering from sensorineural hearing loss and deafness. One such therapy is the cochlear implant. Cochlear implants typically include (1) a microphone which picks up sound from the environment, (2) a speech processor which selects and arranges sounds picked up by the microphone, (3) a transmitter and receiver/stimulator, which receive signals from the speech processor and convert them into electric impulses and (4) an electrode array which collects the impulses from the stimulator and sends them to different regions of the auditory nerve. Cochlear implants provide auditory cues by bypassing the damaged or missing hair cells to electrically stimulate residual spiral ganglion neurons directly.

Manufacturers of cochlear implants include companies such as Cochlear™ Inc., Advanced Bionics, Med-El and Neurelec.

Hair cells are innervated by the neurons of the cochlear ganglion. Since spiral ganglion neurons are the target cells of the cochlear implant, their ongoing loss, as well as the other pathological changes that occur in deafness, may reduce the benefits that patients can derive from these devices. Indeed, animal studies have been carried out which indicate that the efficacy of a cochlear implant may be compromised by ongoing spiral ganglion neuron degeneration (Pfingst BE et al; Relation of psychophysical data to histopathology in monkeys with cochlear implants. *Acta Otolaryngol.* 1981;92:1–13).

Also used to treat deafness are brain stem implants. Brain stem implants are particularly used for those profoundly deaf individuals who do not have cochlear nerves and so are not suitable for cochlear implants. The implant stimulates the auditory pathway through the cochlear nucleus and typically includes electrodes that are placed into the brain, such that the cochlear nerve is bypassed. Brain stem implants carry the risk of side-effects such as leakage of cerebrospinal fluid and meningitis and brain stem implant technology is not widely used at present, at least in the UK.

There is therefore a need for therapies which can help to treat or alleviate hearing loss and deafness in those individuals which suffer from sensorineural hearing loss. In particular, there is a need for treatment of those individuals which have impaired or defective cochlear nerves and therefore who do not benefit fully from traditional cochlear implants.

The maintenance and/or induction of a viable neuron population is likely to enhance the benefits of the cochlear implant and lead to improved outcomes in terms of language acquisition and speech perception in patients.

Embodiments of the present invention therefore aim to at least partly mitigate the problems associated with the prior art and provide agents which could be used to treat sensorineural hearing loss and deafness.

BRIEF SUMMARY OF THE DISCLOSURE

In a first aspect of the present invention, there is provided an agent, which is an agonist of a Protease Activated Receptor (PAR), for use to (a) stimulate neurite growth by an auditory neuronal cell, (b) induce differentiation of auditory stem cells and/or auditory neuronal cells or (c) combinations of (a) and (b). In one embodiment, the agent is for use in treating or preventing sensorineural hearing loss. In one embodiment, the agent is for improving representation of sounds by a patient. The agent may also be for improving language acquisition and/or speech perception in a patient suffering from sensorineural hearing loss. In one embodiment, the agent is an agonist of one or more of the following Protease Activating Receptors (PAR): PAR-1, PAR-2, PAR-3 and PAR-4. Suitable classes of the agents act on a PAR protein which is expressed by an auditory stem cell, an auditory neuron, e.g. a cochlear ganglion, and combinations thereof. In one embodiment, the auditory neuronal cell is a neuron associated with, or previously associated, with a hair cell.

In one embodiment, the auditory neuronal cell is a spiral cochlear ganglion cell.

In one aspect of the present invention, there is provided an agent as described herein of use as a medicament.

The agent may be selected from a protein, a peptide, a small molecule, an antibody, an antibody fragment and a fusion protein. In one embodiment, the agent is an isolated protein, peptide, antibody, antibody fragment and/or fusion protein. An "isolated" or "purified" protein or biologically active fragment thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of the protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced.

In one embodiment, the agent is a protease, a derivative thereof, a precursor form thereof or an active fragment thereof. In an embodiment, the agent is a serine protease. The agent may be selected from tissue plasminogen activator (t-Pa), thrombin, plasmin, Ancrod and fragments, precursors and derivatives thereof.

In one aspect of the present invention there is provided an agent for stimulating neurite growth by an auditory neuronal cell, wherein the agent is a protease, a derivative thereof, a precursor thereof or active fragment thereof.

In one aspect of the present invention, there is provided an agent for treating sensorineural hearing loss wherein the agent is a protease, a derivative thereof, a precursor thereof or active fragment thereof. In one embodiment, the agent is for treating sensorineural hearing loss, such that a patient's representation of sound is improved. The agent may be selected from tissue plasminogen activator (t-Pa), thrombin, plasmin, Ancrod and fragment or derivatives thereof.

A further aspect of the present invention provides an agent as described herein in combination with an intra-auditory device. In one embodiment, the device is suitable for implantation in a patient's ear. In one embodiment, the device is a cochlear implant.

In one embodiment, the device is coated with the agent.

In a further aspect of the present invention, there is provided a method of stimulating neurite growth by an auditory neuronal cell comprising delivering an agent as described herein to an auditory neuronal cell. In one embodiment, the method is an *in vitro* method. In an alternative embodiment, the agent is administered to a patient, and is optionally delivered to a patient's ear e.g. to the patient's cochlear.

The present invention may comprise use of the agents as described herein to treat sensorineural hearing loss in one or both of the patient's ears i.e. the agent may be administrated to one or both of the patient's ears.

In one aspect of the present invention, there is provided a method of treating sensorineural hearing loss comprising administering an agent as described herein to a patient. In one embodiment, the agent is delivered to a patient's cochlear. In one embodiment, the agent is administered in combination with an implantable device.

In one aspect of the present invention, there is provided a pharmaceutical formulation comprising the agent as described herein and a pharmaceutically acceptable excipient.

In one aspect of the present invention, there is provided a kit comprising an agent as described herein and an implantable device. In one embodiment, the implantable device is a cochlear implant.

In one aspect of the present invention, there is provided use of an agent as described herein for the manufacture of a medicament for the stimulation of auditory stem cell differentiation.

In one aspect of the present invention, there is provided use of an agent as described herein for the manufacture of a medicament for the stimulation of auditory neuronal cell differentiation.

In one aspect of the present invention, there is provided use of an agent as described herein for the manufacture of a medicament for the stimulation of growth of a neurite by an auditory neuronal cell.

In one aspect of the present invention, there is provided use of an agent as described herein for the manufacture of a medicament for the treatment of sensorineural hearing loss.

In one embodiment, the medicament is in the form of a device e.g. an implant or is for administration with a device. In one embodiment, the device is a cochlear implant.

The agents of the invention may be, for example, an antibody or fragment thereof, e.g. a Fab fragment. Naturally within the scope of the agents of the invention are antibodies or fragments which are monoclonal, polyclonal, chimeric, human, or humanized. Other agents are encompassed by the present invention.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”, means “including but not limited to”, and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article

is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will be described in more detail below, with reference to the following drawings:

Figure 1: Electron micrographs showing A, C) undifferentiated auditory stem cells and B, D) cells after induction of neuronal differentiation. Bar are 100 micrometers.

Figure 2: Charts showing percentages of bipolar neurons present before (left hand bar) and after dissociation of cells with trypsin (right hand bar) (left chart). Right hand chart shows the percentages before (left hand bar) and after (left hand bar) non-enzymatic dissociation.

Figure 3: A graph showing percentages of bipolar neurons from hFASCs #850 P10 obtained 24 hours after exposure to supernatant from trypsin treated cells. 1) Trypsin treated cells. (2) non-dissociated cells treated with concentrated supernatant obtained from (1); (3) Non-dissociated cells treated by a concentrated preparation of trypsin and soybean trypsin inhibitor (to control any remaining trypsin in (2); (4) cell dissociated non-enzymatically. Conditions (2), (3), (4) were not significantly different, only treatment (1) induced differentiation.

Figure 4: A graph showing thrombin induced neuronal differentiation in hFASC #853 after 24 hours of treatment.

Figure 5: A graph showing plasmin induced neuronal differentiation of hFASC #853 after 24 hours of treatment.

Figure 6: A graph showing tissue plasminogen activator induced neuronal differentiation in human auditory stem cells (hFASC #844).

Figure 7: A graph showing neuronal differentiation induced by PAR-1 activating peptide in hFASC #844. The application of PAR-1 (Figure 7a) and PAR-2 (figure 7b) activating peptides induced the formation of neurites. The effect was more pronounced by Par-2 activating peptides, being clearly detectable within 7 days. The effect of PAR-1 activating peptide was apparent after 10 days.

Figure 8: Experiments were carried out to confirm that human auditory stem cells express PAR-1 and PAR-2. Figure 8 is RT-PCR of PAR-1, PAR-2 and tPA. Jurkat cells were used a positive control. The last lane was a negative control with no template.

Figure 9: Amino acid sequence of a PAR-1 receptor (SEQ ID NO. 1)

Figure 10: Amino acid sequence of a PAR-2 receptor (SEQ ID NO. 2)

Figure 11: Amino acid sequence of a PAR-3 receptor (SEQ ID NO. 3)

Figure 12: Amino acid sequence of a PAR-4 receptor (SEQ ID NO. 4)

Figure 13: Nucleic acid sequence encoding a PAR-1 receptor (SEQ ID NO. 5)

Figure 14: Nucleic acid sequence encoding a PAR-2 receptor (SEQ ID NO. 6)

Figure 15: Nucleic acid sequence encoding a PAR-3 receptor (SEQ ID NO. 7)

Figure 16: Nucleic acid sequence encoding a PAR-4 receptor (SEQ ID NO. 8)

Figure 17: Amino acid sequence of a t-Pa protein (SEQ ID NO. 9)

Figure 18: Amino acid sequence of a human pro-thrombin protein (SEQ ID. No 10)

Figure 19: Time Lapse Imaging of neuronal differentiation. hFetal Auditory Stem Cells were monitored by time-lapse video microscopy during 24hours after dissociation

by trypsin. The induction of neurite projections is shown as a well-coordinated phenomenon and not random stretching of the cells following the dissociation process.

Figure 20: Image showing inhibition of PAR1 causes a decrease in the numbers of bipolar neurons and a shortening in the neurite length. A: Cells incubated with 50nM trypsin, B: cells pre-treated by 100μM of SCH79797 for 2 hours, followed by co-incubation with 50nM of trypsin, C: cells cultured in 100 μM of SCH79797 without trypsin treatment, D: negative control, cells cultured with the trypsin vehicle only.

Figure 21: Graph showing effect of PAR1 inhibition using PAR1 antagonist, SCH79797 .

Figure 22: Image of neurite length following treatment with trypsin and trypsin plus SCH79797 (PAR1 antagonist). F1 refers to the short neurite whilst F2 refers to the long neurite

Figure 23: Graph showing short neurite length following trypsin treatment and PAR1 antagonist treatment.

Figure 24: Graph showing long neurite length following trypsin treatment and PAR1 antagonist treatment.

Figure 25: Semi-quantitative RT-PCR showing expression of PAR1 and PAR2 in mouse fetal auditory cells.

Figure 26: Graph showing 24 hour effect of PAR1 antagonist (SCH79797) treatment in neuronal differentiation of mFASCs.

DETAILED DESCRIPTION

The present invention relates to the stimulation of the production of neurites by auditory neuronal cells. Auditory neuronal cells include, for example, neurons associated with hair cells in the cochlear. The present invention also relates to the differentiation of auditory neuronal cells e.g. the differentiation of auditory stem cells to form auditory neuronal cells. Thus, the agents described herein may be used to induce the production of new neurons in the auditory system, e.g. neurons associated with hair cells. The

agents described herein may also be used to stimulate the generation of projections by existing auditory neurons. In one embodiment, the agent is for the stimulation of the production of bipolar neurons in the auditory system. Agents with one or both functions may be utilised as a therapy for deafness, either alone or in combination e.g. in combination with a second agent or with an implantable hearing device.

Projections or neurites are elongated, membrane-enclosed protrusions of cytoplasm of neurons. They usually don't have a clear function at this point, though most eventually become functional axons. These neurites grow toward other regions of the nervous system or other structures on which the neurons will eventually form synapses, such as glands, muscle, etc; these tissues are usually referred to as the "targets" of the neurons.

In particular, one embodiment of the present invention relates to the use of agents which activate a Protease-Activated Receptor (PAR) protein. The PAR protein may be expressed on auditory stem cells. In one embodiment, the agent may activate more than one member of the PAR protein family. In one embodiment, the agent is for use in treating sensorineural hearing loss. Exemplary agents include, but are not limited to, proteins, peptides, antibodies, peptibodies, carbohydrates and small organic molecules.

Embodiments of the present invention may be used to treat hearing disorders which would benefit from the maintenance of auditory neuronal function and/or stimulation of neurite growth and auditory neuronal function. Thus, the present invention may be used to treat hearing loss and/ or to maintain a certain level of hearing. In one embodiment, application of the present invention does not restore normal hearing but may provide a deaf subject with representations of sounds in the environment. In one embodiment, the agents as disclosed herein may be used to improve language acquisition and/or speech perception in a patient.

The terms “hearing loss” and “deafness” as used herein relate to the partial or total loss of hearing by a subject. Thus, the present invention has applications in the treatment of both total deafness and partial deafness which occurs as a result of loss of auditory neuronal function e.g. loss of function of cochlear ganglion cells. In one embodiment, the present invention may enable a subject to have an improved representation of sounds in the environment but does not reverse loss of hearing in the subject.

Treatment of a hearing disorder e.g. sensorineural hearing loss, includes providing palliation, amelioration or reversal of a hearing disorder. Thus, treatment of a hearing disorder includes provision of partial or complete palliation, amelioration or reversal of hearing loss. In one embodiment, the present invention provides devices, agents and methods for treating deafness such that a level of hearing is substantially maintained and does not deteriorate further. In one embodiment, the present invention provides agents, devices and methods which may improve a patient's level of hearing as compared to their level of hearing without administration of the agents and devices as disclosed herein. In one embodiment, the agent is for the prevention of a sensorineural hearing disorder.

Types of sensorineural hearing loss which may be treated by the methods and agents of the present invention include, without limitation, hereditary sensironeural hearing loss, presbycusis, noise-induced hearing loss (NIHL), drug-induced hearing loss (DIHL), hearing loss as a result of infectious agents, and central auditory processing disorder (CAPD).

Agents as described herein may stimulate neurogenesis or neuritogenesis and therefore have important therapeutic applications, e.g. when combined with current cochlear implants. Embodiments of the present invention may allow auditory implants to be applicable to a larger range of patients, e.g. those patients with aplasia of the auditory nerve. Auditory implants typically do not restore normal hearing. Instead, they may provide a deaf person a useful representation of sounds in the environment and help him or her to understand speech. Thus, in one embodiment, the present invention provides agents which provide a deaf person with an improved representation of sounds in the environment as compared to the sound representation without treatment with the agents disclosed herein.

In one embodiment, the patient is a mammal. In one embodiment, the patient is a human e.g. a human adult, child or infant. The invention may have particular application in the improvement of a child's language acquisition.

In one aspect of the invention, there is provided an agent which is for use in treating sensioneural deafness, which agent is a protease or active fragment thereof. In one embodiment, the agent is t-Pa or a fragment or variant thereof.

In the all of the embodiments of the invention described herein in which the agent is a polypeptide, the amino acid sequence of the agent may be modified by one or more changes in sequence which do not eliminate the underlying biological function and utility of the agents as described herein. Modifications may include substitution of individual amino acids with other naturally occurring or non-naturally occurring amino acids, as described in more detail later on.

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, Genes V, published by Oxford University Press, 1994 (ISBN 0-19- 854287-9); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd. ,1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology : a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). Definitions and additional information known to one of skill in the art in immunology can be found, for example, in Fundamental Immunology, W. E. Paul, ed. , fourth edition, Lippincott-Raven Publishers, 1999.

The term "agonist", as used herein, refers to an agent that, when contacted with a molecule of interest, causes an increase in the magnitude of a certain activity or function of the molecule compared to the magnitude of the activity or function observed in the absence of the agonist. In one embodiment, the agent of the present invention is a PAR agonist e.g. a PAR-1 agonist, a PAR-2 agonist, a PAR-3 agonist and/or a PAR-4 agonist.

"PARs" as used herein relate to "protease-activated receptors". PARs are a family of related receptors in the G-protein coupled receptor family which have been reported to be expressed by *inter alia* platelets, endothelial cells and myocytes. There are four reported members of the PAR family, PAR-1, PAR-2, PAR-3 and PAR-4. The PARs have been indicated as being involved in inflammatory processes and are typically activated upon cleavage of their extracellular NH_2 terminus by serine proteases such as thrombin, plasmin, trypsin, and tryptase.

An exemplary amino acid sequence of PAR-1 is shown in Figure 9 (SEQ. ID. NO. 1) and a nucleic acid sequence which encodes PAR-1 is shown in Figure 13 (SEQ. ID. No. 5) (accession number M62424). An exemplary amino acid sequence of PAR-2 is shown in Figure 10 (SEQ. ID. NO. 2) and a nucleic acid sequence which encodes PAR-2 is shown

in Figure 14 (SEQ. ID. No. 6). An exemplary amino acid sequence of PAR-3 is shown in Figure 11 (SEQ. ID. NO. 3) and a nucleic acid sequence which encodes PAR-3 is shown in Figure 15 (SEQ. ID. No. 7). An exemplary amino acid sequence of PAR-4 is shown in Figure 12 (SEQ. ID. NO. 4) and a nucleic acid sequence which encodes PAR-4 is shown in Figure 16 (SEQ. ID. No. 8).

Included in the present invention are agents which activate a PAR protein. In one embodiment, the agent binds to a PAR protein to activate it. It will be understood that the amino acid sequence of a PAR protein may vary between individuals and therefore the present invention includes agents which bind to a PAR protein which differs in sequence to the proteins whose sequence is shown in Figures 9, 10, 11 and 12 e.g. by way of naturally occurring allelic variation.

Exemplary Agents

One aspect of the present invention relates to the use of agents which stimulate neuritogenesis e.g. which stimulate an auditory neuron such that the neuron differentiates and/or forms neurites. Typically, the PAR receptor is expressed by an auditory nerve cell e.g. an auditory stem cell. In one embodiment, the agent agonises a PAR protein. The agent may activate a PAR protein either directly or indirect e.g. via an intermediary mechanism or agent. In one embodiment, the agent binds to a PAR protein which is expressed by an auditory nerve cell e.g. a cochlear ganglion. In one embodiment, the agent binds to a PAR protein which is expressed by an auditory stem cell. Binding of the agent to the PAR protein may activate the PAR protein and thus its associated downstream mechanisms.

In one embodiment, the agent may be considered to rescue auditory neurons e.g. spiral ganglion neurons, from the degeneration that is typically observed following damage to or loss of the sensory hair cells.

PAR receptors have been described as being expressed by neurons in other systems of the body. However, there has not been any previous disclosure of expression of any PAR receptors by neurons involved in the auditory process. Thus, the present disclosure includes, for the first time, data showing that PAR-1 and PAR-2 are expressed by human auditory stem cells and that these receptors may be involved in the generation of cochlear neurons and also the production of neurites by existing cochlear

neurons. In addition, the present invention discloses that agonists of at least the PAR-1 and PAR-2 receptors may be used as agents of the invention to treat or alleviate sensorineural hearing loss and/ or deafness. The agents of the present invention may also be used to maintain a patient's current level of hearing and prevent the patient losing further hearing capacity.

Previous work relating to applications of supposed PAR agonists and neurons teaches away from the present invention's findings: the application of thrombin, for instance, has been reported to cause retraction of neurites in mouse neuroblastoma cells (Suidan et al, Neuron 8: 363-375, 1992, Jalink et al J. Cell Biol. 118(2): 411-419, 1992) and on dorsal root ganglion neurons (Gill et al Brain Res. 797(2): 321-7, 1998). Olfactory sensory neurons exposed to trypsin, thrombin or peptides that activate the PAR receptors showed rapid neurite retraction (Olianas MC et al,; Neuroscience, 2007).

In one embodiment, the agent is a PAR agonist e.g. selected from a PAR-1 agonist, a PAR-2 agonist, a PAR-3 agonist and a PAR-4 agonist. In one embodiment, the PAR agonist may act to agonise more than one PAR receptor e.g. an agent of the invention may agonise PAR-1 and PAR-2, or for example, PAR-2 and PAR-3. In one embodiment, the agent may agonise all PAR receptors. The PAR protein may be expressed on an auditory cell e.g. an auditory stem cell which has the capacity to differentiate to produce an auditory neuron e.g. a spiral ganglion cell. In one embodiment, the agent may agonise only one PAR family member. Alternatively, the agent may agonise more than one PAR family member.

Without being bound by theory, it is considered that the agents of the present invention activate a PAR protein such that it stimulates growth of projections (i.e. neurites) by an auditory neuronal cell. A subset of agents of the present invention typically acts to stimulate the growth of at least one, e.g. at least two, projections by a neuronal cell on which it acts. Preferably, each projection is longer than the soma of the cell. In some embodiments, the agents may act to stimulate the growth of more than two projections by a neuronal cell e.g. three, four, five or more projections. Furthermore, agents of the present invention may act to stimulate auditory neuronal differentiation and the generation of neurites by auditory neurons. In embodiments of the invention, new auditory neurons may be generated by treatment with agents described herein.

In one embodiment, the agent binds to a PAR protein e.g. a PAR protein expressed on an auditory cell e.g. an auditory neuron. The binding of the agent is optionally binding with an affinity of greater than 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M or 10^{-12} M. The binding may be specific for the PAR protein or non-specific. In some instances there may be a degree of lower affinity non-specific binding to certain other ligands unrelated to the PAR protein.

In one aspect of the present invention, the agent is a protease which activates a cochlear ganglion cell to stimulate growth of projections by the cell. Examples of suitable proteases are described below.

Specific examples of agents of the present invention include:

Proteases

In one embodiment, the protease is a protease e.g. an extracellular protease. The term "protease" is defined herein as an enzyme that hydrolyses peptide bonds. Exemplary proteases are those which activate a PAR protein e.g. PAR-1, PAR-2, PAR-3 and/or PAR-4. In one embodiment, the agent is an isolated protease. In one embodiment, the protease activates a PAR protein by a cleavage at a specific site within the extracellular amino-terminus. This cleavage exposes a new amino terminus that then acts as a tethered ligand domain that will bind to the second extracellular loop of the same molecule, triggering signal transduction.

In one embodiment, the agent is a serine protease (also referred to as a serine endopeptidase). Typically, a serine protease is a peptidase in which the catalytic mechanism depends upon the hydroxyl group of a serine residue acting as the nucleophile that attacks the peptide bond. In one embodiment, the agent is a precursor form of a protease e.g. an inactive precursor which is cleaved e.g. *in vivo,* to form the active protease.

Included in a class of suitable agents are plasminogen activators, for example; streptokinase, a bacterial protein, urokinase, an enzyme synthesized in the kidney and elsewhere, and human tissue plasminogen activator (t-PA), an enzyme produced by the cells lining blood vessel walls, as well as variants and active fragments thereof.

In one embodiment, the agent is t-Pa or a fragment or variant thereof. Natural t-PA is composed of several functional domains: F, E, K1, K2 and P. The term “human tissue plasminogen activator," "human t-PA," and "t-PA" denote human extrinsic (tissue-type) plasminogen activator having two functional regions consisting of a protease domain that is capable of converting plasminogen to plasmin and an N-terminal region believed to be responsible for fibrin binding. These three terms therefore include polypeptides containing these functional domains as part of the overall sequence. t-PA is suitably produced, e.g., by recombinant cell culture systems, in bioactive forms comprising the protease portion and portions of t-PA otherwise native to the source of the t-PA. It will be understood that natural allelic variations exist and occur from individual to individual, demonstrated by (an) amino acid difference(s) in the overall sequence.

T-Pa was first identified as a substantially pure isolate from a natural source, and tested for requisite plasminogen activator activity in vivo, by Collen et al., (U.S. Pat. No. 4,752,603). Subsequently, t-PA was fully identified and characterized by underlying DNA sequence and deduced amino acid sequence based on successful work employing recombinant DNA technology resulting in large quantities of t-PA in a distinct milieu. This work was reported by Pennica et al., Nature, 301:214 (1983)) and in U.S. Pat. No. 4,766,075, issued 23 Aug. 1988, incorporated herein by reference. An amino acid sequence of t-PA is shown in Figure 17.

Thus, in one embodiment, the agent is t-Pa or a variant or active fragment thereof. An active fragment of t-Pa is a polypeptide which is not a full length t-Pa polypeptide but which has the ability to stimulate the differentiation of auditory neurons and/or production of neurites by auditory neurons e.g. spiral ganglion neurons. In one embodiment, the active fragment of t-PA activates a PAR protein e.g. although not limited to PAR-1 and/ or PAR-2. Various t-PA variants which may be suitable as agents of the present invention for use in treating sensorineural deafness have been described in the art e.g. European Patent publication No. 199,574 which discloses t-Pa variants that have amino acid substitutions at the proteolytic cleavage sites at positions 275,276, and 277. These variants, characterized preferentially as t-PA variants having an amino acid other than arginine at position 275, are referred to as protease-resistant one-chain t-PA variants in that, unlike natural t-PA, which can exist in either a one-chain or two-chain form, they are resistant to protease cleavage at position 275 and are therefore not converted metabolically in vivo into a two-chain form. This form is thought to have certain advantages biologically and commercially, in that it is more stable than two-chain t-PA.

Other t-Pa variants and active fragments of t-Pa have been described e.g. in European Patent Publication No. 238,304, as reported by A. J. van Zonneveld et al., Thrombos. Haemostas., 54 (1) 4 (1985) and European Patent No. 240,334. Preferably, the t-Pa derivative has the ability to induce neurite formation by auditory neurons e.g. spiral ganglion cells. The t-Pa derivative may also have the ability to induce auditory neuronal differentiation e.g. differentiation of the spiral ganglion cells. In one embodiment, the agent is a polypeptide which has at least 60% identity with the natural amino acid sequence of t-Pa, as shown in Figure 17 and preferably at least 70%, more preferably at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with the amino acid sequence shown in Figure 17.

In an alternative embodiment of the present invention, the agent is thrombin or a variant or active fragment thereof. Thrombin is a serine protease which has been reported to act on PAR-1, PAR-2 and PAR-4 receptors. However, its role as an agent which can be used to treat sensorineural deafness has not previously been contemplated. Thrombin is a two chain, disulfide-bonded, glycosylated polypeptide which has a pro form – prothrombin. Human prothrombin is known and is described in Friezner et al. (Biochemistry, 22 (1983) 2087-2097). Prothrombin is activated to thrombin by two Factor Xa-complex cleavages releasing the activation peptide and cleaving thrombin into light and heavy chains yielding catalytically active α-thrombin. α-Thrombin is composed of a light chain (A chain)(MW ~ 6,000) and a heavy chain (B chain)(~31,000). These two chains are joined by one disulfide bond.

In one embodiment, the agent is α-Thrombin. In one embodiment, the agent is a variant of thrombin as a result of allelic variations and other deletions, additions or minor amino acid changes that do not destroy thrombin activity.

In one embodiment, the agent is a protein which has at least 60% identity with the amino acid sequence of thrombin e.g. α-Thrombin, and preferably at least 70%, more preferably at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with the published amino acid sequence of human thrombin. In one embodiment, the agent is a protein which has at least 60%,preferably at least 70%, more preferably at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with a sequence of thrombin formed from Factor Xa cleavage of a pro-thrombin protein as shown in Figure 18.

Derivatives of thrombin which may be used as agents of the present invention may comprise amino acid deletions, additions, or substitutions as compared to the wild-type sequence of thrombin. Derivatives useful in the present invention typically retain the function of one or more of the following functions: (a) activating at least one PAR protein, (b) stimulate neurite growth and (c) neuronal differentiation in the auditory system. In one embodiment, the agent is a fragment of thrombin. An example of an active fragment of thrombin is a fragment which includes the protease site. In one embodiment, the agent is a pro-form of thrombin.

In one embodiment, the agent is trypsin or an active fragment or variant thereof.

In one embodiment, the agent is plasmin or an active fragment or variant thereof. In one embodiment, the agent is a precursor form of plasmin (i.e. plasminogen) which is cleavable to form an active protease. Human plasminogen is a single-chain protein containing 791 amino acid residues. Activation of plasminogen to plasmin results from a single cleavage of the Arg561-Val562 peptide bond in the zymogen. The resulting plasmin molecule is a two- chain, disulfide-linked serine protease with trypsin-like specificity (cleaves after Lys and Arg). In one embodiment, the agent is a protein which has at least 60% identity with the amino acid sequence of plasmin and preferably at least 70%, more preferably at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or more identity with the published amino acid sequence of plasmin e.g. the amino acid sequence of the protein following cleavage of plasminogen. The human plasminogen sequence has the UniProt accession number P00747.

In one embodiment, the protease is derivable from the venom of a snake. In one embodiment, the agent is Ancrod, a thrombin-like enzyme from venoms of snakes of the viper/rattlesnake group (Geyer et al, European Journal of Biochemistry 268 (14), 4044–4053). In one embodiment, the agent is an enzyme which has at least 60% identity with the amino acid sequence of Ancrod and preferably at least 70%, more preferably at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or more identity with the amino acid sequence of Ancrod.

Other proteases which may be suitable as agents of the present invention include for example, serine proteases which are members of the S1 family of serine proteases.

A suitable class of agents comprises an active fragment of a protease. In one embodiment, the agent may be a heterologous protein comprising a protease fragment linked to a second protein or fragment thereof. The protease may be linked via a linker e.g. a peptide linker. In one embodiment, the agent is a peptibody. The term "peptibody" refers to a molecule comprising an antibody Fc domain attached to at least one peptide. The production of peptibodies is generally described in PCT publication WO 00/24782, published May 4, 2000.

Antibodies

In one embodiment, the agent may be an antibody which agonises a Protease Activated Receptor (PAR) expressed on an auditory neuron and/or an auditory stem cell. Thus, the antibody may bind to a PAR and cause an agonistic effect. The agents of the invention may be, for example, an antibody or fragment thereof, e.g. a Fab fragment. Preferred antibodies and fragments are Fab fragments or scFv. Naturally within the scope of the agents of the invention are antibodies or fragments which are monoclonal, polyclonal, chimeric, human, or humanized.

In one embodiment, the agent is an antibody. An antibody and immunologically active portions thereof, for instance, are typically molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen.

A naturally occurring antibody (for example, IgG) includes four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. The two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (λ) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Full-length immunoglobulin light chains are generally about 25 Kd or 214 amino acids in length. Full-length immunoglobulin heavy chains are generally about 50 Kd or 446 amino acid in length. Light chains are encoded by a variable region gene at the NH2-terminus (about 110 amino acids in length) and a kappa or lambda constant region gene at the COOH--terminus. Heavy chains are similarly encoded by a variable region gene (about 116 amino acids in length) and one of the other constant region genes.

The basic structural unit of an antibody is generally a tetramer that consists of two identical pairs of immunoglobulin chains, each pair having one light and one heavy

chain. In each pair, the light and heavy chain variable regions bind to an antigen, and the constant regions mediate effector functions. Immunoglobulins also exist in a variety of other forms including, for example, Fv, Fab, and $(Fab')_2$, as well as bifunctional hybrid antibodies and single chains (e.g., Lanzavecchia et al., *Eur. J. Immunol.* 17:105, 1987; Huston et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:5879-5883, 1988; Bird et al., *Science* 242:423-426, 1988; Hood et al., *Immunology*, Benjamin, N.Y., 2nd ed., 1984; Hunkapiller and Hood, *Nature* 323:15-16, 1986).

Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, transplacental mobility, complement binding, and binding to Fc receptors. An immunoglobulin light or heavy chain variable region includes a framework region interrupted by three hypervariable regions, also called complementarity determining regions (CDR's) (see, *Sequences of Proteins of Immunological Interest*, E. Kabat et al., U.S. Department of Health and Human Services, 1983). As noted above, the CDRs are primarily responsible for binding to an epitope of an antigen. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant.

In one embodiment, the antibody is a monoclonal antibody. A monoclonal antibody is produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Generally, a monoclonal antibody is produced by a specific hybridoma cell, or a progeny of the hybridoma cell propaged in culture. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

A suitable class of agents may be chimeric antibodies which bind to a PAR protein expressed by a cochlear neuron. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from

immunoglobulin variable and constant region genes belonging to different species. For example, the variable segments of the genes from a mouse monoclonal antibody can be joined to human constant segments, such as kappa and gamma 1 or gamma 3. In one example, a therapeutic chimeric antibody is thus a hybrid protein composed of the variable or antigen-binding domain from a mouse antibody and the constant or effector domain from a human antibody, although other mammalian species can be used, or the variable region can be produced by molecular techniques. Methods of making chimeric antibodies are well known in the art, e.g., see U.S. Patent No. 5,807,715, which is herein incorporated by reference.

In one embodiment, the agent may be a humanized antibody or fragment thereof. A "humanized" immunoglobulin is an immunoglobulin including a human framework region and one or more CDRs from a non-human (such as a mouse, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor" and the human immunoglobulin providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, such as about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A humanized antibody binds to the same antigen as the donor antibody that provides the CDRs. The acceptor framework of a humanized immunoglobulin or antibody may have a limited number of substitutions by amino acids taken from the donor framework. Humanized or other monoclonal antibodies can have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Exemplary conservative substitutions are those such as gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr (see U.S. Patent No. 5,585,089, which is incorporated herein by reference). Humanized immunoglobulins can be constructed by means of genetic engineering, e.g., see U.S. Patent No. 5,225,539 and U.S. Patent No. 5,585,089, which are herein incorporated by reference.

In one embodiment, the agent is a human antibody. A human antibody is an antibody wherein the light and heavy chain genes are of human origin. Human antibodies can be

generated using methods known in the art. Human antibodies can be produced by immortalizing a human B cell secreting the antibody of interest. Immortalization can be accomplished, for example, by EBV infection or by fusing a human B cell with a myeloma or hybridoma cell to produce a trioma cell. Human antibodies can also be produced by phage display methods (see, e.g., Dower et al., PCT Publication No. WO91/17271; McCafferty et al., PCT Publication No. WO92/001047; and Winter, PCT Publication No. WO92/20791, which are herein incorporated by reference), or selected from a human combinatorial monoclonal antibody library (see the Morphosys website). Human antibodies can also be prepared by using transgenic animals carrying a human immunoglobulin gene (e.g., see Lonberg et al., PCT Publication No. WO93/12227; and Kucherlapati, PCT Publication No. WO91/10741, which are herein incorporated by reference).

Antibodies may also be obtained using phage display technology. Phase display technology is known in the art for example Marks et al J. Mol. Biol. 222: 581-597 and Ckackson et al, Nature 352: 624-628, both incorporated herein by reference. Phage display technology can also be used to increase the affinity of an antibody. To increase antibody affinity, the antibody sequence is diversified, a phage antibody library is constructed, and a higher affinity binders are selected on antigen (see for example Marks et al Bio/ Technology 10:779-783, Barbas et al Proc. Natl. Acad. Sci USA 91:3809-3813 and Schier et al J. Mol. Biol. 263: 551-567, all incorporated herein by reference.

In one embodiment, the agent is an antibody fragment. Various fragments of antibodies have been defined, including Fab, $(Fab')_2$, Fv, dsFV and single-chain Fv (scFv) which have specific antigen binding. These antibody fragments are defined as follows: (1) Fab, the fragment that contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain or equivalently by genetic engineering; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) $(Fab')_2$, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction or equivalently by genetic engineering; (4) $F(Ab')_2$, a dimer of two FAb' fragments held together by disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the

heavy chain expressed as two chains; dsFV, which is the variable region of the light chain and the variable region of the heavy chain linked by disulfide bonds and (6) single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Single chain antibodies may also be referred to as single chain variable fragments (scFv). Methods of making these fragments are routine in the art.

Reference is made to the numbering scheme from Kabat, E. A., et al., Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, MD (1987) and (1991). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in VL domain the two cysteines are typically at residue numbers 23 and 88, and in the VH domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in fig. 5). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

CDR and FR residues are also determined according to a structural definition (as in Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the

residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Fusion Proteins

In one embodiment, the agent is a fusion protein i.e. a protein comprising at least two heterologous peptide sequences. The fusion protein may comprise a linker between each peptide sequence. The fusion protein may be for example, comprised of a peptide which binds to a PAR protein (e.g. PAR-1, PAR-2 or PAR-3) linked to a second peptide. In one embodiment, the fusion protein is an antibody fusion protein. Examples of antibody fusion proteins are detailed in "Antibody Fusion Proteins" (Chamow and Ashenazi, Wiley-Liss 1999). In one embodiment, the agent may be an Fc fusion protein i.e. comprises an Fc portion of an antibody, e.g. linked to an agent which binds to a PAR protein.

In one embodiment, the agent is a fusion protein comprising a PAR activating peptide as defined herein. In one embodiment, the fusion protein comprises a PAR activating peptide and an antibody fragment e.g. an Fc portion. The fusion protein may further contain a linker e.g. a linker which comprises glycine and/or serine residues. Suitable linkers include those disclosed in, for example, Robinson, Proc. Natl. Acad. Sci. USA; 1998, 95, 5929-5934.

PAR activating peptides

A further class of agents which may be used in the present invention to stimulate auditory neuronal growth and e.g. treat sensironeural deafness includes PAR activating peptides. PAR activating peptides are typically short synthetic peptides which mimic the tethered ligand domains of the PARs. Examples of suitable PAR activating peptides include e.g. peptides available from Peptides International, Louisville. Exemplary peptides include peptides which comprise the following sequences:

SFLLR
SFLLR-Amide
SFLLRN
SFLLRNP

SFLLRN-Amide
TFRIFD
TFLLR-NH_2
SLIGKV-NH_2
SLIGRL-NH_2
Trans-cinnamoyl-LIGRLO-NH_2
2-Furoyl-LIGRLO-amide
GYPGKF-NH_2
GYPGQV-NH2
AYPGKF-NH2.
SFLLRNPNDKYEPF-Amide
SFLLRNPNDKYEPF

As discussed above, in one embodiment, the PAR activating peptide may form part of a fusion protein.

The agents of the present invention, if comprising a peptide sequence, for example an antibody, a fusion protein, a peptide or a protein, may be encoded by a nucleic acid sequence. The present invention includes any nucleic acid sequence which encodes an agent as defined herein. The present invention also includes a nucleic acid sequence which encodes the agent of the invention but which differs from the wild-type nucleic acid as a result of the degeneracy of the genetic code.

The present invention also includes nucleic acids that share at least 80% homology with a nucleic acid sequence which encodes an agent of the present invention. In particular, the nucleic acid may have 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% homology to a nucleic acid which encodes an agent of the present invention.

Calculations of sequence homology or identity (the terms are used interchangeably herein) between sequences are performed as follows.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for

comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 75%, 80%, 82%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In one embodiment, the percent identity between two amino acid sequences is determined using the Needleman et al. (1970) *J. Mol. Biol.* 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a BLOSUM 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In one embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is within a sequence identity or homology limitation of the invention) are a BLOSUM 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

The percent identity between two amino acid or nucleotide sequences can be determined using the algorithm of Meyers et al. (1989) *CABIOS* 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

In one aspect of the invention, there is provided a nucleic acid molecule which hybridises under stringent conditions to a nucleic acid molecule which encodes an agent of the present invention. Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following have been found as exemplary for hybridization conditions but without limitation:

Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization:	5x SSC at 65°C for 16 hours
Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization:	5x-6x SSC at 65°C-70°C for 16-20 hours
Wash twice:	2x SSC at RT for 5-20 minutes each
Wash twice:	1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (allows sequences that share at least 50% identity to hybridize)

Hybridization:	6x SSC at RT to 55°C for 16-20 hours
Wash at least twice:	2x-3x SSC at RT to 55°C for 20-30 minutes each.

In one embodiment, the nucleic acids hybridize over substantially their entire length.

In one embodiment the nucleic acid molecule is an isolated nucleic acid molecule. With regards to genomic DNA, the term "isolated" includes nucleic acid molecules that are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences that naturally flank the nucleic

acid (i.e., sequences located at the 5'- and/or 3'-ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived.

Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

In further aspect the invention provides an expression vector comprising a nucleic acid as described above and associated regulatory sequences necessary for expression of a protein or polypeptide in a host cell. Such regulatory sequences include promoters, termination sequences and enhancers, for example.

In another related aspect, the invention provides a host cell comprising a nucleic acid or a vector as described above. Such host cells are transfected or transformed so that they contain the nucleic acid or vector in such a way that they are effective in expressing the desired polypeptide/protein when cultured in appropriate media under the necessary growth conditions. The host cells to be used are not particularly circumscribed so as long as they can be transfected by a vector to be used and can express the DNA of the present invention. For example, bacteria such as *Escherichia coli,* yeast such as *Saccharomyces cerevisiae*, and an animal cell such as a COS cell, a CHO cell, etc. can be used. Examples of prokaryotic host cells appropriate for use with this invention include *E. coli*. Examples of eukaryotic host cells include avian, insect, plant, and animal cells such as COS7, HeLa, and CHO cells.

Alternatively, agents of the invention, when a peptide or polypeptide, can be chemically synthesized using information provided in this disclosure, in conjunction with standard methods of protein synthesis. A suitable method is the solid-phase Merrifield technique. Automated peptide synthesizers are commercially available, such as those manufactured by Applied Biosystems, Inc. (Foster City, Calif.).

For most applications, it is generally preferable that the protein is at least partially purified from other cellular constituents or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, the protein is at least about 50% pure. as a weight percent of total protein. More preferably, the protein is at least

about 50-75% pure. For clinical use, the protein is preferably at least about 80% pure, e.g. 85% or 90% pure.

Aptamers

A further class of agents are aptamers. Aptamers have been defined as artificial nucleic acid ligands that can be generated against amino acids, drugs, proteins and other molecules. They are isolated from complex libraries of synthetic nucleic acids by an iterative process of adsorption, recovery and re-amplification.

RNA aptamers are nucleic acid molecules with affinities for specific target molecules. They have been likened to antibodies because of their ligand binding properties. They may be considered as useful agents for a variety of reasons. Specifically, they are soluble in a wide variety of solution conditions and concentrations, and their binding specificities are largely undisturbed by reagents such as detergents and other mild denaturants. Moreover, they are relatively cheap to isolate and produce. They may also readily be modified to generate species with improved properties. Extensive studies show that nucleic acids are largely non-toxic and non-immunogenic and aptamers have already found clinical application. Furthermore, it is known how to modulate the activities of aptamers in biological samples by the production of inactive dsRNA molecules in the presence of complementary RNA single strands (Rusconi *et al.*, 2002).

It is known from the prior art how to isolate aptamers from degenerate sequence pools by repeated cycles of binding, sieving and amplification. Such methods are described in US 5,475,096, US 5,270,163 and EP0533 38 and typically are referred to as SELEX (Systematic Evolution of Ligands by EX-ponential Enrichment). The basic SELEX system has been modified for example by using Photo-SELEX where aptamers contain photo-reactive groups capable of binding and/or photo cross-linking to and/or photo-activating or inactivating a target molecule. Other modifications include Chimeric-SELEX, Blended-SELEX, Counter-SELEX, Solution-SELEX, Chemi-SELEX, Tissue-SELEX and Transcription-free SELEX which describes a method for ligating random fragments of RNA bound to a DNA template to form the oligonucleotide library. However, these methods even though producing enriched ligand-binding nucleic acid molecules, still produce unstable products. In order to overcome the problem of stability it is known to create enantiomeric “spiegelmers” (WO 01/92566). The process involves initially creating a chemical mirror image of the target, then selecting aptamers to this mirror image and finally creating a chemical mirror image of the SELEX selected

aptamer. By selecting natural RNAs, based on D-ribose sugar units, against the non-natural enantiomer of the eventual target molecule, for example a peptide made of D-amino acids, a spiegelmer directed against the natural L-amino acid target can be created. Once tight binding aptamers to the non-natural enantiomer target are isolated and sequenced, the Laws of Molecular Symmetry mean that RNAs synthesised chemically based on L-ribose sugars will bind the natural target, that is to say the mirror image of the selection target. This process is conveniently referred to as reflection-selection or mirror selection and the L-ribose species produced are significantly more stable in biological environments because they are less susceptible to normal enzymatic cleavage, i.e.they are nuclease resistant.

In one embodiment, the agent is an aptamer which binds to a PAR protein or its gene.

Small Molecule Agonists of PAR

In one embodiment, the agent is a small molecule which directly or indirectly activates a PAR protein which is expressed by an auditory neuronal cell and/or auditory stem cell.

In one aspect of the invention, there is provided a process for preparing a pharmaceutical composition for treating sensorineural hearing loss comprising:

(a) screening a plurality of compounds by a method which utilises an auditory neuronal cell and/or an auditory stem cell, which optionally expresses at least one PAR protein, to obtain IC50 values for each compound;
(b) selecting from the plurality a compound having a binding affinity of greater than a predetermined amount, e.g. having an IC50 of less than 500nM;
(c) synthesising the selected compound; and
(d) incorporating the synthesized compound into a pharmaceutical composition.

Exemplary agents have an IC50 of less than 1000nM, more particularly of less than 500 nM, e.g. less than 100 nM, less than 10 nm, less than 1 nM or less than 0.1 nM.

In a further aspect the invention provides a method of identifying a compound capable of inducing neurite production and/or auditory neuronal cell differentiation comprising assaying the ability of the compound to modulate the nucleic acid expression of a PAR protein by an auditory cell e.g. an auditory stem cell and/or an auditory neuronal cell,

thereby identifying a compound capable of inducing and/or stimulating neurite production and/or auditory neuronal cell differentiation.

Uses of Agents of the Invention

In another aspect of the invention, there is provided the described agents for use as a pharmaceutical. In one aspect of the invention, the agents are for use in treating deafness e.g. full or partial hearing loss. In particular, the agents are for treating sensorineural hearing loss. In some embodiments, the present invention may not restore normal hearing but instead may improve representation of sounds in the environment and help him or her to understand speech.

In one aspect of the invention, there is provided a method of treating deafness comprising delivering an agent as defined herein to a patient's ear. In particular, the method comprises administering the agent to an area of the patient's ear which comprises auditory neurons e.g. spiral ganglion cells and/or auditory stem cells. In one embodiment, the method comprises delivering the agent to the cochlear. In particular, the method is for stimulating growth of neurites by auditory neurons. In one embodiment, the agents of this invention are administered prophylactically. Thus, in one embodiment, the agent may be administered so as to maintain auditory, e.g. cochlear neuronal function.

In one aspect of the present invention, there is provided a method of stimulating growth of least one, e.g. at least two projections by an auditory neuronal cell comprising applying an agent as described herein to the auditory neuronal cell. Preferably, each projection is longer than the cell soma. In some embodiments, the agents may act to stimulate the growth of more than two projections by a neuronal cell e.g. three, four, five or more projections.

In one aspect of the present invention, there is provided a method for stimulating auditory neuronal differentiation comprising administering an agent as described herein to a cell selected from an auditory stem cell and an auditory neuron e.g. a spiral ganglion cell.

In one embodiment, the method comprises administration of the agent in combination with a device. In one embodiment, the device is a cochlear implant, as described in more detail later.

Accordingly, further aspects of the invention provide methods of treatment comprising, administration of an agent as provided herein to a subject suffering from, or at risk of, sensorineural deafness. The invention also provides pharmaceutical compositions comprising such an agent. Also included in the present invention is use of an agent as described herein in the manufacture of a medicament for treating sensorineural hearing loss. In one embodiment, the methods comprises formulating the agent with a pharmaceutically acceptable excipient.

Another aspect of this invention is directed to methods for treating deafness e.g. sensorineural deafness, comprising delivering e.g. administering or applying, to a mammal a therapeutically effective amount of an agent as described herein. In one embodiment, the agent is administered to a mammal's cochlear. In one embodiment, the method does not restore hearing loss but provides a subject with an improved representation of sounds in the environment as compared to the representation without administration of the agents as described herein. In an alternative embodiment, the agent at least partially restores hearing of a patient.

Actual dosage levels of active ingredients in the pharmaceutical compositions of this disclosure may be varied so as to obtain an amount of the active agent(s) that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration (referred to herein as a "therapeutically effective amount"). The selected dosage level will depend upon the activity of the particular agent, the severity of the condition being treated and the condition and prior medical history of the patient being treated. However, it is within the skill of the art to start doses of the compound at levels lower than required for to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

Also included in the present invention is a pharmaceutical formulation comprising an agent as described herein; in embodiments the formulation is a composition comprising the agent and a pharmaceutically acceptable diluent, carrier or excipient. Such formulations may further routinely contain pharmaceutically acceptable concentrations of

salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

The formulations may also include antioxidants and/or preservatives. As antioxidants may be mentioned thiol derivatives (e.g. thioglycerol, cysteine, acetylcysteine, cystine, dithioerythreitol, dithiothreitol, glutathione), tocopherols, butylated hydroxyanisole, butylated hydroxytoluene, sulfurous acid salts (e.g. sodium sulfate, sodium bisulfite, acetone sodium bisulfite, sodium metabisulfite, sodium sulfite, sodium formaldehyde sulfoxylate, sodium thiosulfate) and nordihydroguaiareticacid. Suitable preservatives may for instance be phenol, chlorobutanol, benzylalcohol, methyl paraben, propyl paraben, benzalkonium chloride and cetylpyridinium chloride.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings or animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The present inventive method includes the administration to an animal, such as a mammal, particularly a human, in need of the stimulation of auditory neuronal growth of an effective amount, e. g., a therapeutically effective amount, of one or more of the aforementioned present inventive agents, alone or in combination with one or more other pharmaceutically active agents.

The present inventive method includes the administration to an animal, such as a mammal, particularly a human, in need of the stimulation of auditory stem cell differentiation to produced auditory neurons e.g. spiral ganglion cells, of an effective amount, e. g., a therapeutically effective amount, of one or more of the aforementioned present inventive agents, alone or in combination with one or more other pharmaceutically active agents.

DELIVERY OF AGENTS

Typically the agents of the present invention are administered locally i.e. delivered to the area of the auditory system in which their action is required. In one embodiment, the

agents are localised in an area in which auditory neurons e.g. spiral ganglion cells and/or auditory stem cells are located. In certain embodiments of the invention, a combination of agents as described herein may be administered to a patient.

The anatomy of the cochlea presents several options for the agent delivery; direct infusion into the scala tympani or scala vestibuli (perilymph) or scala media (endolymph); indirect infusion via the vestibular organs which are connected with the cochlea via these fluids; or delivery across the round window membrane. There are also a number of options for the mode of agent delivery, whether it is the pure agent e.g. protein in solution, an agent captured within a polymer, or expression of the agent via cell-based or gene-based therapies. In one embodiment, the agent is encapsulated e.g. in a biocompatible matrix.

In one embodiment, the agent is delivered to the auditory system. Thus, in one aspect of the present invention, there is provided a device comprising an agent of the present disclosure which is suitable for insertion or implantation in the cochlear of the ear.

In one embodiment, an agent of the invention in solution may be infused into the cochlea via a cochleostomy made in either the cochlear bony wall or the round window membrane. In one embodiment, the agent may be delivered to the basal turn of the cochlea, as this is the most surgically accessible region.

In one embodiment, the agent is incorporated into the design of an implantable device. In one embodiment, the device is a cochlear implant. Cochlear implants are neural prostheses which are designed to provide important auditory cues to severely or profoundly deaf patients. Cochlear implants typically rely on transmission by spiral ganglion neurons and thus the ongoing degeneration of the neurons that occurs following a sensorineural hearing loss is therefore considered a limiting factor in cochlear implant efficacy. Cochlear implant typically, although not necessarily, consist of essentially two components, an external component commonly referred to as a processor unit and an internal implanted component commonly referred to as a receiver/stimulator unit. In one aspect of the present invention, there is provided a device e.g. a cochlear implant comprising an agent as described herein. The agent may be comprised as part of, or coated onto, a portion of the cochlear implant which is for implantation in a subject's ear. The agent, when incorporated in a device e.g. a cochlear implant may diffuse throughout the cochlea. In one embodiment, the agent is applied to

a cochlear implant device prior to insertion into a patient's ear/ears. In one embodiment, the agent is applied to the cochlear implant within a polymer. In one embodiment, the agent is formulated for controlled release. In one embodiment, the cochlear implant comprises a reservoir for storing until needed an agent as described herein. In this embodiment, the agent may be in a liquid formulation.

Examples of cochlear implants which may be useful in the present invention include those made by Cochlear Ltd. In addition, US Patent No. 7072717, U.S Patent No. 4,532,930 and U.S. Pat. No. 4,207,441, the contents of which are incorporated herein by reference, provide descriptions of types of cochlear implant system which may be used in the present invention.

Other methods of delivering the agent include drug injection through the tympanic membrane, surgically implanting drug loaded sponges and other drug releasing materials, and/or positioning drug delivering catheters and wicks within the middle ear. In one embodiment, a drug administration system can be used which is configured to administer the agent as described herein to the patient's ear. For example, the system can include a housing which is sized to fit behind a patient's ear to pump the agent as disclosed herein in controlled amounts to the patient's middle ear. A catheter can be used to transport the agent from the housing to the middle ear. Further features of such a system are disclosed in for example US Patent No. 7206639, which is incorporated herein by reference.

In an alternative embodiment, an agent as disclosed herein may be delivered via the round window membrane. The round window membrane offers an alternative site for atraumatic delivery of pharmacological agents to the cochlea, based upon its permeability to a variety of substances.

In a further embodiment, the method for treating deafness comprises administering an agent as defined herein as a part of gene therapy. Gene therapy involves the insertion of genes into cells *in situ* and may be used to replace defective genes, or to induce or increase expression of a desired nucleic acid molecule e.g. a gene or recombinant molecule which encodes for a peptide agent as defined herein.

In one embodiment, the agents may be administered in combination with stem cells to treat sensorineural deafness. In one embodiment, stem cells which express or which

may be induced to express an agent as defined herein, may be administered to a patient's ear. In one embodiment, a vehicle such as a vector may be used to drive gene expression in the cochlear. Exemplary vectors include herpes simplex virus, adeno-associated virus, adenovirus, herpes simplex virus, vaccinia virus and liposomes (Derby et al, Hear Ref. 1999 Aug;134(1-2):1-881).

Examples

The examples described below involved the use of three similar lines of human Fetal Auditory Stem Cells (hFASC #844, #850 and #853).

Example 1

Isolation of human fetal auditory stem cells (hFASCs) and expansion in culture

Cochleae were removed from 9-12 weeks-old human fetuses in sterile Hanks solution. The cartilaginous capsule was removed and the cochlear epithelia was carefully dissected free from surrounding tissue and explanted as small patches on tissue culture plastic-IVF dishes (Corning), in 100 µl of high glucose DMEM (Invitrogen)-10%FCS.

The IVF dish allows the use of a small volume of culture media, while still making the tissue accessible for further dissociation. Given that 3 to 5 hours have normally elapsed since pregnancy termination until the tissue was available for dissection, explants were incubated overnight at 37°C as a precautionary measure to increase cell viability and to diminish the stress should further dissociation be applied immediately after dissection. Epithelial patches were then lifted and dissociated by incubation with 0.125 % trypsin (Sigma) in Hanks solution (supplemented with 5mM HEPES) at room temperature for 10-15 min.

Separation was facilitated by gently mechanical dissociation using initially, a 1,000-µl pipette tip and then, a 200-µl pipette tip. Cells obtained from both cochleae from a single donor were pooled and transferred into two gelatin-coated wells of a 48-well plate (Corning) and grown in OSCFM (otic stem cell full media, which includes high glucose DMEM combined 50:50 with F12 (Invitrogen), including also N2 and B27 supplements and human recombinant bFGF (20 ng ml^{-1}), human recombinant EGF (20 ng ml^{-1}) and human recombinant IGF1 (50 ng ml^{-1}). All the growth factors are from Peprotech.

Cells were then passaged when ~80% confluent, using an enzyme-free dissociation solution (Chemicon) containing EDTA, glycerol and Sodium citrate. Further expansion was done in OSCFM and on gelatin-coated plastic and the cultures established as cell lines.

Results

The cells obtained by culturing dissociated cells from microdissected sensory epithelia from 9 to 11 weeks old foetuses in a serum-free media typically express stem cell markers e.g. NESTIN and SOX2, among others.

Example 2

Comparison of trypsin-induced dissociation vs enzyme-free

Under standard culture conditions, the cells remain undifferentiated, growing as an adherent monolayer and displaying an epithelial-like morphology (Figure 1A, C.) However, when they are treated with trypsin, neuronal differentiation is readily induced. Cells grow processes and elongate. After 24 to 36 hours they display the characteristic bipolar morphology of spiral ganglion neurons (Figure 1B, 1D).

To distinguish whether differentiation was triggered by the mere dissociation of the cells or specifically by the action of trypsin, cells were treated in parallel using either a 0.125% trypsin or a non-dissociating solution. hFASCs were dissociated by rinsing twice with Hanks solution and then incubating with 0.125 % trypsin in Hanks at room temperature for 2-3 min. Trypsin was neutralized by adding an equal volume of 0.1% Soybean Trypsin Inhibitor (Invitrogen). Cells were collected by centrifugation (200 x g), re-suspended in OSCFM and plated at a density of 5,000 cell cm^{-2}. For the enzyme-free dissociation, cells were treated in a similar fashion, but replacing the trypsin with the enzyme-free non-dissociating solution (sodium citrate, EDTA and glycerol) and the soybean inhibitor with Hanks. The generation of a uniform suspension of isolated, single cells was monitored under the microscope. Bipolar neurons were counted 24 hours later and the values expressed as average mm^{-2}. The criteria to select a neuron was established as having two projections, and where each projection was longer that the cell soma.

Results

Both processes (trypsin and non-trypsin) were efficient to produce a suspension of isolated, single cells, a significant difference in the generation of bi-polar cells was evident. Near 80% of the cells treated with trypsin became bipolar, while only 2-3% of cells did when dissociated non-enzymatically (see Figure 2). The example indicates that the induction of differentiation into bipolar phenotypes was due to a direct role of trypsin, rather than to the disruption of cell-cell or cell-extracellular matrix interactions (i.e. cadherin or integrin-mediated).

Example 3

Application of a concentrated supernatant

To ascertain whether trypsin has a direct effect on the cells or if it was mediated by the release of a soluble ligand, the supernatant of trypsin-treated cells was neutralized with 0.4% (high concentration) of soybean inhibitor to secure the inactivation of trypsin and concentrated ten times by using a Centricon Ultracel YM-3000. The small molecular weight cut-off allows the retention of most biologically active compounds. A parallel condition was run using only neutralized trypsin (without having been exposed to the cells) to control for putative residual trypsin activity. Trypsin-treated and enzyme-free treated cells were also included as positive and negative controls.

Results

As shown in Figure 3, the supernatant of trypsin-treated cells did not elicit differentiation, suggesting a more direct action of trypsin.

Example 4

Application of other extracellular proteases and PAR activating peptides

Thrombin, Plasmin (Sigma) and tPA (Actilyse, Boehringer Ingelheim) were diluted as per manufacturer recommendations and applied to the cells (hFASC#844 for experiments relating to Par activating peptides and tPa, hFASCs#853 for plasmin experiments) at different doses. Cell counts were performed 24 hours after the application. PAR-1

activating peptide (SFLLRN-NH2, Sigma) and PAR-2 activating peptide (SLIGKV-NH2, AnaSpec) were resuspended in sterile, distilled water and applied to the cultures at different concentrations. For the long-term culture, a concentration of 100 microMolar was used. Peptides were administered from fresh stocks every other day.

To test whether Protease-activated receptors could be considered to be candidates to mediate the action of extracellular proteases, the effects of PAR activating peptides were tested.

Results

The exposure to thrombin (Figure 4) and plasmin (Figure 5) generated the formation of neurites and projections. The application of tPA induced neural projections (Figure 6). When hFASCs (hFASC#844) were treated with activating peptides from PAR-1 and PAR-2 (Figure 7), differentiation was induced.

Example 5

To confirm that the effect detected could be mediated by PARs, it was established that they are expressed by human auditory stem cells.

Total RNA was extracted using Trizol (Invitrogen). cDNA synthesis was carried out by oligo (dT) primed reverse transcription using M-MLV RT (Stratagene). Primers used were: *PAR1* (for 5'-TGTGAACTGATCATGTTTATG-3', rev 5'-TTCGTAAGATAAGAGATATGT-3'); *PAR2* (for 5'-CCCTTTGTATGTCGTGAAGC-3', rev 5'-TTCCTGGAGTGTTTCTTTGAGG-3');

Figure 8 shows RT-PCT experiments detecting the expression of PAR-1, PAR-2 and t-PA in two hFASCs cell lines.

Example 6

Neuronal differentiation was studied by time-lapse video microscopy. hFetal Auditory Stem Cells were monitored by time-lapse video microscopy for 24hours after dissociation with trypsin. The induction of neurite projections is shown in Figure 19 which indicates that the induction is a well-coordinated phenomenon and not random stretching of the cells following the dissociation process.

Example 7

A non-peptide selective PAR1 antagonist, SCH79797 (available from Axon MedChem, the Netherlands) acts as an irreversible PAR1 inhibitor. To inhibit the effect of PAR1 *in vitro,* hFASCs were treated in parallel as shown in Figure 20. The percentages of bipolar neurons in culture were counted after 48 hours of treatment. Cells were incubated in (A) 50nM of trypsin, (B) pre-treated by 100μM of SCH79797 for 2 hours, following by co-incubation with 50nM of trypsin, (C) cultured in 100μM of SCH79797 without trypsin treatment and (D) cultured with trypsin vehicle only to act as a control.

It was shown that inhibition of PAR1 resulted in shorter neurite length and decreased the number of bipolar neurons (see Figure 21). The percentage of bipolar neurons produced from human Fetal Auditory Stem Cells (hFASCs) co-incubated with 50nM trypsin and 100μM of SCH79797 was significantly lower than in cells treated with trypsin only ($p<0.01$, n=3). This result indicates Par1 as a target of trypsin in the induction of bipolar neuron morphology during neuronal differentiation.

In addition, the length of neurites was compared after treatment by trypsin and trypsin plus SCH79797. Projections were measured using Axio Vision 4.6 Software, 24 hours after the treatment (see Figure 22). F1 of Figure 22 refers to the short neurite whilst F2 represents the longer neurite. FASCs pre-treated by 100μM of SCH79797 before co-incubation with 50nM of trypsin showed shorter F1 and F2 neurites than cells treated with trypsin without PAR1 antagonist (see Figure 23 and 24). The neurite length of F1 with treatment with trypsin was 26.2+/- 11.4 (n=54) whilst the neurite length of F1 with treatment with trypsin and SCH79797 was 17.04 +/- 8.97 (n+25); $p<0.01$. The neurite length of the F2 neurites when treated with trypsin was 46.8+/- 24.7 (n =54) whilst the F2 neurite length when treated with trypsin and SCH79797 was 17.04 +/- 15.1 (n =25) $p<0.01$.

Example 8

Establishment of mouse fetal auditory stem cells as a model to study the response of extracellular proteases.

The use of a mouse model would facilitate certain experiments that cannot be performed in humans. It therefore had to be validated that protease mediated induction of neuritogenesis was also detected in mouse cells. Mouse fetal auditory stem cells (mFASCs) were isolated in the same way to obtain human cells. They were cultured in otic stem cells full media (OSCFM) and demonstrated their ability to differentiate into the two main cell types of the inner ear, sensory hair cells and spiral ganglion neurons, after cultured in differentiating medium for 7 days. When exposed to trypsin, neuritogenesis was induced in a similar fashion as human cells, albeit the response was less remarkable (the neuritis were shorter than in human cells). Expression of PAR1 and PAR2 was detected in mFASCs by semi- quantitative RT-PCR as in hFASCs (see Figure 25), indicating that the mouse system may be a good model. Neuritogenesis in mouse cells was also inhibited by the PAR-1 antagonist (see Figure 26).

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

The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

CLAIMS

1. An agent, which is an agonist of a Protease Activated Receptor (PAR), for use to (a) stimulate neurite growth by an auditory neuronal cell or (b) stimulate differentiation of auditory stem cells and/or auditory neuronal cells or (c) combinations of (a) and (b).

2. An agent according to claim 1, which is for use in treating sensorineural hearing loss.

3. An agent according to claim 1, which is for improving representation of sounds by a patient who suffers from sensorineural hearing loss.

4. An agent according to any preceding claim, which is an agonist of PAR-1.

5. An agent according to any preceding claim, which is an agonist of PAR-2.

6. An agent according to any preceding claim, which is an agonist of PAR-3 and/or PAR-4.

7. An agent according to any of claims 4 to 6, wherein the PAR protein is expressed on the auditory neuronal cell and/or the auditory stem cell.

8. An agent according to any preceding claim, wherein the auditory neuronal cell is a neuron associated with, or previously associated, with a hair cell.

9. An agent according to any preceding claim, wherein the auditory neuronal cell is a spiral cochlear ganglion cell.

10. An agent according to any preceding claim, which is selected from a protein, a peptide, a small molecule, an antibody, an antibody fragment and a fusion protein.

11. An agent according to any preceding claim, which comprises a polypeptide selected from a protease, a derivative thereof, an active fragment thereof and a precursor form thereof.

12. An agent according to claim 11, which is a serine protease.

13. An agent according to claim 12, which is an S1 serine protease.

14. An agent according to claim 12, which is selected from tissue plasminogen activator (t-Pa), thrombin, Ancrod, plasmin and fragments thereof and derivatives thereof.

15. An agent according to claim 10, which is a PAR activating peptide, wherein optionally the agent is selected from a PAR-1 activating peptide and a PAR-2 activating peptide.

16. An agent according to claim 15, which comprises a sequence selected from SFLLRN-NH2 or SLIGKV-NH2.

17. An agent for use to stimulate neuritogenesis, for example, neurite growth by an auditory neuronal cell and/or induce auditory stem cell differentiation, wherein the agent is a protease or active fragment thereof.

18. An agent according to claim 15, which is for treating sensorineural hearing loss, wherein optionally a patient's representation of sound is improved.

19. An agent according to claim 17 or claim 18, which is selected from tissue plasminogen activator (t-Pa), thrombin, plasmin, Ancrod, fragment thereof, precursor forms thereof and derivatives thereof.

20. An agent according to any preceding claim in combination with an intra-auditory device.

21. A device which comprises an agent according to any of claims 1 to 19.

22. A device according to claim 21, which is suitable for implantation in a patient's ear.

23. The device according to claim 21 or claim 22, which is selected from a cochlear implant, a catheter and a wick.

24. The device according to any of claims 21 to 23, which is coated with the agent.

25. A method of stimulating neurite growth by an auditory neuronal cell and/or inducing auditory neuronal cell differentiation comprising delivering an agent according to any of claims 1 to 19 to said auditory neuronal cell.

26. A method according to claim 25, which is an *in vitro* method.

27. A method according to claim 25, wherein the agent is administered to a patient, and is optionally delivered to a patient's ear, wherein the agent is administered in a therapeutically effective amount.

28. A method of treating sensorineural hearing loss comprising administering an agent of any of claims 1 to 19 to a patient.

29. A method according to claim 28, wherein the agent is delivered to a patient's cochlear.

30. A method according to any of claims 25 to 29, wherein the agent is administered in combination with an implantable device.

31. A pharmaceutical formulation comprising the agent of any of claims 1 to 19 and a pharmaceutically acceptable excipient.

32. A kit comprising an agent according to any of claims 1 to 19 and an implantable device.

33. A kit according to claim 32, wherein the implantable device is a cochlear implant.

34. Use of an agent according to any of claims 1 to 19 for the manufacture of a medicament for the stimulation of auditory stem cell differentiation.

35. Use of an agent according to any of claims 1 to 19 for the manufacture of a medicament for the stimulation of auditory neuronal cell differentiation.

36. Use of an agent according to any of claims 1 to 19 for the manufacture of a medicament for the stimulation of growth of a neurite by an auditory neuronal cell.

37. Use of an agent according to any of claims 1 to 19 for the manufacture of a medicament for the treatment of sensorineural hearing loss.

38. Use according to any of claims 34 to 37 wherein the medicament is for administration in combination with a device e.g. an implant.

39. Use according to claim 38, wherein the device is a cochlear implant.

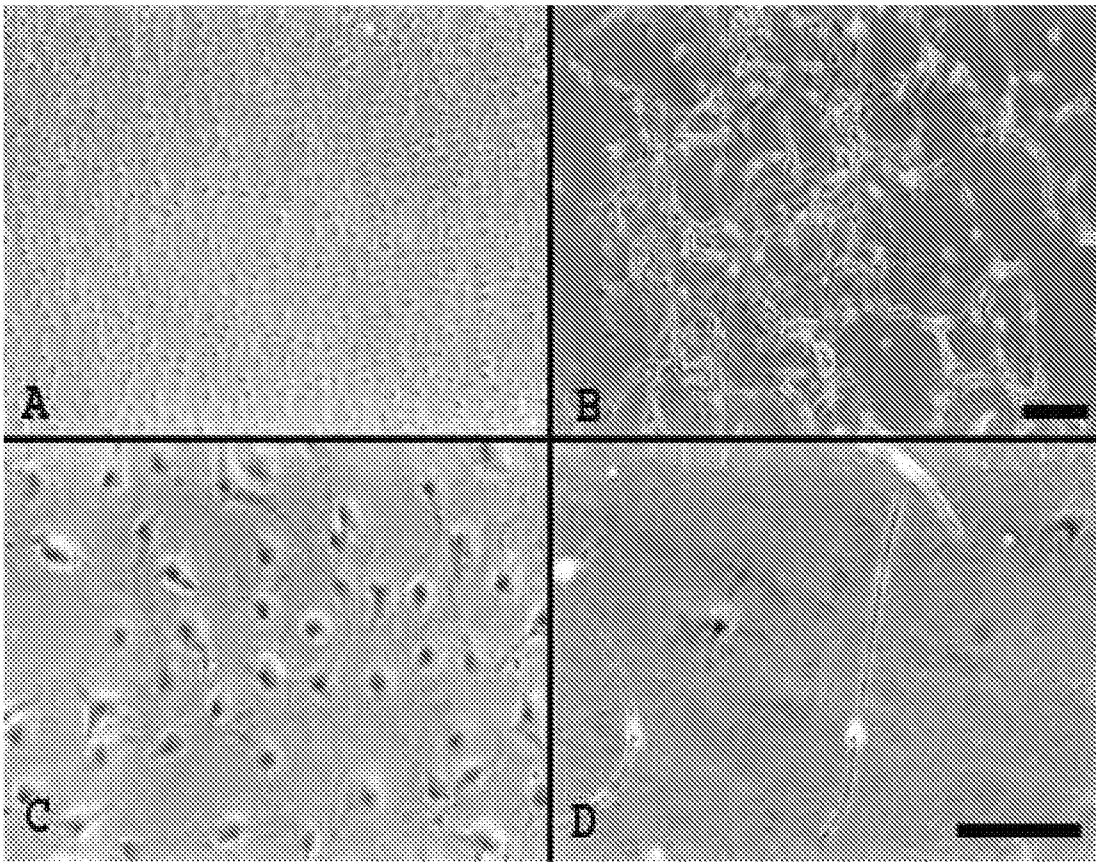
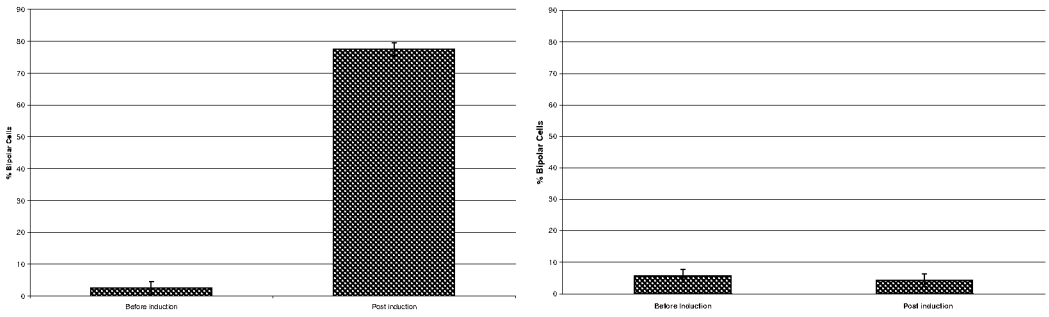


Figure 1



Before induction Post-induction Before induction Post induction

Figure 2

Figure 3

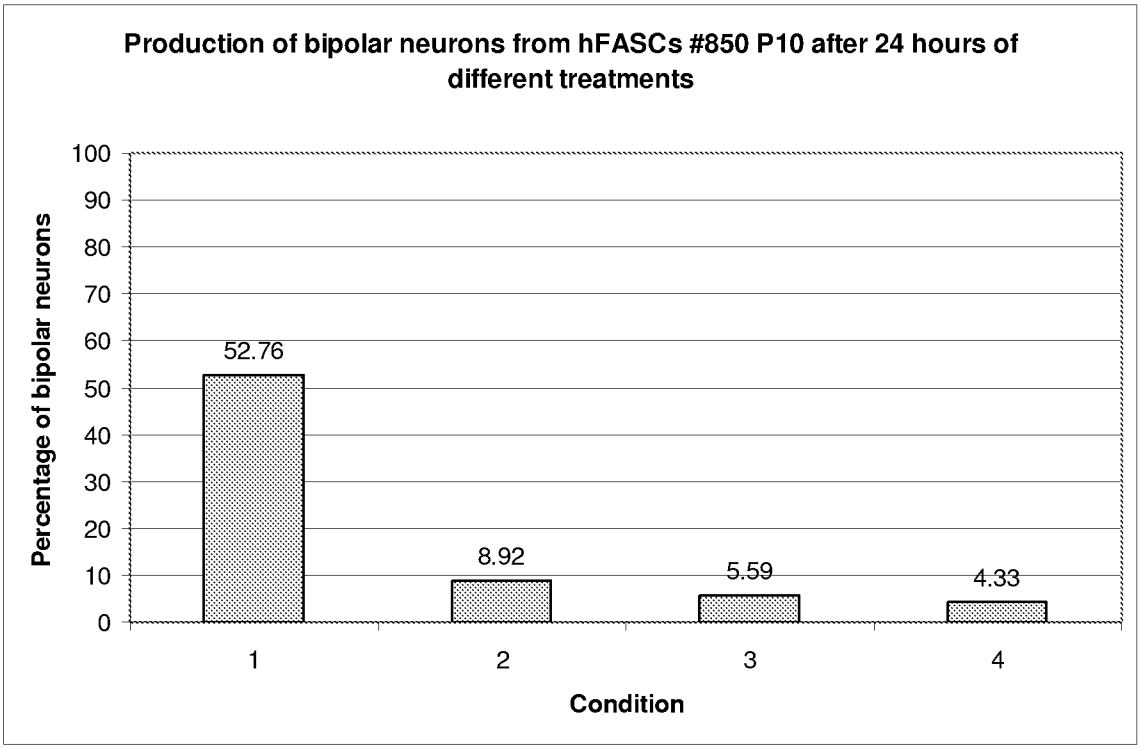

Production of bipolar neurons from hFASCs #850 P10 after 24 hours of different treatments
Percentage of bipolar neurons
100
90
80
70
60
50
40
30
20
10
0
52.76
8.92
5.59
4.33
1
2
3
4
Condition

Figure 4

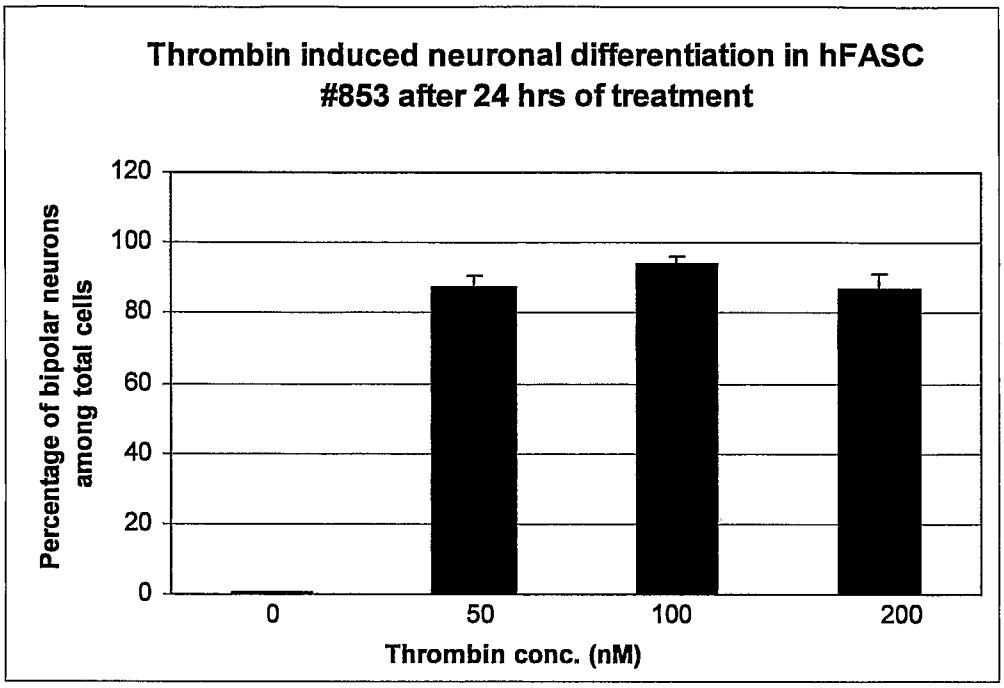

Thrombin induced neuronal differentiation in hFASC #853 after 24 hrs of treatment
Percentage of bipolar neurons among total cells
120
100
80
60
40
20
0
0
50
100
200
Thrombin conc. (nM)

Figure 5

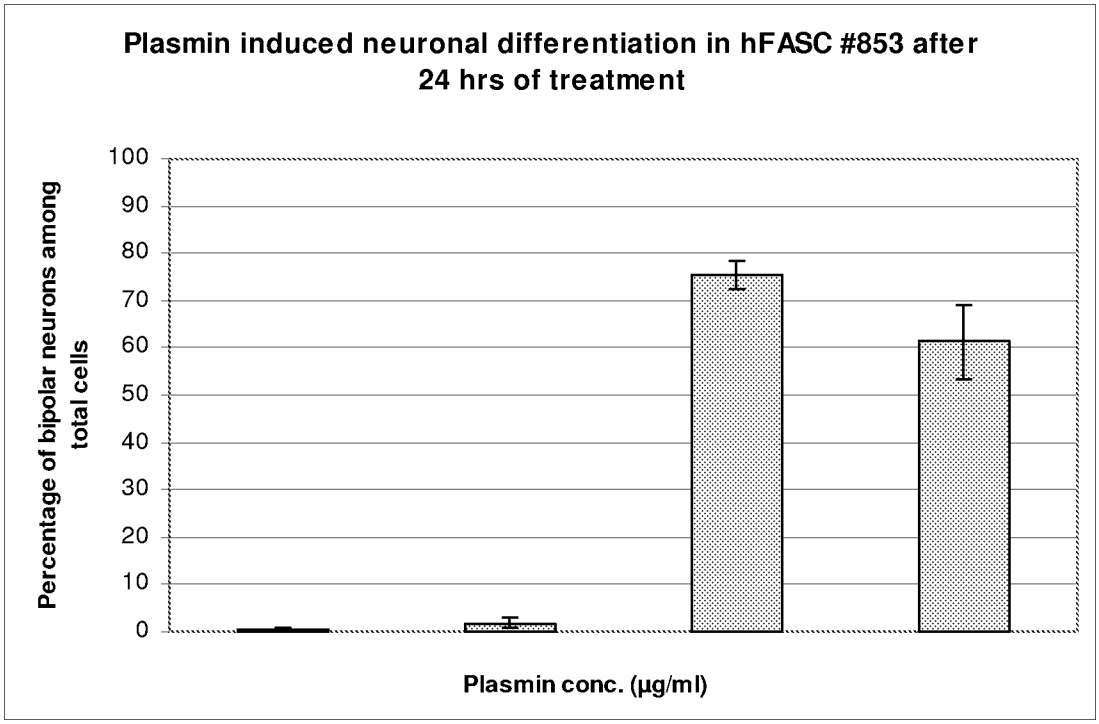

Plasmin induced neuronal differentiation in hFASC #853 after 24 hrs of treatment
Percentage of bipolar neurons among total cells
100
90
80
70
60
50
40
30
20
10
0
Plasmin conc. (µg/ml)

Figure 6

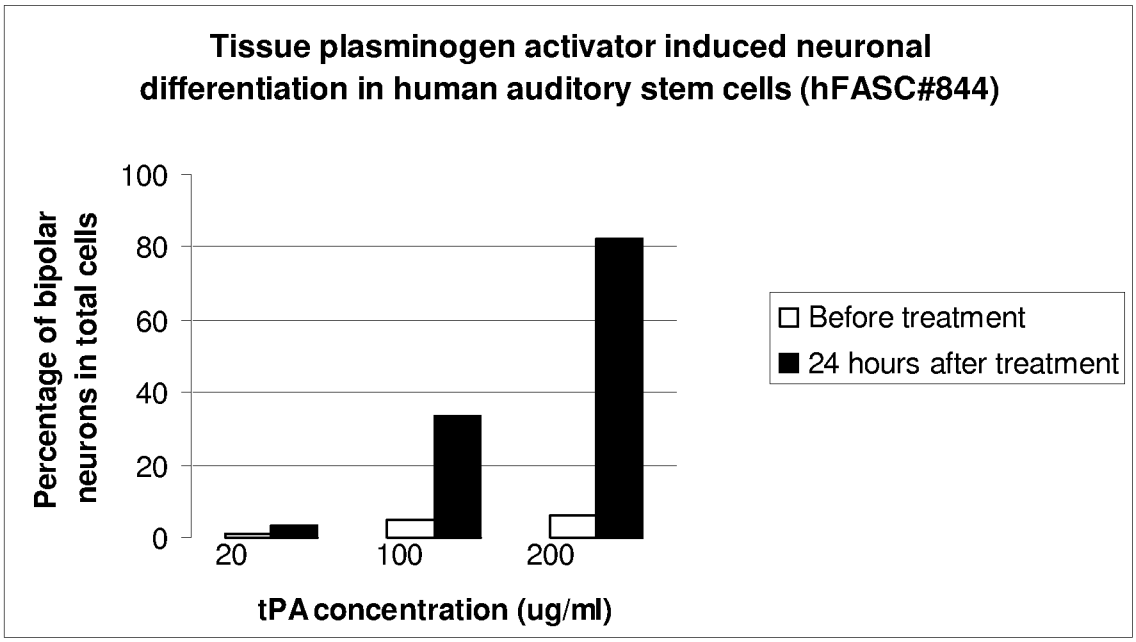

Tissue plasminogen activator induced neuronal differentiation in human auditory stem cells (hFASC#844)
Percentage of bipolar neurons in total cells
100
80
60
40
20
0
20
100
200
tPA concentration (ug/ml)
Before treatment
24 hours after treatment

Figure 7a

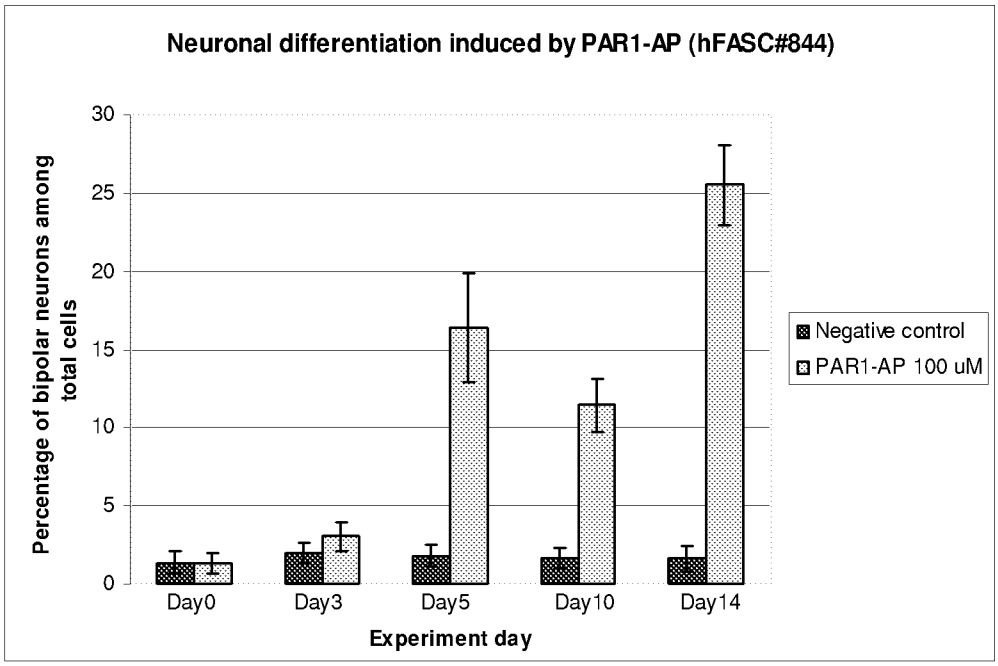

Neuronal differentiation induced by PAR1-AP (hFASC#844)
Percentage of bipolar neurons among total cells
30
25
20
15
10
5
0
Day0
Day3
Day5
Day10
Day14
Experiment day
Negative control
PAR1-AP 100 uM

Figure 7b

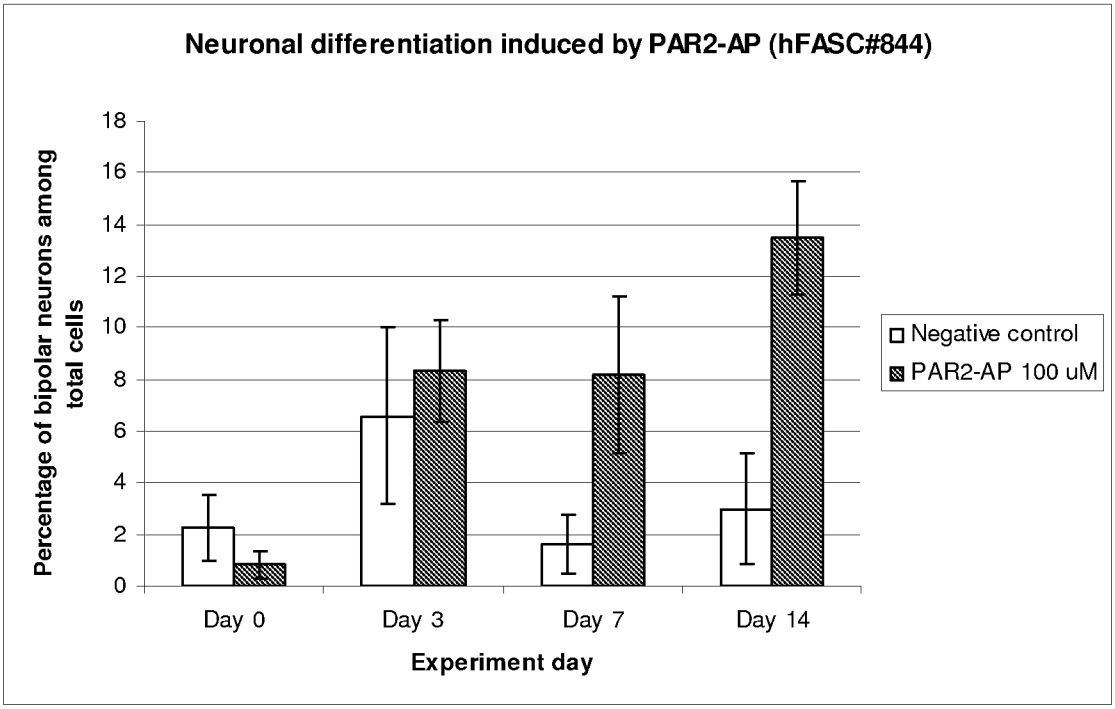

Neuronal differentiation induced by PAR2-AP (hFASC#844)
Percentage of bipolar neurons among total cells
18
16
14
12
10
8
6
4
2
0
Day 0
Day 3
Day 7
Day 14
Experiment day
Negative control
PAR2-AP 100 uM

Figure 8

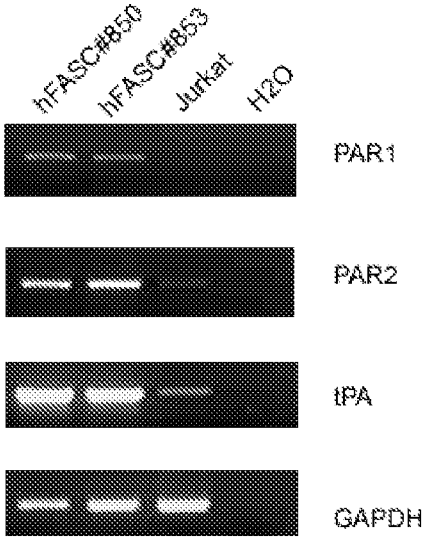


Figure 9

1 mgprrlllva acfslcgpll sartrarrpe skatnatldp rsfllrnpnd kyepfwedee
61 knesglteyr lvsinksspl qkqlpafise dasgyltssw ltlfvpsvyt gvfvvslpln
121 imaivvfilk mkvkkpavvy mlhlatadvl fvsvlpfkis yyfsgsdwqf gselcrfvta
181 afycnmyasi llmtvisidr flavvypmqs lswrtlgras ftclaiwala iagvvplllk
241 eqtiqvpgln ittchdvlne tllegyyayy fsafsavfff vpliistvcy vsiirclsss
301 avanrskksr alflsaavfc ifiicfgptn vlliahysfl shtstteaay fayllcvcvs
361 sisccidpli yyyassecqr yvysilccke ssdpssynss gqlmaskmdt cssnlnnsiy
421 kkllt

SEQ ID. No. 1

Figure 10

1 mrspsaawll gaaillaasl scsgtiqgtn rsskgrslig kvdgtshvtg kgvtvetvfs
61 vdefsasvlt gklttvflpi vytivfvvgl psngmalwvf lfrtkkkhpa viymanlala
121 dllsviwfpl kiayhihgnn wiygealcnv ligffygnmy csilfmtcls vqrywvivnp
181 mghsrkkani aigislaiwl lillvtiply vvkqtifipa lnittchdvl peqllvgdmf
241 nyflslaigv flfpafltas ayvlmirmlr ssamdensek krkraikliv tvlamylicf
301 tpsnlllvvh yfliksqgqs hvyalyival clstlnscid pfvyyfvshd frdhaknall
361 crsvrtvkqm qvsltskkhs rksssyssss ttvktsy

SEQ. ID. NO. 2

Figure 11

1 mkalifaaag llllptfcq sgmendtnnl akptlpiktf rgappnsfee fpfsalegwt
61 gatitvkikc peesashlhv knatmgylts slstklipai yllvfvvgvp anavtlwmlf
121 frtrsicttv fytnlaiadf lfcvtlpfki ayhlngnnwv fgevlcratt vifygnmycs
181 illlacisin rylaivhpft yrglpkhtya lvtcglvwat vflymlpffi lkqeyylvqp
241 dittchdvhn tcessspfql yyfislaffg flipfvliiy cyaaiirtln aydhrwlwyv
301 kasllilvif ticfapsnii liihhanyyy nntdglyfiy lialclgsln scldpflyfl
361 msktrnhsta yltk

SEQ ID. No. 3

Figure 12

1 mwgrlllwpl vlgfslsggt qtpsvydesg stgggddstp silpaprgyp gqvcandsdt
61 lelpdssral llgwvptrlv palyglvlvv glpanglalw vlatqaprlp stmllmnlaa
121 adlllalalp priayhlrgq rwpfgeaacr lataalyghm ygsvlllaav sldrylalvh
181 plraralrgr rlalglcmaa wlmaaalalp ltlqrqtfrl arsdrvlchd alpldaqash
241 wqpaftclal lgcflpllam llcygatlht laasgrrygh alrltavvla savaffvpsn
301 lllllhysdp spsawgnlyg ayvpslalst lnscvdpfiy yyvsaefrdk vraglfqrsp
361 gdtvaskasa eggsrgmgth ssllq

SEQ ID. No. 4

Figure 13

1 ggcgggggggc gcacagagcc agaggggcttt gcgagcggcg gctgagggac cgcggggagg
61 gggcgccgag cggctccagc gcagagactc tcactgcacg ccggaggccc cttcctcgct
121 ccgcccgcgc gaccgcgcgc cccagtcccg ccccgccccg ctaaccgccc cagacacagc
181 gctcgccgag ggtcgcttgg accctgatct tacccgtggg caccctgcgc tctgcctgcc
241 gcgaagaccg gctccccgac ccgcagaagt caggagagag ggtgaagcgg agcagcccga
301 ggcggggcag cctcccggag cagcgccgcg cagagcccgg gacaatgggg ccgcggcggc
361 tgctgctggt ggccgcctgc ttcagtctgt gcggcccgct gttgtctgcc cgcacccggg
421 cccgcaggcc agaatcaaaa gcaacaaatg ccaccttaga tccccggtca tttcttctca
481 ggaaccccaa tgataaatat gaaccatttt gggaggatga ggagaaaaat gaaagtgggt
541 taactgaata cagattagtc tccatcaata aaagcagtcc tcttcaaaaa caacttcctg
601 cattcatctc agaagatgcc tccggatatt tgaccagctc ctggctgaca ctctttgtcc
661 catctgtgta caccggagtg tttgtagtca gcctcccact aaacatcatg gccatcgttg
721 tgttcatcct gaaaatgaag gtcaagaagc cggcggtggt gtacatgctg cacctggcca
781 cggcagatgt gctgtttgtg tctgtgctcc cctttaagat cagctattac ttttccggca
841 gtgattggca gtttgggtct gaattgtgtc gcttcgtcac tgcagcattt tactgtaaca
901 tgtacgcctc tatcttgctc atgacagtca taagcattga ccggtttctg gctgtggtgt
961 atcccatgca gtcctctcc tggcgtactc tgggaagggc ttccttcact tgtctggcca
102 1 tctgggcttt ggccatcgca ggggtagtgc ctctcgtcct caaggagcaa accatccagg
1081 tgcccgggct caacatcact acctgtcatg atgtgctcaa tgaaaccctg ctcgaaggct
1141 actatgccta ctacttctca gccttctctg ctgtcttctt ttttgtgccg ctgatcattt
1201 ccacggtctg ttatgtgtct atcattcgat gtcttagctc ttccgcagtt gccaaccgca
1261 gcaagaagtc ccgggctttg ttcctgtcag ctgctgtttt ctgcatcttc atcatttgct
1321 tcggacccac aaacgtcctc ctgattgcgc attactcatt cctttctcac acttccacca
1381 cagaggctgc ctactttgcc tacctcctct gtgtctgtgt cagcagcata agctcgtgca
1441 tcgacccccct aatttactat tacgcttcct ctgagtgcca gaggtacgtc tacagtatct
1501 tatgctgcaa agaaagttcc gatcccagca gttataacag cagtgggcag ttgatggcaa
1561 gtaaaatgga tacctgctct agtaacctga ataacagcat atacaaaaag ctgttaactt
1621 aggaaaaggg actgctggga ggttaaaaag aaaagtttat aaaagtgaat aacctgagga
1681 ttctattagt ccccacccaa actttattga ttcacctcct aaaacaacag atgtacgact
1741 tgcatacctg ctttttatgg gagctgtcaa gcatgtattt ttgtcaatta ccagaaagat
1801 aacaggacga gatgacggtg ttattccaag ggaatattgc caatgctaca gtaataaatg
1861 aatgtcactt ctggatatag ctaggtgaca tatacatact tacatgtgtg tatatgtaga
1921 tgtatgcaca cacatatatt atttgcagtg cagtatagaa taggcacttt aaaacactct
1981 ttccccgcac cccagcaatt atgaaaataa tctctgattc cctgatttaa tatgcaaagt
2041 ctaggttggt agagtttagc cctgaacatt tcatggtgtt catcaacagt gagagactcc
2101 atagtttggg cttgtaccac ttttgcaaat aagtgtattt tgaaattgtt tgacggcaag
2161 gtttaagtta ttaagaggta agacttagta ctatctgtgc gtagaagttc tagtgttttc
2221 aattttaaac atatccaagt ttgaattcct aaaattatgg aaacagatga aaagcctctg
2281 ttttgatatg ggtagtattt tttacatttt acacactgta cacataagcc aaaactgagc
2341 ataagtcctc tagtgaatgt aggctggctt tcagagtagg ctattcctga gagctgcatg
2401 tgtccgcccc cgatggagga ctccaggcag cagacacatg ccagggccat gtcagacaca
2461 gattggccag aaaccttcct gctgagcctc acagcagtga gactggggcc actacatttg
2521 ctccatcctc ctgggattgg ctgtgaactg atcatgttta tgagaaactg gcaaagcaga

2581 atgtgatatc ctaggaggta atgaccatga aagacttctc tacccatctt aaaaacaacg
2641 aaagaaggca tggacttctg gatgcccatc cactgggtgt aaacacatct agtagttgtt
2701 ctgaaatgtc agttctgata tggaagcacc cattatgcgc tgtggccact ccaataggtg
2761 ctgagtgtac agagtggaat aagacagaga cctgccctca agagcaaagt agatcatgca
2821 tagagtgtga tgtatgtgta ataaatatgt ttcacacaaa caaggcctgt cagctaaaga
2881 agtttgaaca tttgggttac tatttcttgt ggttataact taatgaaaac aatgcagtac
2941 aggacatata ttttttaaaa taagtctgat ttaattgggc actatttatt tacaaatgtt
3001 ttgctcaata gattgctcaa atcaggtttt cttttaagaa tcaatcatgt cagtctgctt
3061 agaaataaca gaagaaaata gaattgacat tgaaatctag gaaaattatt ctataatttc
3121 catttactta agacttaatg agactttaaa agcatttttt aacctcctaa gtatcaagta
3181 tagaaaatct tcatggaatt cacaaagtaa tttggaaatt aggttgaaac atatctctta
3241 tcttacgaaa aaatggtagc attttaaaca aaatagaaag ttgcaaggca aatgtttatt
3301 taaaagagca ggccaggcgc ggtggctcac gcctgtaatc ccagcacttt gggaggctga
3361 ggcgggtgga tcacgaggtc aggagatcga gaccatcctg gctaacacgg tgaaacccgt
3421 ctctactaaa aatgcaaaaa aaattagccg ggcgtggtgg caggcacctg tagtcccagc
3481 tactcgggag gctgaggcag gagactggcg tgaacccagg aggcggacct tgtagtgagc
3541 cgagatcgcg ccactgtgct ccagcctggg caacagagca agactccatc tc

SEQ ID No. 5 (PAR-1)

Figure 14

1 tgaaacctaa cccgccctgg ggaggcgcgc agcagaggct ccgattcggg gcaggtgaga
61 ggctgacttt ctctcggtgc gtccagtgga gctctgagtt tcgaatcggc ggcggcggat
121 tccccgcgcg cccggcgtcg gggcttccag gaggatgcgg agccccagcg cggcgtggct
181 gctgggggcc gccatcctgc tagcagcctc tctctcctgc agtggcacca tccaaggaac
241 caatagatcc tctaaaggaa gaagccttat tggtaaggtt gatggcacat cccacgtcac
301 tggaaaagga gttacagttg aaacagtctt ttctgtggat gagttttctg catctgtcct
361 cactggaaaa ctgaccactg tcttccttcc aattgtctac acaattgtgt ttgtggtggg
421 tttgccaagt aacggcatgg ccctgtgggt ctttcttttc cgaactaaga agaagcaccc
481 tgctgtgatt tacatggcca atctggcctt ggctgacctc ctctctgtca tctggttccc
541 cttgaagatt gcctatcaca tacatggcaa caactggatt tatggggaag ctctttgtaa
601 tgtgcttatt ggctttttct atggcaacat gtactgttcc attctcttca tgacctgcct
661 cagtgtgcag aggtattggg tcatcgtgaa ccccatgggg cactccagga agaaggcaaa
721 cattgccatt ggcatctccc tggcaatatg gctgctgatt ctgctggtca ccatcccttt
781 gtatgtcgtg aagcagacca tcttcattcc tgccctgaac atcacgacct gtcatgatgt
841 tttgcctgag cagctcttgg tgggagacat gttcaattac ttcctctctc tggccattgg
901 ggtctttctg ttcccagcct tcctcacagc ctctgcctat gtgctgatga tcagaatgct
961 gcgatcttct gccatggatg aaaactcaga gaagaaaagg aagagggcca tcaaactcat
1021 tgtcactgtc ctggccatgt acctgatctg cttcactcct agtaaccttc tgcttgtggt
1081 gcattatttt ctgattaaga gccagggcca gagccatgtc tatgccctgt acattgtagc
1141 cctctgcctc tctaccctta acagctgcat cgacccctttt gtctattact ttgtttcaca
1201 tgatttcagg gatcatgcaa agaacgctct cctttgccga agtgtccgca ctgtaaagca

1261 gatgcaagta tccctcacct caaagaaaca ctccaggaaa tccagctctt actcttcaag
1321 ttcaaccact gttaagacct cctattgagt tttccaggtc ctcagatggg aattgcacag
1381 taggatgtgg aacctgttta atgttatgag gacgtgtctg ttatttccta atcaaaaagg
1441 tctcaccaca taccatgtgg atgcagcacc tctcaggatt gctaggagct cccctgtttg
1501 catgagaaaa gtagtccccc aaattaacat cagtgtctgt ttcagaatct ctctactcag
1561 atgaccccag aaactgaacc aacagaagca gacttttcag aagatggtga agacagaaac
1621 ccagtaactt gcaaaaagta gacttggtgt gaagactcac ttctcagctg aaattatata
1681 tatacacata tatatatttt acatctggga tcatgataga cttgttaggg cttcaaggcc
1741 ctcagagatg atcagtccaa ctgaacgacc ttacaaatga ggaaaccaag ataaatgagc
1801 tgccagaatc aggtttccaa tcaacagcag tgagttggga ttggacagta gaatttcaat
1861 gtccagtgag tgaggttctt gtaccacttc atcaaaatca tggatcttgg ctgggtgcgg
1921 tgcctcatgc ctgtaatcct agcactttgg gaggctgagg caggcaatca cttgaggtca
1981 ggagttcgag accagcctgg ccatcatggc gaaacctcat ctctactaaa aatacaaaag
2041 ttaaccaggt gtgtggtgca cgtttgtaat cccagttact caggaggctg aggcacaaga
2101 attgagtatc actttaactc aggaggcaga ggttgcagtg agccgagatt gcaccactgc
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2281 aaacctgcat ggtgtttatg cacacagaga tttgagaacc attgttctga atgctgcttc
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2401 tgttcagctt ataatgaaat ctgtttgttg acttattagg actttgaatt atttctttat
2461 taaccctctg agtttttgta tgtattatta ttaaagaaaa atgcaatcag gattttaaac
2521 atgtaaatac aaattttgta taacttttga tgacttcagt gaaattttca ggtagtctga
2581 gtaatagatt gttttgccac ttagaatagc atttgccact tagtatttta aaaaataatt
2641 gttggagtat ttattgtcag ttttgttcac ttgttatcta atacaaaatt ataaagcctt
2701 cagagggttt ggaccacatc tctttggaaa atagtttgca acatatttaa gagatacttg
2761 atgccaaaat gactttatac aacgattgta tttgtgactt ttaaaaataa ttattttatt
2821 gtgtaattga tttataaata acaaaatttt ttttacaact taaaaaaaaa aaaaaa

SEQ ID No. 6

Figure 15

1 gtggattaca tcctttccct gcggaggacc agggcaagtt tcctgcctgc acggcacagg
61 agagcaaact tctacagaca gaccaaggct tccatttgct gctgacacat ggaactgagg
121 tgaaattgtg ctccatgatt ttacagattt cataacgttt aagagacggg actcaggtca
181 tcaaaatgaa agccctcatc tttgcagctg ctggcctcct gcttctgttg cccacttttt
241 gtcagagtgg catggaaaat gatacaaaca acttggcaaa gccaacctta cccattaaga
301 cctttcgtgg agctccccca aattcttttg aagagttccc cttttctgcc ttggaaggct
361 ggacaggagc cacgattact gtaaaaatta agtgccctga agaaagtgct tcacatctcc
421 atgtgaaaaa tgctaccatg gggtacctga ccagctcctt aagtactaaa ctgatacctg
481 ccatctacct cctggtgttt gtagttggtg tcccggccaa tgctgtgacc ctgtggatgc

541 ttttcttcag gaccagatcc atctgtacca ctgtattcta caccaacctg gccattgcag
601 attttctttt ttgtgttaca ttgcccttta agatagctta tcatctcaat gggaacaact
661 gggtatttgg agaggtcctg tgccgggcca ccacagtcat cttctatggc aacatgtact
721 gctccattct gctccttgcc tgcatcagca tcaaccgcta cctggccatc gtccatcctt
781 tcacctaccg gggcctgccc aagcacacct atgccttggt aacatgtgga ctggtgtggg
841 caacagtttt cttatatatg ctgccatttt tcatactgaa gcaggaatat tatcttgttc
901 agccagacat caccacctgc catgatgttc acaacacttg cgagtcctca tctcccttcc
961 aactctatta cttcatctcc ttggcattct ttggattctt aattccattt gtgcttatca
1021 tctactgcta tgcagccatc atccggacac ttaatgcata cgatcataga tggttgtggt
1081 atgttaaggc gagtctcctc atccttgtga tttttaccat ttgctttgct ccaagcaata
1141 ttattcttat tattcaccat gctaactact actacaacaa cactgatggc ttatatttta
1201 tatatctcat agctttgtgc ctgggtagtc ttaatagttg cttagatcca ttcctttatt
1261 ttctcatgtc aaaaaccaga aatcactcca ctgcttacct tacaaaatag tgaaatgatc
1321 ttagagaaca aggacagcca tcacagagaa cgtctgtttt caagaacaac ataagcatag
1381 tgcaaggagc tccatttccg agctcctaag aaatatgctt caaaggtcaa acattacaaa
1441 agcattagta gtttgtttgt ttgtttttga gactgagtct cactttatca cccagactgg
1501 cgtgcagtgg cactatcttg gctcattgca acctctgcct cccaggtcag cctcccaagt
1561 agctgggatt acaccaccat gcccagctac taaaaatact tgtatttttа gtagagacgg
1621 ggtttcacca tgttgaccag gctggtcttg aactcctgac ctcaagtgat cttccggcct
1681 cagcctccca aagtgctgga ttacaggcgt gagccactga gccagccagc attagtaatt
1741 tttaaaaaca ctttatcagt attttaaaaa tgttaatgca ggagaaaaga tatcacaact
1801 ctatggaaaa tgacatttcc atttgcctta ttgctacttc aagctcttta aatcaccatc
1861 ttccctattt ctgtgagtgg tattgccatc cttgacattt gacatcattt tttattcttt
1921 ggtctctttt gactctcatg ctggtggctg cctcatcaac tgattctatc tttgtagggt
1981 ccctcaccag ggtcttttat ctagtttcac ctttgccttc ttttttcctt tttttaatag
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2161 caggcatgca ccaccatacc caactaattt tatttttttat tttttgcaga gatggagtct
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2281 ccaaagtgct gggactacag gcatgagcca ctacgtccag gccacctcta ccttctaacc
2341 attctctcca ctgccagcct accctccacc acttcacaag tcctgccaga ttaatcttcc
2401 ttagatatca gttaggatac atcactaaaa catttccact aactccactg tttctataat
2461 aaatcataaa tctttagcct ggcattcaag gctaacttgc taatcacact tcccattaaa
2521 ccttgcttat gatataggca ccaaccaaac ctcttgctct gctatatctc cactctgcct
2581 cagactgaaa agctagtcct aacgtctttg ccttaattct gttcattttt tcaagtcctt
2641 gaaggaccat tcttccctga ttctcaggct agaagtgtca cttttcttat ctgtacttcc
2701 aaagcacttt cgtatatttt tattatggca tttatatata gttcatttat atttaaattt
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3001 gcatacaaaa aagtaaggaa gctaaactaa gactatactc accaggccat ttagaagttt
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3181 ttagacagga aggaagacat tagtgattag acaggaggga agatattagt gattagacag

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3301 aactttaagc ataaaagatt cctgagatga ctttacaagt ctgtacgaat ctgccttgac
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3421 aaaaaaa

SEQ ID NO. 7

Figure 16

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121 ggtccggcga ggcaggaagc ctgaggccac agcccagagc agcctgagtg cagtcatgtg
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301 cctgcctgcc ccccgcggct acccaggcca agtctgtgcc aatgacagtg acaccctgga
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1021 gaggctgacc gcagtggtgc tggcctccgc cgtggccttc ttcgtgccca gcaacctgct
1081 gctgctgctg cattactcgg acccgagccc cagcgcctgg ggcaacctct atggtgccta
1141 cgtgcccagc ctggcgctga gcaccctcaa cagctgcgtg gatcccttca tctactacta
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1381 acttcacgtc cttcctggga cctcagaatg tgaccttatt tggaaatagg gttgttacaa
1441 ctgtcactag cggaggtcac tttggagaag ggtgggcctt acatccagtg tgggtggtgt
1501 cctcataaga taaggagagg ccaggcctgg tggctcacgc ctgtaatccc agcactttaa
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1621 taaaacccca tctctaccaa aaatacaaaa attagctggg cttggtggct ggcgcctgta
1681 atcccagcta ctcaggagac tgaggcagaa ggatcgcttg aacctgggag gcagaggttg
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1801 ttaattaatt aattaattta attcaatttt aaaaagacga aaagtgacgg ccaggtgcag
1861 tggctcacgc ctataatctc agcactctgg gaggccaaga tggaggattg cttgaagcca
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2041 gggaggatca cttgagcctg ggaggttgtg gttgcagtga gctatgattg taccactgca
2101 ctccagcctg ggcaacagag caagaccttg tctcaaaaat aaacaaacta aaattaaaaa
2161 aagaagacga gagatagtgg gtgtggtggc tcacacctgc aatcccagca ctttggaagg
2221 ccgaggtggg cagatcatct gaggccagga gttcaagacc agcctggcta acatggtgaa
2281 atcctatctc taccaaaaat acaaaaatta gccaggcgtg gtggtgggca cctgtactgg
2341 ggaggtgccc acccagctac tggggaggct gagtcaggag aatcgcttga acctgggagg
2401 cggaggttgc ggtcagctga gatggtgcca ctgcactcca gcctgggcga aagagcgact
2461 ctgtctccaa aaaaaagaga agaggagagg acacagagac acacagagaa gaaagccatg
2521 tggcggcaga ggcagagatg ggagtgatgc ggacggacac aaactaaggg atgccacgat
2581 gccaagcaca gccaacagcc accagcagcc aggagacagg cctgggacgg gctctccctc
2641 acagcctcca gagggaacca gccctgccac caccttgacc ctggacttct ggcctgcaga
2701 actgtgagac aataaactct cattgtttta a

SEQ ID. NO. 8

Figure 17

1 rrgarsyqvi crdektqmiy qqhqswlrpv lrsnrveycw cnsgraqchs vpvkscsepr
61 cfnggtcqqa lyfsdfvcqc pegfagkcce idtratcyed qgisyrgtws taesgaectn
121 wnssalaqkp ysgrrpdair lglgnhnycr npdrdskpwc yvfkagkyss efcstpacse
181 gnsdcyfgng sayrgthslt esgasclpwn smiligkvyt aqnpsaqalg lgkhnycrnp
241 dgdakpwchv lknrrltwey cdvpscstcg lrqysqpqfr ikgglfadia shpwqaaifa
301 khrrspgerf lcggilissc wilsaahcfq erfpphhltv ilgrtyrvvp geeeqkfeve
361 kyivhkefdd dtydndiall qlksdssrca qessvvrtvc lppadlqlpd wtecelsgyg
421 khealspfys erlkeahvrl ypssrctsqh llnrtvtdnm lcagdtrsgg pqanlhdacq
481 gdsggplvcl ndgrmtlvgi iswglgcgqk dvpgvytkvt nyldwirdnm rp

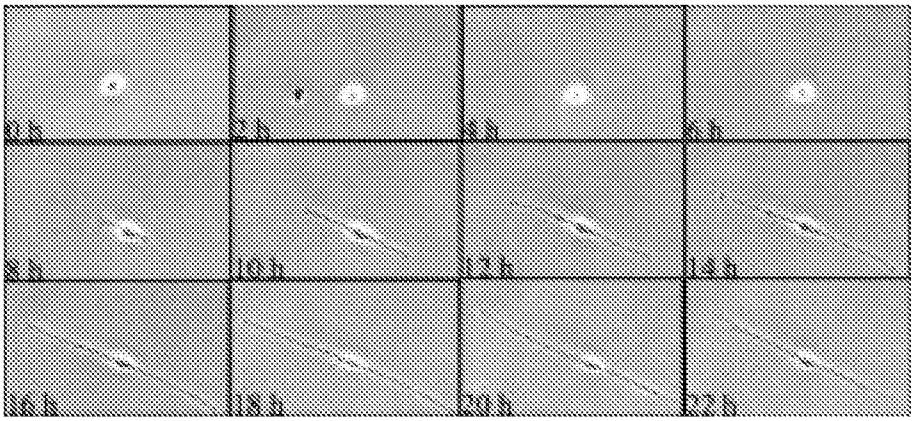
SEQ ID. No. 9 (t-Pa)

Figure 18

Prothrombin

MAHVRGLQLP GCLALAALCS LVHSQHVFLA PQQARSLLQR
VRRANTFLEE VRKGNLEREC VEETCSYEEA FEALESSTAT
DVFWAKYTAC ETARTPRDKL AACLEGNCAE GLGTNYRGHV
NITRSGIECQ LWRSRYPHKP EINSTTHPGA DLQENFCRNP
DSSTTGPWCY TTDPTVRRQE CSIPVCGQDQ VTVAMTPRSE
GSSVNLSPPL EQCVPDRGQQ YQGRLAVTTH GLPCLAWASA
QAKALSKHQD FNSAVQLVEN FCRNPDGDEE GVWCYVAGKP
GDFGYCDLNY CEEAVEEETG DGLDEDSDRA IEGRTATSEY
QTFFNPRTFG SGEADCGLRP LFEKKSLEDK TERELLESYI
DGRIVEGSDA EIGMSPWQVM LFRKSPQELL CGASLISDRW
VLTAAHCLLY PPWDKNFTEN DLLVRIGKHS RTRYERNIEK
ISMLEKIYIH PRYNWRENLD RDIALMKLKK PVAFSDYIHP
VCLPDRETAA SLLQAGYKGR VTGWGNLKET WTANVGKGQP
SVLQVVNLPI VERPVCKDST RIRITDNMFC AGYKPDEGKR
GDACEGDSGG PFVMKSPFNN RWYQMGIVSW GEGCDRDGKY
GFYTHVFRLK KWIQKVIDQF GE

Figure 19



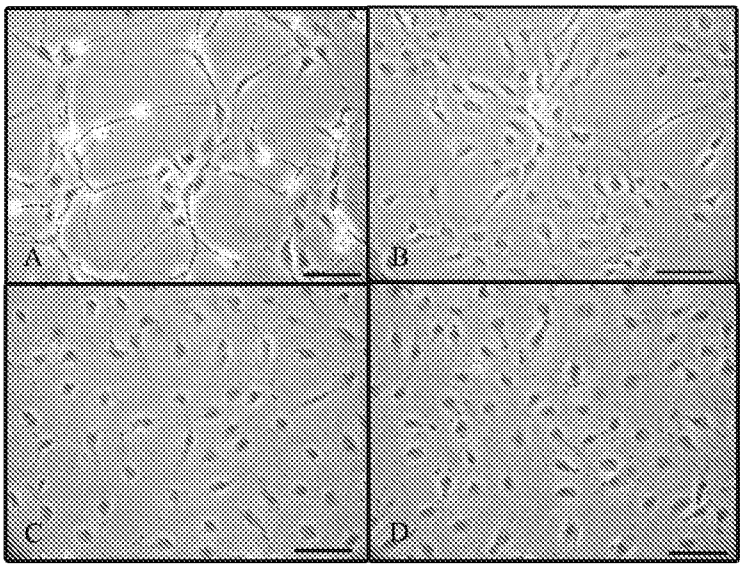


Figure 20

Figure 21

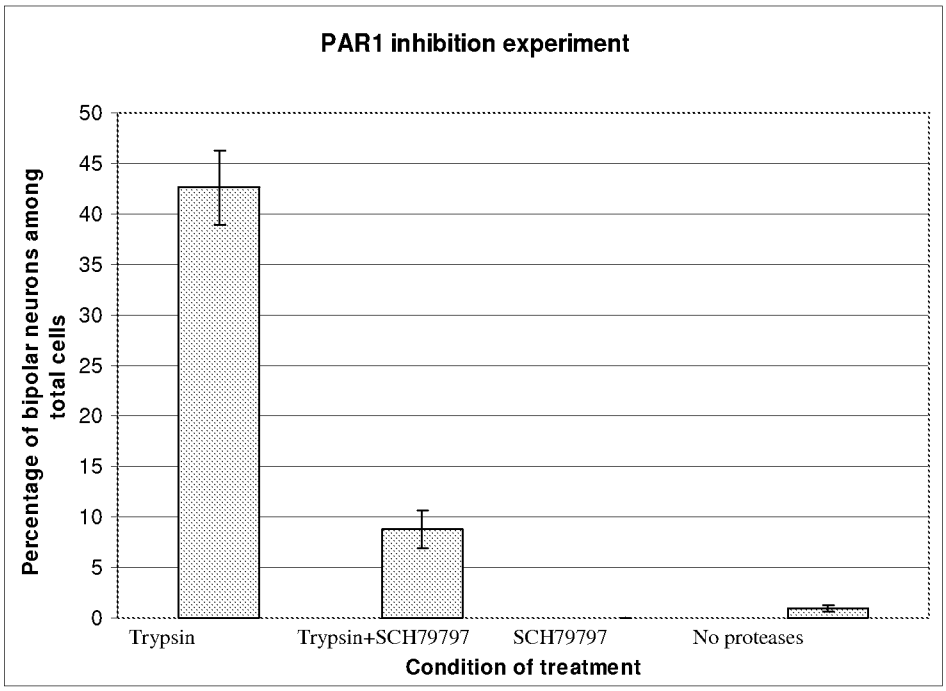

PAR1 inhibition experiment
Percentage of bipolar neurons among total cells
50
45
40
35
30
25
20
15
10
5
0
Trypsin
Trypsin+SCH79797
SCH79797
No proteases
Condition of treatment

Figure 22

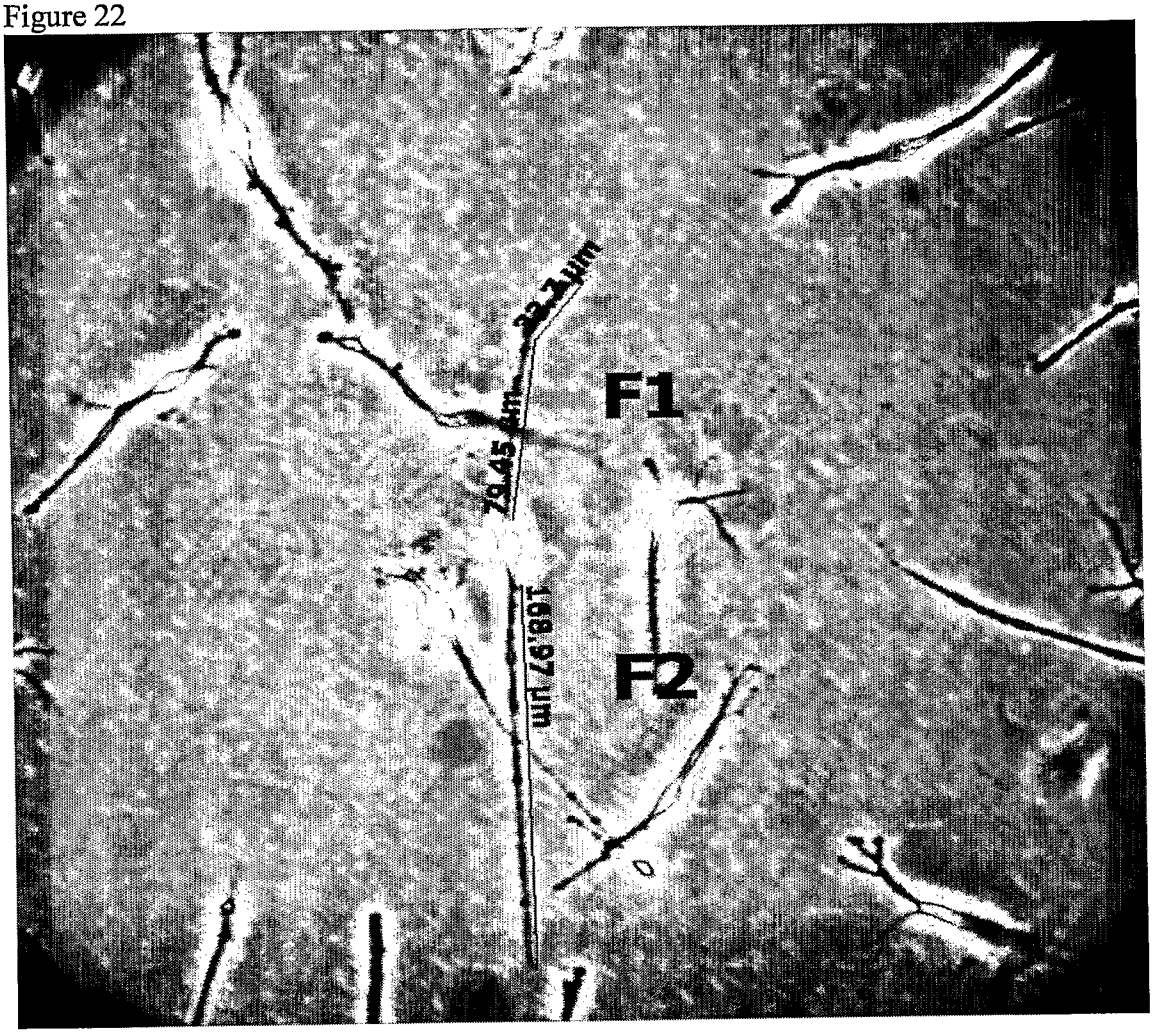


Figure 23

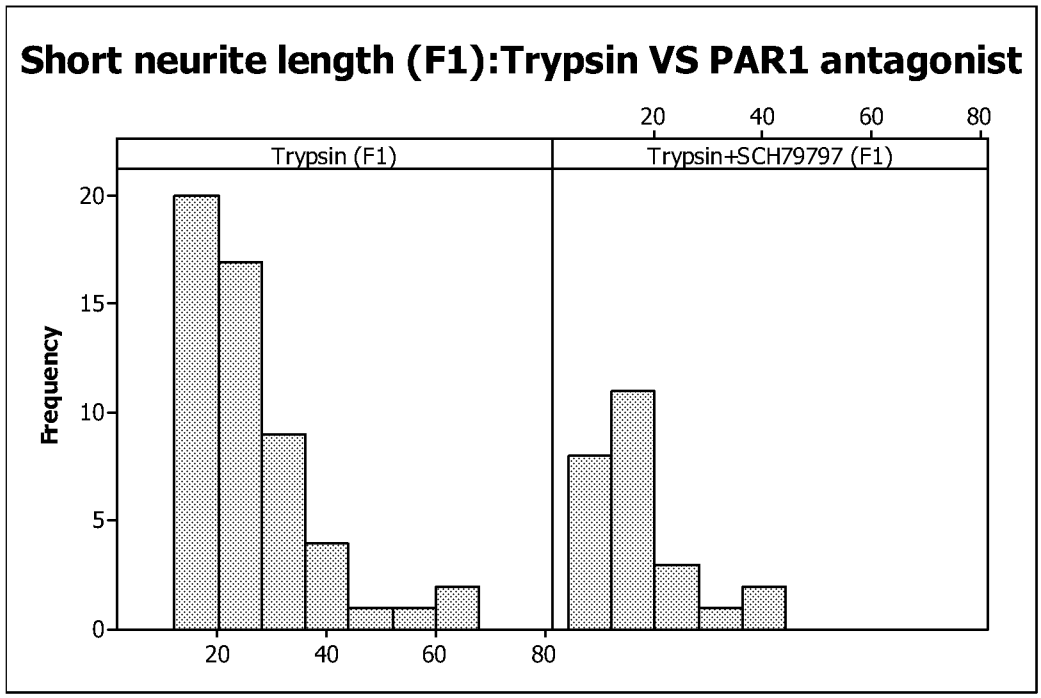


Figure 24

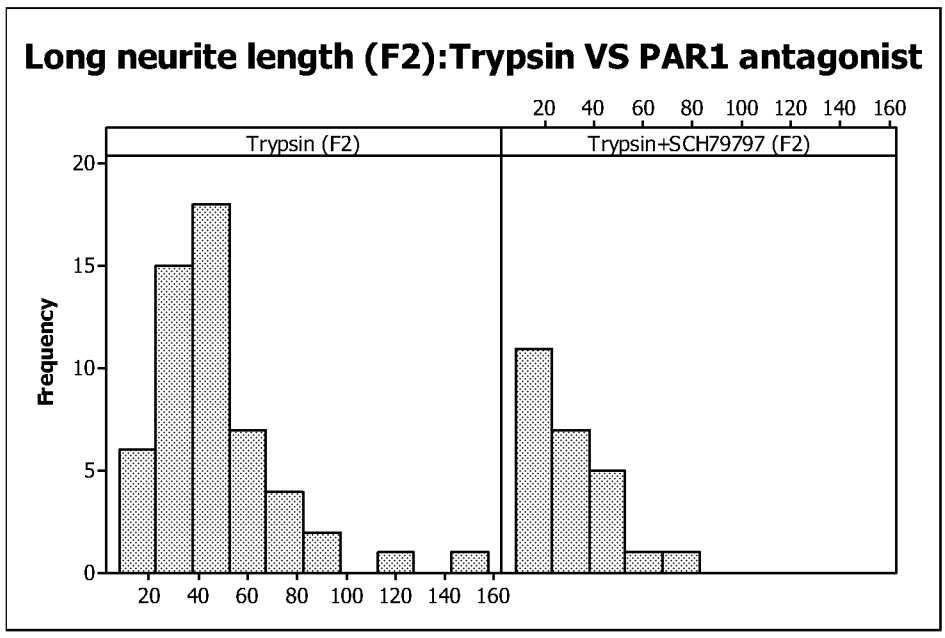


Figure 25

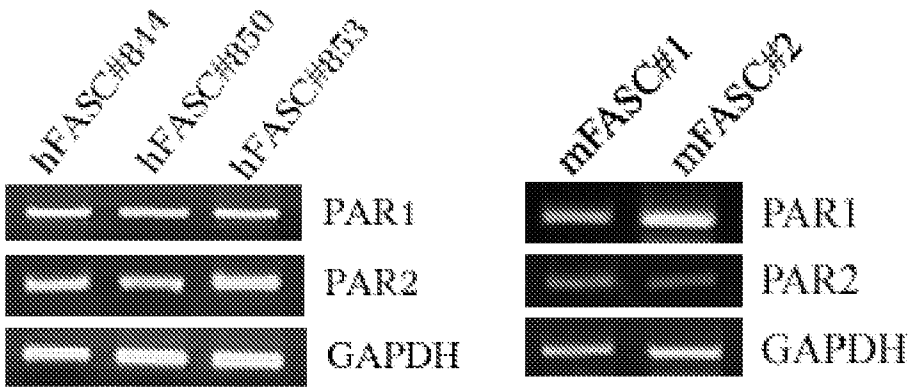


Figure 26