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MEDICINAL PRODUCT AND METHOD FOR TREATMENT OF CONDITIONS AFFECTING NEURAL STEM CELLS OR PROGENITOR CELLS

Field of invention

The present invention relates to use of substances that upon administration to a patient will lead to increased concentrations of growth hormone for the production of medicinal products.

The present invention also relates to a method for treatment of abnormal conditions affecting neural stem cells or progenitor cells.

Background of the invention

The result of traumatic, asfyxial, hypoxic, ischemic, toxic, infectious, degenerative or metabolic insults to the central nervous system (CNS) of man may involve a certain degree of damage in several different cell types. Damage to the brain by trauma, asphyxia, toxins, ischemia or infections are frequently causing neurological and cognitive deficits. Degenerative diseases may cause loss of specific populations of cells. For instance Parkinson's disease is associated by specific loss of dopaminergic neurons in the Substantia nigra, similarly, multiple sclerosis is associated with loss of myelin and oligodendrocytes. Other examples of degenerative disorders caused by selective loss of a specialized type of neurons is Alzheimer's disease associated with loss of cholinergic neurons. There are many other instances in which CNS injury or disease can cause damage to oligodendroglia, astroglia or neuronal cells.

Furthermore, axonal regeneration and sprouting after injury to axons in the CNS white mater tracts and injury to the spinal cord has been shown to be inhibited by surface molecules expressed by oligodendrocytes.

Progenitor cells have been grown and propagated with growth factors like epidermal growth factor (EGF), which is a substance belonging to a different class than GH.

In general, replacement of neurons following degeneration or damage is not a characteristic of the mammalian brain. Neuronal loss is thus considered permanent. Prolonged postnatal neurogenesis has been described in the granule cell layer of the hippocampal formation (Altman, J., and Das, G.D. , J. Comp. Neurol. 124: 319-335 (1965); Altman, J. and Das, G.D. Nature 214: 1098-1101 (1967); Caviness, V. S. jr., J. Comp Neurol. 151: 113-120 (1973); Gueneau, G., Privat, A., Drouet, J., and Court, L., Dev. Neurosci. 5, 345-358(1982); Eckenhoff, M.F., and Rakic, P., J. Neurosci. 8: 2729-2747(1988)). Neurogenesis has recently been shown to persist well into adulthood in man (Eriksson, P.S., Perfilieva, E., Björk-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D.A., Gage, F.H., Nature Med. in press). Neuronal progenitor cells reside in the subgranular zone (SGZ) of the dentate gyrus where they continuously proliferate, migrate into the granulae cell layer and differentiate into granule cells (Kuhn, H., Dickinson-Anson, H., and Gage, F.H., J. Neurosci. 16: 2027-2033 (1996); Cameron, H.A., Woolley, C.S., McEwen, B.S., and Gould, E., Neuroscience 56: 337-344 (1993); Seki, T. and Arai, Y., J. Neurosci. 13: 2351-2358 (1993)). These newborn neurons in the granule cell layer express markers of differentiated neurons and have morphological characteristics corresponding to differentiated granulae cells (Kaplan, M.S. and Bell, D.H., J. Neurosci. 4: 1429-1441 (1984); Cameron, H.A., Woolley, C.S., McEwen, B.S. and Gould, E. Neuroscience 56: 337-344 (1993); Cameron, H.A., Woolley, C.S., and Gould, E., Brain Res. 611: 342-346 (1993)). Furthermore, they establish axonal processes into the mossy fiber pathway and form synaptic connections with their targets in hippocampus CA3 (Seki, T. and Arai, Y., J. Neurosci. 13: 2351-2358 (1993); Stanfield, B.B. and Trice, J.E. Exp. Brain

Res. 72: 399-406 (1988)). The hippocampus is associated with spatial learning and memory (McNamara, R.K, and Skelton, R.W., Brain Res. Rev. 18: 33-49 (1993)). The proliferation of progenitor cells can be influenced by the administration of n-methyl-d-aspartate (NMDA) receptor antagonists or by the removal of the adrenal glands (Cameron, H.A. and Gould, E. Neuroscience 61: 203-209 (1994); Cameron, H.A., Tanapat, P., and Gould, E., Neuroscience 82: 349-354 (1998)). Plasticity is reduced with increasing age, and recent studies have demonstrated that proliferation of progenitor cells also is decreased but not completely abolished with age (Kuhn, H., Dickinson-Anson, H., and Gage, F.H., J. Neurosci. 16: 2027-2033 (1996)). Stem cells isolated from the adult rodent brain has recently been transplanted into the brain of adult animals where they differentiate into cells with neuronal characteristics (Suhonen, J.O., Peterson, D.A., Ray, J. And Gage, F.H., Nature 383:624-627 (1996)).

Furthermore, neurogenesis in the dentate gyrus in young mice has been shown to be facilitated by enriched environments. It was shown that exposure to enriched environments leads to an increased number of surviving newly formed granulae cell neurons and an increased total number of neurons in the dentate gyrus (Kempermann, G., Kuhn, H. G., and Gage, F. H., Nature 386: 493-495 (1997)).

Summary of the invention

It has now been found that by using growth hormone, or an analogue thereof, or another substance leading to increased concentrations of growth hormone or analogues thereof, it is possible to modulate the proliferation and/or differentiation of neural stem cells and progenitor cells from the adult CNS. The present invention thus provides new possibilities to treat injuries to or diseases of the central nervous system that predominantly affect oligodendroglia, astroglia or neuronal cells by

modification of proliferation cell genesis and/or differentiation of neuronal stem cells or progenitor cells in the central nervous system.

It has also been found that it is possible to control the propagation in vitro of stem cells, progenitor-cells and other cells, especially cells derived from the central nervous system, with the potential to generate neurons, astrocytes or oligodendrocytes. Such cells may e.g. be used for therapeutic purposes in patients.

Thus, the present invention relates to the use of a substance that upon administration to a patient will lead to an increased concentration of growth hormone or a functionally equivalent analogue thereof for the production of a medicinal product for treatment of an abnormal condition affecting neural stem cells and/or progenitor cells.

The invention also relates to a method for treatment of an abnormal condition affecting neural stem cells and/or progenitor cells, wherein a pharmaceutically active amount of a substance that will lead to an increased concentration of growth hormone or a functionally equivalent analogue thereof is administered to a patient.

Furthermore, the invention relates to a method of inducing lineage determination, propagating and/or inducing or maintaining the genesis of neurons, oligodendrocytes, astroglial cells from progenitor cells, stem cells and/or cells derived from said cells by administration of an effective amount of growth hormone or a functionally equivalent analogue thereof to stem cells, progenitor cells, neurons astroglial cells and/or oligodendrocytes in vitro.

Another aspect of the invention relates to abnormal conditions in the CNS due to too high concentrations of growth hormone in the CNS.

The invention thus also relates to the use of a substance that upon administration to a patient will lead to a decreased concentration of growth hormone or a func-

tionally equivalent analogue thereof for the production of a medicinal product for treatment of an abnormal condition affecting stem cells, progenitor cells and/or cells derived from stem cells or progenitor cells, as well as to a method of reducing the genesis of oligodendrocytes, neurons, astroglial cells from progenitor cells or stem cells in, or derived from, the central or periferal nervous system in a patient, wherein a pharmaceutically effective amount of a substance that will lead to a decreased concentration of growth hormone or a functionally equivalent analogue thereof is administered to said patient.

The characterizing features of the invention will be evident from the following description and the appended claims.

Detailed description of the invention

The mammalian brain, including the human brain, retains its ability to generate neurons throughout life in certain brain regions. New neurons and astroglial cells and oligodendrocytes are generated by cell genesis from stem or progenitor cells. During the research leading to the present invention it was found that growth hormone (below denoted GH) induces an increase in cell genesis from progenitors/stem cells in the adult brain. It was also found that increased number of new cells in the hippocampus is associated with improvement in learning and memory. These findings lead to the insight that it is possible to manipulate neurological deficits, such as memory and learning deficits, in patients by manipulating the amount of GH present in the environment surrounding the cells.

It was thus found that it is possible to treat a CNS damage or deficit after an insult by increasing the number of stem cells or progenitor derived cells including neurons, astroglial cells and oligodendrocytes.

It was also found that it is possible to treat neural loss suffered after a CNS insult by increasing the number of stem cells or progenitor derived cells including neurons, astroglial cells and oligodendrocytes in a patient by increasing the concentration of GH in the patient to induce proliferation and/or differentiation of stem cells with a concomitant increase in cell genesis.

Finally it was found that it is possible to treat neural loss suffered after a CNS insult by increasing the number of stem cells or progenitor derived cells including neurons and/or astroglial cells and/or oligodendrocytes in a patient by increasing the concentration of GH in the patient to induce proliferation and/or differentiation of stem cells with a concomitant increase in cell genesis in order to facilitate the isolation through surgical removal of small samples of brain tissue containing said cells for further expansion in vitro and concomitant re-transplantation into the patient.

Thus, the present invention relates to the use of a substance that upon administration to a patient will lead to an increased concentration of growth hormone, or of an analogue thereof, for the production of a medicinal product for treatment of an abnormal condition affecting neural stem cells, progenitor cells and/or cells derived from neural stem cells or progenitor cells, as well as to a method for treatment of an abnormal condition affecting neural stem cells, progenitor cells and/or cells derived from neural stem cells or progenitor cells, wherein a pharmaceutically active amount of a substance that will lead to an increased concentration of growth hormone is administered to a patient.

The substance that will lead to an increased concentration of growth hormone or analogue thereof may e.g. be growth hormone itself, or a functionally equivalent analogue thereof. The term "functionally equivalent analogue thereof" relates to all substances that upon administration to a patient will have essentially the same biologi-

cal and pharmaceutical effect as GH. Such an analogue may e.g. be a synthetic GH mimetic. It is also possible to use a compound that upon administration to a patient will give rise to an elevated active concentration of GH or of a natural occurring GH analogue or its mediators in the CNS of the patient, e.g. by giving rise to an increased release of endogenous GH. For example, positively regulating binding proteins of GH may be used, such as the GH releasing substance growth hormone releasing peptide (GHRP) and analogous thereof.

The medicinal product according to the invention preferably comprises the active substance in a pharmacologically acceptable carrier or diluent such as those known in the art.

The medicinal product or the substance used according to the invention is preferably administered via intravenous periferal infusion or via intramuscular or subcutaneous injection into the patient. It is also possible to administer the medicinal product or the pharmaceutically active substance through a surgically inserted shunt into a cerebral ventricle of the patient.

Preferably, the administered subcutaneous dosage range of the pharmaceutically active substance is about 0.01-1 IE/kg body weight of the patient per week.

The term "patient", as used herein, relates to any human or non-human mammal in need of treatment according to the invention.

The term "treatment" used herein relates to both treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The term treatment also refer to the affecting of cell genesis from stem cells or progenitor cells, by inducing the genesis of neurons and/or glial cells after either neuronal, oligodendroglial or glial cell loss in the CNS or PNS (periferal nervous system) or to prevent the normal age related deterioration in the CNS or PNS, the term also relates to

the cultivation of stem or progenitor cells for concomitant transplantation to the CNS or PNS in patients. The treatment may either be performed in an acute or in a chronic way.

As stated above the pharmaceutically active substance used according to the invention is suitable for treatment of abnormal conditions affecting neural stem cells, progenitor cells and/or cells derived from neural stem cells or progenitor cells. It can thus be used to prevent, treat or ameliorate damages, diseases or deficits of central nervous system (CNS). The pharmaceutically active substance used according to the invention is especially suitable for treatment of conditions affecting the oligodendroglia, astroglia, and/or neuronal cells. Such conditions may e.g. be a CNS damage or deficit, neuronal cell loss or memory loss. Such conditions may be caused by a number of different factors or diseases, such as multiple sclerosis, hypoxic injury, ischemic injury, traumatic injury, Parkinson's disease, and demyelition disorder.

The effect the pharmaceutically active substances used according to the invention is due to their ability to either induce cell genesis, proliferation and/or differentiation of progenitor derived cells in or from the central nervous system.

According to another embodiment of the invention it is possible to use growth hormone or a functionally equivalent analogue thereof in order to propagate progenitor cells or stem cells or other neural cells in a tissue culture or a cell culture. Such cells may thereafter be used for cell transplantation into a patient suffering from neuronal cell loss or a condition due to lack of endogenous cells of this type. The cells used to start the culture may either originate from the patient itself of from human or animal donors.

When cells are to be removed from a patient for in vitro propagation it may be advantageous to first in-

crease the number of progenitor cells in the patient. This facilitating the subsequent isolation of said cells from patients facilitates the subsequent isolation of said cells from patients. The number of progenitor cells are increased by use of the method or medicinal product according to the invention, i.e. by the use of substance that upon administration to a patient will lead to an increased concentration of growth hormone or a functionally equivalent analogue thereof.

Growth hormone, or a functionally equivalent analogue thereof, may be used alone or in junction with other medicaments or growth factors such as epidermal growth factor (EGF) or fibroblast growth factor 2 (FGF2) designed to induce in cell genesis or proliferation in the CNS or PNS. Growth hormone, or a functionally equivalent analogue thereof, alone or in conjunction with other medicaments, peptides, growthfactors, steroids, lipids, glycosylated proteins or peptides, either simultaneous or in sequence, may be used in order to facilitate cell genesis or the generation of specific cell types in vivo or in vitro. It may also be used to induce immature, or multipotent cells to active specific developmental programs as well as specific genes in the aforementioned cells.

By the above mentioned cell genesis is meant the generation of new cells such as neurons oligodendrocytes schwancells and astroglial cells from multipotent cells, progenitor or stem cells within the adult CNS or PNS or in vitro.

Furthermore, the invention also relates to the therapeutic use of substances that decrease the amount of active GH or naturally occurring analogous of GH in the patient and thus decrease the genesis of oligodendrocytes in patients with axonal or spinal cord injury. Examples of such substances are negatively regulating binding proteins, GH-receptor antagonists, drugs or antibodies or compounds or peptides. Axonal regeneration and spinal

cord injury have been shown to be inhibited by certain molecules expressed by oligodendrocytes. Furthermore, drugs or antibodies or compounds or peptides, that increase endogenous peptides, or proteins that decrease the biological activity of endogenous GH can also be used.

The invention will be more fully understood when reading the following example. It should not, however, be considered to limit the scope of the invention.

Brief description of the drawings

In the examples below, reference is made to the appended drawings on which:

Fig. 1 shows the density of BrdU-positive cells after 7 days in the dentate gyrus of hypophysectomized (Hx) rats treated according to the invention with growth hormone (GH), together with cortisol (C), and L-thyroxine (T) compared to hypophysectomized rats treated with only C and T, and to a control group.

Fig. 2 shows that animals treated with cortisone, thyroxine and GH, according to the invention, had significantly more granulae cell neurons than hypophysectomized animals treated with only cortisone and thyroxine four weeks after the last BrdU injection the hypophysectomized animals

Fig. 3 shows that animals with increased number of new born cells according to the invention (○) performed significantly better in the hidden-platform version of the water maze task, used to assess spatial performance, than a control group (●).

Examples

In this example, the density of BrdU-positive cells in the dentate gyrus of hypophysectomized (Hx) rats treated according to the invention with growth hormone (GH), cortisol (C), and L-thyroxine (T) was compared to

the density of BrdU-positive cells in the dentate gyrus of hypophysectomized rats treated with cortisol (C), and L-thyroxine (T), and to the density of the same cells for an untreated unoperated control group.

Fisher rats (Harlan Sprague Dawley) which were intact or hypophysectomized at 50 days of age were maintained under standardized conditions of temperature (24-26°C), humidity (50-60%) and with lights on between 0500 and 1900 h.

The rats had free access to standard laboratory chow and water. Hormonal treatment started 7-10 days after hypophysectomy. All the hypophysectomized rats were given cortisol phosphate (400 µg/kg/day; Solu-Cortef, Upjohn, Puurs, Belgium) and L-thyroxine (10 µg/kg/day; Sigma, USA) diluted in saline as a daily subcutaneous injection (at 0800 h). Recombinant bovine GH (bGH) was diluted in 0.05 M phosphate buffer, pH 8.6, with 1.6% glycerol and 0.02% sodium azide. GH 1 mg/kg/day was given as one daily subcutaneous injection at 24 h intervals. The treatment continued for seven days. Thereafter the rats were sacrificed and the brains taken out and prepared for immunohistochemistry.

Ten hypophysectomized rats were substituted with only cortisole and L-thyroxine. Fifteen hypophysectomized rats were substituted with cortisole, L-thyroxine and GH. Ten rats weighing 120 g were assigned to a control group. During the seven days of the treatment period all animals received a daily intraperitoneal injection (50 mg/kg bodyweight) of bromodeoxyuridine (BrdU; Sigma). The thymidine analog BrdU is incorporated into the genetic material upon mitotic division whereafter it can be detected immunohistochemically in the resulting cells. On the twentieth day all animals were sacrificed by a lethal dose of anesthetics and transcardially perfused with 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 24h. and thereafter stored in 30% sucrose solution. Coronal freezing microtome sections

(40μm) were stored in cryoprotectant (25% ethylene glycol, 25% glycerin, 0.05 M phosphate buffer) at -20°C until processing for immunohistochemistry or immunofluorescence.

The number of BrdU positive cells in the dentate gyrus of the hippocampus were counted using unbiased counting techniques. For detection of BrdU-labeled nuclei in tissue sections, the following DNA denaturation steps preceded the incubation with mouse anti-BrdU antibody 1:400 (Boeringer Mannheim) : 2 h incubation in 50% formamide/2X SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, 5 min. Rinse in 2 x SSC, 30 min incubation in 2N HCl at 37°C, and 10 min. Rinse in 0.1M boric acid, pH 8,5. All stainings were performed on free floating 40 mm sections. Free-floating sections were treated with 0.6% H_2O_2 in tris buffered saline (TBS) (0.15M NaCl, 0.1M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase. Several rinses in TBS were then followed by incubation in TBS/0.25% Triton X-100/3% normal horse serum (TBS-TS) for 30 min and incubation with primary antibody in TBS-TS overnight at 4°C. After rinsing in TBS-TS, the sections were incubated for 3 hr with biotinylated horse anti-mouse IgG, 1:160 secondary antibodies (Vector Laboratories, USA). After TBS rinsing avidin-biotin-peroxidase complex was applied for 1 h followed by peroxidase detection for 5 minutes (0.25 mg/ml diaminobenzidine, 0.01% H_2O_2, 0.04% NiCl).

For the immunofluorescence, sections were treated for DNA denaturation as described above, followed by incubation in TBS-TS for 30 min. Thereafter the sections were incubated with mouse-anti-Calbindin-D28k, 1:2000 (Sigma) for 16 h at 4°C and was detected with a Texas red conjugated donkey anti-mouse IgG. BrdU was detected with a FITC conjugated rabbit anti-BrdU antibody. Fluorescent signals were detected and processed using a confocal scanning laser microscope (Bio-Rad MRC1024, Richmond, CA).

The total number of BrdU positive cells in the granule cell layer, the subgranular layer and the hilus and their corresponding sample volumes were determined in 7-9 coronal sections, 240 mm apart, that contained the dentate gyrus. Cell counting was done according to an optical dissector method to avoid over sampling errors.

The results are shown in figure 1. After 7 days, the number of newborn cells in the dentate gyrus is significantly increased in hypophysectomized animals substituted with GH, cortisone, and thyroxine compared to animals substituted with only cortisone and thyroxine. Furthermore, the rate of proliferation was significantly increased after administration of GH to hypophysectomized animals treated with cortisol and L-thyroxine as quantified after one week of substitution. These results clearly show that GH increase the proliferative rate of progenitor cells in the dentate gyrus in the hippocampus.

Furthermore, the rate of proliferation was significantly increased after administration of GH to hypophysectomized animals treated with cortisol and L-thyroxine as quantified after one week of substitution. This result suggest that GH affect the proliferative rate of progenitor cells in the dentate gyrus of the hippocampus.

Furthermore, the proliferation was increased in normal animals receiving treatment for one week with GH compared with normal controls and compared with the hypophysectomized animals that were substituted for one week and thereafter unsubstituted during the following 3 weeks. The number of BrdU positive cells were estimated one month after treatment with either cortisone and L-thyroxine or cortisone, L-thyroxine and GH. The results are shown in figure 2.

The results suggest that GH either direct or indirect promote proliferation or survival of cells resulting from neural cell progenitor proliferation in the dentate gyrus.

The inventors of the present invention are the first to show that growth hormone can regulate the proliferation and subsequent generation of neurons in the adult brain.

Rats with increased number of newborn cells were tested and compared with rats that had lower number of newborn cells four weeks after BrdU injection during four consecutive days. The rats were tested in a water maze with a video-tracking system. The time to reach the platform (latency) were monitored. The escape platform was hidden 1 cm below the surface of the water at a fixed position. The water was made opaque by adding dry milk powder to the water. The water temperature was kept constant at 22°C throughout the test. Each animal was tested in four trials each day. Each trial lasted 45 s. Animals that failed to find the hidden platform within 45 s were designated as having a 45-s latency and were put on the platform and allowed to stay there for 15 s.

The latency in finding the platform during the water maze test was analyzed with a two-way ANOVA, and repeated postcomparative tests at each monitored time interval were performed using the Scheffe *F*-test. The results are shown in figure 3. There were no significant difference in swim speed. It is evident that animals with increased number of newborn cells in the dentate gyrus, due to treatment according to the invention, performed significantly better in the spatial learning task. These group of animals represent the data denoted with O in the figure. The data for the rats with the lower number of newborn cells are denoted with ● in the figure.

CLAIMS

1. Use of growth hormone or a functionally equivalent analogue thereof for the production of a medicinal product for treatment of a CNS damage affecting neural stem cells, progenitor cells and/or cells derived from stem cells or progenitor cells.

2. Use according to claim 1, wherein said CNS damage affects the oligodendroglia, astroglia, and/or neuronal cells.

3. Use according to any one of the claims 1-2, wherein said CNS damage affects non-cholinergic neuronal cells, cholinergic neuronal cells, or glial cells.

4. Use according to any one of the claims 1-3, wherein said CNS damage is neural cell loss.

5. Use according to any one of the claims 1-4, wherein said CNS damage is caused by hypoxic injury, ischemic injury, and/or traumatic injury.

6. Use according to any one of the claims 1-5, wherein said medicinal product is formulated for intravenous infusion, intramuscular injection or subcutaneous injection.

7. Use according to any one of the claims 1-6, wherein said medicinal product is formulated so that the active substance will pass into the ventricles of the patient's brain when it is administered to a patient.

8. Use according to any one of the claims 1-7, wherein said medicinal product is formulated so that the active substance will pass into the cerebrospinal fluid of the patient when it is administered to a patient.

9. Use of a substance that upon administration to a patient will lead to a decreased concentration of growth hormone or a functionally equivalent analogue thereof for the production of a medicinal product for treatment of an abnormal condition affecting the central nervous system, wherein said abnormal condition is the consequence of ax-

onal damage caused by concussion, axonal damage caused by head trauma, axonal damage caused by small vessel disease in the CNS, damage to the spinal cord after disease and/or trauma.

10. Use according to claim 9, wherein said substance is a negatively regulating growth hormone binding protein, a functionally equivalent analogous thereof, an antibody against growth hormone, a biologically active growth hormone receptor inhibitor, and/or an inhibitor of endogenous growth hormone release.

11. A method of propagating progenitor cells, stem cells and/or cells derived from said cells by administration of an effective amount of growth hormone or a functionally equivalent analogue thereof to stem cells, progenitor cells, neurons astroglial cells and/or oligodendrocytes in vitro.