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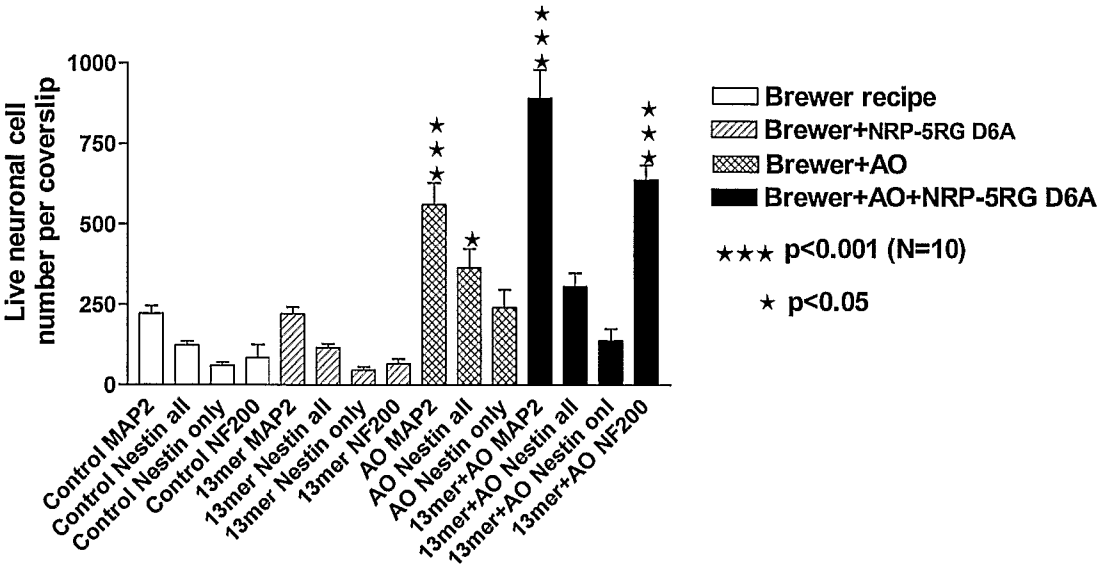
(54) **Title:** NEURAL REGENERATION PEPTIDES AND ANTIOXIDANTS PROTECT NEURONS FROM DEGENERATION



(57) **Abstract:** Aspects of this invention include compositions for treating conditions in which neurons would otherwise degenerate or die as a result of an insult to the nervous system. Other aspects include methods for treating conditions in which neurons would otherwise degenerate or die as a result of an insult to the nervous system. Compositions include a neural regeneration peptide (NRP) and an antioxidant. The methods include administering to a subject in need thereof, a composition comprising a neural regeneration peptide and an antioxidant. Compositions including one or more NRPs and one or more antioxidants can also be used in vitro, to promote growth and differentiation of neural cell cultures. Kits include one or more NRPs, one or more antioxidants, solvents for preparing solutions, a mixing vial and instructions for use.

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NEURAL REGENERATION PEPTIDES AND ANTIOXIDANTS PROTECT NEURONS FROM DEGENERATION

CLAIM OF PRIORITY

This application claims priority to United States Provisional Patent Application No: 60/714,916, filed September 7, 2005 and to United States Provisional Patent Application No: 60/699,642, filed July 15, 2005. Each of the above applications is expressly incorporated herein fully by reference.

FIELD OF THE INVENTION

This application relates to compositions and methods for protecting neurons from degeneration or death using a combination of a neural regeneration peptide (NRP) and an antioxidant (AO). Additionally, this invention relates to compositions and methods for stimulating neuronal growth using an NPR and an AO.

BACKGROUND

There are many conditions associated with loss of neurons. Such conditions include hypoxia, ischemia, degenerative neuronal diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, as well as traumatic injury to the brain. There are several methods useful for decreasing the degeneration of neurons faced with an insult. These include use of the tripeptide Gly-Pro-Glu (GPE), NRPs and others. However, there is a continuing need to improve compositions and methods for treating neurodegeneration.

SUMMARY

We have unexpectedly found that a combination of a NRP and an AO can substantially and significantly decrease neurodegeneration in the face of an insult. Therefore, combination therapy using an NRP and an AO can aid patients in recovering from insults to the central nervous system (CNS). When used before, during and/or after an insult, the combination therapy can lead to more rapid recovery with fewer long-term neuronal deficits.

BRIEF DESCRIPTIONS OF THE FIGURES

This invention is described with reference to specific embodiments thereof. Other features of this invention can be appreciated with reference to the Figures, in which:

Figure 1A shows a graph of effects of tested media (Brewer's, Brewer's supplemented with NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG), Brewer's supplemented with anioxidant (AO) and Brewer's supplemented with AO + NRP). NRP-5RG D6A is labelled as 13 mer.

Figure 1B shows the percentage of NG2 positive cells after 7DIV culturing in the tested media. Significance was measured at *** $p<0.001$ by one-way ANOVA followed by Bonferroni's post hoc test.

Figures 1C and 1D show the effects of the tested media on the neuronal survival represented as the percentage of MAP2 positive cells within the adult hippocampal cell culture after 7DIV. Figure 1C shows the results of an experiment where NRP (SEQ ID NO: 1) was added at the start of the experiment only. Figure 1D shows the results of an experiment where the NRP was added at the start of the experiment and at 5DIV. Significance was measured at ** $p<0.01$ by one-way ANOVA followed by Bonferroni's post hoc test.

Figure 2 shows a graph of the effects of Brewer's medium supplemented by antioxidant alone or antioxidant plus NRP-5RG D6A on the neurite outgrowth of dissociated adult cerebellar neurons at 7 DIV. Significance was measured at *** $p<0.001$ by one-way ANOVA followed by Bonferroni's post hoc test.

Figure 3 shows the number of nestin-positive cells per coverslip in four different cultivation mediums. Significance was measured at *** $p<0.001$ by one-way ANOVA followed by Bonferroni's post hoc test.

Figure 4 shows a graph of neurite outgrowth of dissociated hippocampal neurons cultivated for 7DIV in the presence of NRP-5RG D6A and/or antioxidants. Significance was measured at *** $p<0.001$ by one-way ANOVA followed by Bonferroni's post hoc test.

Figures 5A and 5B show the effects of AO+NRP supplemented medium on neuronal proliferation rate in adult hippocampal cells. In Figure 5A NRP was added at the start of the experiment only. In Figure 5B, NRP was added at 4 different time points (0DIV, 2DIV, 4DIV 6DIV). Significance was determined at *** $p<0.001$, ** $p<0.01$; $n=6$) by one-way ANOVA followed by Bonferroni's post hoc test.

Figures 6A and 6B show the percentage of MAP2 positive cerebellar (6A) and hippocampal (6B) neurons that are α-synaptophysin positive. Data were analysed using one-way ANOVA followed by a Bonferroni's post hoc test (significance was determined at ** $p<0.01$; $n=6$).

DETAILED DESCRIPTION

This invention is based upon the surprising discovery that treating neurons with an antioxidant and a neural regeneration peptide together produce neuroprotection and stimulate neurite growth in a synergistic fashion.

This discovery was made using an *in vitro* cell culture system, in which cerebellar neurons were cultured under different conditions. Previous studies have shown that *in vitro* effects of neuroprotective agents are highly predictive of neuroprotective effects *in vivo*. Thus, studies of neuroprotection in cerebellar neuronal cultures are highly predictive of neuroprotective effects

expected in humans and other animals exposed to insults likely to result in neurodegeneration and symptoms associated with neurodegeneration.

The study of pathological events like injuries or CNS diseases within the mature brain can advantageously be carried out with the use of appropriate *in vitro* systems. One such system consists of cultures of adolescent/adult neurons. Apart from the hippocampal formation (Brewer, G. J. Isolation and culture of adult rat hippocampal neurons. *Journal of Neuroscience Methods, 71*(2), 143-155 (1997). Prior to the disclosures herein, there had been no *in vitro* systems that comprise medium-term (> 5 days) cultivated neurons derived from dissociated mature brain tissue. In attempt to establish such *in vitro* system, we have found that generation of dissociated cells by mechanical titration and enzymatic papain treatment under antioxidant control (quercetin and neuronal NOS-inhibitor 7-nitroindazole) leads to substantial neuronal survival improvement. Significant further increase of cell survival and subsequent neuronal differentiation can be achieved by supplementation of the medium with a Neuronal Regeneration Peptide (NRP) (Gorba et al. Neural regeneration protein is a novel chemoattractive and neuronal survival-promoting factor, *Experimental Cell Research* (2006), Sieg et al., PCT/US02/26782; Sieg et al., US Patent Application No. 10/976,699, filed October 29, 2004, entitled: Neural Regeneration Peptides and Methods for Their Use in Treatment of Brain Damage; Attorney Docket No: NRNZ 1023 US2), each publication expressly incorporated herein fully by reference. The NRPs promote neuronal cell migration, proliferation, differentiation, survival and neurite outgrowth.

Neural Regeneration Peptides

Embodiments of this invention include the use of Neural Regeneration Peptides (NRPs) previously disclosed in U.S. Patent Applications: 10/225,838 titled "Neural Regeneration Peptides and Methods for Their Use in Treatment of Brain Damage" filed August 22, 2002, Publication No: US 2003/0211990; 10/976,699 titled, "Neural Regeneration Peptides and Methods for Their Use in Treatment of Brain Damage" filed October 29, 2004; US 60/678,302 titled "Neural Regeneration Peptides and Methods for Their Use in Preventing Obstetric Complications" filed May 6, 2005; US 60/699,642 titled "Neural Regeneration Peptides and Antioxidants Protect Neurons From Degeneration" filed July 15, 2005; US 60/714,916 titled "Neural Regeneration Peptides and Antioxidants Protect Neurons from Degeneration" filed September 7, 2005; US 60/726,904 titled "Neural Regeneration Peptides and Methods for Their Use in Treatment of Autoimmune Disorders of the Brain" filed October 14, 2005; PCT International Patent Applications: PCT/US02/26782 titled "Neural Regeneration Peptides and Methods for Their Use in Treatment of Brain Damage" filed August 22, 2002, Publication No: WO 03/018754; and PCT/US2004/036203 titled "Neural Regeneration Peptides and Methods for Their Use in Treatment of Brain Damage" filed November 1, 2004, Publication No: WO 2005/042,561; all for prevention of degeneration or death of neurons and

other cell types. Each of the aforementioned patent applications is expressly incorporated herein fully by reference.

Definitions

The term "homolog" includes one or more genes whose gene sequences are significantly related because of an evolutionary relationship, either between species (ortholog) or within a species (paralog). Homolog also includes genes related by descent from a common ancestral DNA sequence. Homolog also includes a relationship between genes separated by a speciation event, or to a relationship between genes by the event of genetic duplication (see paralog). As used herein, the term "homolog" also includes gene products related to each other by way of an evolutionary relationship. NRPs having conserved amino acid sequence domains are examples of homologs.

The term "paralog" includes one of a set of homologous genes that have diverged from each other as a consequence of genetic duplication. For example, the mouse alpha globin and beta globin genes are paralogs. As used herein, the term "paralog" also includes gene products related to each other by way of an evolutionary relationship. Human NRPs having conserved amino acid sequence domains are examples of paralogs.

The term "ortholog" includes one of a set of homologous genes that have diverged from each other as a consequence of speciation. For example, the alpha globin genes of mouse and chick are orthologs. As used herein, the term "ortholog" also includes gene products related to each other by way of an evolutionary relationship. Human and mouse NRPs having conserved amino acid sequence domains are examples of homologs.

The term "paralog peptide" includes a peptide encoded by a paralog nucleotide sequence.

The term "peptide" and "protein" include polymers made of amino acids.

The term "prodrug" includes molecules, including pro-peptides which, following enzymatic, metabolic or other processing, result in an active NRP, an active NRP analog or a NRP paralog.

The term "NRP compound" includes NRPs, NRP homologs, NRP paralogs, NRP orthologs, NRP analogs, and prodrugs of NRP.

The term "NRP" includes peptides having functions including one or more of neural migration, neuroblast migration, neural proliferation, neuronal differentiation, neuronal survival and neurite outgrowth, regardless of evolutionary relationship. The term NRP also refers to peptides having sequences defined herein. It is understood that a "sequence" or "SEQ ID NO: " includes both C-terminal OH and C-terminal amidated peptides.

Amino acids are represented by the standard symbols where alanine is represented by "A" or "Ala", arginine by "R" or "Arg", asparagine by "N" or "Asn", aspartic acid by "D" or "Asp", cysteine by "C" or "Cys", glutamic acid by "E" or "Glu", glutamine by "Q" or "Gln", glycine by "G" or "Gly", histidine by "H" or "His", isoleucine by "I" or "Ile", leucine by "L" or "Leu", lysine by "K" or "Lys", methionine by "M" or "Met", phenylalanine by "F" or "Phe", proline by "P" or "Pro", serine

by "S" or "Ser", threonine by "T" or Thr", tryptophan by "W" or "Trp", tyrosine by "Y" or "Tyr", and valine by "V" or "Val". Carboxy terminally amidated peptides are indicated by $-NH_2$.

"Disease" includes any unhealthy condition of CNS or peripheral nervous system of an animal, including particularly Parkinson's disease, Lewy Body, Huntington's disease, Alzheimer's disease, multiple sclerosis, motor neuron disease, muscular dystrophy, peripheral neuropathies, metabolic disorders of the nervous system including glycogen storage diseases.

"Insult" includes any disease or injury that can cause a brain or other cell to degenerate or die.

"Injury" includes any acute damage of an animal, including particularly stroke, traumatic brain injury, hypoxia, ischemia, perinatal asphyxia associated with fetal distress such as following abruption, cord occlusion or associated with intrauterine growth retardation, perinatal asphyxia associated with failure of adequate resuscitation or respiration, severe CNS insults associated with near miss drowning, near miss cot death, carbon monoxide inhalation, ammonia or other gaseous intoxication, cardiac arrest, coma, meningitis, hypoglycaemia and status epilepticus, episodes of cerebral asphyxia associated with coronary bypass surgery, hypotensive episodes and hypertensive crises, cerebral trauma and spinal cord injury.

Description of Specific Embodiments

Certain embodiments of this invention include compositions and methods for the treatment of brain damage, comprising administering neural regeneration peptides (NRPs) to mammals in need of such treatment.

NRPs are characterized by the presence of one or more peptide domains, including a [A]PG[R,S] domain, such as APGS, APG, APGR, APGS, PGR or PGS. Additionally, NPRs may have other domains, including ARG, ARR, a C-terminal GG domain, an [A,G]RR domain, including ARR or GRR domain. NRPs may also have a PE domain. Thus, NRPs may have one or more of the above domains.

A series of NRPs was described in U.S. Patent Applications Nos: 10/225,838 and 10/976,699. One of those NRPs, NRP-5 (SEQ ID NO: 11 in U.S. 10/976,669), includes the single letter amino acid sequence REGRRDAPGRAGG (SEQ ID NO:30 in U.S. 10/976,669; and also called "NRP-5RG") was used to develop a novel 13-mer NRP analogue having the amino acid sequence REGRRAAPGRAGG (SEQ ID NO:1; also called "NRP-5RG D6A" or "NRP-5RG analogue D6A"), comprising the sequence:

REGRRAAPGRAGG SEQ ID NO: 1

SEQ ID NO:1 has a GRR domain, a APGR domain and a C-terminal GG domain.

Another embodiment of the invention is an 11-mer analogue of NRP-5 (SEQ ID NO: 11 in U.S. Patent Application Number 10/976,699), herein termed NRP-5 segment GG analogue D4A, comprising the following sequence:

GRRAAPGRAGG SEQ ID NO: 2

SEQ ID NO:2 has a GRR domain, an APG domain and a C-terminal GG domain.

Additional embodiments of the invention include use of NRPs to treat functional neurological deficits resulting from autoimmune disorders of the brain, including multiple sclerosis. In certain of these embodiments, several NRPs were found to be effective.

A 13-mer NRP-5 segment RG (also known as SEQ ID NO: 30 disclosed in U.S. Patent Application Number 10/976,699) is REGRRDAPGRAGG SEQ ID NO: 3

As with SEQ ID NO:1 and SEQ ID NO:2, SEQ ID NO:3 has a GRR domain, an APGR domain and a C-terminal GG domain.

Additionally, an NPP, herein called 25-mer NRP-4 GG, also known as (SEQ ID NO: 29 disclosed in U.S. Patent Application Number 10/976,699) is

GTPGRAEAGGQVSPCLAASCSQAYG SEQ ID NO: 4

24-mer NRP-7 SW, also known as SEQ ID NO: 24 in U.S. Patent Application Number 10/976,699 is SEPEARRAPGRKGGVVCASLAADW SEQ ID NO: 5

are useful.

Additionally, the following sequences are neuroprotective and are useful for incorporation into compositions including an antioxidant.

YDPEAASAPGSGNPCH	SEQ ID NO:6
KDPEARRAPGSLHPCLAASCSAAG	SEQ ID NO:7
RRAPGSLHPCLAASCSAAG	SEQ ID NO:8
KDPEARRAPGSLHPCLAAS	SEQ ID NO:9
KDPEARRAPGS	SEQ ID NO:10
GTPGRAEAGGQ	SEQ ID NO:11
GTPGRAEAG	SEQ ID NO:12
TPGRAEAGG	SEQ ID NO:13
PGRAEAGGQ	SEQ ID NO:14
GRAEAGGQV	SEQ ID NO:15
RAEAGGQVS	SEQ ID NO:16
GRAEAGG	SEQ ID NO:17
REAAADAPGRAGG	SEQ ID NO:18
AAARRDAPGRAGG	SEQ ID NO:19
SDSFKSQARGQVPPFLGGVGCPWF	SEQ ID NO:20
SDSFKSQARGQ	SEQ ID NO:21
SEPEARRAPGR	SEQ ID NO:22
SEPEARRAP	SEQ ID NO:23
EPEARRAPG	SEQ ID NO:24
PEARRAPGR	SEQ ID NO:25
EARRAPGRK	SEQ ID NO:26

ARRAPGRKG	SEQ ID NO:27
SEVDARRAKKSLHCILSDTSHPRG	SEQ ID NO:28
SEPEARRAQGGQIPSERVLSD	SEQ ID NO:29

Each of the above NPRs can have either: (1) a free carboxyl group on the C-terminal end or (2) an amidated carboxyl terminus. Both amidated and non-amidated NPRs are effective.

Certain embodiments of this invention include use of NRPs to prevent degeneration or death of peripheral neurons. In certain of these embodiments, the NRP-5 segment GG analogue D4A (SEQ ID NO:2) was found to be effective.

Other embodiments include the use of NRPs to treating disorders involving reduced trophoblast migration including pre-eclampsia, HELLP or IUGR. Such embodiments include peptides that can induce the migration or survival of trophoblasts (for example, SEQ ID NO:1).

We also unexpectedly found that NRPs can decrease TNF-alpha- and interferon-gamma-induced injury *in vitro* in both human term placental trophoblast cells and in a placental cell line (for example, SEQ ID NO:1).

It can be appreciated that NRPs can be effective either as C-terminal free OH peptides or as C-amidated peptides. Both free C-terminal OH peptides and C-terminal amidated peptides are effective, and both are included within the scope of this invention.

Therapeutic Uses of NRP Compounds

NRPs of this invention can be used to treat neurological disorders and obstetric complications. NPRs have been unexpectedly effective in treating neural degeneration associated with autoimmune disorders of the brain, peripheral neuropathy and toxic injury to neural cells. Additionally NPRs have been unexpectedly effective in promoting survival of trophoblast cells.

Thus, the invention includes embodiments which relate to NRPs, peptides encoded by NRPs, homologs, orthologs or paralogs of NRPs, analogs of NRPs, and prodrugs of NRPs, where a prodrug of an NRP is a molecule that may be enzymatically, metabolically or otherwise modified to become an NRP, a NRP homolog, NRP paralog, an NRP ortholog or an NRP analog. Such molecules are collectively termed as "NRP compounds" or "NRPs." NRP compounds may be encoded for by nucleotide sequences, which may be DNA or RNA and which may be single stranded or double stranded. It will be understood that the invention includes sequences complementary to the sequences described in this application as well as the sequences themselves. It is also to be understood that there may be alternatively spliced forms of NRPs, in which case, those alternatively spliced forms of NRP RNA, and the proteins and peptides they may encode are also considered to be part of this invention.

As indicated above, embodiments of the present invention are based upon the inventors' surprising finding of novel NRPs that can induce neurons and neuroblasts to proliferate, migrate, differentiate, produce neurite outgrowth and can protect neurons against damage caused by neural

insults. Proliferation and migration of neural cells into areas of damage caused by acute brain injury or chronic neurodegenerative disease can result in improvement in neural functioning. Further, NRPs can promote neuronal survival, neuronal differentiation, and/or neurite outgrowth. Thus, NRP compounds may be used to treat a variety of disorders and conditions where brain tissue degenerates, is at risk of degeneration or death, or has died.

As indicated above, other embodiments of the present invention are based upon the inventor's surprising finding that NRPs can attenuate motor impairment and body weight loss associated with peripheral neuropathy by preventing degeneration or death of peripheral neurons.

As indicated above, yet other embodiments of the present invention are based upon the inventor's surprising finding that NRPs are useful in treating obstetric complications.

Cells can also use NRP oligonucleotides to stimulate production of NRPs after transfection. In some cases, transfection can be in a replicable vehicle, and in others, the NRP oligonucleotide can be introduced as naked DNA.

Disorders and Conditions Treatable with NRPs and Antioxidants

Disorders and conditions in which NRP compounds of this invention can be of benefit include the following.

Nervous system conditions treatable with NRPs include infections of the central nervous system including bacterial, fungal, spirochetal, parasitic and sarcoid including pyrogenic infections, acute bacterial meningitis, leptomeningitis.

Cerebrovascular diseases include stroke, ischemic stroke, atherosclerotic thrombosis, lacunes, embolism, hypertensive haemorrhage, ruptured aneurysms, vascular malformations, transient ischemic attacks, intracranial haemorrhage, spontaneous subarachnoid haemorrhage, hypertensive encephalopathy, inflammatory diseases of the brain arteries, decreased perfusion caused by, for example, cardiac insufficiency (possibly resulting from coronary bypass surgery) and other forms of cerebrovascular disease.

Craniocerebral traumas include basal skull fractures and cranial nerve injuries, carotid-cavernous fistula, pneumocephalus, aerocele andrhinorrhea, cerebral contusion, traumatic intracerebral haemorrhage, acute brain swelling in children.

Demyelinating diseases include neuromyelitis optica, acute disseminated encephalomyelitis, acute and subacute necrotizing haemorrhagic encephalitis, diffuse cerebral sclerosis of Schilder and multiple sclerosis in conjunction with peripheral neuropathy. Degenerative diseases of the nervous system including syndrome of one or more of progressive dementia, diffuse cerebral atrophy, diffuse cortical atrophy of the non-Alzheimer type, Lewy body dementia, Pick's disease, fronto-temporal dementia, thalamic degeneration, non-Huntingtonian types of Chorea and dementia, cortico-spinal degeneration (Jakob), the dementia-Parkinson-amyotrophic lateral sclerosis complex (Guamanina and others).

Peripheral neuropathy is a common and disabling condition characterised by damage to or loss of peripheral neurons. There are more than 100 types of peripheral neuropathy, each with its own characteristic set of symptoms, pattern of development, and prognosis. Peripheral neuropathy may be either inherited or acquired. Inherited forms of peripheral neuropathy can be caused by genetic mutations. Acquired peripheral neuropathy may result from: physical injury (trauma) to a nerve, tumors, toxins (including chemotherapy), autoimmune responses, nutritional deficiencies, alcoholism, vascular and metabolic disorders (e.g. diabetic neuropathy). The HIV-associated peripheral neuropathy is a common side effect of drugs targeting the reverse transcriptase of the HIV virus. The symptoms of peripheral neuropathy can vary from temporary numbness, tingling, and pricking sensations, sensitivity to touch or muscle weakness, to more extreme symptoms such as burning pain, muscle wasting, paralysis, organ or gland dysfunction.

Metabolic Disorders

Acquired metabolic disorders of the nervous system including metabolic diseases presenting as a syndrome comprising one or more of confusion, stupor or coma-ischemia-hypoxia, hypoglycaemia, hyperglycemia, hypercapnia, hepatic failure and Reye syndrome, metabolic diseases presenting as a progressive extrapyramidal syndrome, metabolic diseases presenting as cerebellar ataxia, hyperthermia, celiac-sprue disease, metabolic diseases causing psychosis or dementia including Cushing disease and steroid encephalopathy, thyroid psychosis and hypothyroidism and pancreatic encephalopathy. An example of a metabolic disorder that can result in neuropathy is pyridoxine excess described more fully below.

Diseases of the nervous system due to nutritional deficiency, alcohol and alcoholism.

Disorders of the nervous system due to drugs and other chemical agents include opiates and synthetic analgesics, sedative hypnotic drugs, stimulants, psychoactive drugs, bacterial toxins, plant poisons, venomous bites and stings, heavy metals, industrial toxins, anti-neoplastic and immunosuppressive agents, thalidomide, aminoglycoside antibiotics (ototoxicity) and penicillin derivatives (seizures), cardioprotective agents (beta-blockers, digitalis derivatives and amiodarone).

As illustrated by the preceding list, compositions and methods of the invention can find use in the treatment of human neural injury and disease. Still more generally, the compositions and methods of the invention find use in the treatment of human patients suffering from neural damage as the result of acute brain injury, including but not limited to diffuse axonal injury, perinatal hypoxic-ischemic injury, traumatic brain injury, stroke, ischemic infarction, embolism, and hypertensive haemorrhage; exposure to CNS toxins, infections of the central nervous system, such as, bacterial meningitis; metabolic diseases such as those involving hypoxic-ischemic encephalopathy, peripheral neuropathy, and glycogen storage diseases; or from chronic neural injury or neurodegenerative disease, including but not limited to Multiple Sclerosis, Lewy Body Dementia, Alzheimer's disease, Parkinson's disease

and Huntington's disease. Patient's suffering from such diseases or injuries may benefit greatly by a treatment protocol able to initiate neuronal proliferation and migration, as well as neurite outgrowth.

Still more generally, the invention has application in the induction of neuronal and neuroblast migration into areas of damage following insult in the form of trauma, toxin exposure, asphyxia or hypoxia-ischemia.

Uses of NRPs in Treating Obstetric Complications

Trophoblasts are essential in maintaining early pregnancy. They are among the first cells to differentiate to form the outer layer of the blastocyst, they secure its implantation in the uterine wall and subsequently develop into a placenta. The differentiation of trophoblasts following the implantation of the blastocyst results in the creation of extravillous trophoblast cells (EVT) that migrate and invade the uterine stroma. The trophoblast stem cells fuse to form syncytiotrophoblasts, which form anchoring villous trophoblasts. The villous trophoblasts give rise to a sub-population known as extravillous trophoblasts. Extravillous trophoblasts invade the uterine wall and its blood vessels and remodel the maternal spiral arteries by displacing smooth muscle and endothelial cells. As a result, blood vessels that are characterised by a larger diameter, an increased blood flow and a reduced resistance are produced. This step is essential for providing for the higher blood supply requirements of the fetus later in the pregnancy and, as a consequence, for maintaining a normal pregnancy.

Trophoblasts differentiate into endothelial-like cells in the spiral arteries of the endometrium where they remodel the arteries by replacing the smooth muscle and the endothelial cells to achieve a similar effect: an increase in vessel diameter, increase in blood flow and decrease in resistance zone.

In vitro studies suggest that in normal pregnancy, maternal cells may play a role in controlling the trophoblast invasion, although the exact nature of the regulatory interactions between these cells is unknown (Campbell et al., 2003). Deficient human trophoblast invasion into the maternal decidua appears to be a major feature of the pregnancy-associated pre-eclampsia. A failure to remodel the maternal spiral arteries, for example, is thought to restrict the blood flow to the developing foetus and contribute to the onset of pre-eclampsia or intrauterine growth restriction. The reasons for the failure are not known, but it is postulated that they may include an increase in the apoptosis of trophoblasts or compromised invasiveness of the trophoblasts.

Pre-eclampsia is characterized by a sudden onset of maternal hypertension, proteinuria and edema. In a pre-ecalmptic patient the cytotrophoblast invasion is shallow and vascular transformation is incomplete. Pre-eclampsia has been the leading cause of maternal mortality in the developed countries. Worldwide the disease is responsible for approximately 150,000 deaths per year. It also leads to considerable mortality and morbidity in newborn children and is expected to carry health implications in adult life, including increased risk of hypertension, heart disease and diabetes.

Intra-uterine growth restriction (IUGR), paired with permanent hypoxic placental conditions associated with the pathological condition of pre-eclampsia, lead to a retarded placental growth, putative birth complications and/or damages to the human foetus (e.g. a necessity for a pre-mature caesarean section resulting in a very low birth weight). A rare outcome of pre-eclampsia is a syndrome characterized by hepatic and renal failure with putative fatal outcome, so called "HELLP" syndrome (Haemolysis, Elevated Liver enzymes, Low Platelets) syndrome (Volz et al., 1992).

Patients with inherited thrombophilias developing pre-eclampsia during pregnancy have been shown to respond to a low molecular weight heparin treatment (LMWH-therapy) that can reverse some clinical symptoms (Saisto et al., 2004). Nevertheless, other forms of pre-eclampsia do not respond to LMWH-therapy.

It is therefore beneficial to establish a treatment or prophylaxis, which would increase the migration and invasiveness of trophoblasts during pregnancy in order to prevent the development of pre-eclampsia, HELLP syndrome or IUGR.

Administration of NRPs

NRP compounds, including NRP-1, its orthologs, analogs, paralogs, the NPRs disclosed herein and prodrugs containing the identified NRP peptide domains, can be used to promote neuronal and neuroblast migration. Most conveniently, this can be affected through direct administration of NRP compounds to the patient.

However, while NRPs can be advantageously used, there is no intention to exclude administration of other forms of NRP compounds. For example, human paralog forms or peptide fragments of NRP can be administered in place of NRP. By way of example, the effective amount of NRP in the CNS can be increased by administration of a pro-drug form of NRP that comprises NRP and a carrier, NRP and the carrier being joined by a linkage that is susceptible to cleavage or digestion within the patient. Any suitable linkage can be employed which will be cleaved or digested to release NRP following administration.

Another suitable treatment method is for NRP levels to be increased through an implant that is or includes a cell line that is capable of expressing NRP or analogs, paralogs or pro-peptides of an NRP in an active form within the central nervous system of the patient.

An NRP can be administered as part of a medicament or pharmaceutical preparation. This can involve combining NRP compounds with any pharmaceutically appropriate carrier, adjuvant or excipient. Additionally an NRP compound can be used with other non-NRP neuroprotective, proliferative, or other agent. The selection of the carrier, adjuvant or excipient will of course usually be dependent upon the route of administration to be employed.

The administration route can vary widely. An NRP may be administered in different ways: intraperitoneal, intravenous or intracerebroventricular. The peripheral application may be the way of choice because then there is no direct interference with the central nervous system.

Any peripheral route of administration known in the art can be employed. These can include parenteral routes for example injection into the peripheral circulation, subcutaneous, intraorbital, ophthalmic, intraspinal, intracisternal, topical, infusion (using eg. slow release devices or minipumps such as osmotic pumps or skin patches), implant, aerosol, inhalation, scarification, intraperitoneal, intracapsular, intramuscular, intranasal, oral, buccal, pulmonary, rectal or vaginal. The compositions can be formulated for parenteral administration to humans or other mammals in therapeutically effective amounts (eg. amounts which eliminate or reduce the patient's pathological condition) to provide therapy for the neurological diseases described above.

One route of administration includes subcutaneous injection (e.g., dissolved in 0.9% sodium chloride) and oral administration (e.g., in a capsule).

It will also be appreciated that it may on occasion be desirable to directly administer NRP compounds to the CNS of the patient by any appropriate route of administration. Examples include administration by lateral cerebroventricular injection or through a surgically inserted shunt into the lateral cerebral ventricle of the brain of the patient.

Therapeutic Doses of NRPs

In some embodiments of this invention, methods for treating brain damage comprise administering one or more NRPs in a dose range of from about 0.01μg/kg body weight to about 100 μg/kg body weight. In other embodiments, a dose of 1 μg/kg body weight to about 10 μg/kg body weight can be useful. We have found that at a dose of about 4.16μg/kg, mice with EAE showed significant improvement in motor function compared to control animals treated with saline only (see Example 3). In further embodiments, a dose of an NRP can be in the range of about 0.01 μg/kg body weight to about 0.1mg/kg.

In other embodiments, the determination of an effective amount of an NRP to be administered is within the skill of one of ordinary skill in the art, and will be routine to those persons skilled in the art. In certain embodiments, the amount of an NRP to be used can be estimated by *in vitro* studies using an assay system as described herein. The final amount of an NRP to be administered will be dependent upon the route of administration, upon the NRP used and the nature of the neurological disorder or condition that is to be treated. A suitable dose range may for example, be between about 0.1μg to about 15μg per 1kg of body weight or in other embodiments, about 20 μg/kg to about 30 μg/kg body weight per day.

For inclusion in a medicament, NRP can be directly synthesized by conventional methods such as the stepwise solid phase synthesis method of Merrifield *et al.*, 1963 (J. Am. Chem. Soc. 15:2149-2154) or Goodman M. (ed.), "Synthesis of Peptides and Peptidomimetics" in Methods of organic chemistry (Houben-Weyl) (Workbench Edition, E22a,b,c,d,e; 2004; Georg Thieme Verlag, Stuttgart, New York), expressly incorporated herein fully by reference. Such methods of peptide

synthesis are known in the art, and are described, for example, in Fields and Colowick, 1997, Solid Phase Peptide Synthesis (Methods in Enzymology, vol. 289), Academic Press, San Diego, CA, expressly incorporated herein fully by reference. Alternatively synthesis can involve the use of commercially available peptide synthesizers such as the Applied Biosystems model 430A.

As a general proposition, the total pharmaceutically effective amount of an NRP administered parenterally per dose will be in a range that can be measured by a dose response curve. For example, an NRP in the blood can be measured in body fluids of the mammal to be treated to determine dosing. Alternatively, one can administer increasing amounts of an NRP compound to the patient and check the serum levels of the patient for the NRP. The amount of NRP to be employed can be calculated on a molar basis based on these serum levels of the NRP.

One method for determining appropriate dosing of the compound entails measuring NRP levels in a biological fluid such as a body or blood fluid. Measuring such levels can be done by any means, including RIA and ELISA. After measuring NRP levels, the fluid is contacted with the compound using single or multiple doses. After this contacting step, the NRP levels are re-measured in the fluid. If the fluid NRP levels have fallen by an amount sufficient to produce the desired efficacy for which the molecule is to be administered, then the dose of the molecule can be adjusted to produce maximal efficacy. This method can be carried out *in vitro* or *in vivo*. This method can be carried out *in vivo*, for example, after the fluid is extracted from a mammal and the NRP levels measured, the compound herein is administered to the mammal using single or multiple doses (that is, the contacting step is achieved by administration to a mammal) and then the NRP levels are remeasured from fluid extracted from the mammal.

NRP compounds are suitably administered by a sustained-release system. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, for example, films, or microcapsules. Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), poly(2-hydroxyethyl methacrylate) (Langer *et al.,* 1981), ethylene vinyl acetate (Langer *et al.,* supra), or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also include a liposomally associated compound. Liposomes containing the compound are prepared by methods known to those of skill in the art, as exemplified by DE 3,218,121; Hwang *et al.,* 1980; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appln. 83-118008, U.S. Pat. Nos. 4,485,045 and 4,544,545 and EP 102,324. In some embodiments, liposomes are of the small (from or about 200 to 800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the most efficacious therapy. All U.S. parents referred to herein, both *supra* and *infra*, are hereby expressly incorporated by reference in their entirety.

PEGylated peptides having a longer life than non-PEGylated peptides can also be employed, based on, for example, the conjugate technology described in WO 95/32003 published November 30, 1995.

In some embodiments, the compound can be formulated generally by mixing each at a desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically, or parenterally, acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides. It can be appreciated that the above doses are not intended to be limiting. Other doses outside the above ranges can be determined by those with skill in the art.

In some embodiments, formulations can be prepared by contacting a compound uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if desired, the product can be shaped into the desired formulation. In some embodiments, the carrier is a parenteral carrier, alternatively, a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, a buffered solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are desirably non-toxic to recipients at the dosages and concentrations employed, and include, by way of example only, buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; glycine; amino acids such as glutamic acid, aspartic acid, histidine, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, trehalose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counter-ions such as sodium; non-ionic surfactants such as polysorbates, poloxamers, or polyethylene glycol (PEG); and/or neutral salts, e.g., NaCl, KCl, $MgCl_2$, $CaCl_2$, and the like. In certain embodiments, a peptide of this invention can be stabilized using 0.5 M sucrose or 0.5 M trehalose. Using such sugars can permit long-term storage of the peptides.

An NRP compound can be desirably formulated in such vehicles at a pH of from about 6.5 to about 8. Alternatively, the pH can be from about 4.5 to about 8. It will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of salts of the compound. The final preparation may be a stable liquid or lyophilized solid.

In other embodiments, adjuvants can be used. Typical adjuvants which may be incorporated into tablets, capsules, and the like are a binder such as acacia, corn starch, or gelatin; an excipient such as microcrystalline cellulose; a disintegrating agent like corn starch or alginic acid; a lubricant such as magnesium stearate; a sweetening agent such as sucrose or lactose; a flavoring agent such as peppermint, wintergreen, or cherry. When the dosage form is a capsule, in addition to the above materials, it may also contain a liquid carrier such as a fatty oil. Other materials of various types may be used as coatings or as modifiers of the physical form of the dosage unit. A syrup or elixir may

contain the active compound, a sweetener such as sucrose, preservatives like propyl paraben, a coloring agent, and a flavoring agent such as cherry. Sterile compositions for injection can be formulated according to conventional pharmaceutical practice. For example, dissolution or suspension of the active compound in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants, and the like can be incorporated according to accepted pharmaceutical practice.

Desirably, an NRP compound to be used for therapeutic administration may be sterile. Sterility can be readily accomplished by filtration through sterile filtration membranes (e.g., membranes having pore size of about 0.2 micron). Therapeutic compositions generally can be placed into a container having a sterile access port, for example an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

In other embodiments, an NRP compound can be stored in unit or multi-dose containers, for example, sealed ampules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-mL vials are filled with 5 ml of sterile-filtered 0.01% (w/v) aqueous solution of compound, and the resulting mixture is lyophilized. The infusion solution can be prepared by reconstituting lyophilized compounds using bacteriostatic water or other suitable solvent.

In still further embodiments, a kit may contain a predetermined amount of lyophilized NRP, a physiologically compatible solution for preparation of a dosage form, a mixing vial, a mixing device, and instructions for use. Such kits can be manufactured and stored according to usual practices in the industry.

An NRP-containing composition may be administered by one or more of a variety of routes. By way of example, intravenous, intraperitoneal, intracerebral, intraventricular, inhalation, lavage, rectal, vaginal, transdermal, subcutaneous administration can be used.

Antioxidants

Antioxidants are compounds that prevent cellular damage due to the action of reactive oxygen species, such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) or theoxyl radical ($\cdot OH$) as well as reactive nitrogen species, e.g. peroxynitrate ($ONOO^-$) by binding to a radical and thereby resulting in its inactivation. Antioxidants participate also in the major signaling pathways of cells. The human brain utilises large quantities of oxygen and is especially susceptible to oxidative stress resulting from formation of oxidants during mitochondrial electron transport, auto-oxidation of some neurotransmitetrs and initiation of events during hypoxia or ischemia (Warner et al. (2004). Oxidants, antioxidants and the ischemic brain. *Journal of Experimental Biology, 207*, 3221-3231). It is accepted in the literature that the increase in oxidative stress during aging is one of the factors contributing to the development of neurodegenerative diseases in the aging brain (Casetta et al. (2005) Oxidative

Stress, Antioxidants and Neurodegenerative Diseases. Current Pharmaceutical Design 11, 2033-2052).

Antioxidants useful for administration with an NRP include but are not limited to the following:

(a) Vitamins: vitamin A (retinol), vitamin C (ascorbic acid), vitamin E (tocotrienol, tocopherol);

(b) Vitamin cofactors: coenzyme Q10 (idebenone);

(c) Antioxidant proteins: catalase, thioredoxins, thioredoxin reductase 1, peroxiredoxin 2, peroxiredoxin 4, peroxiredoxin 6, Cu/Zn superoxide dismutase, Mn superoxide dismutase, glutathione and L-carnosine;

(d) Carotenoids: lycopene, lutein, alpha-carotene, beta-carotene, zeaxanthin, and astaxanthin

(e) Polyphenol antioxidants:

(i) Flavonoids including:

Flavanols: resveratrol, kaempferol, myricetin, isorhamnetin, and proanthocyanidins

Flavones: quercetin, rutin, luteolin, apigenin and tangeritin

Flavanones: hesperetin, naringenin and eriodictyol

Flavan-3-ols: catechin, gallocatechin, epicatechin and epigallocatechin, Theaflavins, thearubigin

Isoflavone phytoestrogens: genistein, daidzein and glycitein;

Anthocyanins;

(ii) Phenolic acids and their esters: rosmarinic acid, cinnamic acid, chlorogenic acid, chicoric, gallotannins and ellagitannins;

(iii) Other nonflavonoid phenolics: curcumin

(f) Other antioxidants: acetylcysteine, lipoic acid (α-lipoic acid) and methionine.

Persons of ordinary skill can select other antioxidants that have similar effects, and all of the antioxidants known in the art are included within the scope of this invention.

The summary of protective effects of flavonoids (genistein, epigallocatechin-gallate, quercetin, silymarin, wogonin, kaempferol, patuletrin, flavopiridol, naringenin and nepitirin) on neurons or PC12 cells in cultures is available in Dajas et al. Current Neuropharmacology, 3: 193-205, at 197 (2005). Neuroprotective effects of isoflavonoids formononetin, 9,10-dimethoxypterocarpan 3-O-beta-D-glucoside, ononin, calycosin 7-O-glc and calycosin, on PC12 cells were described by Yu et al, (J. Ethnopharmacol. 98(1-2): 89-94, 2005).

Quercetin is a plant flavonoid found in many fruits (e.g. apples) and vegetables (Graefe et al. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *The Journal of Clinical Pharmacology,* 37, 219-233 (1999) . It is a free radical scavenger and has anti-inflammatory activity.

Many studies have shown that quercetin decreases neuronal injury in cultured hippocampal cells by inhibiting the production of nitric oxide and by reducing the activity of nitric oxide synthase (Bastianetto, Zheng, & Quirion, 2000; Raso, Meli, Di Carlo, Pacilio, & Di Carlo, 2001).

Antioxidant proteins involved in neuronal diferentiation of neuroblastoma cell lines into neuronal phenotype have been identified in the literature (Oh et al. The neural differentiation process involves a series of antioxidant proteins. Amino Acids 2005).

Although desirable effects on neurons of antioxidants have been observed, our finding of a totally unexpected syngergic effect of NRPs and antioxidants provides an improved strategy for treating a variety of neurological and other deficits.

Additionally, we found that cell culture medium supplemented with an NRP and an antioxidant can improve growth and differentiation of neurons *in vitro*. Thus, this invention provides improved methods for studying properties and responses of cultures of neural cells under conditions of neural cell growth and/or differentiation.

Media containing one or more NRPs and/or one or more antioxidants can be either: (1) a cellular dissociation medium or (2) a cultivation medium. These media can have different amounts or species of NRPs or antioxidants depending on the conditions in which the medium is used.

Thus, in some embodiments, a dissociation medium can contain the usual constituents known in the art, plus glucose and can be augmented with one or more NRPs and/or one or more antioxidants. In one embodiment, the amount of glucose can be 25 mM and the NRP can be NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG). In some embodiments, the amount of NRP can be 100fM. In other embodiments, the amount of NRP can be higher. Based on the disclosures and teachings in this application, persons of ordinary skill can readily determine an appropriate amount of an NRP in a particular situation.

A culture medium can also contain one or more antioxidants as disclosed herein. In ome embodiment, a cultivation medium can include the NOS-inhibitor 7 nitroindazole (7-NI), and/or Quercetin. In one embodiment, the amount of 7-NI can be 500μM and the amount of Quercetin can be 100μM. Based on the disclosures and teachings in this application, persons of ordinary skill can readily determine an appropriate amount of an NRP in a particular situation.

In certain embodiments, to demonstrate synergism between an NRP and antioxidant, an amount of NRP can be chosen to not produce a large effect alone. In some cases, the effect may be so small as to not be observable (a "sub-threshold" amount of the agent). Thus, as shown in Example 2, a sub-threshold amount of NRP was selected that alone had little or no effect on neuronal cell number. The amount of antioxidant was selected to show a measurable effect on neuronal cell number.

One would expect that if an NRP and an antioxidant did not act synergistically, the effect of the two agents together would have a similar magnitude as the sum of the individual effects. On the other hand, if the two agents did act synergistically, then one would find that the effects of the two agents in combination would have a greater effect than the sum of the individual effects. As show in

Example 2 below, the NRP and antixoidant together had a combined effect greater than the sum of the individual effects, thereby demonstrating an unexpected synergistic effect.

Kits

Additional embodiments include kits for modifying cell culture media comprising an NRP in a vial, an antioxidant in a vial, solvents for preparing solutions of the NRP and the antioxidant, a mixing vial, a stirring device, and instructions for use. In some embodiments, the NRP and/or the antioxidant can also include preservatives to decrease or prevent degradation of the compound. Alternative kits include combinations of at least one NRP and at least one antioxidant, a solvent for preparing a solution of said NRP(s) and antioxidant(s) and instructions for use.

To use a kit of this invention, one provides a basic cell culture medium known in the art, optionally containing glucose, then one can prepare the solution(s) of NRP and antioxidant, then mix the NRP/antioxidant solution with the cell culture medium, and then apply the NPR/AO-augmented cell culture medium to cells in culture.

It can be appreciated that the materials used for *in vitro* cell culturing are desirably sterilized before use. Alternatively, the NRP(s) and antioxidant(s), solvent, mixing vial, stirring rods and other equipment can be provided in sterile form so that subsequent sterilization of the final cell culture midium may not be required.

EXAMPLES

The examples that follow are presented to illustrate aspects of this invention. The scope of this application is not intended to be limited to the examples shown. Rather, persons of ordinary skill can modify the teachings of these examples to arrive at other embodiments. Each of these embodiments is considered to be part of this invention.

Example 1: Cell Culture System

The studies described below were designed to develop a cell culture medium that would supplement and support the growth of adult neurons from different parts of the brain for up to 7 days in vitro (DIV). In the studies below, the Brewer's medium was used as a control and then supplemented with quercetin, NRP and selective nNOS inhibitor, 7-nitroindazole.

Methods and Materials

Media

Four different medium conditions were used. All medium conditions contained: Cultivation medium of Brewer's medium consisting of Neurobasal A, 2% B27, 1% Glutamine, 1% Penicillin / Streptomycin, 5ng/ml bFGF, and additionally:

1 Control Conditions:

Dissociation Medium: 25mM Glucose;

Cultivation Medium:

2 NRP Alone:

Dissociation Medium: 25mM Glucose, 100fM NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG);

Cultivation Medium: plus 100fM NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG).

3 Antioxidant Alone:

Dissociation Medium: 25mM Glucose, 500μM 7-NI (NOS-inhibitor 7 nitroindazole), 100μM Quercetin;

Cultivation Medium:

4 Antioxidant and NRP:

Dissociation Medium: 25mM Glucose, 500μM 7-NI, 100fM NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG), 100 μM Quercetin;

Cultivation Medium: plus 100fM NRP-5RG D6A (SEQ ID NO:1; REGRRAAPGRAGG).

Cultivation Plate Preparation

18x18 glass coverslips were soaked in 100% absolute ethanol (15-20min) per side, then rinsed with milliQ water and left to dry (1h). Dry tops of the coverslips were coated with 0.1mg/ml PDL and incubated at 37°C/5%CO_2 overnight. The following day the coverslips were rinsed out with milliQ, dried and placed into a 6-well cell cultivation plate. The cells were then seeded onto glass coverslips and incubated at 34°C 5%CO_2, 100% humidity, for maximum 1h. Approximate seeding density was one cerebellar hemisphere or one hippocampal hemisphere per 6-well plate. Coverslips were then drained and rinsed twice with 1ml of cultivation or control medium to remove debris. Cells were then reconstituted with 1ml of cultivation medium at 34°C 5%CO_2 and 100% humidity for up to 7 days.

Cell Extraction and Preparation for Survival Assay

All studies were performed the hippocampus and the cerebellum. Sprague-Dawley rats (postnatal day 38-42) were obtained from University of Auckland Animal Resource Unit. All the ethical approvals were obtained from the Ethics Committee of the University of Auckland.

Using modified Brewer's procedure (Brewer, G. J. Regeneration and Proliferation of Embryonic and Adult Rat Hippocampal Neurons in Culture. *Experimental Neurology, 159*(1), 237-247; 1999), animals were anaesthetized by CO_2 and killed by spinal dislocation. The animals were

decapitated and the cerebella and hippocampi of both hemispheres were removed using aseptic technique in a biohazard class 2 hoods. The cerebella and hippocampi were placed in either control medium or dissection medium. Only dissociation medium contained antioxidants (quercetin and 7-NI). They were not present in the cultivation medium due to the possible long-term toxic effect they could have on the neurons. The tissue was then finely chopped with small scissors and placed into sterile 50ml tubes using a 23 gauge needle. 5ml of 2000units/ml of papain containing 250µl of 2000units/mL DNase was added to each tube and the cells were incubated in a 37°C incubator for half an hour under constant agitation. Once the tissue had been digested the cells were triturated with 10mL pipette 10 times and filtered through 300µm followed by 200µm filters and centrifuged at 300 x g for 5 minutes at room temperature. Once the supernatant was removed the pellet was resuspended in medium of either 2.7ml of dissociation medium or control with 300µl of 10mg/ml albumin-ovomucoid inhibitor solution and 150µl of 2000units/ml DNase. The cells were then layered on top of 5ml of albumin-ovomucoid inhibitor solution and centrifuged at 70 x g for 6 minutes at room temperature. The interface between the two layers (pellet containing neuronal cells and the debris on the top) of the gradient was clear. Once centrifuged the dissociated cells were in the pellet and the debris and other membrane fragments remained at the interface. The supernatant was discarded and the cell pellet was resuspended in cultivation or control medium.

After 7 days *in vitro* (7DIV), cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. Cells were permeabilised with 0.3% Triton X-100 in PBS for 30 minutes at room temperature. Nonspecific sites were blocked by incubation with 1% normal goat serum, 2% BSA and 1% Triton X-100 in PBS (blocking solution) for 30 minutes. Cells were then washed with three 10-minute washes of PBS. Rabbit antibodies, generated against MAP2 (1:750) and mouse antibodies against nestin (1:1000) were incubated with the cells at 4°C overnight. All dilutions were done in blocking solution.

The following day the cells were washed with 3 washes of PBS plus 0.1% Tween 20. Secondary goat-anti-mouse Cy3-red (1:300) and goat-anti-rabbit-Biotin (1:300) were diluted in the blocking solution and incubated with the cells for 3 hours at room temperature in the dark. Once the unbound secondary antibodies were removed, cells were washed three times with PBS, ExtrAvidin-FITC conjugate green (1:250) was added and the cells were incubated for a further hour in the dark, followed by 3 PBS washes. After the staining procedure was completed, the cells on the coverslips were mounted onto slides and analyzed under Zeiss axiophot microscope, equipped with Axoivision software. The total number of red and green stained cells was counted at 20x magnification.

Differentiation Assay

Two sets of experiments were done to analyse the effects of antioxidants and NRPs on neuronal differentiation. The first was analyzing the length of neurite outgrowth. The 5 longest

dendrites within one coverslip were measured using the axiovision software. The neurons that had longer dendrites were classified as being more differentiated.

The second set of experiments was designed to evaluate the expression of α-synaptophysin in neurons. After 7 DIV cells were fixed with 4% paraformaldehyde, and permeabilised with 0.3% Triton X-100 in PBS for 30 minutes at room temperature. Nonspecific sites were blocked by incubation with 1% normal goat serum, 2% BSA and 1% Triton X-100 in PBS (blocking solution) for 30 minutes. Cells were then washed three times with PBS. Rabbit antibodies against MAP2 (1:750) and mouse antibodies against α-synaptophysin (1:200) were incubated at 4°C overnight. All dilutions were done in blocking solution.

The following day the cells were washed 3 times in PBS (0.1% Tween 20). Secondary goat-anti-mouse Cy3-red (1:300) and goat-anti-rabbit-Biotin (1:300) were diluted in the blocking solution and incubated with the cells for 3 hours at room temperature in the dark. Once the unbound secondary antibodies were removed and the cells were washed with 3x10minutes PBS, ExtrAvidin-FITC conjugate green (1:250) was added and the cells were incubated for a further hour in the dark. The total number of red and green stained cells was counted at 20x magnification.

Assay for Neuronal Proliferation Rate

The hippocampal and cerebellar neurons were prepared as described above. Cells were then reconstituted in 1ml of cultivation medium. 0.05μM BrdU was added for 24hrs at different time points: 1 hour after seeding, 2 days, 4 days and 6 days after seeding. The cells were fixed with 4% PFA after 24hrs from BrdU treatment and stained for BrdU positive cells. In one set of experiments NRP was added only at start of the cultivation. In another set of experiments NRP was added at different time points, simultaneous with BrdU. PBS was added as a control into the conditions which did not contain any NRP.

After the cells were fixed with 4% PFA the cells were incubated with 2N HCl for 30 min to permeabilise the nuclear membrane. HCl was neutralized by 2% Borax, employing 2 washes for 5min. Nonspecific sites were blocked by incubation with 1% normal goat serum, 2% BSA and 1% Triton X-100 in PBS (blocking solution) for 30 minutes. Cells were then washed 3 times with PBS. Rabbit antibody generated against MAP2 (1:750) and mouse antibodies against BrdU (1:50) were incubated at 4°C overnight. All dilutions were done in blocking solution.

The following day the cells were washed with 3x10minutes PBS (0.1% Tween 20). Secondary goat-anti-mouse Cy3-red (1:300) and goat-anti-rabbit-Biotin (1:300) were incubated with the cells for 3 hours at room temperature in the dark. Once the secondary antibodies were removed, cells were washed with three washes of PBS. ExtrAvidin-FITC conjugate green (1:250) was then added and the cells were incubated for a further hour in the dark. After the staining procedure was completed the cells on coverslips were mounted onto slides and analyzed under a Zeiss axiophot

microscope, equipped with Axiovision software. Both total number of MAP2-positive cells and BrdU positive cells were counted and the percentage of MAP2/BrdU double labelled cells was graphed. Total number of red and green stained cells was counted at 20x magnification.

Data Analysis

Data were analyzed using statistical software Graph Prism. One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's test was used to determine statistical significance. Significance refers to results where $p<0.05$ was obtained. All the graphs were normalised to the control condition.

Example 2: Promotion of Neuronal Survival by NRPs and Antioxidants

Adult Cerebellar Cells

The overall MAP2-positive neuronal cell number was increased 301.8% when medium was supplemented with NRP-5RG D6A (SEQ ID NO: 1), quercetin and 7-nitroindazole. Comparing the AO condition versus AO+NRP there was a 58.4% ($p<0.001$) increase in the number of MAP-2 positive cells in the AO+NRP cultures. The neuronal subtype of NF-200 (phosphorylated epitope) positive cells in the AO+NRP condition was increased by 665.0% over the control condition. The undifferentiated cell marker nestin was only increased within the AO condition versus the Brewer control medium. We noted that compared to control conditions (Control MAP2), treatment with the NRP did not increase cell number. Treatment with AO significantly increased cell number. Surprisingly however, the combination of NRP+AO exhibited a synergistic effect, i.e., the effect of NRP+AO was greater than the sum of each effect separately (Figure 1A).

Adult Hippocampal Cells

NG2 positive cells

NG2 positive cells were quantified using an assay for a chondroitin sulphate proteoglycan, a marker for oligodendrocytic precursor cells.

Both AO and NRP+AO supplementation of the medium produced a significant increase in survival (387.6 ± 63.2% and 427.8 ± 98.52%, respectively compared to the Brewer's control) of NG2 positive cells (Figure 1B). NG2 expressing cells have been shown to manage to survive neurotoxic insults and retain their ability to divide (Dzwonek, 2005). Other studies have also demonstrated that oligodendrocytic precursor cells can differentiate into either mature oligodendrocytes or type II astrocytes but there is a subpopulation of precursor cells that do not differentiate and form synaptic junctions with CA3 pyramidal neurons in the hippocampus (Lin & Bergles, 2002).

MAP2 Positive Hippocampal Cells

AO or NRP supplementation showed a slight trend to increase the survival of MAP2-positive, mature, neurons (Figure 1C). When NRP was administered at the start and then again at 5DIV, the survival of mature neurons was increased by 97.5 ± 19.1% ($p<0.01$) compared to Brewer's control (Figure 1D).

As mentioned above for cerebellar cultures, NRP does not need to be added at 5DIV to significantly induce neuroprotection. One possible explanation is that endogenous NRP-like protein sequences (i.e. CAPS2) are expressed in the cerebellum but not in the hippocampus. An alternative hypothesis is that administration of NRP to cerebellar cells might cause an autocrine activation that leads to further synthesis of CAPS2, thus sustaining a prolonged neuroprotective effect until 7DIV analysis. Because CAPS2 is expressed only in small amounts in the hippocampus, this could explain the need for constant administration (Speidel et al., 2003). Alternatively, hippocampal cells may specifically express and release a CAPS-2 and NRP cleaving enzyme that is not expressed by cerebellar cells. However, regardless of the mechanism of action, the synergistic effects of NRPs and antioxidants can be useful to promote neuronal survival.

Nestin Positive Cells

Cultivation of dissociated cells from the hippocampal formation in the presence of the NRP-5RG D6A (SEQ ID NO:1: REGRRAAPGRAGG), quercetin and 7-nitroindazole resulted in a 386% increase in nestin-positive precursor cells, in comparison with the Brewer medium based control (Figure 3). One possible explanation for this effect is that hippocampal cells represent oligodencrocytic precursor cells (OPCs). This finding is different from the results found with cerebellar cell cultivation where the number of nestin-positive cells is similar between all tested conditions.

Example 3: NRPs and Antioxidants Promote Neuronal Differentiation and Neurite Outgrowth

The effects of AO+NRP combination on neuronal differentiation were assessed by determining the longest neurite within one experimental sample (Figures 2 and 4). Through differentiation neurons adopt their phenotype. One of the ways of examining differentiation is to examine neurite outgrowth. The process of extension of neurites and establishing connections with other is a staring point for morphological and functional maturation of the neuron.

Figure 2 depicts the increase in neurite length when cerebellar cell cultivation was performed in the presence of NRP-5RG D6A. The five longest neurites of MAP2-positive cells were analysed under AO-conditions versus AO+NRP conditions. A 300% increase ($p<0.001$) in neurite length was observed in the NRP+AO condition in comparison with the AO condition after 7DIV.

Similar findings were observed in the hippocampus after cultivation for 7DIV. Increase in neurite outgrowth length was 44.3 ± 10.5μm with neurons cultured with NRP plus AO compared to Brewer's control and an increase of 19 ± 8.8% ($p<0.05$) with cells cultured with AO+NRP compared to AO alone (Figure 4). The effect of antioxidant plus NRP was greater than the sum of effects of NRP alone plus the effect of antioxidant alone. Thus, the greater response to NRP plus antioxidant was completely unexpected.

Example 4: Promotion of Neuronal Proliferation by NRPs and Antioxidants

In order to facilitate neuroregenerative capacity within a cell culture system one of the major biological activities of a neurorepair molecule is the promotion of neuronal proliferation. The neuronal proliferation rate can be conveniently measured by incorporation of BrdU (bromodeoxyuridine, a thymidine analogue) into dividing DNA for the period of 24h and subsequence measures of proliferation rate at regular time points. In the first set of experiments the cells were exposed to 100fM of NRP (SEQ ID NO: 1) at the start of the experiment (day 0). In the second group of experiments the 100fM NRP was added together with BrdU. Following fixation, BrdU labelled cells were analysed for MAP2.

Hippocampal Cells

NRP Added at the Start Only

No significant difference was observed between the AO-supplemented and AO+NRP-supplemented media (Figure 5A). There was a significant increase of 74 ± 19.7% ($p<0.01$) in BrdU positive cells between Brewer's control and AO conditions when analysed at 7DIV. There was a trend in increase of 53.63 ± 24.73% in BrdU positive cells in NRP+AO condition at 7DIV in comparison to Brewer's control. The number of proliferating cells within AO condition increased by 137.2 ± 23.8% from 1DIV to 7DIV (Figure 5A) and by 198.1 ± 27.3% within NRP+AO from day 1 to day 7 of cultivation.

NRP Added with BrdU

At 5DIV the proliferation rate of MAP-2 positive cells within NRP+AO condition increased 183.5 ± 35.27% ($p<0.001$) when compared to Brewer's control and 125 ± 28.36% ($p<0.01$) when compared to AO alone condition. A significant increase in NRP+AO cells proliferation was also detected at 7DIV, 218.7 ± 43.15% ($p<0.001$) when compared to Brewer's control and 82.5 ± 26.9% ($p<0.01$) when compared to AO alone condition (Figure 5B). A boost of NRP 24 hours before fixation at 5DIV and 7 DIV caused an increase in proliferation rate.

Example 5: NRPs and antioxidants affect *α*-Synaptophysin Levels

Another way to assess neuronal differentiation in cultured neurons is to study the expression of α - synaptophysin. Synaptophysin is an integral presynaptic vesicle protein that is expressed throughout the brain (Fykse et al., 1993; Marqueze-Pouey, Wisden, Malosio, & Betz, 1991). It plays a role in synapse formation and the stabilization of newly formed synapses (Tarsa & Goda, 2002). Immunohistochemical staining with an anti α - synaptophysin antibody was used in the experiments.

In both investigated brain regions, cerebellum (Figure 6A) and hippocampus (Figure 6B), the α-Synaptophysin expression in cultures supplemented with NRP+AO, was increased by 40% in comparison with cells in antioxidants only condition.

We conclude from these studies that compositions containing an antioxidant and a neural regeneration peptide can be useful for improving neuroprotective effects in the face of neuronal insults, including cardiac bypass graft surgery, chronic neurodegenerative diseases, traumatic injury to the brain or other conditions in which neurons are at risk for degeneration or death. We also conslude that a combination of one or more NRPs and one or more antioxidants can be useful additions to cell culture medium to promote neural cell growth and/or differentiation *in vitro*.

We claim:

1. A composition for promoting neuroprotection in a mammal having a disease or condition likely to result in neurodegeneration or neural cell death, comprising:

a neural regeneration peptide (NRP); and

an antioxidant (AO).

2. The composition of claim 1, wherein said NRP is selected from the group consisting of peptides having the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29.

3. The composition of claim 1, wherein said antioxidant is selected from the group consisting of but no limited to vitamin A (retinol), vitamin C (ascorbic acid) and vitamin E (tocotrienol or tocopherol), coenzyme Q10 (idebenone), catalase, thioredoxins, thioredoxin reductase 1, peroxiredoxin 2, peroxiredoxin 4, peroxiredoxin 6, Cu/Zn superoxide dismutase, Mn superoxide dismutase, glutathione, L-carnosine, lycopene, lutein, alpha-carotene, beta-carotene, zeaxanthin, astaxanthin, resveratrol, kaempferol, myricetin, isorhamnetin, proanthocyanidins, quercetin, rutin, luteolin, apigenin, tangeritin, hesperetin, naringenin, eriodictyol, catechin, gallocatechin, epicatechin, epigallocatechin, theaflavins, thearubigin, genistein, daidzein, glycitein; rosmarinic acid, cinnamic acid, chlorogenic acid, chicoric, gallotannins, ellagitannins, curcumin, acetylcysteine, lipoic acid (α-lipoic acid) and methionine.

4. A method for protecting neurons from degeneration or death, comprising:

administering to a subject having a condition in which neurons would be destined to degenerate or die, a composition comprising a neural regeneration peptide (NRP) and an antioxidant (AO).

5. The method of claim 4, wherein said NRP is selected from the group consisting of peptides having the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ

ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29.

6. The method of claim 4, wherein said AO is selected from the group consisting of but not limited to vitamin A (retinol), vitamin C (ascorbic acid) and vitamin E (tocotrienol or tocopherol), coenzyme Q10 (idebenone), catalase, thioredoxins, thioredoxin reductase 1, peroxiredoxin 2, peroxiredoxin 4, peroxiredoxin 6, Cu/Zn superoxide dismutase, Mn superoxide dismutase, glutathione, L-carnosine, lycopene, lutein, alpha-carotene, beta-carotene, zeaxanthin, astaxanthin, resveratrol, kaempferol, myricetin, isorhamnetin, proanthocyanidins, quercetin, rutin, luteolin, apigenin, tangeritin, hesperetin, naringenin, eriodictyol, catechin, gallocatechin, epicatechin, epigallocatechin, theaflavins, thearubigin, genistein, daidzein, glycitein; rosmarinic acid, cinnamic acid, chlorogenic acid, chicoric, gallotannins, ellagitannins, curcumin, acetylcysteine, lipoic acid (α-lipoic acid) and methionine.

7. The method of any of claims 4-6, wherein said disease or condition is selected from the group consisting of bacterial, viral, spirochetal or parasitic infections of the central nervous system, pyrogenic infections, acute bacterial meningitis, leptomeningitis, stroke, ischemic stroke, atherosclerotic thrombosis, lacunes, embolism, hypertensive haemorrhage, ruptured aneurysms, vascular malformations, transient ischemic attacks, intracranial haemorrhage, spontaneous subarachnoid haemorrhage, hypertensive encephalopathy, inflammatory diseases of the brain arteries, decreased perfusion caused by, for example, cardiac insufficiency craniocerebral trauma, basal skull fractures, cranial nerve injuries, carotid-cavernous fistula, pneumocephalus, aerocele andrhinorrhea, cerebral contusion, traumatic intracerebral haemorrhage, acute brain swelling, neuromyelitis optica, acute disseminated encephalomyelitis, acute and subacute necrotizing haemorrhagic encephalitis, diffuse cerebral sclerosis of Schilder, multiple sclerosis in conjunction with peripheral neuropathy, dementia, diffuse cerebral atrophy, diffuse cortical atrophy of the non-Alzheimer type, Lewy body dementia, Pick's disease, fronto-temporal dementia, thalamic degeneration, non-Huntingtonian types of Chorea and dementia, cortico-spinal degeneration (Jakob), the dementia-Parkinson-amyotrophic lateral sclerosis complex, guamanina, peripheral neuropathy, autoimmune disorders, nutritional deficiencies, alcoholism, vascular and metabolic disorders, diabetic neuropathy, confusion, stupor, coma-ischemia-hypoxia, hypoglycaemia, hyperglycemia, hypercapnia, hepatic failure and Reye syndrome, metabolic diseases presenting as a progressive extrapyramidal syndrome, ataxia, hyperthermia, celiac-sprue disease, psychosis, Cushing disease and steroi-nduced encephalopathy, thyroid psychosis and hypothyroidism and pancreatic encephalopathy, nervous system due to nutritional deficiency, alcohol, alcoholism, opiates and synthetic analgesics, sedative hypnotic drugs, stimulants, psychoactive drugs, bacterial toxins, plant poisons, venomous bites and stings, heavy metals, industrial toxins, anti-neoplastic and immunosuppressive agents, thalidomide, aminoglycoside

antibiotics (ototoxicity) and penicillin derivatives, cardioprotective agents, beta-blockers, digitalis derivatives and amiodarone.

8. A method for protecting neurons in cell culture, comprising:

preparing and culturing neuronal cells in the presence of a neural regeneration peptide (NRP) and/or an antioxidant (AO).

9. The method of claim 8, wherein said NRP is selected from the group consisting of peptides having the sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29.

10. The method of claim 8, wherein said antioxidant is selected from the group consisting of but no limited to vitamin A (retinol), vitamin C (ascorbic acid) and vitamin E (tocotrienol or tocopherol), coenzyme Q10 (idebenone), catalase, thioredoxins, thioredoxin reductase 1, peroxiredoxin 2, peroxiredoxin 4, peroxiredoxin 6, Cu/Zn superoxide dismutase, Mn superoxide dismutase, glutathione, L-carnosine, lycopene, lutein, alpha-carotene, beta-carotene, zeaxanthin, astaxanthin, resveratrol, kaempferol, myricetin, isorhamnetin, proanthocyanidins, quercetin, rutin, luteolin, apigenin, tangeritin, hesperetin, naringenin, eriodictyol, catechin, gallocatechin, epicatechin, epigallocatechin, theaflavins, thearubigin, genistein, daidzein, glycitein; rosmarinic acid, cinnamic acid, chlorogenic acid, chicoric, gallotannins, ellagitannins, curcumin, acetylcysteine, lipoic acid (α-lipoic acid) and methionine.

11. A method for protecting neurons in cell culture, comprising:

dissociating neuronal cells in a cell culture medium containing an antioxidant; and

culturing said cells in a cell culture medium containing an NRP.

12. A kit for culturing neuronal cells, comprising:

at least one NRP;

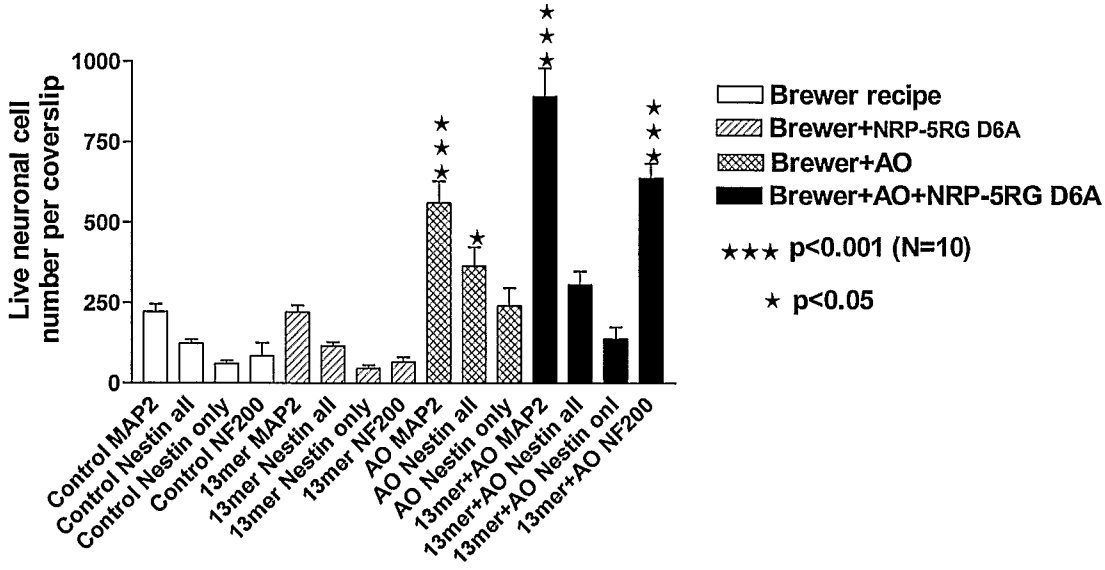
at least one AO;

a mixing vial;

a solvent for dissolving said NRP and said AO;

and instructions for use.

13. The kit of claim 12, further comprising a cell culture medium.



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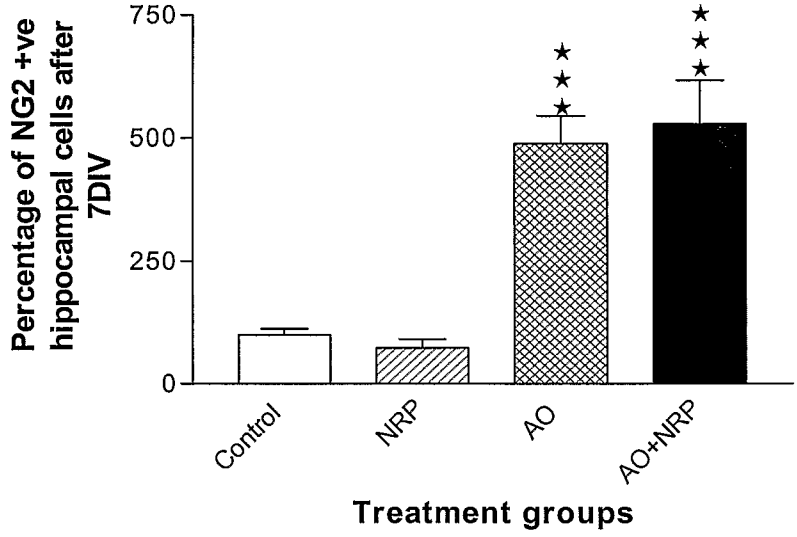

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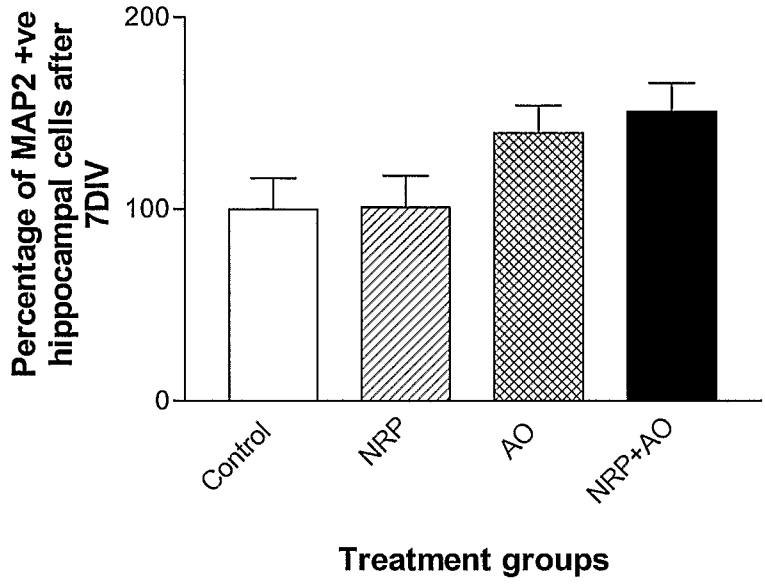

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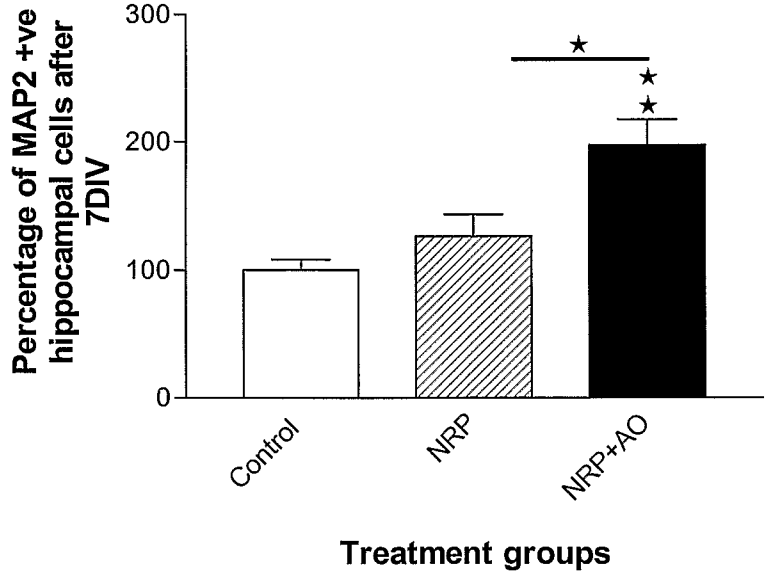

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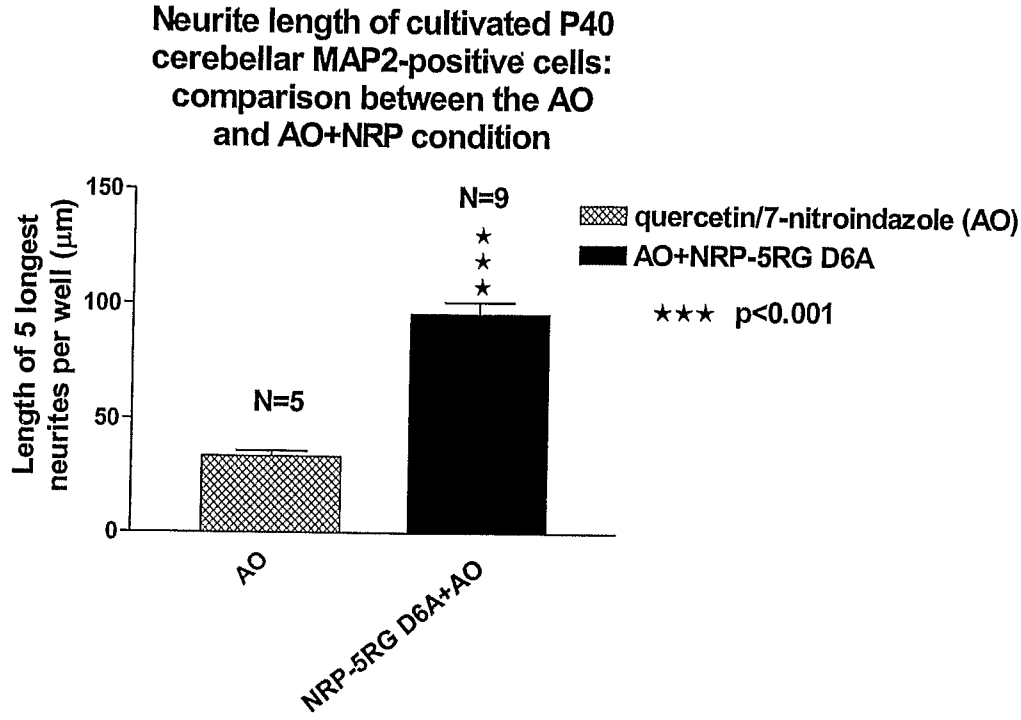

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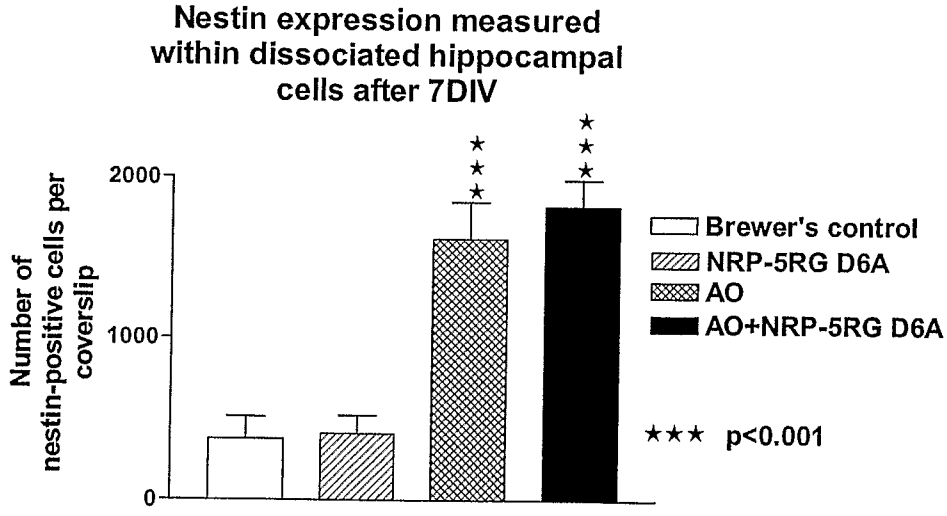

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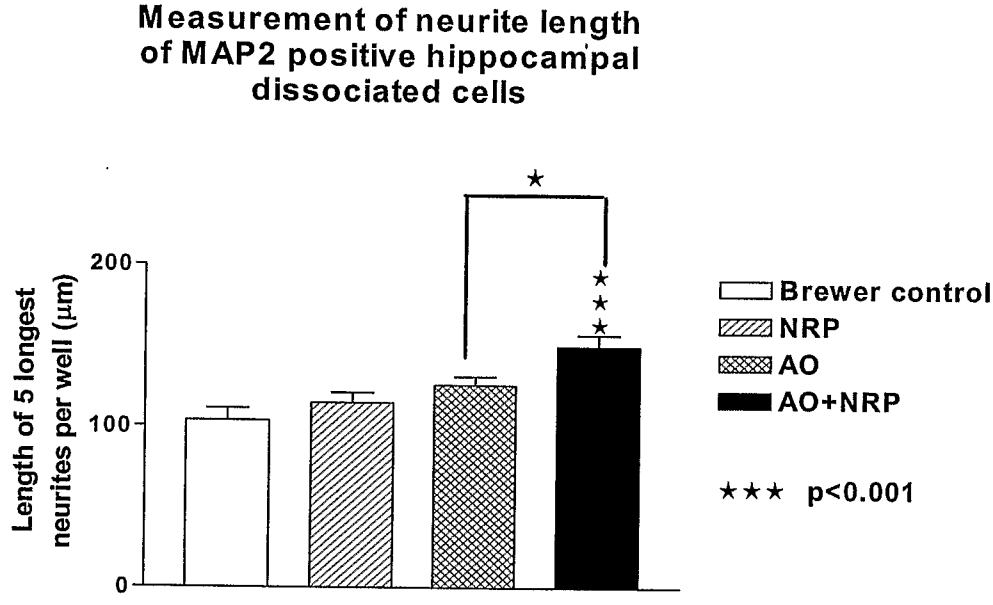

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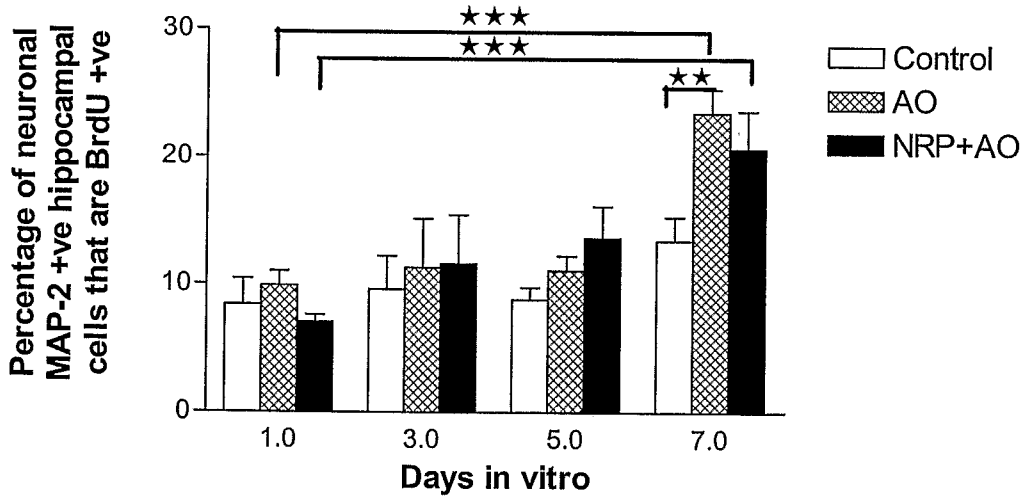

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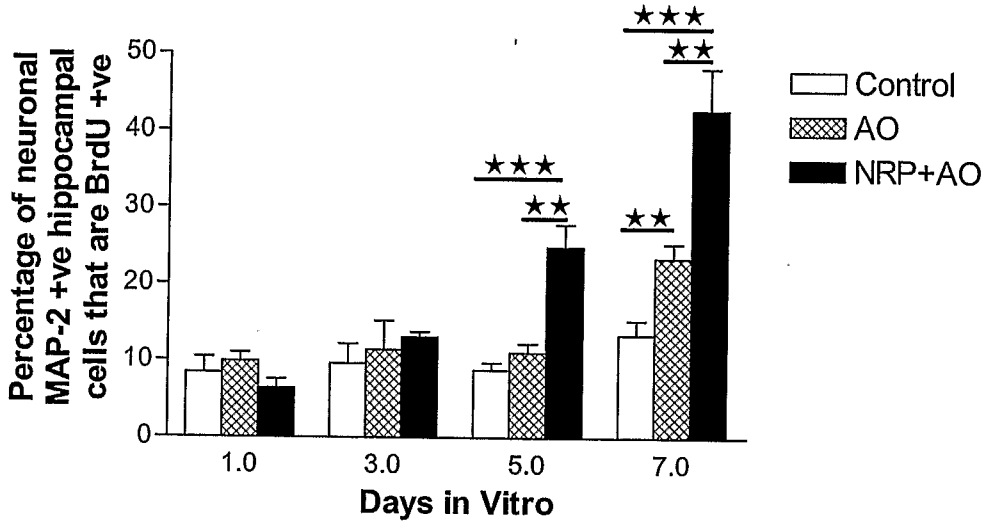

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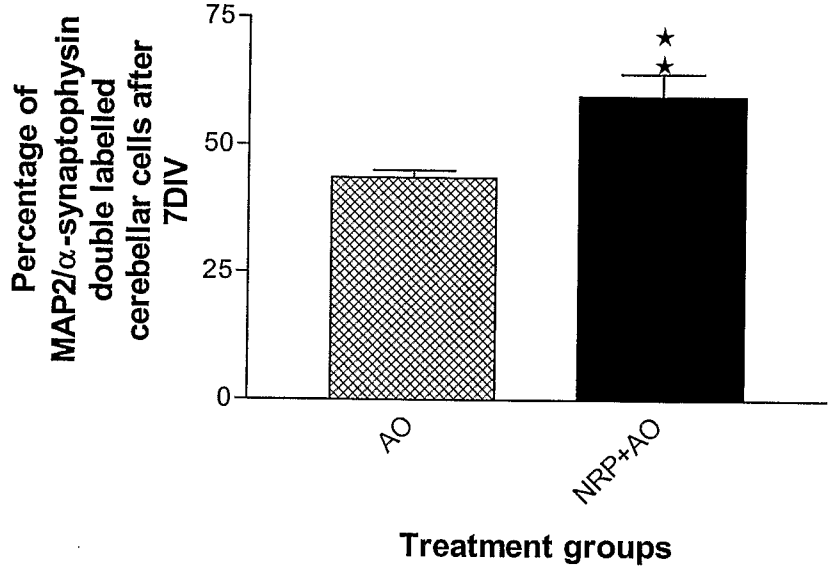

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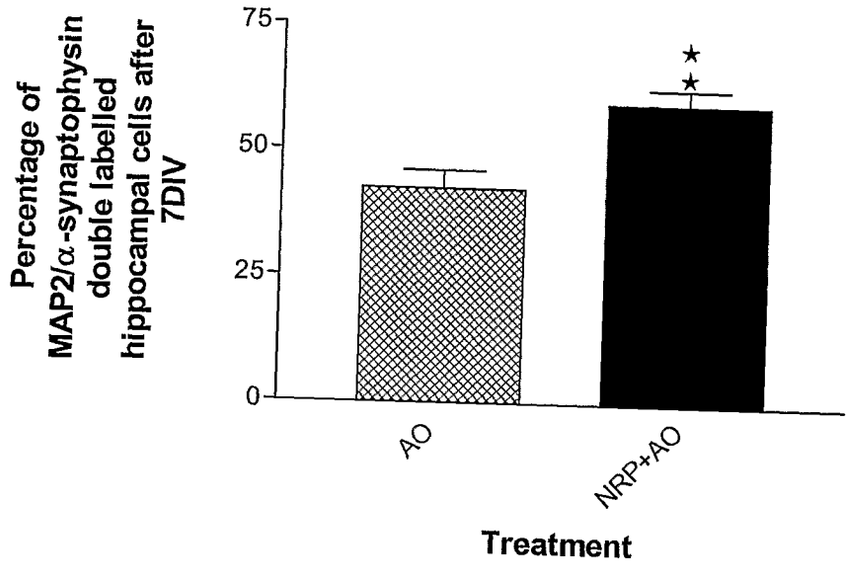
Percentage of MAP2/α-synaptophysin double labelled hippocampal cells after 7DIV
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AO
NRP+AO
Treatment

Figure 6B

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Neuren Pharmaceuticals Inc.

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