The ALS MicroNeurotrophin Research Consortium

About MicroNeurotrophins
UNLOCKING THE PROMISE OF NEUROTROPHINS
Most researchers now agree that ALS and other neurodegenerative diseases including Multiple Sclerosis, Parkinson’s and Alzheimer’s are remarkably complex, almost certainly involving a combination of different biological, genetic and external triggers. Yet, time and again, drug development for these multi-faceted diseases has focused almost exclusively on medications that only target one factor likely causing the disease. It’s not surprising that these single-target drugs have uniformly failed in previous clinical trials.

With this in mind, esteemed pharmacologist Dr. Achilleas Gravanis and his team of scientists from Bionature, a spin-off of the University of Crete in Greece, began searching for a way to effectively address the multiple triggers contributing to the onset and progression of neurodegenerative diseases.

Their investigation led them to study neurotrophins, proteins naturally secreted in the brain that transmit signals between motor neurons by binding to and activating two different types of receptors, TrkA and p75, found on the surface of nerve cells.

Twenty years of research have shown that neurotrophins help motor neurons develop, grow, function, signal and even repair themselves. Neurotrophins also appear to protect motor neurons from stress and death. By contrast, ALS causes motor neurons to degenerate and die and ALS patients experience a profound loss of neurotrophins as the disease progresses. However, numerous clinical trials have been unable to prove the efficacy of neurotrophins to treat ALS and other neurodegenerative diseases.

TWO STARTLING DISCOVERIES
To understand why, Dr. Gravanis and his colleagues carefully examined past studies on neurotrophins, and two astonishing observations became apparent to them. Neurotrophins, because of their polypeptide structure, are too large to pass through the blood-brain barrier, a filtering mechanism that controls which substances enter and exit the brain.

Secondly, many research studies administered the trial drugs in pill or liquid form, but neurotrophins cannot be given orally because they are degraded in the stomach or intestine. Simply put, it was impossible for the neurotrophins to demonstrate efficacy in past clinical trials because the molecules were never able to reach their intended neuronal targets.

MICRONEUROTROPHINS: AN UNPRECEDENTED BREAKTHROUGH
Armed with this profound new insight, Dr. Gravanis and his team developed therapeutic molecules, called MicroNeurotrophins, which mimic the beneficial properties of neurotrophins, but are also small enough to pass through the blood brain barrier and reach the motor neurons.

To avoid the oral delivery problems associated with past drug trials, a pill coating has been developed by chemists under Dr. Gravanis’ supervision that can withstand the churning actions
and acidic environment found in the stomach, enter the bloodstream, cross the blood brain barrier and target the appropriate neuronal receptors.

**MICRONEUROTROPHINS AND THE POTENTIAL FOR TREATING ALS**

After rigorous laboratory testing, MicroNeurotrophins have proven highly effective and non-toxic in experimental animal models of three major human neurodegenerative diseases: Multiple Sclerosis, Parkinson’s disease and Alzheimer’s disease. This initial research clearly indicates that MicroNeurotrophins hold great promise for treating ALS because they target the multiple triggers of motor neuron degeneration that are characteristic of the disease.

**How do MicroNeurotrophins address the multiple triggers of ALS?**

**Anti-Apoptotic**
Apoptosis, programmed cell death, is responsible for motor neuron degeneration in ALS. In both *in vitro* (cell culture) and *in vivo* (animal model) studies, MicroNeurotrophins have been shown to combat apoptosis by protecting and strengthening neurons.

**Anti-Inflammatory and Autoimmune Inhibitor**
Inflammation is the immune system’s defense against bacteria, viruses, and substances that are foreign and harmful. However, there is increasing evidence that inflammation accompanies the death of motor neurons in ALS. MicroNeurotrophins’ powerful anti-inflammatory properties may help reduce, prevent, or even reverse this destructive autoimmune response.

**Glutamate Modulator**
ALS patients are unable to properly regulate glutamate, a chemical that carries messages throughout the brain and spinal cord. Too much glutamate in the brain can be toxic and cause motor neurons to die. MicroNeurotrophins serve as an indirect protectant against excess glutamate through their ability to promote cell survival processes, increase neuronal signaling strength and as an autoimmune modulator.

**Neuronal Signaling**
In ALS, loss of neurotrophic signaling causes neurons to die. MicroNeurotrophins prevent cell death by increasing neuroprotective signals that help neurons survive, differentiate, and grow.
THE CRITICAL NEXT STEP: TESTING MICRONEUROTROPHINS FOR ALS

Scientists require collaborators with diverse research expertise, skills, and specializations to advance their discoveries. Yet, too often researchers work in complete isolation, in competition with one another, often wasting novel discoveries, time and resources without producing results.

That’s why ALS Worldwide, a nonprofit organization working to accelerate innovative research and support patients and their loved ones in more than 85 countries, introduced some of the world’s best and brightest ALS neuroscientists to Dr. Gravanis and his team’s findings to foster collaboration among them.

After meticulously analyzing the initial research, leaders from several prestigious medical research institutions enthusiastically agreed to work together as part of an international research consortium that will test the drug in laboratory models of ALS.

In addition to Bionature and ALS Worldwide, the Consortium includes renowned research teams from Harvard University, Virginia Commonwealth University, Penn State Hershey, The University of Sheffield’s Institute for Translational Neuroscience (SITraN) and University of Crete.

Consortium members have generously agreed to waive their institution’s facilities and administrative fees to show their shared commitment to the research effort and to reduce the total project budget. By doing so, the partnering universities have collectively contributed roughly $1.3 million to the overall fundraising campaign.

The traditional approach to drug development is often very slow and inefficient because research is conducted in a sequential fashion. To accelerate the process, each member of the Consortium will simultaneously study the efficacy of MicroNeurotrophins in treating ALS. At the same time, the FDA required independent safety testing will be conducted by Covance, one of the world’s largest and most comprehensive drug development services companies. As a result, we are confident the MicroNeurotrophin-based medication can be brought to an Investigational New Drug (IND) Trial in 12-18 months.
The ALS MicroNeurotrophin Research Consortium

About the Consortium
ACHILLEAS GRAVANIS, PH.D.

Dr. Achilleas Gravanis, PhD, Professor of Pharmacology at University of Crete’s School of Medicine will continue his work as principal investigator of MicroNeurotrophins, leading his team of scientists while providing technical assistance to the other members of the consortium.

Dr. Gravanis has served as Chair of the Department of Basic Sciences at University of Crete’s Medical School. He is also a collaborating Researcher at the Institute Molecular Biology-Biotechnology, Foundation of Research & Technology-Hellas. He obtained his Diploma in Pharmacy from the University of Athens in 1980, his PhD in Pharmacology in 1983 from the University Pierre Marie Curie, Paris 6. Dr. Gravanis has worked as a post-doctoral fellow at the School of Medicine at Mount Sinai in New York from 1983 to 1986.

He served as a member of the European Commission’s Project Review Board of BIOMED 2, the 5-year Assessment Committee of BIOMED 1, the Monitoring Committee of BIOMED 2, and Co-Chair of the BIOMED 2 Assessment Committee; and participated as Chairman and member in numerous research committees of the European Union, including the Programme Committee of Framework Programmes FP6 and FP7.

Dr. Gravanis has also served as vice-president of the Hellenic Pharmacological Society, the Hellenic Biochemical Society, and as a member of the steering committee of the European Pharmacology Network. He is a member of the Board of the Hellenic Quality Assurance Agency for Higher Education and serves as the Chairman of the Biosciences Committee of the Hellenic Research and Technology Council. He is a member of the Hellenic Council of Public Health.

Dr. Gravanis has published 120 papers in peer-reviewed journals, cited in PubMed. He is the co-founder of Bionature, established in 2003, as a spin-off of the University of Crete.
Animal testing of MicroNeurotrophins using an ALS mouse model will be conducted under the direction of Dr. Ghazaleh Sadri-Vakili, an Assistant Professor of Neurology at Harvard Medical School and the Director of the NeuroEpigenetics Laboratory at Massachusetts General Hospital.

She graduated from the University of California, Irvine in 1997 with a BS in Biological Science and then received her MS and PhD in Pharmacology and Biomedical Neuroscience from Boston University School of Medicine in Boston, Massachusetts.

Dr. Sadri-Vakili completed her post-doctoral training in the department of Neurology at Massachusetts General Hospital/Harvard Medical School. Her laboratory is focused on identifying the molecular mechanisms underlying alterations in gene expression that contribute to neurodegeneration and addiction. Her previous work on Huntington’s disease (HD) identified enzymes involved in histone acetylation as a potential novel therapeutic target.

Recently, she has started a new collaboration with Dr. Achilleas Gravanis from the University of Crete and colleagues at Harvard Medical School and Massachusetts General Hospital to screen an exciting and novel group of compounds, called MicroNeurotrophins, for the treatment of Amyotrophic Lateral Sclerosis (ALS).

Dr. Sadri-Vakili has authored multiple publications in the fields of neurodegeneration, epigenetics, and drug addiction. She is the co-founder of the Professional Women’s Nexus on LinkedIn, and a member of several professional societies, including the Society for Neuroscience, New York Academy of Sciences, American Association for the Advancement of Science, and the International Society for Neurochemistry.
Dr. James Bennett Jr., Bemiss Professor of Neurology, Psychiatry and Physiology/Biophysics at Virginia Commonwealth University’s School of Medicine will lead the testing of MicroNeurotrophins in stem cells derived from living ALS patients, genetically modified and used to generate motor neurons in a dish.

James P. Bennett, Jr. is a native of St. Petersburg, FL. He received a B.S. in Chemistry with Honors from the University of Florida (1970), and then attended Johns Hopkins University School of Medicine for his M.D. (1974) and Ph.D. (Pharmacology, 1977) degrees. He worked in the laboratory of Solomon Snyder, M.D. for six years while in medical and graduate schools and trained in neuropharmacology.

He then trained clinically for two years in Internal Medicine (1978-80), followed by Neurology residency at the University of Virginia (1980-83) where he was Chief Resident (1982-83). He then became a faculty member in the departments of Neurology, Psychiatry and Pharmacology and held the Ebbert Chair in Medical Science.

In 2009 he moved to Virginia Commonwealth University, where he is the Bemiss Professor, was Chair of Neurology (2009-2013) and is founding Director of the Parkinson’s Disease and Movement Disorder Center.

Dr. Bennett has held multiple NIH grants and directed the NIH-funded Udall Parkinson’s Center at UVA. He investigates mitochondrial dysfunction in adult neurodegenerative diseases. He holds multiple patents related to experimental therapies of degenerative disorders.
PROFESSOR DAME PAMELA SHAW, DBE MBBS MD FRCP FAAN FANA FMedSci

Professor Dame Pamela Shaw, Professor of Neurology at the University of Sheffield and Director of the Sheffield Institute for Translational Neurosciences (SITraN), one of the world leading centers for ALS research, will oversee testing of MicroNeurotrophins in both ALS mouse and zebrafish animal models.

Professor Shaw graduated in Medicine with 1st Class Honors from the University of Newcastle in 1979. She undertook her MRCP and Specialist Training in Neurology training in Newcastle. In 1988, she was awarded an MD with commendation for her work on the neurological complications of coronary bypass surgery.

After an intermediate fellowship award from the Wellcome Trust, she was awarded a Wellcome Senior Fellowship in Clinical Science which she held from 1991 to 2001, underpinning her program of work investigating molecular mechanisms of motor neuron injury and new therapeutic approaches in motor neuron disease.

In 1997 she was appointed Professor of Neurological Medicine at the University of Newcastle and in 2000 was appointed as Professor of Neurology at the University of Sheffield. As an undergraduate in Newcastle she was awarded the Stephen Scott; Gibb; Mary Gordon; Mona McNaughton and Phillipson Prizes/Scholarships.

Recent post-graduate awards include: Association of British Neurologists Sir Charles Symonds award (1991; 1996; 2001); American Academy of Neurology Sheila Essey award 2001; UK Royal College of Physicians Jean Hunter award 2006; the International ALS/MND Forbes Norris award 2007; Fellowship of the Academy of Medical Sciences 2007. In 2013 she was awarded the Dame Commander of the Order of the British Empire (DBE) for services to Neuroscience in the HM the Queen’s New Year’s Honors.
GLENN S. GERHARD, M.D.

Glenn S. Gerhard, M.D. is a Professor in the Departments of Biochemistry and Molecular Biology and Pathology and Laboratory Medicine at the Penn State College of Medicine and Co-Director of the Institute for Personalized Medicine.

Dr. Gerhard earned his B.S. and medical degrees from Penn State with post-doctoral research training in the cell biology of aging at the Wistar Institute and in Human Genetics at the University of Pennsylvania. He completed residency training in Pathology at Dartmouth.

He previously held faculty positions at the Penn State Hershey Medical Center, Dartmouth Medical School, and the Geisinger Clinic. He is currently site Principal Investigator for two NIH funded Center Grants and Principal Investigator on two NIH R01 grants.

His research interests are in personalized medicine and using cutting edge genomics technologies to define diseases at the molecular level in order to discover new drug targets and to create cellular and animal models that can be used for drug development.

Dr. Gerhard has been a pioneer in the use of zebrafish as an animal model to study aging and related disorders. His laboratory published the first papers using zebrafish as a model for aging, has a long track record in oxidative stress research, and has been developing zebrafish models for Amyotrophic Lateral Sclerosis using next generation DNA sequencing to identify new mutations from an ongoing research cohort of ALS patients.

Dr. Gerhard has authored or coauthored more than 80 papers in peer-reviewed journals, cited in PubMed.
ALS Worldwide is a nonprofit organization that works to advance and accelerate groundbreaking research and provides guidance, compassion and hope to thousands of ALS patients and their loved ones in more than 80 countries around the world. ALS Worldwide is the official fundraising and gift-receiving organization for the ALS MicroNeurotrophin Research Consortium.

Stephen and Barbara Byer
Co-Executive Directors

After Ben Byer received an ALS diagnosis in 2002, his parents, Stephen and Barbara, sought to help their son by consulting with the world’s top neuroscientists and neurologists, along with other ALS patients and their families, to learn about and advance promising scientific research, drug trials, medical devices and symptom relief.

In addition to sharing each new discovery with Ben, Stephen and Barbara Byer created a global circle of support via internet chat rooms, email, Skype and telephone to pass on what they learned to others struggling with the devastating effects of the disease.

After Ben’s death in 2008 at the age of 37, Stephen and Barbara Byer formalized their efforts by launching ALS Worldwide. To date, they have helped more than 11,000 patients in over 80 countries.

Barry M. Wein, MSW
Director of Philanthropy & Communications

Barry M. Wein has more than 15 years of fundraising and communications experience in the nonprofit sector. Prior to joining ALS Worldwide, Mr. Wein directed fundraising communications for the Wisconsin Historical Society, including their successful $77 million capital campaign.

He earned his Bachelor of Arts Degree from the University of Wisconsin-Madison and his Masters in Social Work from the University of Washington.
Bionature E.A. Ltd, inventor of MicroNeurotrophins, was established in 2003 as a spin-off of the University of Crete in Greece. The Company's founders are Professors Achilleas Gravanis and Elias Castanas, both of the University of Crete Medical School. Bionature's stakeholders also include the University of Crete and the private investment group Emergo. The Faculty of Medicine of the University of Crete is presently ranked among the best medical schools in Europe.

Bionature focuses on the development of novel, proprietary small molecules for the treatment of neuroinflammatory and neurodegenerative diseases, such as Multiple Sclerosis, Retinopathy and Amyotrophic Lateral Sclerosis. Bionature owns a robust portfolio and holds broad international intellectual property coverage for MicroNeurotrophins that includes structure, composition of matter, function and method of use claims.

Bionature has completed pre-clinical proof of efficacy studies in various animal models and has established the safety profile of MicroNeurotrophins. Bionature is currently collaborating with a consortium, led by ALS Worldwide, which includes renowned research teams from Harvard University, Virginia Commonwealth University, The University of Sheffield’s Institute for Translational Neuroscience (SITraN) and Penn State Hershey College of Medicine for pre-clinical development of MicroNeurotrophins that will lead to Phase I/II studies. Bionature's MicroNeurotrophins hold significant promise to become first in class therapeutic agents of tremendous clinical importance and value.

Constantinos Neophytou, Ph.D., CIMA®, Cert. Director (IoD)

Dr. Constantinos Neophytou is the managing director of Bionature E.A. Ltd. He has worked with several emerging companies in the biomedical sector and in the information and communication technology sectors. Prior to joining Bionature, he worked as the General Manager of a financial services group in Nicosia.

Dr. Neophytou served as the Vice President for International Operations of nutraceuticals company, Yasoo Health Inc., where he was responsible for international business development and R&D. He holds a B.A. and an M.A. in Natural Sciences from Cambridge University and a Ph.D. in Neurobiology from University College London, both on full merit-based scholarships. Dr. Neophytou served as a post-doctoral fellow at Harvard Medical School as a Welcome Trust Prize Scholar. He regularly serves as an independent expert evaluator and reviewer for the European Commission on life sciences projects.
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
We hypothesize that the MicroNeurotrophin drug compounds invented by Dr. Achilleas Gravanis are potential therapeutic candidates for the treatment of Amyotrophic Lateral Sclerosis (ALS). We will determine this by conducting the following studies using the SOD1 Mouse Mutant Model of ALS and tissue samples donated by ALS patients.

Proposed Study 1
- Determine whether enhancement of neurotrophic signaling can improve the phenotype in the SOD1 ALS mouse model
- Assess the molecular and biochemical changes in pre- and post-symptomatic SOD1 mice
  - Determine whether MicroNeurotrophins treatment of SOD1 mice improves the survival and phenotypes associated with ALS
    - Assess the expression of key proteins
    - Evaluate effects on survival
    - Determine effects on phenotype (motor and behavior)
    - Measure neuroprotection

Proposed Study 2
- Determine whether neurotrophic signaling is altered in brain, spinal cord, and cerebrospinal fluid of ALS patients
- Perform longitudinal assessment of key proteins in samples from ALS patients
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
Our project is focused on determining the responses of human motor neurons exposed to either an active, or inactive analog of MicroNeurotrophin drug compounds designed by Dr. Achilleas Gravanis. Through blinded testing, we will create the human motor neurons in culture with special stem cell technologies and measure their responses to the MicroNeurotrophin drugs with a state-of-the-art technology called next-generation RNA sequencing.

Proposed Study 1
- Determine whether MicroNeurotrophins treatment will enhance the survival of iPS cell derived motor neurons
  - Create iPSC-derived neurons from sporadic ALS and CTL subjects, starting with peripheral blood mononuclear cells. Characterize their neuronal phenotype using both molecular and electrophysiological properties
  - Expose ALS/CTL iPSC-derived neurons to oxidative, proteosomal and excitotoxic stresses. Characterize survival using molecular and morphological approaches
  - Measure neuroprotection by MicroNeurotrophins of ALS/CTL iPSC-derived neurons exposed to oxidative, proteosomal and excitotoxic stresses
  - Share proteins from ALS/CTL iPSC-derived neurons exposed to MicroNeurotrophins and oxidative, proteosomal and excitotoxic stresses with other Consortium investigators
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
We plan to test the efficacy of the MicroNeurotrophin drug compounds designed by Dr. Achilles Gravanis in the SOD1 Zebrafish Mutant Model of ALS, the SOD1 Mouse Mutant Model of ALS and in cellular models to test their utility in treating ALS.

Proposed Study 1
- Perform screening of MicroNeurotrophins and use efficacy of such compounds to inform SAR of Neurosteroids through multiple interactions of synthesis and testing
- Perform efficacy testing of selected MicroNeurotrophins in preventing disease spread to motor neurons and damage to neuromuscular junction using our adult motor neuron stress model
- Perform dose-range and combination studies to identify most suitable compounds for pre-clinical screening in rodent models

Proposed Study 2
- Evaluate dose range and efficacy testing of lead compounds using the SOD1 G93A mouse model of ALS
- Perform histopathological and MRI analysis of denervation and muscle atrophy and efficacy of lead compounds
- Evaluate the effects of lead compounds on the Nrf2-ARE pathway

Proposed Study 3
- Test the effects of lead compounds on NMDA, AMPA, and GABA-A receptors by electrophysiological evaluation of cell models, including primary murine motor neuron cultures from normal and SOD1G93A mice
- Test the capacity of the lead compounds to ameliorate the toxic properties of astrocytes expressing mutant SOD1, in murine astrocyte-motor neuron co-cultures.
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
Dehydroepiandrosterone (DHEA) appears to interact with the nerve growth factor (NGF) receptors TrkA and p75NTR to induce neuroprotective effects. DHEA is an intermediate in the biosynthesis of estrogens and androgens that affects the endocrine system. Dr. Gravanis has synthesized 17-spiro analogs of DHEA that lack estrogenic or androgenic properties but can still bind to and activate NGF receptors, thus exerting potent neuroprotective effects without hormonal side effects. These synthetic DHEA derivatives or MicroNeurotrophins have potential applications in the treatment of neurodegenerative diseases.

Zebrafish offer numerous advantages for in vivo ALS drug screening including a prominent and easily accessible nervous system with native neuronal and non-neuronal cells, small size, aqueous environment, simultaneous assessment of toxicity and teratogenicity, and economical husbandry.

Our goal is to test whether the MicroNeurotrohin drug compounds designed by Dr. Achilleas Gravanis exert neuroprotective effects in the SOD1 Zebrafish Mutant Model of ALS.

Proposed Study 1
- Perform screening of 20 DHEA analogs on zebrafish models of 10 ALS mutations using dose-response and structure-activity analysis

Proposed Study 2
- Characterize effects of selected DHEA analogs in preventing motor neuron death through imaging of spinal cord and motor neurons in situ

Proposed Study 3
- Determine the effects of selected DHEA analogs on apoptosis, reactive oxygen species (ROS), and mitochondrial morphology through the use of in vivo fluorescent probes in living zebrafish embryos
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
Our team has recently shown that MicroNeurotrophins effectively protect sympathetic and sensory neurons from apoptosis, increasing the levels of neuroprotective and anti-apoptotic Bcl-2/Bcl-xL proteins, while decreasing the activation of pro-death bad protein. Additionally, in the same neurons, MicroNeurotrophins strongly induce the activity of pro-survival Akt kinase.

We will use a mouse model to test the neuroprotective properties of MicroNeurotrophins and the efficacy of MicroNeurotrophins to specifically block apoptotic death of motor neurons, increasing pro-survival Bcl-2 and Akt proteins.

Proposed Study 1
- Test the efficacy of MicroNeurotrophins to protect mouse motor neurons in culture against various noxious challenges (oxidative stress, excitotoxic amino acids, serum and trophic factor deprivation)
- Test the efficacy of MicroNeurotrophins to increase pro-survival anti-apoptotic Bcl-2 and Akt proteins in mouse motor neurons in culture
Pre-clinical Evaluation of MicroNeurotrophins as a Potential Neuroprotective Therapy for Amyotrophic Lateral Sclerosis

SCIENTIFIC PROTOCOL

Background
With headquarters in Princeton, New Jersey, Covance is one of the world's largest and most comprehensive drug development services companies. Covance has helped pharmaceutical and biotech firms develop one-third of all prescription medicines in the market today. Covance will serve as the Consortium's contract research organization and will conduct the following FDA required IND-enabling safety studies.

Proposed Study 1: Design, Synthesis and Formulation
- Synthesis of MicroNeurotrophins for in vitro and in vivo studies. Synthesis of further molecules for lead generation and lead optimization
- Test raw materials, APIs, and excipients used in formulation as well as stability studies and container/material compatibility to evaluate the impact of material compositions on the formulation

Proposed Study 2: Bioanalysis
- Provide an accurate quantitative measure of the active compound in biological samples for the purpose of toxicokinetics, pharmacokinetics, bioequivalence and exposure–response

Proposed Study 3: Dose–Ranging Study
- Conduct testing of different doses of lead compounds against each other to establish which dose is most efficacious and/or safe

Proposed Study 4: Genetic and Molecular Toxicology
- Assess genetic damage caused by MicroNeurotrophins as well as the response to this damage via in vitro, in vivo and exploratory screening genetic toxicology assays designed to meet established scientific standards and evolving regulatory mandates

Proposed Study 5: Toxicology
- Test MicroNeurotrophins on animals to identify doses causing no adverse effect and doses causing major (life-threatening) toxicity when administered in one or more doses during a period not exceeding 24 hours

Proposed Study 6: Safety Pharmacology
- Test lead compounds to identify if they have any side effects on the major physiological systems of the body
THE SCIENCE BEHIND MICRONEUROTROPHINS
By Achilleas Gravanis, Ph.D.

Background
Neurotrophins hold a key role in brain development and maintenance in adulthood. During ageing and in neurodegenerative diseases they decline, leaving the neural system unprotected against various challenges. Neurotrophins offer one of the most compelling opportunities to significantly improve the treatment of serious age-related, neurological diseases such as Alzheimer's and Parkinson's, as well as Huntington's and Amyotrophic Lateral Sclerosis (Bartus et al, 2013). The therapeutic potential of neurotrophins to alleviate the symptoms and slow or even halt disease progression in neurodegenerative diseases, is widely recognized (Apfel et al., 2000, Eriksdotter Jönhagen et al., 1998, Mufson et al., 1999 and Seiger et al., 1993). A major therapeutic advantage of neurotrophic factors is that they tangle both the symptoms of a disease (improving clinical status) as well as its pathogenesis (delaying disease progression) without any prerequisite, deep insight into the etiology or specific pathogenic variables driving the disease process.

Neurotrophins are endogenous proteins that have consistently demonstrated that under conditions of neurodegeneration they are able to activate neuronal repair genes, inducing morphological and functional restoration of the degenerating neurons, significantly slowing further neurodegeneration and protecting against cell death (Hefti et al., 1989).

Neurotrophins appear to provide functional and morphological benefit to their responsive neurons, no matter how the neurons are damaged or impaired. Investigators have consistently shown benefit of neurotrophic factors against cutting and/or crushing axons, exposure to neurotoxins, free radical donors, inflammatory agents and other cytotoxic agents, genetic mutations, protein processing defects, and the effects of age. Thus, neurotrophins seem to represent a final common therapeutic pathway to achieve neuronal restoration and protection, likely providing potential benefit independent of which of many possible pathogenic cascade(s) are truly responsible for the disease and thus free of theoretical insight, assumptions, or uncertainties surrounding those issues.

The possibility of reversing and slowing disease progression represents the “key target” for neurological diseases and neurotrophins arguably provide the best opportunity to accomplish this in the foreseeable future. Numerous clinical trials, testing many different neurotrophic factors in several different neurodegenerative diseases, have been conducted over the past 20 years (Apfel,
2002, Apfel et al., 1998, Apfel et al., 2000, Eriksdotter Jönhagen et al., 1998, Gill et al., 2003, Lang et al., 2006, Marks et al., 2008, Miller et al., 1996, Nutt et al., 2003, Penn et al., 1997, Slevin et al., 2005, Tuszynski et al., 2005 and Wellmer et al., 2001), with mixed results. The major reason of these inconsistent therapeutic effects is related to obstacles in effective delivery. Neurotrophins, because of their polypeptidic nature, cannot pass the blood-brain barrier. Many investigators have agreed that delivery problems produce poor distribution of the protein throughout the brain, limiting their therapeutic effectiveness (Morrison et al., 2007, Salvatore et al., 2006 and Sherer et al., 2006).

**Synthetic MicroNeurotrophins: Brain Bioavailable Neurotrophic Compounds**

Scientists of Bionature E.A. Ltd, established in 2003 as a spin-off of the University of Crete in Greece, have shown that neurosteroid dehydroepiandrosterone (DHEA) exerts part of its well described neuroprotective and neurogenic properties (Charalampopoulos et al, PNAS 2004), through binding and activation of neurotrophin receptors, TrkA and p75NTR (Lazaridis et al, 2011). However, DHEA is converted to androgen and estrogen and their metabolites. Long term use of this steroid may scramble the endogenous endocrine system, in addition to its capacity to bind and activate estrogen and androgen receptors, increasing the incidence of hormone-dependent tumors (breast, endometrium, prostate), particularly in genetically predisposed patients (Fourkala et al, 2012, Engdahl et al, 2013).

Bionature’s scientists “chemically dissected” the neuroprotective (3-Carbon) from the endocrine (17-Carbon) moieties of DHEA, synthesizing C17-derivatives of DHEA (BNNs) (Calogeropoulou et al, 2009), shown to interact with both NGF receptors. These derivatives are deprived of any estrogenic or androgenic effects, described for endogenous DHEA. They are small, highly lipophilic molecules, which can effectively pass the blood-brain barrier, mimicking the neuroprotective and neurogenic properties of neurotrophins.

The lead molecule BNN27 acts as a synthetic MicroNeurotrophin, interacting with NGF receptors, TrkA and p75NTR (IC50: 1.86 and 3.9 nM respectively). Pull-down assays and NMR studies confirmed the direct physical interaction of BNN27 with recombinant TrkA and p75NTR receptors. BNN27 dose-dependently induced TrkA tyrosine phosphorylation in the functionally relevant tyrosine residues (Tyr490, Tyr674/675 and Tyr785), affecting downstream pro-survival signaling pathways Akt and MAPKs in primary neurons. However, BNN27 was ineffective in triggering or maintaining neurite outgrowth in primary neurons. BNN27 was also effective in associating p75NTR receptor with its effector proteins RhoGDI, RIP2 and TRAF6, in primary Schwann cells. BNN27 rescues from apoptosis TrkA positive sensory neurons of Dorsal Root Ganglia (DRG) in ngf-/- mouse embryos at the age of E13.5 when naturally occurring programmed cell death begins.
BNN27 is ineffective in inducing cell death in p75NTR-expressing Schwann cells, in contrast to proNGF. It is of note that while BNN27 binds and dissociates from p75NTR receptor its effector protein RhoGDI, is ineffective in inducing axonal growth and elongation, a p75NTR-mediated phenomenon, controlled by the post-receptor inactivation of RhoA through its blocking by liberated RhoGDI. It appears that small molecules like BNNs may act as p75NTR blockers, rendering the receptor ineffective and desensitized to cell death stimuli. MicroNeurotrophins interacting with specific NT receptors may represent lead molecules to develop BBB-permeable, synthetic neurotrophin agonists with potential therapeutic applications in neurodegenerative diseases and brain trauma (Gravanis et al, 2012).

Effective in Experimental Models of Neurodegenerative Diseases
BNN27 effectively reverses the established phenotype of Experimental Allergic Encephalomyelitis (EAE) mice (an animal model for Multiple Sclerosis) through activation of transcription factor FoxP3, induction of anti-inflammatory Interleukin 10 and blocking of neurotoxic Interleukin 17. As shown in the left panel of the figure below, BNN27 strongly reduces spinal cord inflammation and gliosis and blocks the entry of neurotoxic Th17 lymphocytes in the brain (blue lagoons). It appears that BNN27 reverses early paralysis by inducing remyelination processes: BNN27 completely reverses the demyelinating effect of neurotoxin cuprizone (right panel of the figure below).
Experiments in a Parkinson’s mouse model (Weaver mice) have shown a significant protective effect of BNN50 in dopaminergic neurons of striatum, a well-characterized neuronal population for the expression of Ret and TrkB receptors. Collaborating scientists with Bionature investigate now the potential, dose-dependent activation of these receptors by BNN50 (and other analogs), or its ability to act as specific agonist/antagonist on TrkB, Ret and p75NTR receptors.

MicroNeurotrophins in Experimental Models of ALS and Motor Neuron Protection

Several observations indicate that neurotrophin dysfunction may represent key clues to or intermediates within the pathogenesis of ALS: 1) mutations in at least 3 neurotrophin genes cause motor neuron disease, 2) expression of several target-derived neurotrophins is reduced in ALS, 3) downstream signaling initiated by other target-derived neurotrophins is blocked in ALS and 4) centrally, intrathecally and/or virally delivered neurotrophins often exhibit superior BNN27 effectively decreased neuro-inflammation in mice injected with LPS as well as in mouse microglia cells in primary culture, inhibiting the production of pro-inflammatory Interleukin 6.
neuroprotective effects when compared to systemically administered neurotrophins (the route used for neurotrophin delivery in ALS clinical trials), due to poor blood-brain barrier penetrance (Could & Oppenheim, 2011).

Viral-mediated expression of a constitutively active form of pro-survival kinase Akt prevents motor neurons from neonatal axotomy (Namikawa et al, 2000). Transgenic overexpression of pro-survival, anti-apoptotic Bcl-2 or Bcl-XL (Dubois-Dauphin et al, 1994. Parsadanian et al, 1998) as well as deletion of pro-death, pro-apoptotic Bax (Deckwerth et al, 1996, Sun & Oppenheim, 2003) also inhibits this form of motor neuron death. The death of adult motor neurons in response to avulsion is also prevented by Bax deletion (Martin and Liu, 2002; but see Park et al., 2007) or viral-mediated Bcl-2 overexpression (Yamada et al., 2001).

GDNF is transported to motor neurons in both retrograde and anterograde directions, indicating a wide variety of potential cellular sources (Yan et al, 1995, Leitner et al, 1999, Rind et al, 2002). Treatment with exogenous GDNF prevents the apoptotic death of neonatal MNs in response to axotomy (Henderson et al, 1994, Perrelet et al, 2002) and blocks motor neurons from undergoing death through apoptosis (Oppenheim et al, 1995, Houenou et al, 1996). Deletion of GDNF in mice causes a 20–30% loss of spinal motor neurons (Oppenheim et al, 2000), while more than 90% of lumbar fusimotor axons are missing in adult mice lacking the GDNF receptors Ret or GFRα1 (Gould et al, 2008).

These findings suggest a more complicated neuromuscular scenario underlying ALS, in which neurotrophins and their receptors are dynamically expressed by different subcellular regions of motor neurons as well as by many other interacting cell types such as glia, muscle and endothelial cells. Neurotrophic factors delivered from target muscles are essential for motoneuronal survival, mainly during development and early postnatal maturation (Morcuende et al, 2013). Neurotrophic factors like NGF, and GDNF were revealed as the most effective neuroprotective agents in motor neuron axotomy models. Loss of survival-promoting neurotrophic signaling has been proposed as a contributing factor to motor neuron demise in ALS. In support of this view, CNTF knockout produces progressive motor neuron death in mice (Macu et al, 1993) and exacerbates neurodegeneration in the SOD1G93A model (Giess et al, 2002) Muscle-specific overexpression of glial cell-derived neurotrophic factor (GDNF) in the G93A mouse preserves NMJs and improves motor neuron survival (Li et al, 2007) suggesting therapeutic potential of neurotrophic factor supplementation.

Bionature’s invention, MicroNeurotrophins, offers a new pharmacological approach of neurotrophin supplementation, using blood-brain-barrier permeable, synthetic small molecules, with strong anti-apoptotic/neuroprotective, anti-neuroinflammatory and neurogenic activity. Bionature’s synthetic MicroNeurotrophins may overcome the brain bioavailability and distribution problems of endogenous polypeptidic neurotrophins. MicroNeurotrophins interact
with NGF receptors, inducing the expression of anti-apoptotic Bcl-2 proteins and the activation of pro-survival Akt kinases. Both neuronal signaling pathways hold a pivotal role in motor neuron survival.

MicroNeurotrophins were proven very effective in various experimental animal models of human neurodegenerative diseases, such as Experimental Allergic Encephalomyelitis (EAE) mice (Multiple Sclerosis), Weaver rats (Parkinson’s) and APP5xFAD transgenic mice (Alzheimer’s). MicroNeurotrophins were proven non-toxic in cell culture and small animal model tests. Bionature plans to test the efficacy of MicroNeurotrophins in vivo, using ALS animal models (mice and zebrafish), as well as their capacity in boosting iPS from ALS patients. In addition their ability to activate GDNF/Ret/GFRα1 receptors will be also tested. GDNF and its receptors are central players in motor neuron protection and function.

To achieve these goals Bionature and University of Crete are collaborating with ALS Worldwide as part of a consortium that includes renowned research teams from Harvard University/Massachusetts General Hospital, Virginia Commonwealth University, University of Sheffield’s Institute for Translational Neuroscience (SITraN) and Penn State Hershey College of Medicine.
The ALS MicroNeurotrophin Research Consortium

Project Budget
# ALS Microneurotrophin Research Consortium Project Budget

Funds granted by ALS Worldwide to each member of the ALS Microneurotrophin Research Consortium will only be allocated to the project’s direct costs. Participating research institutions have agreed to provide the required physical facilities and administrative services for this project.

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| ALS Worldwide                                        | $331,000.00 |             | $331,000.00 |

| Bionature                                            | $467,160.00 |             | $467,160.00 |

| Contingency                                          | $500,000.00 |             | $500,000.00 |

| Philanthropic Contributions to Date                  | ($600,000.00)|             | ($600,000.00)|

**Grand Total Required**: $3,900,000.00