EuroNeurotrophin Newsletter April 2021

Computer-Aided Drug Design in the EuroNeurotrophin Project

Neurotrophins are protein growth factors that are present in the central and peripheral nervous system. Neurotrophins and their receptors play a key role in the proper function of the nervous system, as they modulate several signaling pathways that regulate neuronal survival, axonal and dendritic network maintenance, as well as synaptic plasticity (Gomez et al. 2013). Neurotrophins could serve as a treatment in a number of neurological disorders, such as Alzheimer's disease and amyotrophic lateral sclerosis (Allen et Dawbarn, 2006), (Chao et al. 2006). However, their suboptimal pharmacological properties have hindered them from being used as therapeutics against neurodegenerative diseases. A solution to this problem is the development of small molecule neurotrophin mimetics that specifically target the neurotrophin receptors. This approach is pursued in the EuroNeurotrophin consortium by concerted efforts in computer-aided drug design, medicinal chemistry, structural and molecular biology. The contributions of *in silico* methodologies to the quest for neurotrophin mimetics will be described.

Computer-aided drug design has successfully contributed to the discovery of agents against brain diseases. For example, compounds that inhibit and reverse amyloid-β aggregation and neurotoxicity, as well as inhibitors against β-secretase (BACE-1) have been identified as lead molecules for the treatment of Alzheimer's disease by pharmacophore modeling, database screening and molecular docking methods (Zeng and Wu, 2016). In the EuroNeurotrophin consortium, computational work is performed for the discovery of neurotrophin mimetics. The PhD students working on the computational aspects of the project evaluate lead-molecule optimization efforts by medicinal chemists in the consortium and identify new chemical scaffolds with neurotrophin mimetic activity by virtual screening of compound libraries and isolated marine products provided by other partners in the consortium. They also predict or calculate the Absorption, Distribution, Metabolism, Excretion (ADME) properties of various small molecules tested within the project in order to identify potentially undesired properties of a compound that should be addressed in the compound design phase. Molecular dynamics simulations of the neurotrophin receptors and the compounds are employed to study the dynamical behavior of these systems. All of these calculations enable the prioritization of the most promising compounds, thus establishing a compound design process that is more efficient than serendipitous drug discovery efforts.
Some of the results of this work have been presented at the EFMC-ASMC'19 8th EFMC International Symposium on Advances in Synthetic and Medicinal Chemistry, the EFMC-YSMC'19 6th EFMC Young Medicinal Chemist Symposium, and the 65th Biophysical Society Annual Meeting 2021.

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In addition to research in computer-aided drug design, the EuroNeurotrophin members at Heidelberg Institute for Theoretical Studies (HITS) provided the PhD students in the consortium with training in computational techniques, an aspect that is important for both the well-rounded doctoral training of the students, and for the efficient collaboration between the computational and experimental groups in the consortium. Specifically, a workshop on computer-assisted drug design was held during the 1st EuroNeurotrophin Training Week in Athens, Greece in 2018, in the context of which the participants received training on molecular docking and molecular dynamics simulation techniques. Furthermore, a workshop on bioinformatic analysis of protein structures and the prediction of protein binding properties was held during the online 3rd EuroNeurotrophin Training Week in 2020. Finally, HITS has been a host institute for secondments of three other PhD students from the consortium, who had the opportunity to become familiar with *in silico* methodologies and do computational research related to their PhD project.

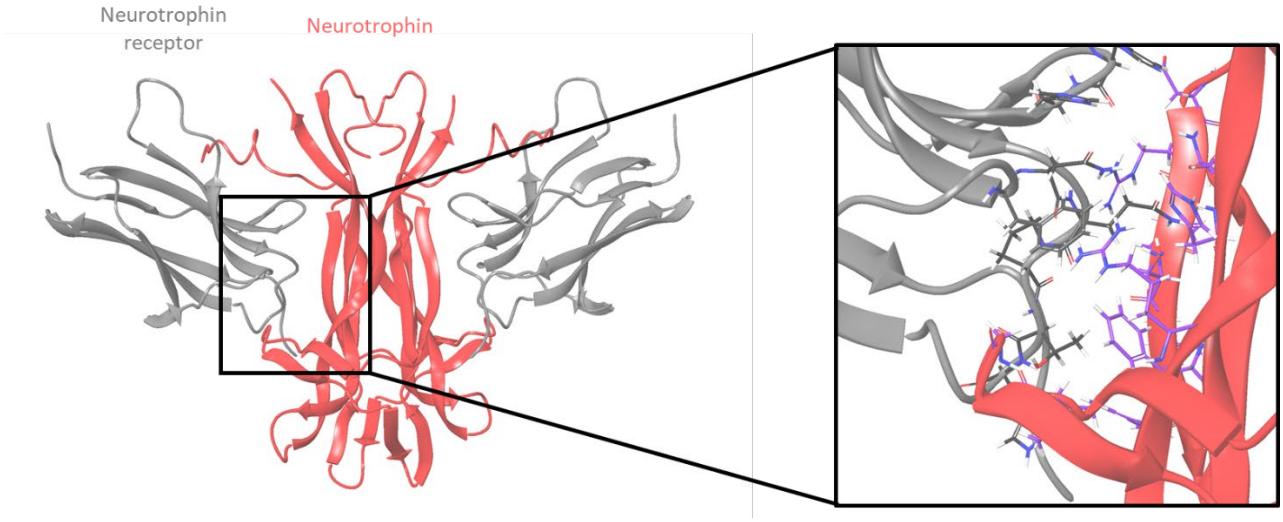

***Figure 1**: Crystal structure of a neurotrophin (in red) bound to its receptor (in gray), showing the amino acid residues in the interface that participate in interactions between the two proteins. Mimetic compounds can be designed to form similar interactions with the neurotrophin receptors or to enhance the effects of neurotrophins, e.g. by stabilizing neurotrophin-receptor complexes. Original structure PDB ID 1WWW (Wiesmann, C., Ultsch, M., Bass, S. et al. Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature 401, 184–188 (1999). https://doi.org/10.1038/43705).*

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Structural Biology for Neurodegenerative Diseases

In all living organisms, proteins are the machineries that carry out all the biological processes inside and outside the cells. The understanding of their structure is an important step in figuring out how they work and why an alteration in their structure, and in turn their function, results in disease. Indeed, the protein functions are strictly related to their structures even when proteins are without a defined three-dimensional structure, as in the case of intrinsically disorder proteins. But how do scientists unravel the structures of such macromolecules?

Structural biology is the answer. Structural biology is a branch of biochemistry that studies the structure of macromolecules, such as proteins and nucleic acids. Using and integrating different techniques, structural biology allows the determination of the atomic-detail structure of molecules that otherwise would not be visible. The most commonly used methods are X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (EM). Structural studies can impact on our understanding of neurodegenerative diseases. Amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease among others, are characterised by the gradual loss of neuronal function due to protein aggregation and/or dysfunction. Clarifying the pathophysiological mechanisms behind these diseases is the first step towards the development of effective drugs that can prevent or treat such diseases.

X-Ray Crystallography: the old but gold method

In 1953, Kendrew and Perutz determined the crystal structure of myoglobin and haemoglobin. Since then, X-ray crystallography has been the dominant method in structural biology research, with the deposition of thousands of protein structures per year. Using X-ray beams and packing the biomolecules in crystals, X-ray crystallography gives a “photograph” at atomic resolution of the molecules in the crystal lattice. All the structural details are revealed: from structure interfaces to protein-protein interactions, as well as the binding sites of small molecules. For example, Wherman et al. determined the crystal structure of the extracellular domain of TrkA in complex with NGF, elucidating the residues involved in the interaction with the neurotrophin and providing a model for molecular docking in receptor-based drug design.

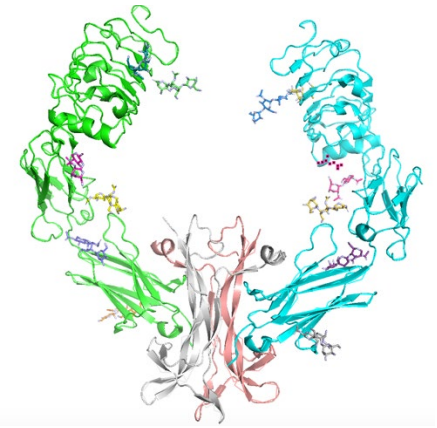

***Figure 2**: Crystal structure of the TrkA extracellular domain in complex with NGF. Image of 2IFG (Wehrman et al. (2007) Structural and mechanistic insights into nerve growth factor interaction with TrkA and p75 receptors”. Neuron 53:25-38) created with PyMol Molecular Graphics System, Version 2.0 Schrödinger, LLC.*

Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy provides protein three-dimensional structures at high resolution, as well as information on conformational dynamics and interactions that take place both in solution and in living cells. Thus, proteins that are too dynamic, disordered or not amenable to crystallisation can be examined by NMR spectroscopy. However, the application of this technique is limited to proteins with molecular weights of less than 50 kDa.

In addition, NMR spectroscopy is a powerful tool in screening the binding of compounds to target proteins for drug discovery research. Recently, Franco *et al.* determined the first NMR structure of the TrkA transmembrane domain (TM), showing the dimer length, the angle formed between the two helices, and the contact surface areas characterized by a conserved sequence motif. This information is fundamental to understanding the mechanism underlying receptor activation upon NGF binding, laying the groundwork for studying molecules that may trigger the receptor activation.

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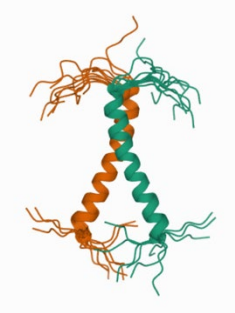

***Figure 3**: NMR structure of TrkA-TM dimers. Image of 2N90 (Franco et al. (2020) Structural basis of the transmembrane do-main dimeriza-tion and rotation in the activation mechanism of the TRKA re-ceptor by nerve growth factor. Journal of Biological Chemistry 295:275-286)) created with PyMol Molecular Graphics System, Version 2.0 Schrödinger, LLC.*

Single-Molecule Cryo-Electron Microscopy

The structure of large macromolecular complexes or proteins that are difficult to crystallise can be determined by cryo-elec-tron microscopy (cryo-EM). Cryo-EM uses a beam of electrons, whose wavelength is shorter than that of light, enabling the atomic image of molecules at high resolution to be determined. With the advent of new detectors and developments in sample preparation (protein vitrification), the use of this technique has exploded in the last few years. An example of the advantage of Cryo-EM for studying neurodegenerative diseases is the struc-ture of γ-secretase, a multi-subunit complex of about 270 kDa involved in the formation of amyloid plaques in Alzheimer's dis-ease (AD). In AD brains, γ-secretase cleaves amyloid precursor protein (APP), generating peptides prone to aggregation and forming amyloid plaques.

In 2019, Zhou et al. have determined the cryo-EM structure of the γ-secretase in complex with the APP fragment, providing a model that is useful, not only to better understand the disease progress, but also for the rational design of substrate-specific inhibitors.

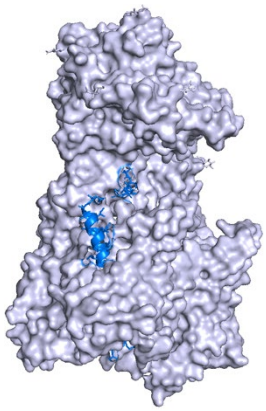

***Figure 4**: Cryo-EM density map of human γ-secretase (grey) in complex with APP fragment (marine). Image of 6IYC (R. Zhou et al. (2019) Recognition of the amyloid precursor protein by hu-man γ-secretase. Science 363(6428)) created with PyMol Molecu-lar Graphics System, Version 2.0 Schrödinger, LLC.*

Structural biology in the EuroNeurotrophin Network

Within the "EuroNeurotrophin" network, we aim to crystallise and determine the structures of neurotrophin receptors in complex with small molecules by X-ray crystallography, in order to drive the optimization of lead compounds. Also, we want to integrate this structural information with functional studies by biophysical methods.

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The marine environment: a challenging source of novel drugs against neurodegeneration

An "unlimited" and "underexploited" library of new drugs from the sea

Did you know that 63% of the drugs on the pharmaceutical market nowadays are related to terrestrial and marine natural products? Have you ever heard about the marine environment, its richness in terms of biodiversity, and its potential as a treasure trove for new drugs? *If you want to learn more about these topics, check them out in this article.*

The oceans cover more than 70% of our planet's surface and host an estimated 1-2 million different species.

When most people think about ocean life, they tend to think solely about fish, but there is a lot more to marine life than just fish. Marine organisms, such as sponges, tunicates, fish, soft corals, molluscs, echinoderms, bryozoans, crustaceans, bivalves, and many other species live in symbiosis with marine microorganisms, such as *bacteria* and *fungi*, applying specialized strategies that allow them to survive in different habitats.

***Table 1** The marine drug pipeline adapted fromHarshad Mal, J Pharm Bioallied Sci., **2016**, 8(2): 83–91.*

Clinical status	Compound name	Marine organism	Chemical class	Disease area
Approved	Cytarabine, ara-C	Sponge	Nucleoside	Cancer, leukemia
	Brentuximab vedotin (SGN-35)	Mollusk/cyanobacterium	ADC (MMAE)	Cancer, lymphoma
	Vidarabine, ara-A	Sponge	Nucleoside	Anti-viral
	Omega-3-acid ethyl esters	Fish	Omega-3 fatty acid	Hypertriglyceridemia
	Ziconotide	Cone snail	Peptide	Pain
	Eribulin mesylate (E7389)	Sponge	Macrolide	Breast cancer
	Trabectedin (ET-743)	Tunicate	Alkaloid	Cancer
Phase III	Plitidepsin	Tunicate	Depsipetide	Cancer
	Tetrodotoxin	Pufferfish	Guanidinium alkaloid	Chronic pain
	Soblidotin (TZT-1027)	Bacterium	Peptide	Cancer
Phase II	DMXBA (GTS-21)	Worm	Alkaloid	Cognition, AD, schizophrenia
	Plinabulin (NPI-2358)	Fungus	Diketopiperazine	Cancer
	Glembatumumab vedotin	Mollusk/cyanobacterium	ADC (MMAE)	Breast cancer, melanoma
	Elisidepsin	Mollusk	Depsipetide	Cancer
	PM1004	Nudibranch	Alkaloid	Cancer
	Tasidotin, synthadotin (ILX-651)	Bacterium	Peptide	Cancer
	Pseudopterosins	Soft coral	Diterpene glycoside	Wound healing
Phase I	Bryostatin 1	Bryozoa	Polyketide	Cancer
	Pinatuzumab vedotin (DCST-2980S) and (DCDS-4501A)	Mollusk/cyanobacterium	ADC (MMAE)	Non-Hodgkin lymphoma, chronic lymphocytic leukemia
	Hemiasterlin (E7974)	Sponge	Tripeptide	Cancer
	HuMax®-TF-ADC	Mollusk/cyanobacterium	ADC (MMAE)	Cancer for ovary, endometrium, cervix, prostate
	Marizomib (salinosporamide A)	Bacterium	Beta-lactone-gamma lactam	Cancer
Preclinical	Chrysophaentin A	Alga *Halobacillus salines*	Shikimate	Bacterial infections
	Phenethylamine	Bacterium lyngnyoic acid	Shikimate	Bacterial infections
	Geodisterol sulfates	Sponge	Peptide	Fungal infections
	Pseudoalteromonas sp. metabolites	Bacteria	Polyketide	Bacterial infections
	Peziza vesiculosa β-carboline	Bryozoa	Alkaloid	Fungal infections
	Bromophycolides	Alga	Terpene	Malaria
	Plakortin	Sponge	Polyketide	Malaria
	Homogentisic acid	Sponge	Shikimate	Malaria
	Cladonia cervicornis diterpene	Alga	Terpene	Protozoal infections
	Hymenidin	Sponge	Alkaloid	Tuberculosis
	Ggyrosanols	Soft coral	Terpene	Viral infections
	Dysidine	Sponge	Terpene	Diabetes
	Arenamides A and B	Bacteria	Peptide	Inflammation
	Capnellene	Soft coral	Terpene	Inflammation
	Floridosides	Alga	Glycolipid	Inflammation
	Grassystatins A-C	Bacteria	Peptide	Immunity
	Callyspongidiol	Sponge	Polyketide	Immunity
	Calyculin A	Sponge	PKS/NRPS	Nervous system
	Pulicatin A	Bacteria	Alkaloid	Nervous system
	Dysideamine	Sponge	Terpene	Nervous system

AD: Alzheimer's Disease, ADC: Antibody drug conjugate, MMAE: Monomethylauristatin E, PKS: Poliketide synthases, NRPS: Nonribosomal peptide synthases, DMXBA: 3-(2,4-dimethoxy)-benzylidene-anabaseine

Some marine organisms can live in sub-zero waters in polar regions, whereas others live in hydrothermal vents where temperatures can reach up to 400°C. Some organisms live in shallow waters, where there is high exposure to sunlight, while others prosper in the deep sea, where there is no light and pressure is much higher. The huge biodiversity found in various marine habitats is echoed in the molecular diversity of unique chemical entities that can be extracted from marine animals, plants and microbes (which are poorly studied compared to their terrestrial relatives), known as *Marine Natural Products.*

In the early 1900s, the idea of investigating marine ecosystems as the potentially largest source for new chemicals began to be explored systematically. Since then, more than 36,000 new molecules with a wide range of pharmaceutically relevant bioactivities (including antibacterial, antifungal, antiviral, anticancer, and anti-inflammatory properties) have been discovered from the marine environment. A list of marine derived drugs, along with their therapeutic uses and their chemical class, is displayed in Table 1. Unfortunately, sampling of marine organisms, is often more difficult compared to sampling terrestrial organisms, and this consequently makes the exploration and collection of marine samples (e.g., deep-sea organisms that are more susceptible to pressure and light changes) cumbersome and expensive.

Nevertheless, progress in marine technologies, such as easily accessible scuba diving equipment, as well as remotely operated vehicles (ROVs), facilitates the investigation of the marine environment.

Inspired by its extraordinarily promising capital, we strongly believe that the *Marine Environment* has the potential to be key in finding useful treatments for *Neurodegenerative Diseases.*

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Alzheimer's Disease: Current therapies and future directions

Alzheimer's disease is a devastating neurodegenerative disorder, which currently affects more than 35 million people worldwide. It is estimated that by 2050 this figure will increase by 204% to a total of 106 million people affected by the disease. (Prince, 2015) Additionally, there is a significant economic and psychological cost associated with the disease that includes the people suffering from it but also carers and family members. Furthermore, the cost for dementia, including direct medical costs and social and informal care, will reach 2 trillion US dollars by 2030 (Prince, 2015).

Clinically, the disease is characterized by memory loss, cognitive impairment, and difficulties in performing simple everyday tasks. In the later stages, depression and aggressive behaviour are also common symptoms. The pathological features of the disease are attributed to the malformation of two proteins that form aggregates inside and outside cells. The former are caused by a hyperphosphorylated form of the protein Tau, while the latter occur after misprocessing of the amyloid precursor protein (APP), producing toxic Aβ oligomers. These aggregates then cause neuronal cells to malfunction or even die, while inhibiting the ability of the brain to produce new neurons (Long and Holtzman, 2019).

Currently, there are no drugs or predictive prognostic markers available that can alter or detect the onset or the course of the disease, and the approved therapies can only alleviate some of the pathological symptoms of the disease. At present, there are four drugs that are used for people suffering from Alzheimer's disease. Three of these (donepezil, galantamine and rivastigmine) act as acetylcholinesterase inhibitors and the other one (memantine) as an N-methyl-D-aspartate receptor (NMDA) antagonist. The inhibitors of the enzyme acetylcholinesterase help increase the availability of acetylcholine, an important neurotransmitter, at the synapse, whereas the drug that blocks the NMDA receptor reduces the excitotoxicity effects of L-glutamate (Lane, Hardy and Schott, 2018). Even though these drugs can alleviate some of the symptoms of the disease, it is important for future efforts to be directed to discovering novel disease-modifying drugs or therapies, which reverse or fully block the progress of the disease. It is estimated, for example, that if a drug can slow the onset of the disease by 5 years, this would translate into 469,000 fewer people living with dementia by 2030 and £21.2bn/year less money spent by 2050 in the UK (Lewis *et al.*, 2014).

In the EuroNeurotrophin Consortium, we have focused our research on developing and testing novel small molecules, mimetics of endogenous neurotrophins, as well as isolated natural products from marine bacteria and/or fungi from the East Mediterranean basin that act as mimetics of neurotrophins, that can be used as drugs against Alzheimer's disease. Furthermore, evidence clearly shows that neurotrophin processing and expression levels are deregulated in Alzheimer's Disease (AD), and this has been postulated to contribute to the disease pathology (J. Allen, J. Watson and Dawbarn, 2011). To this end, we use a variety of Alzheimer's disease models (*in vitro* and *in vivo*), as well as novel techniques and interdisciplinary approaches, in order to design and biologically identify a lead drug that will hinder disease progression.

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Improving drug development for Alzheimer's disease and other neurodegenerative diseases by using human induced stem cells

Drug development for neurodegenerative diseases has largely depended on the availability of humanized animal models, as well as cell lines and culture systems derived from animals. Such models attempt to simulate complex human conditions, but their success in therapeutics has been under extensive debate. Unfortunately, the availability of optimal human material for such work is negatively impacted by the difficulty in sourcing neuronal tissue from patients. Undeniably, traditional models, such as rodents, have led the progress in uncovering the underlying mechanisms behind neurodegenerative disorders for decades.

However, it has become increasingly evident that there are key differences between rodent and human neurobiology which impact our ability to translate drug efficacy from rodent systems to humans. For example, many rodent models only exhibit Alzheimer's Disease (AD) pathophysiology after a large accumulation of mutations associated with the human disease, while they still lack important phenotypes, such as the extensive loss of neurons, that is characteristic in humans (Drummond & Wisniewski, 2017) (LaFerla & Green, 2012). What is more, a species-specific genetic background can limit their pharmacological application in many cases. Such issues are thought to be the underlying factors behind the limited success rates of drugs developed through animal model testing, reported at less than 12% at clinical trial stages (Paul et al., 2010).

Progress in stem cell research and regenerative biomedicine provide a new path for carrying out drug testing on human cells. Initially, the potential of this work was limited by the difficulties associated with sourcing and working with human embryonic stem cells (ESC), such as the consideration of ethical aspects and the difficulty of accessing patient specific material. However, the discovery of the "Yamanaka factors" revolutionised the field. Takahashi, Yamanaka and colleagues showed that only four transcription factors can be used to reprogram fibroblasts and blood cells to pluripotent cells that are highly similar to ESC (Takahashi et al., 2007)(Takahashi & Yamanaka, 2006). Human induced pluripotent stem cells (iPSC) have since provided an unprecedented opportunity for drug discovery, bringing down multiple barriers in acquiring efficient human cell culture systems (Shi et al., 2016). Most importantly, it is easy to source the material needed for iPSC reprogramming and it has become possible to acquire patient specific cells, paving the road to personalised medicine, while avoiding the ethical issues of ESC (Kondo et al., 2017) (Shi, Yanhong Inoue, Haruhisa Wu, Joseph C. Yamanaka, 2017).

In recent years, the use of iPSC has powered cutting-edge developments on AD research and drug discovery. Currently there is a focus on the development of complex combinatorial systems based on iPSC-derived cell populations that include 3D cultures and mini-brain organoids that provide simplified proxies of the human brain (Tan et al., 2021) (Choi et al., 2016). Nevertheless, iPSC systems not only provide a research and development platform, but are also potential therapies that could be administered through the transplantation of stem cells or derived cell populations in patients (Hayashi et al., 2020).
There is now available multiple protocols for the differentiation of iPSC towards various specific neuronal fates, including CA3 hippocampal neurons (Sarkar et al., 2018), cerebral cortical neurons (Shi et al., 2012) , medium spiny neurons (Naujock et al., 2016) and dopaminergic neurons (Zhang et al., 2014) (Mahajani et al., 2019). In addition, iPSC-derived neurons, astrocytes, and microglia derived from familial AD patients have been shown to successfully replicate AD-related pathophysiology, such as tau phosphorylation, endosome enlargement or impaired amyloid beta clearance, which are associated with APOE polymorphisms (Lin et al., 2018) (Wang et al, 2018). Genetic engineering experiments have also demonstrated the value of iPSC-derived neurons for both understanding and treating familial AD. For example, correction of PSEN1 and FAD1 mutations in induced neurons leads to a reversal of amyloid beta phenotypes. The above also highlight the potential of iPSC-derived systems for personalized drug screening, targeted to the genetic background of different patients (Kondo et al., 2017). Finally, while much progress has been made in the field, there is still vibrant ongoing work on optimizing iPSC-derived systems, such as obtaining neuronal populations with high purity and specificity (Kondo et al., 2017), as well as defining small molecules that could control cell reprogramming, differentiation or survival of iPSC, increasing their potential as druggable agents.

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In the scope of EuroNeurotrophin, iPSC technology presents an opportunity for testing the action and efficacy of promising candidates on a human cell system. Hence, efforts have focused on testing the neurogenic or neuroprotective action of selected microneurotrophin compounds on human iPSC-derived cortical neurons. This innovative approach can help us assess a new way to test compounds for their ability to induce neurogenesis on human tissue by the selective activation of neurotrophin receptor pathways, inducing specific differentiation of precursor cells and decreasing cell death of human neuronal populations. This work provides a testing platform that is much more translational for potential human applications and promotes a key goal of the consortium, adhering to the 3R principle: Replacement, Reduction and Refinement in animal research.

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About the Authors

The EuroNeurotrophin March 2021 Newsletter was written by five of the Early Stage Researchers (ESRs) working on individual projects as part of the EuroNeurotrophin project.

ESR3: Athanasios-Alexandros Tsengenes

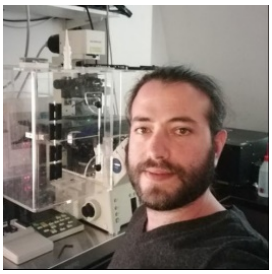
Alexandros is hosted at the HITS gGmbH, Heidelberg Institute for Theoretical Studies and he works on the design and optimization of small molecule mimetics and potentiators of neurotrophins, using a combination of *in silico* ligand-based and receptor-based drug design approaches

ESR5: Federica Carucci

Federica is hosted at the University of Siena. Her research activities are focused on the production and crystallization of neurotrophin receptors in complex with selected compounds. The structural studies will clarify the determinants for ligands binding and hence drive receptor-based drug design.

ESR7: Paolo Giaccio

Paolo is hosted at the National and Kapodistrian University of Athens and the aim of his research is the isolation of natural products from marine bacteria and/or fungi from the East Mediterranean basin that act as mimetics of neurotrophins using a bioassay-guided isolation protocol.



ESR11: Thanasis Rogdakis

Thanasis's research focuses on novel synthetic or naturally derived microneurotrophins that he is testing for their ability to activate neurotrophin receptors. Compounds that present favourable pharmacokinetic profiles will be further studied in Alzheimer's Disease models *in vitro* and *in vivo*.

ESR12: Despoina Charou

Despoina is hosted at the Foundation for Research and Technology Hellas and her work focuses on testing synthetic and natural neurotrophin mimetics on specific neurotrophin-dependent cellular populations. The molecular mechanisms and functions leading to an increase in adult neurogenesis through the induction of endogenous neural stem cell proliferation and survival, which is affected in AD, will be assessed.

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