Eighth European Workshop and InNerMeD Information Network

First Open Conference

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European Task Force on Brain and Neurodegenerative Lysosomal Storage Diseases

Fondazione BRAINS FOR BRAIN- Onlus Via Giustiniani 3 c/o Dipartimento di Pediatria Salus Pueri - 35128 Padova - Italy
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Lysosomal Storage Disorders (LSDs) are inherited metabolic disorders due to the deficit of lysosomal enzymes causing accumulation of undegraded material which is responsible for cell apoptosis with time.
Since lysosomal enzymes are ubiquitous molecules, their deficiency has important effects in all organs, in particular the central nervous system (CNS), liver, spleen, heart and bones.
With the advent of recombinant DNA technology, the identification and cloning of all the known lysosomal enzymes has been recently achieved, and therefore, expression and purification of recombinant proteins is now possible and enzyme replacement therapy (ERT) is now available for a growing number of storage disorders.

However, although ERT has proven to be valuable to possibly change the clinical history of the disease it has been evident that the recombinant proteins do not have any effect on the CNS, as they are unable to cross the blood brain barrier.
Furthermore, the mechanisms and etiology of CNS pathology in LSDs are still poorly understood.

We still do not know whether storage is really the “primum movens” of the metabolic disaster or whether other processes might be more important (inflammation, alteration of ion channel activity, lack of chaperone molecules etc.). The understanding of these basic aspects might be extremely valuable to unravel why most of the LSDs have an attenuated and a severe form without and with CNS involvement, respectively, depending on whether there is a total enzymatic deficiency or not.

Co-funded by the Health Programme of the European Union

INNERMED
BRAINS FOR BRAIN

THE BRAINS FOR BRAIN TASK FORCE

The task force takes advantage from the expertise of the most distinguished european scientists, leaders in basic and applied neurotechnology and neurology grouped together to create a coordinate effort toward the comprehension of the pathophysiological processes of the neurological disorders, the implementation of knowledge on the blood brain barrier and the development of new molecular and or biochemical strategies to overcome the blood brain barrier and treat neurological disorders. The B4B nickname of the group has been created to acknowledge the effort of the 4 initial industrial sponsors (ACTELION, BIOMARIN, GENZYME and SHIRE Human Genetic Therapies) without the support of which this brainstorming pannel could not have been created.

Brains For Brain (B4B) was formally founded in March 2007 as a research group formed by international specialists and leaders on clinical and basic research in the field of neuro-pediatrics and neuroscience.
The group has attracted interest from major biotech companies working on the development of new therapeutical strategies for lysosomal diseases, and furthermore has a strong interaction with international family associations, involved in taking care of the needs of lysosomal patients, and has stimulated collaborations toward coordinate actions to disseminate knowledge about the diseases.

B4B has also collaborated with International Scientific Associations, such as the European Study Group for Lysosomal Diseases (ESGLD) and the International Blood Brain Barriers Society (IBBS) and it is a member of the European Brain Council.

THE BRAINS FOR BRAIN FOUNDATION

The BRAINS FOR BRAIN FOUNDATION is a no-profit international organization addressed to disabled children who are affected (or healthy carriers) by rare neurological diseases.
The purposes of the FOUNDATION are:
- scientific research;
- dissemination of knowledge;
- social and socio-medical assistance;
- health assistance.

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In the field of Neurodegenerative Lysosomal Disorders the aims of the FOUNDATION are:

- to support medical and scientific research with regard to paediatric rare neurodegenerative diseases (with particular regard to Lysosomal Storage Disorders and genetic pathologies);
- to support medical and scientific research with regard to paediatric rare neurodegenerative diseases (with particular regard to Lysosomal Storage Disorders and genetic pathologies);
- to increase public awareness and interest on such diseases;
- to organize and promote national and international research activities;
- to coordinate and promote preclinical and clinical trials;
- to organize conferences and workshops on the abovementioned topics;
- to share cultural and scientific backgrounds with different stakeholders to implement knowledge on Neurodegenerative Disorders;
- to raise funds to support research;
- to fund fellowships or prizes;
- to campaign to increase public and stakeholders awareness to Neurodegenerative Disorders and for public fund raising.

THE EUROPEAN PARLIAMENT MEETINGS

Rare neurological diseases of childhood pose a serious medical health issue in Europe. Although individually uncommon, collectively there are thousands of rare diseases that affect a large number of people. The need to collaborate to focus on these disorders was highlighted during the meeting: RARE NEUROLOGICAL DISEASES OF CHILDHOOD: WE TREAT THE CHILD TO TREAT THE ADULT organized by the Brains for Brain Foundation at the European Parliament in Brussels on December 2nd 2010. The main aim of the meeting was to acknowledge the growing interest of the European Union Commission in both rare and neurological disorders.

To reinforce the necessity to work together and center attention on rare neurological disorders of infants and children the B4B Foundation has more recently organized the roundtable: “PAVING THE WAY FOR A COMPETITIVE AND DYNAMIC EU KNOWLEDGE ECONOMY: THE WAY FORWARD IN RARE DISEASES" which was held again at the EU Parliament in Brussels, on November 26, 2013. The meeting rallied numerous relevant stakeholders to discuss initiatives aiming to create a model of intersectoral cooperation that could facilitate the set-up of a European PhD Programme in the area of rare neurological diseases of children. In line with the core principles established by “Towards a Maastricht for Research”, the Brains for Brain Foundation has in fact created a network of Universities and Scientific Societies to start a doctorate programme aimed at furthering the knowledge on neurometabolic diseases amongst young physicians and scientists in order to establish an European Network of specialized experts and maintain excellence in Europe. Such initiative intends to enhance an advance awareness and knowledge about rare diseases via cross-border collaboration and to enable better diagnosis and management of patients affected by these diseases. Holding these meetings, B4B wished to demonstrate the unity of intent of family associations, biotechnology and pharmaceutical industries and the scientific community in stimulating interest in rare neurological diseases and advance care for affected children.

The B4B EP Roundtable in particular represents a major step toward the establishment of a successful EU cross border collaboration and cooperation to raise awareness about rare diseases of childhood and keep them on the health-care agenda. Although individually rare by definition, rare diseases collectively affect millions of people worldwide. Joint forces to tackling them are essential to ensure that affected children are given the priority they deserve and that their needs are met.

INHERITED NEUROMETABOLIC DISEASES INFORMATION NETWORK

The Inherited NeuRoMetabolic Diseases INFORMATION NETWORK (InNerMeD-I-Network) has been funded by the Executive Agency for Health & Consumers (DG-SANCO) under the Second Programme of Community action in the field of Health, 2008-2013 (contract id 20121212) to be the first European Network on paediatric neurometabolic diseases. InNerMeD-I-network wants to create a network of information targeted on diagnosis and treatment of iNMDs based on the collection and exchange of proper information among scientific community, health professionals, patients, patient associations and all interested stakeholders. The project aims to increase current knowledge on iNMDs and speed up the timely and precise identification of patients, who may benefit of the the available (experimental and marketed) treatments. The network will also favour biomedical research, straightening research capacities and fostering innovative therapeutic tools derived from the recent scientific advancements based on biomarkers use and personalised approaches. The InNerMeD-I-Network, coordinated by the Brains for Brain Foundation, includes five associated partners (Gianni Benzi Pharmacological Research Foundation, Center for Metabolic Disorders at the University of Copenaghen, Center for lysosomal storage disorders at the University of Mainz, University of Zagreb School of Medicine, Hospital Sant Joan de Déu) plus fifteen collaborating partners, including clinical and research centres, patients and parents associations and scientific societies.

Co-funded by the Health Programme of the European Union
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AIMS OF THE WORKSHOP

The aims of the eighth Meeting of the Brains For Brain Foundation are:

- to discuss research achievements in the field of neurodegenerative disorders at clinical and basic science level in the field of neurodegenerative lysosomal storage disorders and Blood Brain Barrier;
- to discuss new recent advances on natural history and pathophysiology of LSDs particular attention to the important role of an early intervention in preventing the morbidity and mortality associated with each of the disorders;
- to discuss factors which control the entry into the brain of medicines and other therapeutic agents which may be helpful in treating central nervous disease;
- to discuss how B4B might collaborate with the European Union to stimulate interest in the research on LSDs and BBB. For this reasons representatives from EU Commission will be invited;
- to discuss collaborations with international family associations and corporations to increase knowledge about storage diseases and research projects;
- to discuss the role of the industries in driving innovation for new therapeutical approaches for true unmet needs.

This Workshop arises from the project Inherited NeuRoMetabolic Diseases Information Network (InNerMeD-I-Network, agreement no. 2012 12 12) which has received funding from the European Union, Executive Agency for Health and Consumers, in the framework of the Second Health Programme.

Organization
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SCIENTIFIC PROGRAMME 2014

March 7th 2014

14.00-14.10
WELCOME AND OPENING

14.15-15.00
OPENING PLENARY LECTURE
R. DANEMAN, *University of California San Francisco, USA*
The Regulation of the BBB in Health and Disease
Discussion.

15.00-16.45
BASIC ASPECTS
Chair Discussants:
I.E. BLASIG, DE - B. BIGGER, UK

15.00-15.25
S. GARBUZOVA-DAVIS, *University of South Florida, USA*
Blood-Brain Barrier changes in Sanfilippo Syndrome (MPSIII)
Discussion.

15.35-16.00
T. MOOS, *Aalborg University, DK*
Iron and Copper transport at the brain barriers
Discussion.

16.10-16.35
R. CECCHELLI, *University of Lille Nord de France, FR*
Generation of new human models of the Blood Brain Barrier from stem cells
Discussion.

Coffee

17.00-17.25
F. GOSSELET, *University of Lille Nord de France, FR*
Effects of oxysterols at the blood-brain barrier: Implications for Alzheimer's disease
Discussion.

17.35-18.00
A. BALLABIO, *Telethon Institute of Genetics and Medicine, IT*
An efficient toolkit to detect sequence variations of genes involved in lysosomal-autophagic pathways
Discussion.

18.10-18.35
T. M. COX, *University of Cambridge, UK*
Clinical gene therapy targeted to the brain
Discussion.

19.30 DINNER

March 8th 2014

8.45-13.30
PATHOPHYSIOLOGY AND LSDs
Chair Discussants:
T. MARQUARDT, DE - M. VANIER, FR

8.45-9.10
T. MARQUARDT, *University of Münster, DE*
Understanding Niemann-Pick C
Discussion.

9.25-9.50
K. ÖLLINGER, *University of Linköping, SE*
The lysosome: from waste bag to potential therapeutic target
Discussion.

10.00-10.25
J. BERGER, *Center for Brain Research, Medical University, Vienna, AT*
Pathophysiology of X-linked Adrenoleukodystrophy
Discussion.

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10.35-11.00
G. MAZZOCCOLI, *IRCCS Scientific Institute and Regional General Hospital "Casa Sollievo della Sofferenza", S. Giovanni Rotondo, IT*
Altered functioning of the biological clock in Hunter syndrome
Discussion.

Coffee

11.15-13.00
PATHOPHYSIOLOGY AND LSDs 2
Chair Discussants:
T. FUTERMAN, IL - R. GABATHULER, USA

11.15-11.40
S. HEALES, *University College London, UK*
The link between Gaucher & Parkinsonism
Discussion.

11.50-12.15
M. N. G JAMES, *University of Alberta, USA*
The X-ray crystallographic structure and enzymatic mechanism of α-l-iduronidase and its relationship to Mucopolysaccharidosis I
Discussion.

12.25-12.50
G. MANN, *Kings College London, UK*
Nrf2-mediated neurovascular protection against oxidative stress in experimental stroke
Discussion.

13.00 LUNCH

14.00-16.20
CROSSING THE BLOOD BRAIN BARRIER AND THERAPEUTIC OPTIONS
Chair Discussants:
S. GARBUZOVA-DAVIS, USA - G.W. FRICKER, DE

14.00-14.25
F. PLATT, *University of Oxford, UK*
New approaches for the treatment of NPC1
Discussion.

14.35-15.00
A. H. FUTERMANN, *Weitzman Institute, IL*
RIPK3 as a novel therapeutic target for Gaucher disease
Discussion.

15.10-15.35
O. MARTIN, *Université d'Orléans, FR*
Novel Inhibitors of GCase as Pharmacological Chaperones for Type 1 and 2 Gaucher disease
Discussion.

15.45-16.10
L. DE FILIPPIS, *University Milan Bicocca, IT*
Murine neural stem cells model Hunter disease in vitro: glial cell-mediated neurodegeneration as a possible mechanism involved
Discussion.

Coffee

16.45-19.30
CROSSING THE BLOOD BRAIN BARRIER AND THERAPEUTIC OPTIONS 2
Chair Discussants:
T. MOOS, DK - F. PLATT, UK

16.45-17.10
J. YU, *Genentech, California, USA*
Delivering antibody therapeutics across the blood-brain barrier
Discussion.

17.20-17.45
S.N. WADDINGTON, *University College London, UK*
Perinatal gene therapy rescues acute neonatal lethal Neuronopathic Gaucher Disease in mice
Discussion.

17.55-18.20
I. E. BLASIG, *Leibniz-Institut für Molekulare Pharmakologie FMP, Berlin*
Claudin peptidomimetics as potential drug enhancers for the blood-brain barrier
Discussion.

18.30-19.15
PLENARY LECTURE
K. SANDHOFF, *University of Bonn, DE*
Metabolic and cellular bases of Sphingolipidoses
Discussion.

20.30 DINNER

March 9th 2014

09.00-11.00
B4B AND INNERMED EUROPEAN ACTIONS
Chair Discussants:
D. BEGLEY, UK - M. SCARPA, IT

9.00-9.15
M. SCARPA and **D. BEGLEY,** *Brains For Brain Foundation, EU*
Update on the activity of B4B at EU
Discussion.

9.25-9.50
M. SCARPA, IT - F. (Duccio) BONIFAZI, IT
on behalf of All InNerMed Partners
Presentation of the Inherited NeuroMetabolic Diseases Information Network (InNerMeD-I-Network) and website platform to increase awareness on Neurometabolic Diseases
Discussion.

10.00-10.25
A. CECI, *European Medicine Agency (EMA)*
European Regulation on Clinical Trials and drug development in neurometabolic disorders
Discussion.

Coffee

11.15-13.30
B4B AND BIOTECH COLLABORATIONS
Chair Discussants:
T. EAGLETON, USA - S. GELPERINA, RU

11.15-11.40
S. H. CHENG, *Genzyme, a Sanofi Company, Framingham, USA*
Augmenting CNS glucocerebrosidase activity as a therapeutic strategy for Parkinsonism and other Gaucher-related synucleinopathies
Discussion.

11.50-12.15
R. GABATHULER, *biOasis Technologies Inc, USA*
Using targetors to direct and deliver Biologics such ERT to the CNS
Discussion.

12.25-12.50
A. CHRISTIANSEN, *Shire*
Mechanisms of blood brain barrier bypass in K16ApoE mediated lysosomal enzyme delivery to the brain
Discussion.

13.00-13.25
S. BUNTING, *BioMarin Pharmaceutical Inc, USA*
Clearance of accumulated substrate from the brain of Sanfilippo B mice by intracerebroventricular delivery of glycosylation-independent lysosomal targeted NAGLU
Discussion.

LUNCH AND FAREWELL
to the next 2015 meeting

Co-funded by the Health Programme of the European Union
Co-funded by the Health Programme of the European Union

NOTES

THE REGULATION OF THE BBB IN HEALTH AND DISEASE

RICHARD DANEMAN
Department of Anatomy, UCSF.

The blood vessels of the central nervous system (CNS) form a barrier that greatly restricts the movement of molecules, ions and cells between the blood and the brain. This blood-brain barrier (BBB) is crucial to allow for proper brain function and to protect the CNS from injury and disease, however it also provides a stubborn obstacle for drug delivery to the CNS. Therefore determining the mechanisms regulating BBB formation and function may prove vital to develop therapeutics to restore the BBB to prevent neuronal damage during neurological disease but also identify transport mechanisms to bypass the BBB to aid in drug delivery. A key aspect of the BBB is that endothelial cells which form the walls of blood vessels in the CNS have different properties than endothelial cells in non-neural tissues, and many of these properties are regulated by interactions with the underlying neural tissue. To understand the molecular mechanisms that regulate BBB formation and function, we have used microarray analysis to compare the gene expression of endothelial cells purified from the CNS with endothelial cells purified from non-neural tissue, and thus have generated a comprehensive resource of transcripts that are enriched in the BBB forming endothelial cells of the CNS. Through this comparison we have identified novel tight junction proteins, transporters, metabolic enzymes, signaling components, and unknown transcripts whose expression is enriched in CNS endothelial cells. This analysis has led to the identification that neural stem cell-derived Wnt/beta-catenin signaling is required for CNS angiogenesis, but not angiogenesis in non-neural tissue, and also induces BBB-specific gene expression. Furthermore, we have identified a role for pericytes in regulating the permeability of CNS vessels by inhibiting the expression of molecules that increase vascular permeability. In particular, pericytes limit the expression of leukocyte adhesion molecules in CNS endothelial cells, which limits CNS immune infiltration. This has led to a model for BBB formation in which CNS endothelial cells are induced to express BBB-specific genes during angiogenesis, and then the functional properties of the BBB are regulated by pericytes and astrocytes. We have further utilized gene-profiling to identify the molecular changes to the CNS endothelial cells during BBB dysfunction. This has identified novel genes and pathways that are regulated in response to injury and disease and lead to increased BBB permeability.

BLOOD-BRAIN BARRIER CHANGES IN SANFILIPPO SYNDROME (MPS III)

SVITLANA GARBUZOVA-DAVIS AND PAUL R. SANBERG
Department of Neurosurgery and Brain Repair, Center of Excellence for Aging and Brain Repair, University of South Florida, Morsani College of Medicine, Tampa, USA.

Mucopolysaccharidosis type III (MPS III), or Sanfilippo syndrome, is an autosomal recessive disorder caused by deficiency of a specific enzyme leading to accumulation of heparan sulfate within cells and to eventual progressive cerebral and systemic organ abnormalities. Different enzyme deficiencies in the heparan sulfate degradation pathway comprise the four MPS III subcategories (A, B, C, D). Since neuropathological manifestations are common to all MPS III types, determining blood-brain barrier (BBB) condition may be critical to understand potential additional mechanisms of this devastating disorder. Recently, we showed BBB structural and function impairment of various brain structures (cerebral cortex, hippocampus, striatum, and cerebellum) known to experience neuropathological changes in a MPS III B mouse model. Damaged endothelial and pericyte cells and degenerated astrocytes compromise the BBB even at early disease stage, resulting in vascular leakage. Edematous spaces around microvessels and highly vacuolated perivascular macrophages have been noted. Also, a microaneurysm was observed adjacent to a ruptured endothelium. Accumulation of GM3 ganglioside, a secondary storage product, was determined in the endothelium microvasculature of multiple brain structures. These results indicate severe BBB breakdown which might lead to cerebral hemorrhage and accelerate neuronal damage. Finding BBB damage in an animal model of MPS IIIB prompted us to investigate BBB competence in MPS III patients. Analysis of BBB integrity was performed in post-mortem brain tissues from patients with MPS III A (the most common subtype) and MPS III D (the rarest subtype) and age-matched controls. Capillary ultrastructure revealed endothelial and pericyte cell damage in the primary motor cortex, hippocampus, putamen, and cerebellum in both MPS III A and MPS III D patients. Mucopolysaccharide bodies were determined in a majority of endothelial cells and pericytes, rupturing cell membranes. Severe edematous space surrounded microvessels. Endogenous IgG microvascular leakage was clearly indicated in multiple brain structures. Reductions of tight junction proteins (occludin and claudin-5) via Western immunoblot were shown with variations between MPS III types. Additionally, extensive lysosomal accumulation was determined in brain microvasculature endothelium. These new findings of BBB impairment, shown in both an animal model and in MPS III patients, may have implications for disease pathogenesis and should be considered in development of treatments for this disease. Special attention should be given to endothelial cell function in view of possible deterioration of influx and efflux transport systems needed to maintain CNS homeostasis.

Supported by: The Children's Medical Research Foundation.

IRON AND COPPER TRANSPORT AT THE BRAIN BARRIERS

TORBEN MOOS*, TINA SKJØRRINGE*•, ANNETTE BURKHART*, MAJ SCHNEIDER THOMSEN*, LISBETH BIRK MØLLER•
**Section of Neurobiology, Biomedicine, Institute of Medicine and Health Technology, Aalborg University, Aalborg, Denmark.*
• Center for Applied Human Molecular Genetics, The Kennedy Center, Glostrup, Denmark.

Iron and copper are important co-factors for a number of enzymes in the brain, including enzymes involved in neurotransmitter synthesis and myelin formation. Both the absence and an excess of iron or copper will affect the brain. The transport of iron and copper into the brain from the circulation is strictly regulated, and concordantly protective barriers i.e. the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barriers have evolved to separate the brain environment from the circulation.
Iron and copper are mainly taken up at the BBB and transported further into the brain, but the blood-CSF barrier also plays a vital role in the homeostasis of the two metals, in terms of sequestering and efflux of iron and copper from the brain.

The uptake mechanisms of the two metals interact. The divalent metal transporter-1 (DMT1) is probably involved in the uptake and transport across the BBB of both iron and copper. Ferroportin transport ferrous iron from the brain capillary endothelial cells into the brain where ferrooxidases denoted by copper-containing molecules like ceruloplasmin expressed by astrocytes acts as ferrooxidases making ferric iron available for binding to transferrin inside the brain.

Further inside the brain, iron and copper are taken up by neurons and glia cells that express various transporters. Both iron deficiency and overload lead to altered copper homeostasis in the brain. Copper is an essential co-factor in numerous proteins that are vital for iron homeostasis and affects the binding of iron-regulatory proteins to iron-responsive elements in the mRNA of the transferrin receptor, DMT1 and ferroportin, all highly involved in iron transport. A better understanding of the regulation of iron and copper transport at the brain barriers and inside the brain will improve attempts to design drugs for treatment of neurological diseases related to impaired metal homeostasis.

GENERATION OF NEW HUMAN MODELS OF THE BLOOD BRAIN BARRIER FROM STEM CELLS

R. CECCHELLI[1]*, S. ADAY[2], E. SEVIN[1], C. ALMEIDA[2], M.-P. DEHOUCK[1], L. FERREIRA[2]
[1] *University Lille Nord de France, U. Artois, BBB laboratory, EA 2465, 62307 Lens, France.*
[2] *CNC - Center of Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal.*

BBB models can provide a valuable tool for studying mechanistic aspects related to the transport of drugs at the brain, as well as biological and pathological processes related to the BBB. Although in vitro models were established from various species, the most widely used being rat, mouse, pig and bovine, the establishment of a stable human BBB model is very important to account for differences between species. Primary human brain endothelial cells (hBECs) and immortalized human cells have been used as in vitro models ; however, several issues prevent their general use including constraints in obtaining human tissue, loss of hBEC phenotype during immortalized cell culture, or lack of important tight junctions and low transendothelial electrical resistance (TEER) values as shown in human cell lines. Recently, hBECs have been differentiated from induced pluripotent stem cells (iPSCs). However, the reproducibility of paracellular permeability and TEER across replicates was relatively low. In addition, it is unclear whether the reproducibility of the model is affected by the type and history of iPSC line used to derive the hBECs and the stability of the in vitro BBB model for periods of time above 7 days, which might preclude its general use for drug screening and toxicology studies. Also recently, it has been reported a human in vitro BBB model based in the co-culture of cord blood-derived ECs with astrocytes. However, the BBB model presents low TEER values and relatively high permeability.
Here we report a general and relatively easy method to generate a human BBB model using cord blood-derived hematopoietic stem cells, which can be obtained non-invasively. The cells were initially differentiated into endothelial cells (ECs) followed by the induction of BBB properties by co-culture with pericytes. The model is very reproducible (similar paracellular permeability for cells derived from 3 different donors and in 3 different laboratories) and stable (for at least 20 days). Our results show for the first time a good correlation between the in vitro predicted ratio of concentrations of unbound drug in brain and plasma obtained with our model and the in vivo ratio of concentrations of unbound drugs in cerebrospinal fluid (CSF) and plasma reported in humans. Finally, we show that Wnt signalling pathway mediates in part the BBB inductive properties of pericytes.

EighthEuropeanWorkshop

EFFECTS OF OXYSTEROLS AT THE BLOOD-BRAIN BARRIER: IMPLICATIONS FOR ALZHEIMER'S DISEASE

J. SAINT-POL[1], P. CANDELA[1], M.C. BOUCAU[1], L. FENART[1] & F. GOSSELET[1]
[1] *University Lille Nord de France, U. Artois, BBB laboratory, EA 2465, 62307 Lens, France.*
Corresponding author: ***fabien.gosselet@univ-artois.fr***

Alzheimer's disease (AD) is a neurodegenerative disease closely linked with the cholesterol metabolism and mainly characterized by the abnormal accumulation of amyloid-ß (Aß) peptides in several brain areas. Unfortunately, to date there is no efficient therapeutic approach for AD patients. However, in several in vitro and animal models, the stimulation of the nuclear receptor Liver X Receptors (LXRs) by agonists gives some promising results. Thus, when this signalling pathway is activated, the brain cholesterol metabolism is modulated, and the amyloid burden and the inflammatory process are reduced. With the hope to develop a similar therapeutic approach in human, several studies have been focused on the cellular and molecular mechanisms behind these effects. For this reason, role of the LXRs is mainly investigated at the brain level and effects of LXR ligands are under intense scrutiny on neurons and glial cells.
However, it is noteworthy that the brain is separated from the whole body by the blood-brain barrier (BBB) which is composed of brain capillary endothelial cells (BCECs) and brain pericytes (BPs). We and others have previously reported that this barrier is involved in the exchanges of Aß peptides and cholesterol between the blood and the brain. To date, the role of the LXRs in these exchanges remains poorly characterized.
With these considerations in mind, our projects aim to highlight the influence of both natural and synthetic agonists of LXR on BCECs and BPs, focusing particularly on their effects on LXRs target gene regulation, on Aß peptide transport and on cholesterol metabolism.
Our results demonstrate that LXR natural agonists (named oxysterols) induce the expression of ATP-Binding Cassette sub-family A member 1 (ABCA1, in BCECs and BPs) and ABCG1 (only in BCECs), correlating with an increase of cellular cholesterol efflux to (apo)lipoproteins. Our further experiments suggest that ABCA1 is a key player of this lipoprotein genesis process. In addition, these oxysterols do not modify Aß peptide accumulation in BPs but decrease their influx across the BCECs. This latter process is ABCA1-independent and seems to be mediated by the P-gp (P-glycoprotein, also named ABCB1) efflux pump.
These results highlight the importance of the BBB in the LXR-mediated effects in brain cholesterol metabolism and in Aß peptide clearance and, thus, in AD and reinforce the notion that the BBB may be a therapeutic target in neurological diseases.

AN EFFICIENT TOOLKIT TO DETECT SEQUENCE VARIATIONS OF GENES INVOLVED IN LYSOSOMAL-AUTOPHAGIC PATHWAYS

DI FRUSCIO G.[1,2], SCHULZ A.[3], DE CEGLI R.[1], MUTARELLI M.[1], SAVARESE M.[1,2], SINGHMARWAH V.[1], FILOCAMO M.[4], DI BERNARDO D.[1], BANFI S.[1,2], BRAULKE T.[3], NIGRO V.[1,2] AND BALLABIO A.[1,5,6,7]

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[5] *Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, USA.*
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The aim of this study was to develop the first Next Generation Sequencing (NGS)-based workflow for the identification of exon-variations in genes involved in lysosomal function and autophagy. Our NGS-based workflow was designed using a Haloplex-based enrichment protocol, a method based on digestion, hybridization, and followed by an extension step, which we named Lysoplex, followed by sequencing by an IlluminaHiSeq platform. Lysoplex represents the most complete collection of genes involved in lysosomal function, autophagy and endocytosis to date. As proof of sensitivity and specificity, a set of 15 DNA samples from patients affected by several types of Lysosomal Storage Disorders (LSDs) was tested. We analyzed samples from patients with MPS I, MPS II, MPS IIIB, Fabry, NPC, Mucolipidosis III, Batten, Gaucher's, MSD, Pompe, Danon and MLD. In 13 of these patients causative mutations had been previously identified, while in the remaining two the mutations were unknown. Using our protocol we were able to detect all the known mutations and identified putative causative variations in the previously molecularly unresolved patients. Moreover, a subset of additional variations in other genes not directly correlated with the disease was also identified in each sample. The same strategy was also used to identify disease-causing mutations in approximately 50 patients clinically diagnosed as affected by neuronal ceroid lipofuscinoses (NCL). Preliminary results of this analysis will be presented at the meeting. The Lysoplex strategy is therefore a valid tool for the genetic diagnosis of lysosomal gene mutations. This strategy also provides a comprehensive view of variants in genes involved in the lysosomal-autophagic pathway. Finally, due to the crucial role that the lysosomal-autophagic pathway plays in health and disease (e.g. neurodegenerative diseases), Lysoplex can also be used to identify causative or predisposing mutations in a variety of debilitating human conditions such as common neurodegenerative diseases.

CLINICAL GENE THERAPY TARGETED TO THE BRAIN

TIMOTHY M COX
University of Cambridge, UK.

After four decades, gene therapy is now back on the clinical stage; but it is, nonetheless, surprising that the unique therapeutic position of the inborn errors affecting the lysosomal function has yet to receive the scientific attention it deserves.
Lysosomal diseases have always been at the focus of Brains4Brain and it is timely to review progress and the immediate future beyond animal experimentation and into patients.
While the biopharmaceutical industry re-sets its clinical priorities, and hitherto has provided great benefit for some orphan diseases, its commitment to gene therapy appears to be one of equivocation and paralysis.
While internal debates about "one-off cost models" continue, huge opportunities are overlooked. At the same time, academic investigators, who are usually slower to act, have kept the faith, so that sooner or later those who plough the fields will eventually reap rich therapeutic harvests.
Here, I will attempt to show the special opportunities that all in Brains4Brain share to develop gene therapy for patients with severe lysosomal conditions affecting the brain, and also highlight those areas which require intensive thought and further investigation.
Ahead lie the outcomes of several Phase I/II clinical trials, but a clinical trial remains an experiment and nothing more than a beginning, but without which, however, there would be little hope: "Now this is not the end. It is not even the beginning of the end. It is perhaps the end of the beginning".

THE LYSOSOME: FROM WASTE BAG TO POTENTIAL THERAPEUTIC TARGET

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Lysosomes are the major digestive compartment within cells and were long regarded as simple waste bags jeopardizing the cell survival if ruptured and their content released. Research during the last decades has, however, recognized them as advanced organelles that are involved in many cellular processes including degradation, cell death, plasma membrane repair and cholesterol homeostasis, which make them crucial regulators of cell homeostasis. The function of lysosomes is critically dependent on the acidic interior regulated by vacuolar H+-ATPase, soluble lysosomal hydrolases as well as lysosomal membrane proteins, LAMPs (lysosome associated membrane proteins).
Lysosome-mediated cell death has been extensively studied. The executors of lysosome-mediated apoptosis are specific lysosomal hydrolases, namely the cathepsins. A critical step in the cell death inducing process is the release of cathepsins from the lysosomal lumen to the cytosol. The cathepsins are often mediating the apoptosis signal upstream mitochondrial events and the proteolytic activation of the pro-apoptotic protein Bid has been identified as one possible mechanism of action. Cathepsins are released from lysosomes in a process known as lysosomal membrane permeabilization (LMP). The mechanism of LMP is poorly understood but seems to be heavily dependent on the composition of the lysosomal membrane. Reduction of the amount of LAMP-2 protein in the membrane is found to sensitize cells to LMP-mediated cell death. Moreover, the lysosomal cholesterol content also modulates the membrane stability. In fibroblasts from Niemann-Pick disease type C (NPC), one ofthe cholesterol transporting proteins NPC-1 or NPC-2 are mutated, which cause massive cholesterol accumulation within the lysosomes. NPC-1 fibroblasts show reduced LMP when exposed to lysosomotropic weak bases with apoptosis inducing ability. Reduction of the cholesterol content by treatment with methyl-β-cyclodextrin or 25-hydroxy cholesterol sensitizes the cells to a cell death-inducing stimuli.
Lysosomal dysfunction contributes to diseases, such as lysosomal storage disorders, neurodegenerative disorders and cancer and therapeutic interventions aiming at restoring lysosomal function may be useful for the treatment. Future therapeutic approaches could be to enhance lysosomal degradation activity by addition of certain small molecules to remove toxic deposits from cells and to retard the progression of devastating neurodegenerative diseases. Moreover, restoration of lysosomal acidification by therapeutic intervention could represent an efficient way to promote the degradation and clearance of accumulating macromolecules.

PATHOPHYSIOLOGY OF X-LINKED ADRENOLEUKODYSTROPHY

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Currently the molecular basis for the clinical heterogeneity of X-linked adrenoleukodystrophy (X-ALD) is poorly understood. The genetic bases for all different phenotypic variants of X-ALD are mutations in the gene encoding the peroxisomal ATP-binding cassette (ABC) transporter, ABCD1 (formerly adrenoleukodystrophy protein, ALDP). We show that ABCD1 transports CoA-activated very long-chain fatty acids from the cytosol into the peroxisome for degradation. Furthermore, we show that the peroxisomal β-oxidation defect inX-ALD is directly caused by ABCD1 dysfunction as blocking ABCD1 function with a specific antibody reduced peroxisomal β-oxidationin controls to levels observed in X-ALD patients.The phenotypic variability is remarkable ranging from cerebral inflammatory demyelination of childhood onset, leading to death within few years, to adults remaining presymptomatic through more than five decades. There is no general genotype-phenotype correlation in X-ALD. The default manifestation of mutations in ABCD1 is adrenomyeloneuropathy, a slowly progressive dying-back axonopathy affecting both ascending and descending spinal cord tracts, and causing sphincter disturbances as well as a peripheral neuropathy in some cases. In about 60% of male X-ALD patients, either in childhood (35-40%) or in adolescence and adulthood (20%), an initial, clinically silent, myelin destabilization converts to a devastating, rapidly progressive form of cerebral inflammatory demyelination. Here, ABCD1 remains a susceptibility gene, necessary but not sufficient for inflammatory demyelination to occur. Although the accumulation of very long-chain fatty acids appear to be essential for the pathomechanism of all phenotypes, the molecular mechanisms underlying these phenotypes are fundamentally different. Cell autonomous processes such as oxidative stress and energy shortage in axons as well as non-cell autonomous processes involving axon-glial interactions seem pertinent to the dying-back axonopathy. Various dynamic mechanisms may underlie the initiation of inflammation, the altered immune reactivity and the propagation of inflammation. When the main immune cells are investigated for metabolic defects macrophage/monocytes displayed the severest biochemical phenotype with a 6-fold accumulation of C26:0 a very long-chain fatty acids and a striking 70% reduction in peroxiosmal β-oxidation activity. In contrast, very long-chain fatty acid metabolism was close to control values in B cells and T cells. Thus, the vulnerability of the main immune cell types is highly variable in X-ALD. Based on these results, we propose that in X-ALD the halt of inflammation after allogeneic hematopoietic stem cell transplantation relies particularly on the replacement of the monocyte lineage. An improved understanding of the molecular mechanisms involved in these events is required for future development of urgently needed therapeutic strategies.

ALTERED FUNCTIONING OF THE BIOLOGICAL CLOCK IN HUNTER SYNDROME

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Hunter syndrome (HS) or Mucopolysaccharidosis type II (MPSII) is an hereditary X-linked recessive genetic disorder and a metabolic and lysosomal storage disease caused by mutations in the IDS gene, encoding the enzyme iduronate-2-sulfatase (IDS), which decrease or completely shut down the function of the IDS enzyme. The patients present with coarsening of facial features, bone and joint abnormalities, short stature, changes in the heart, respiratory system, hearing and vision, and in more severe forms with altered motor function, progressive learning difficulties and behavioral abnormalities. IDS deficiency causes loss of ability to break down and recycle the glycosaminoglycans heparan and dermatan sulfate, leading to cellular engulfment, impairment of cellular processes and cell death. Cellular processes, such as cell cycle, proliferation, DNA damage response, autophagy, apoptosis, metabolism, redox equilibrium and functioning of intracellular organelles are controlled by the biological clock. At the molecular level the biological clock functions by way of a transcriptional-translational feedback loop revolving in about 24 hours and working through a set of genes, defined clock genes, encoding circadian proteins that in turn suppress circadian gene expression. The loop is activated by the transcription factors CLOCK/NPAS2 and BMAL1/2 that heterodimerize and rhythmically activate the transcription of PER1, PER2, PER3, CRY1, CRY2, encoding PER and CRY proteins that upon posttranslational modification by Casein Kinase I-II move into the nucleus and suppress the transcriptional activity of the heterodimer. The biological clocks are entrained by an internal timing system synchronizing the oscillation frequency with 24-hour periodicity (circadian). The mammalian circadian timing system is composed by central oscillators in the suprachiasmatic nuclei (SCN) of the brain entrained to the environmental light/dark alternation through the retino-hypothalamic tract and timely organizing bodily functions via sympathetic and parasympathetic nerve fibers or hormones such as cortisol and melatonin. We evaluated the expression of clock genes and clock controlled genes in HS, and assessed the circadian pattern of variation and the effects of the treatment with IDS on the expression of clock genes and clock controlled genes at different time points taking advantage of an in vitro model of HS. Using Next Generation Sequencing we performed transcriptome analysis in fibroblasts from healthy subjects and fibroblasts from HS patients before, 24 hours and 144 hours after IDS treatment. We also assessed by qRT-PCR the expression of clock genes upon serum-shock induced synchronization in normal human fibroblasts and fibroblasts of patients affected by HS before and after 24 hours of treatment with IDS. The results show severe alteration of expression and uneven response to IDS treatment of many clock genes and clock controlled genes regulating vital cellular processes, such as DNA transcription, post-translational modification and degradation, lipid and glucose metabolism and biosynthetic pathways, molecular processing, molecular transport, DNA damage response, endoplasmic reticulum stress and unfolded protein response, xenobiotic response, autophagy, cell cycle control, and tissue processes, such as inflammation, hemocoagulation and fibrinolysis. A semantic hypergraph-based analysis highlighted five gene clusters significantly associated to important biological processes or pathways, and five genes, AHR, HIF1A, CRY1, ITGA5 and EIF2B3, proven to be central players in these pathways. After synchronization by serum shock and 24h treatment with IDS the expression of ARNTL2 at 10h ($p=0.036$), PER1 at 4h ($p=0.019$), PER2 at 10h ($p=0.041$) and 16h ($p=0.043$) changed in HS fibroblasts. In conclusion, in human HS fibroblasts the expression of circadian genes is changed and some modifications in the expression levels are evidenced upon IDS treatment suggesting a direct involvement of the biological clock in the pathological mechanisms underlying HS.

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THE LINK BETWEEN GAUCHER & PARKINSONISM

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The association between mutations affecting glucocerebrosidase (GBA1) and Parkinson's Disease (PD) is well documented. It is also becoming apparent that a range of diverse mechanisms are being proposed to explain the link between loss of lysosomal function and PD. However, not all individuals harbouring GBA1 mutations necessarily go on to get PD. Other disease modifying factors are therefore likely to be involved. Amongst these is the potential involvement of the non-lysosomal glucocerbrosidase (GBA2). Recently we have demonstrated that GBA2 activity is particularly high in brain and that GBA1 deficient mice have significantly elevated levels of GBA2 [1]. With regards to Gaucher patients, we also demonstrated, in a proportion of patients, increased leukocyte GBA2 activity. However, for some individuals there was no increase in activity [1]. Whether GBA2 can substitute, in some patients, for GBA1 and minimise disease severity and PD risk in not yet known. Considering further the potential role of GBA1 in the neurodegenerative process, we have assessed GBA1 activity in neuronal and glial cells. Our data show that GBA1 activity is significantly greater in neuronal cell lines when compared to an astrocyte cell lines. This result may point to a particular requirement of neuronal cells for GBA1 and raises the possibility that loss of activity could be particularly detrimental to this cell type. Intriguingly, data are now emerging to suggest that loss of GBA1 activity may occur in PD, irrespective of whether a patient has a GBA1 mutation or not [2].

Loss of mitochondrial function has been implicated in the pathogenesis of PD. The mechanisms responsible for this loss are not known. Recently, data from the GBA1 deficient mouse has shown significant losses of brain mitochondrial complex activities [3]. These findings therefore illustrate an intriguing link between compromised lysosomal GBA1 activity and energy metabolism.

PD is associated with loss of brain dopaminergic function. Identifying pathogenic mechanisms associated with PD should therefore have some focus on the metabolism of this and other neurotransmitters such as serotonin. There are few data reporting the integrity of neurotransmitter (dopamine and serotonin) metabolism in Gaucher. However, we have demonstrated undetectable levels of dopamine and serotonin metabolites in the cerebrospinal fluid of a 66 year old male patient with Gaucher/PD [4].

The association between loss of GBA1 activity and the risk of developing PD is a fine example of a rare disease providing valuable insight into the pathogenesis of a more common disorder. However, it is becoming increasingly clear that if new disease mechanisms are to be identified and effective treatments developed an integrated approach to evaluating brain metabolism in a GBA deficiency state needs to be adopted.

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THE X-RAY CRYSTALLOGRAPHIC STRUCTURE AND ENZYMATIC MECHANISM OF α-L-IDURONIDASE AND ITS RELATIONSHIP TO MUCOPOLYSACCHARIDOSIS I

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Mucopolysaccharidosis (MPS) I is a lysosomal storage disease caused by a deficiency of α-L-iduronidase (IDUA) (EC 3.2.1.76); currently, enzyme replacement therapy (ERT) is the conventional treatment for this genetic disease. We have developed a seed-based platform in complex glycan (cgl) mutant seeds of Arabidopsis thaliana for the production of recombinant human IDUA for structural studies and the potential treatment of MPS I. Enzyme kinetic data showed that cgl-IDUA has similar enzymatic properties to the commercially available recombinant IDUA derived from cultured Chinese hamster ovary (CHO) cells(AldurazymeTM). Using the techniques of X-ray Crystallography, our group has recently determined the 3D structure of human α-L-iuronidase alone and bound by three active site directed inhibitors. IDUA has three prominent domains: a TIM (βα)8 barrel extending from Arg48 to Ala394 that contains the active site residuesGlu182 and Glu299; a β-sandwich domain (Glu398 to Ala542) that contains the N terminus of the enzyme and the first β-strand, β1 (His30 toLeu43); and a third domain (Thr552 to Glu640) that resembles the fold of a type III fibronectin domain and consists of the C-terminal residues of the enzyme. Through crystal soaking and co-crystallization, we obtained structures of complexes of IDUA with each of the three substrate analog inhibitors5-fluoro-α-l-idopyranosyluronic acid fluoride (5F-IdoAF), 2-deoxy-2-fluoro- α-l-idopyranosyluronic acid fluoride (2F-IdoAF) and [2R, 3R, 4R, 5S]-2-carboxy-3,4,5-trihydroxy-piperidine (IdoADNJ).The refined electron density maps clearly revealed that both 5F-IdoAF and IdoADNJ are bound in a2So skew-boat conformation in the active site of IDUA, most likely mimicking an enzyme-substrate Michaelis complex in the normal catalytic pathway. The 2F-IdoAF molecule forms a covalent adduct with the carboxylate group of Glu299, and the resulting 2F-IdoA adduct adopts a distorted 2,5B conformation, thereby illuminating the key glycosyl-enzyme intermediate on the catalytic pathway.

IDUA is anα-retaining glycoside hydrolase with strict substrate specificity for l-iduronate (l-IdoA). As shown for many other configuration-retaining glycosidases, the hydrolysis of polysaccharide substrates by IDUA most likely proceeds through a double displacement mechanism. Our crystal structures have confirmed that the general acid/base in this reaction is Glu182. Furthermore, the covalent complex of IDUA–2F-IdoA formed with Glu299 is consistent with the general catalytic mechanism of retaining glycosidases. To our surprise, the N-glycan attached to Asn372 interacts with the iduronate analogs bound in the active site of IDUA and is required for enzymatic activity.The enzymatic activityof the deglycosylated IDUA (IDUA treated with carbohydrate binding module-peptide N glycosidase F (CBM-PNGase F)) was only 11.2% of that of the untreated (glycosylated) IDUA. The Km and Vmax, of deglycosylated IDUA were roughly three times and 50% of the Km and Vmax of the untreated IDUA, respectively, suggesting that N-glycosylation is critical for IDUA activity.

Our present structures of human IDUA offer, for what is to our knowledge the first time, insights into the association between the clinical phenotypes and defects in the IDUA structure caused by the corresponding missense mutations. As an example of this, we will discuss some recent results obtained for the effects that the P533R mutation has on the IDUA structure and enzymatic activity.

NRF2-MEDIATED NEUROVASCULAR PROTECTION AGAINST OXIDATIVE STRESS IN EXPERIMENTAL STROKE

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Disruption of the blood-brain barrier (BBB) and cerebral edema are the major pathogenic mechanisms leading to neurological dysfunction and death after ischemic stroke. The brain protects itself against infarction via activation of antioxidant defense mechanisms, and we recently reported that sulforaphane induced activation of the redox sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream target heme oxygenase-1 (HO-1) in the cerebral vasculature is critical for preventing brain injury after stroke. Sprague-Dawley rats were subjected to 70 min middle cerebral artery occlusion (MCAo) followed by 4, 24 or 72 h reperfusion. HO-1 was predominantly induced in the peri-infarct region after transient MCAo and preferentially associated with perivascular astrocytes rather than the cerebrovascular endothelium. Nrf2 levels were upregulated in cerebral microvessels of peri-infarct regions after 4-24 h. Pretreatment with sulforaphane for 1 h led to a rapid increase in Nrf2 expression with levels declining over 4 - 24 h. Upregulation of Nrf2 by sulforaphane treatment prior to MCAo was associated with increased HO-1 expression in peri-vascular astrocytes within peri-infarct regions and in the cerebral endothelium within the infarct core. BBB disruption, lesion progression analysed by MRI, and neurological deficits were reduced by sulforaphane pretreatment. Sulforaphane pretreatment led to a moderate increase in peroxynitrite generation, suggesting that hormetic preconditioning underlies sulforaphane-mediated protection against stroke. We propose that pharmacological and/or dietary interventions aimed to precondition the brain via activation of the redox sensitive Nrf2 defence pathway in the brain microvasculature provides a novel therapeutic approach to prevent BBB breakdown and neurological dysfunction in stroke.

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NEW APPROACHES FOR THE TREATMENT OF NPC1

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Niemann-Pick type C (NPC) disease is an autosomal recessive, neurodegenerative lysosomal storage disorder (LSD) that occurs at an estimated frequency of 1:120,000 live births. Clinical features include hepatosplenomegaly in infants, eye movement abnormalities, dysphagia, dysarthria, ataxia and cognitive decline leading to dementia. The cellular pathology of NPC is complex involving the storage of multiple classes of lipids (including cholesterol and sphingolipids), defects in lysosomal calcium regulation that results in a block in late endosome:lysosome fusion. Miglustat, an inhibitor of glycosphingolipid biosynthesis modifies the course of NPC disease in patients and is approved (EMA) for treating CNS manifestations of this disease.

NPC disease is caused by mutations in two genes, NPC1 or NPC2. Mutations in the NPC1 gene are the most common and account for approximately 95% of cases. The disease is clinically and cell biologically indistinguishable irrespective of which gene is defective. This has led to the hypothesis that the NPC1 and NPC2 proteins function in a common cell biological pathway. NPC1 is a transporter localized to the limiting lysosomal membrane where as NPC2 is a soluble mannose-6-phosphorylated lysosomal protein. Both proteins bind cholesterol but whether they function primarily in cholesterol transport or are cholesterol regulated and serve another transport function remains unresolved.

The pathogenic cascade in NPC is particularly complex and has led to the discovery of multiple potential clinical intervention points that can be targeted with a range of different therapies. I will discuss the current status of experimental therapies for NPC and discuss the need for combination therapy in this severe progressive neurodegenerative disorder.

RIPK3 AS A NOVEL POTENTIAL THERAPEUTIC TARGET FOR GAUCHER DISEASE

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Gaucher disease, an inherited metabolic disorder caused by mutations in the glucocerebrosidase (GBA) gene, is the most common lysosomal storage disease. Heterozygous mutations in GBA are a major risk factor for Parkinson's disease. Gaucher disease is divided into three clinical sub–types based on the absence (type 1) or presence (types 2 and 3) of neurological signs. Type 1 Gaucher disease was the first lysosomal disease for which enzyme therapy became available, and although infusions of recombinant glucocerebrosidase ameliorate the systemic effects of Gaucher disease, lack of efficacy for the neurological manifestations, along with the significant expense and inconvenience of enzyme therapy for patients, renders the search for alternative or complementary therapies paramount. Glucosylceramide and glucosylsphingosine accumulation in the brain leads to massive neuronal loss in neuronopathic Gaucher disease patients and in neuronopathic Gaucher disease mouse models. However, the mode of neuronal death is not known. I will discuss our recent data (Vitner et al., Nat Med. 2014 Jan 19. doi: 10.1038/nm.3449)that shows that modulating the receptor–interacting protein kinase 3 (Ripk3) pathway markedly improves neurological and visceral disease in a mouse model of Gaucher disease. Importantly, Ripk3 deficiency dramatically improved the clinical course of Gaucher disease mice with increased survival, motor coordination and salutary effects on cerebral as well as hepatic injury.

NOVEL INHIBITORS OF GCASE AS PHARMACOLOGICAL CHAPERONES FOR TYPE 1 AND 2 GAUCHER DISEASE

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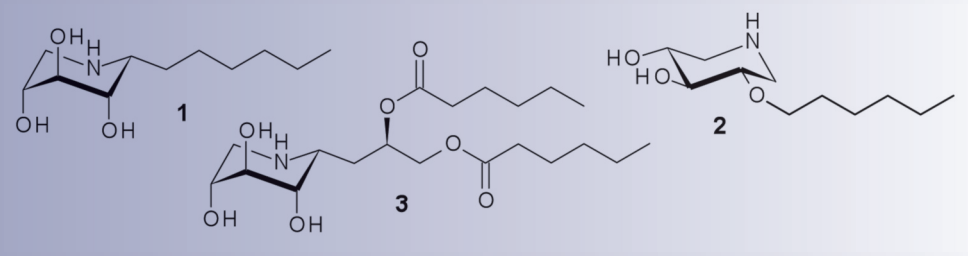
Pharmacological Chaperone Therapy (PCT) is gaining increasing attention as a promising approach to treat rare metabolic diseases such as, in particular, LSDs. The potential of using small organic 'correctors' for the treatment of these genetic diseases is extremely attractive both in terms of efficiency (high potential for BBB crossing) and costs. Such molecules are designed to have a high affinity for the protein, and are most frequently potent inhibitors of the deficient enzyme. This approach has already met with significant success: a drug candidate, 1-deoxy-galactonojirimycin (migalastat) is undergoing phase III clinical trials for Fabry disease, a LSD due to the deficiency of a-galactosidase A. A great deal of effort has been dedicated to the search of PCs for Gaucher disease (GD) in recent years. GD, which is the most prevalent LSD, is caused by a mutation in the gene encoding for b-glucocerebrosidase (GCase); this enzyme is responsible for the hydrolysis of a-glucosylceramide (GlcCer) into glucose and ceramide. Mutations in the GCase gene lead to aberrant folding in the ER, resulting in impaired trafficking and degradation of the defective protein by the ER quality control system. While a large number of mutations have been identified, those involving the N370S or L444P single amino acid exchange are by far the most common. Both of these defective enzymes exhibit residual hydrolytic activity (30% and 10-12% respec-tively) which is a requirement for PCT to be effective. Gaucher disease is characterized by three clinical subtypes: type 1 (bone pain, skeletal lesions, anemia and enlarged liver or spleen), is due largely to the N370S mutation; types 2 and 3 are neuronopathic forms of the disease, type 2 being an acute, infantile form with a median patient lifespan of 9 months while type 3 is more viable. The L444P allele is most frequently associated with these neuronopathic forms. Our investigations have shown that α-1-C-alkyl derivatives of imino-D-xylitol (DIX) such as 1 are potent inhibitors of β-glucocerebrosidase (GCase) and act as effective pharmacological chaperones of the N370S mutant form of GCase from Gaucher disease patients. [1] Shifting the alkyl chain from the pseudo-anomeric position to the O-2 of the DIX backbone, such as in 2, led to very active and more easily accessible iminosugars. [2] We have further explored the field of "iminoglucolipids" (*gluco*-like iminosugars carrying a lipophilic substituent) with the goal of finding new derivatives with improved biological activity towards mutant GCase. We have thus prepared a series of DIX derivatives carrying a ceramide-like aglycone, which are much closer glucosyl-ceramide analogues, such as 3. These new compounds were found to be extremely po-

tent GCase inhibitors (nM range) and to exhibit significant and selective chaperone effects, including on L444P GCase.



The design of these compounds and the results of biological investigations will be described in the presentation.

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MURINE NEURAL STEM CELLS MODEL HUNTER DISEASE IN VITRO: GLIAL CELL-MEDIATED NEURODEGENERATION AS A POSSIBLE MECHANISM INVOLVED

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Mucopolysaccharidosis type II (MPS II or Hunter syndrome) is a lysosomal storage disorder (LSD) caused by the deficit of iduronate-2-sulfatase (IDS) activity and characterized by progressive systemic and neurological impairment, although the causes underlying the devastating neurodegenerative phenotype remain unclear. Since endogenous neural stem cells (NSCs) are essential for central nervous system homeostasis and no data is currently available on the NSC compartment of MPS II patients, the aim of our study was to isolate and characterize NSCs from the subventricular zone (SVZ) of IDS-knockout (IDS-ko) mice in order to establish an in vitro model of the disease and to shed light on the mechanisms underlying the development of the neuropathology. Results of our in vitro analysis have been confirmed by parallel in vivo evaluations, both performed in IDS-ko mouse brain sections and, most importantly, for the first time conducted on a Hunter patient brain.
Our study has elucidated some pivotal steps of MPSII pathogenesis through the main following findings:

1) by using NSCs from adult IDS-ko mouse brain at an early symptomatic stage, as a tool to model MPSII disease ex vivo, we showed that IDS deficit appears not to affect their proliferation, but the late differentiation of glial progenitors and the neurogenesis of the post-natal brain in IDS-ko mice.

2) Together with recapitulation of MPSII disease *ex vivo* by exploiting NSCs, we have provided a previously undocumented characterization of the IDS-ko adult mouse brain and a unique parallelism with the analysis of a human brain from a Hunter patient. We showed that glial degeneration likely precedes that of neuronal cells. In particular, a reduced percentage of PDGFR-a+ cells is present in the IDS-ko mouse and Hunter human brains as in IDS-ko NSC-derived progeny, suggesting a potential mechanism for the development of the MPS II pathology.

3) We were able to modulate the mutant phenotype of IDS-ko NSCs in vitro by enzyme supplementation or PDGF treatment, suggesting NSCs as a valid tool for drug screening and PDGF, or other "gliotrophines", as candidate molecules for a combined therapeutic approach to MPS II.

To note, our study is the first report suggesting a possible abnormality of NSC-derived glial progenitor differentiation in Hunter disease and demonstrating the unique promise of NSCs for evaluations on pathogenesis and treatment of LSDs. The similarity of brain tissue disorganization evidenced in both the IDS-ko mouse model and the human Hunter brain emphasizes the clinical relevance of our study and the reliability of the IDS-ko animal model for neuropathogenetic evaluations.
Importantly, MPS II shares many features with more common neurodegenerative disorders such as Parkinson's and Alzheimer's disease, thus, preclinical research on MPS II could also be helpful to unravel the pathophysiology of neurodegenerative diseases with higher prevalence.

DELIVERING ANTIBODY THERAPEUTICS ACROSS THE BLOOD-BRAIN BARRIER

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Receptor-mediated transport is a promising approach for boosting the delivery of therapeutic antibodies to the brain. We have shown that reducing the affinity of an antibody targeting the transferrin receptor (TfR) can substantially increase its uptake in brain. Bispecific antibodies were generated that target both TfR and β-secretase (BACE1), an enzyme that cleaves amyloid precursor protein leading to the generation of beta amyloid (Aβ) peptides associated with Alzheimer's disease. A single systemic injection can significantly accumulate in the brain and reduce Aβ in the brain of both wild-type and transgenic mouse models of Alzheimer's disease. We explore the potential therapeutic utility of this approach with additional anti-TfR/BACE1 affinity variants. Whereas administration of high affinity anti-TfR/BACE1 decreases total brain TfR expression, lower affinity anti-TfR/BACE1 does not affect TfR levels. Furthermore, lowering the affinity to TfR also improves pharmacokinetics of anti-TfR/BACE1 in vivo. Cellular studies utilizing live imaging and colocalization revealed that high affinity TfR bispecific antibodies accelerated trafficking of TfR to lysosomes, thus actively driving degradation of TfR. These studies suggest that high affinity TfR antibodies may modulate trafficking and stability of TfRs in vivo, which may dramatically impact its capacity for BBB transport and therapeutic utility.

PERINATAL GENE THERAPY RESCUES ACUTE NEONATAL LETHAL NEURONOPATHIC GAUCHER DISEASE IN MICE

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Background
Type II Neuropathic Gaucher Disease (nGD) is caused by mutation of the glucocerebrosidase gene. Patients exhibit acute and aggressive neurodegeneration and die before two years of age; no treatment exists. A neonatal lethal mouse model of nGD exhibits neurodegeneration and dies before 14 days of age. We conducted a temporospatial analysis of disease progression in the brain. We used this data to measure the efficacy of gene therapy in utero and in neonatal mice.

Methods
Brains from wild type, heterozygous and knockout nGD mice were collected at 1 day of age (P1), P9 (pre-symptomatic) and P12 (symptomatic). Microglial activation, astrogliosis, lysosomal content and neurodegeneration were measured.
For rescue of the model, adeno-associated virus serotype 9 carrying therapeutic glucocerebrosidase gene was administered to the brains of mice by intracranial injection at E15 gestation or on P1. Survival, behavior and indices of brain pathology were analysed.

Results
Microglial activation and astrogliosis at P1 in the brain stem of KO mice was observed. Global and progressive spread of CNS pathology was evident at P9 and P12; the most affected regions were the brain stem, VPL/VPM and layer V of the cortex. This was accompanied by severe and rapid neurodegeneration.

A single injection of AAV9 vector in utero dramatically ameliorated astrogliosis, microglial activation, lysosomal accumulation and neuronal loss and extended the lifespan of all treated KO mice by at least nine-fold (day 130, n=5, $p<0.005$). Nevertheless performance on rotarod and foot-fault tests was subnormal. Neonatal gene therapy was equally effective; treated knockouts were fertile and we have been able to maintain the mice as a colony of treated knockouts. As expected, visceral pathology remained uncorrected by the intracranial treatment.

Conclusion
Perinatal gene therapy is successful in profoundly ameliorating an early neonatal lethal mouse model of nGD. To date, this is the most severe model of a neurodegenerative lysosomal storage disorder to be rescued by gene therapy.

CLAUDIN PEPTIDOMIMETICS AS POTENTIAL DRUG ENHANCERS FOR THE BLOOD-BRAIN BARRIER

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We found two claudin (Cld) petidomimetics as potential drug enhancer of the blood-brain barrier (BBB) formed by endothelial cells. Clds are transmembrane proteins of the tight junctions (TJs) limiting the permeation of solutes through the intercellular space between the cells. The tightening is mainly achieved by interaction of the extracellular loops of Clds from two opposing cells. Consequently, modification of the integrity Clds by peptidomimetics is a promising strategy to enhance drug delivery through the BBB. Cld1- and Cld5-derived peptides, C1C2 and Pep5, respectively, were analyzed concerning their barrier modulating properties in vitro and in vivo. C1C2 transiently increased the permeability in a murine cell culture model of the BBB as well as monolayers of human colorectal adenocarcinoma and Madin-Darby canine kidney cells. Redistribution of the staining of different Clds form cell-cell contacts to a more intracellular localization suggested an interaction of C1C2 with various Clds. Further analysis with TJ-free human embryonic kidney-293 cells, transfected with Cld1, -2, -3, -4 or -5-YFP, identified Cld1 and Cld5 as targets of C1C2. Binding measurements with full-length Clds and recombinant extracellular loops confirmed these findings. Freeze-fracture electron microscopy revealed alteration in the TJ-architecture of Cld5-YFP by a drastic P- to E-face transition, whereas the TJ network of Cld1-YFP was altered in shape with increased number of parallel TJ strands. Pep5 increased the permeability of rat brain endothelial cell monolayer. Transmission electron microscopy showed opening of interendothelial TJs. The binding of Pep5 to Cld5-YFP showed a high affinity to Cld5. Pep5 injected i.v. in mice monitored via magnetic resonance imaging, resulting in a concentration dependent opening of the BBB. Similarly, the blood-retina barrier showed increased permeability and reduction of Cld5 after peptide treatment. In summary, the Cld peptidomimetics C1C2 and Pep5 transiently increase the paracellular permeability of Cld1 and Cld5 expressing cell barriers by affecting the TJ localization and network structure. These findings recommend Cld peptidomimetics as potential candidates to improve drug delivery to the central nervous system.

METABOLIC AND CELLULAR BASES OF SPHINGOLIPIDOSES

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Lysosomes are cellular stomachs. They degrade macromolecules and release their components as nutrients into the cytosol. Digestion of sphingolipids and other membrane lipids occurs at luminal, intraendosomal vesicles and membranes (IMs). Sphingolipid and membrane digestion needs catabolic hydrolases with the help of lipid-binding proteins (SAPs) and anionic lipids such as bis(monoacylglycero)phosphate (BMP). Inherited defects of hydrolases or SAPs or uptake of cationic amphiphilic drugs cause lipid accumulation, eventually leading to death, especially in inherited sphingolipid storage diseases.
IMs are formed during endocytosis and their lipid composition is adjusted for degradation. Their cholesterol content, which stabilizes membranes, decreases and the level of negatively charged BMP, which stimulates sphingolipid degradation, increases. At the level of late endosomes, cholesterol is transported out of the luminal vesicles preferentially by cholesterol binding proteins, NPC-2 and NPC-1. Their defects lead to an endolysosomal accumulation of cholesterol and sphingolipids in Niemann-Pick disease type C.
Anionic lipids (PA, PG, BMP) and ceramide stimulate NPC-2 mediated cholesterol transfer and sphingomyelin inhibits. Anionic membrane lipids also activate sphingomyelin degradation by acid sphingomyelinase facilitating cholesterol export by NPC-2. ASM is a non-specific phospholipase C and degrades more than 23 phospholipids.
SAPs are membrane-perturbing proteins which, activated by anionic membrane lipids, solubilize lipids, facilitate glycolipid digestion by presenting them to soluble catabolic enzymes at acidic pH values. High BMP and low cholesterol levels favour lipid extraction and membrane disintegration by saposin A and B. The simultaneous inherited defect of saposins A-D causes a severe membrane and sphingolipid storage disease, also disrupting the water permeability barrier of the skin.

INHERITED NEUROMETABOLIC DISEASES INFORMATION NETWORK

MAURIZIO SCARPA
Project Coordinator and President of the Brains for Brain Foundation, Main Project Partner.
FEDELE BONIFAZI
Project Manager and vice-President of the Gianni Benzi Foundation.

Brains for Brain Foundation c/o Dept. of Pediatrics University of Padova Italy.

The Project "Inherited NeuRoMetabolic Disease Information Network" (InNerMeD-I-Network), is an European project funded by the Directorate-General for Health & Consumers (DG SANCO) within the Second Health Programme of the European Commission(2012 12 12, Second Health Programme 2008-2013). It started on the 1stof April 2013 but was officially kicked off in Luxemburg on May 21st.

InNerMeD-I-Network represents the first European Information Network on Inherited NeuroMetabolic Diseases (iNMDs).

iNMDs are a group of rare genetic metabolic diseases that impact on the brain causing mental retardation and progressive neurodegeneration which, if not promptly treated, could end in early death.
Lack of information on these conditions can lead to delayed diagnosis and treatment, withconsequent tragic results. Increasing awareness is therefore the first crucial step in fighting these pathologies.
InNerMeD-I-network wants to create a network of information targeted on diagnosis and treatment of iNMDs based on the collection and exchange of proper and validated information among scientific communities, health professionals, patients, patient associations and all interested stakeholders. The project aims to increase current knowledge on iNMDs and speed up the timely and precise identification of patients to which apply the available treatments. The network will also favour biomedical research, straightening research capacities and fostering innovative therapeutic tools derived from the recent scientific advancements based on biomarkers use and personalised approaches.
Brains for Brain Foundation, coordinating partner of the project, will particularly assure the partners coherence and capacity to share decisions and to put together their relevant knowledge and research capacity.
Partnership also includes 5 both public and private international clinical centres: Gianni Benzi Foundation (Italy), Copenhagen University Hospital (Denmark), University of Mainz (Germany), University of Zagrebu (Croatia) and Hospital Sant Joan de Déu (Spain), together with 15 collaborating partners. Thanks to partners specific expertise, InNerMeD-I networking will create a formidable concentration of competences in such a complex and heterogeneous medical field.

EUROPEAN REGULATION ON CLINICAL TRIAL AND DRUG DEVELOPMENT IN NEUROMETABOLIC DISORDERS

ADRIANA CECI
Gianni Benzi Pharmacological Research Foundation

The European scenario of clinical trials is going to change, as demonstrated by the Proposal of a new European Regulation on Clinical Trials repealing the current Directive 2001/20/EC. This Proposal aims to overcome the existing drawbacks of clinical research in Europe, by proposing the simplification of procedures and reduction of costs, including the creation of a centralised supranational systems responsible of assess trials in term of 'risk level', distinguishing procedures for "low-interventional clinical trial" from other trials. The expected positive results are to reduce delays in the approval and to strength the biomedical research competitiveness in Europe.
This will strongly influence the clinical research in Europe, including the research in 'small populations', such as the paediatric and rare disease ones, that pay more difficulties in term of economic, ethical and methodological issues. However it is to be recommended that the existing requirements of the European Paediatric Regulation EC 1901/2006 and of the EC Paediatric Ethical Recommendations (2008) would be integrated in the Regulation, that will be voted by the European Parliament next April.
In this context, it should be considered that inherited neurometabolic disorders are rare, life-threatening, paediatric, genetic diseases for which the research activities are very challenging. Their development also refers to the innovative therapies and orphan drugs requirements. The current status of both the approved drugs and the planned research in the field suggests that more incentives are needed.
In December 2013, Horizon 2020 calls for proposals have been launched. An ad hoc call is addressed to speed up research and development of new or improved therapeutic approaches, namely PHC-14-2015 "New therapies for rare diseases". The preclinical research, animal model development, GMP production, as well as clinical trials will be supported.
Therefore, Horizon 2020 appears as a good chance for companies, researchers, clinicians, and patients to develop drugs for these rare conditions.

AUGMENTING CNS GLUCOCEREBROSIDASE ACTIVITY AS A THERAPEUTIC STRATEGY FOR PARKINSONISM AND OTHER GAUCHER-RELATED SYNUCLEINOPATHIES

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Mutations of GBA1, the gene encoding glucocerebrosidase, represent a common genetic risk factor for developing the synucleinopathies Parkinson's disease (PD) and dementia with Lewy bodies (DLB). PD patients with or without GBA1 mutations also exhibit lower enzymatic levels of glucocerebrosidase in the central nervous system (CNS), suggesting a possible link between the enzyme and the development of the disease. Previously, we have shown that early treatment with glucocerebrosidase can modulate a-synuclein aggregation in a pre-symptomatic mouse model of Gaucher-related synucleinopathy ($Gba1^{D409V/D409V}$) and ameliorate the associated cognitive deficit. To probe this link further, we have now evaluated the efficacy of augmenting glucocerebrosidase activity in the CNS of symptomatic $Gba1^{D409V/D409V}$ mice and in a transgenic mouse model overexpressing A53T a-synuclein. Adeno-associated virus-mediated expression of glucocerebrosidase in the CNS of symptomatic $Gba1^{D409V/D409V}$ mice completely corrected the aberrant accumulation of the toxic lipid glucosylsphingosine and reduced the levels of ubiquitin, tau and proteinase-K-resistant a-synuclein aggregates. Importantly, hippocampal expression of glucocerebrosidase in $Gba1^{D409V/D409V}$ mice (starting at 4 or 12 months of age) also reversed their cognitive impairment when examined using a novel object recognition test. Correspondingly, overexpression of glucocerebrosidase in the CNS of A53T a-synuclein mice reduced the levels of soluble a-synuclein, suggesting that increasing the glycosidase activity can modulate a-synuclein processing and may modulate the progression of a-synucleinopathies. Hence, increasing glucocerebrosidase activity in the CNS represents a potential therapeutic strategy for GBA1-related and non-GBA1-associated synucleinopathies, including PD.

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EighthEuropeanWorkshop

USING TARGETORS TO DIRECT AND DELIVER BIOLOGICS SUCH ERT TO THE CNS

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biOasis Technologies Inc. is a biopharmaceutical company focused on the treatment of CNS diseases and disorders. The Company is developing proprietary peptide vectors based on melanotransferrin (MTf , Transcend) for the delivery of therapeutics to the CNS.
Drug delivery into the CNS remains a significant challenge for clinical neuroscientists as most drugs show limited penetration in the CNS caused by the blood-brain barrier (BBB). The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions and express high levels of active efflux transport proteins. Specific receptors and transport systems are highly expressed at the BBB to provide essential substances to brain cells. These important characteristics provide a natural defense against toxic or infective agents circulating in the blood. Therefore, the development of new technology to cross the BBB for brain parenchyma uptake is of great interest for the treatments of neurological disorders and genetic diseases. We will summarize some if these technologies that use the endogenous receptors expressed at the BBB and show that therapeutic drugs such as biologics ''piggybacked'' as conjugates can be shuttled across the BBB for treatment of brain diseases. Specific description will be dedicated to the technology developed by biOasis Technologies (MTf) on the CNS delivery of biologics.
MTf is very rapidly transported across the BBB into the brain parenchyma following intravenous injection as demonstrated by fluorescent derivatives of MTf conjugated molecules. Using MTf-cy5.5, we show by fluorescence microscopy that MTf conjugates are rapidly and efficiently transported in the brain parenchyma and in the lysosomal compartment in neurons and astrocytes. Using antibodies labeled with fluorescent molecules we demonstrated that antibodies against Her2 (Traztuzumab), against ßA1-42 peptides (6E10) and other mAbs or alduryzyme (IDU) as an example of lysosomal enzymes have shown transport across the BBB in brain cells after conjugation to MTf.
BT2111 (MTf-Trastuzumab) and Trastuzumab were labeled with fluorescent markers, either Cy5.5 or rhodamine. The fluorescent conjugates were also injected IV into mice and the distribution of BT2111 and Trastuzumab to endothelial cells and brain parenchyma was compared with laser scanning confocal microscopy. To aid in the colocalization of BT2111 and Trastuzumab to the brain vasculature, mice were injected with tomato lectin-FITC or anti CD31 IgG-FITC prior to sacrifice. When BT2111 and Trastruzumab were injected into mice, laser scanning confocal microscopy showed increased distribution of BT2111 in the brain parenchyma when compared to trastuzumab. 10 to 15 times more Trastuzumab was delivered in the brain parenchyma when conjugated to MTf.In addition, using a mice model characterized by the formation of brain metastasis after intracardiac administration of MDA-MB 231BR we show that BT2111 is homogenously distributed in normal brain and can reach therapeutical concentration in the brain. 0.2 to 1.2 µM of Trastuzumab is reached with one tenth of the therapeutic dose injected in mice. Further studies have shown efficacy of BT2111 in decreasing the amount and size of brain metastasis in this mice model. It was found that BT2111 reduced the number of human HER2+ breast cancer metastases in the brain by 68% when compared to control animals. The tumours that remained after treatment were 57% smaller than those in controls. In contrast, Trastuzumab alone had no effect on reducing the number of metastases and was associated with only a slight reduction in tumour size when compared to untreated controls.
These studies provide the proof of concept that MTf can be used as a carrier capable of shuttling a variety of compounds ranging from small anti-cancer agent to larger biologics across the BBB into the brain parenchyma in therapeutic doses that enable treatment of neurological disorders.

MECHANISMS OF BLOOD BRAIN BARRIER BYPASS IN K16APOE MEDIATED LYSOSOMAL ENZYME DELIVERY TO THE BRAIN

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Delivery of enzyme replacement therapy to the brain and central nervous system remains an area of great unmet medical need for many patients with lysosomal storage disorders. In the field, there has been significant interest in using Apolipoprotein E (ApoE) as a carrier to shuttle large molecules across the blood brain barrier via low density lipoprotein receptor mediated transport. The current studies aimed to determine 1) the amount of enzyme delivered to the brain using a novel ApoE peptide, K16ApoE, and 2) if the delivery was specifically mediated through receptor mediated transport or a more general permeability of the barrier. The results indicate that the K16ApoE peptide doubles the amount of enzyme delivered to the brain of normal mice from a single intravenous dose in vivo. Data from in vitro and in vivo studies suggest that this enhancement of delivery may result from a general increase in permeability of the blood brain barrier instead of, or in addition to receptor mediated transport. These data invoke an interesting discussion around the specificity of blood brain barrier bypass strategies and the risk/benefit balance of compromising the barrier to deliver drugs.

NOTES

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