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**PROCEEDINGS OF THE  
24<sup>TH</sup> NATIONAL CONGRESS  
“GRUPPO ITALIANO PER LO STUDIO  
DELLA NEUROMORFOLOGIA” G.I.S.N.**

*Bologna, November 28-29, 2014  
Accademia delle Scienze dell'Istituto di Bologna  
Università di Bologna  
Via Zamboni, 31*

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# *European Journal of Histochemistry*

## *a journal of functional cytology*

The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published until 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the University of Pavia and of the Ferrata Storti Foundation, Pavia, Italy.

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The Journal publishes Original Papers, Technical Reports, Reviews, Brief Reports, Letters to the Editor, Book Reviews, Views and Comments, concerning investigations performed with the aid of biophysical, biochemical, molecular-biological, enzymatic, immunohistochemical, cytometric, and image analysis techniques.

Areas of particular interest to the *European Journal of Histochemistry* include:

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*In memoriam Prof. Ruggero Bortolami*

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## MAIN LECTURES

### GENE THERAPY FOR MITOCHONDRIAL NEUROGASTROINTESTINAL ENCEPHALOMYOPATHY (MNGIE)

R. Martí

*Research Group on Neuromuscular and Mitochondrial Disorders, Vall d'Hebron Research Institute, Barcelona, Spain*

MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) is a mitochondrial disorder caused by mutations in the nuclear gene *TYMP*, encoding thymidine phosphorylase (TP). In recent years, knowledge gained from basic research on the biochemical mechanisms involved in this disorder has allowed us to design plausible therapy approaches. In MNGIE patients, TP dysfunction leads to systemic overload of the nucleosides thymidine and deoxyuridine, which results in alteration of the homeostasis of mitochondrial deoxyribonucleoside triphosphate (dNTP) pool. This imbalance interferes the correct replication of mitochondrial DNA (mtDNA). As a consequence, mtDNA depletion, multiple deletions and somatic point mutations occur in several tissues in patients, ultimately leading to mitochondrial dysfunction. As the clinical phenotype of MNGIE is the result of the toxic accumulation of thymidine and deoxyuridine, therapy approaches have focused on clearing the systemic overload of these nucleosides. First attempts to use hemodialysis failed to reduce nucleoside overload because of the high rate of endogenous production of these compounds by human metabolism. By contrast, hematopoietic stem cell transplantation restored nucleoside homeostasis in patients with successful engraftment, and led to slow clinical improvement. However, the high morbidity and mortality rates associated to the procedure encouraged us to find alternatives, and the most obvious one, gene therapy, has given very promising results in a murine model of the disease. Two different vectors carrying the human *TYMP* gene (a lentiviral vector transduced to hematopoietic stem cells, and an adeno-associated virus vector with targeted expression in liver) have been tested in a murine model of the disease. In both cases, successful and long-term stable expression of the transgene was achieved, resulting in permanent reduction of nucleoside overload *in vivo*. These results indicate that gene therapy is a feasible option for MNGIE patients; therefore, clinical trials should be implemented to investigate the safety and efficacy of this option.

### ROLE OF CALLOSAL CONNECTIONS IN VISUAL CORTEX DEVELOPMENT AND PLASTICITY

M. Caleo

*CNR Neuroscience Institute, Pisa, Italy*

The mammalian visual cortex represents a paradigmatic model for the study of experience-dependent changes in neural connectivity. During a "critical period" in early life, the visual cortex matures its anatomical and functional properties, and this developmental process is tightly dependent upon visual experience. Sensitivity of visual cortical circuits to experience is strongly downregulated in adulthood. This seminar will focus on the role of callosal connections in experience-dependent plasticity during the critical period. I will review data showing that deprivation of sensory experience can modify the morphology of callosal fibres, thus altering the communication between the two hemispheres. More importantly, manipulation of callosal input activity during the critical period alters developmental maturation of functional properties in visual cortex, and modifies its ability to remodel in response to experience. I will also discuss recent data in rat visual cortex, demonstrating that the corpus callosum plays a role in binocularity of cortical neurons, and is involved in the plastic shift of eye preference that follows a period of monocular eyelid suture (monocular deprivation) in early age. Thus, experience can modify the fine connectivity of the corpus callosum, and callosal connections represent a major pathway through which experience can mediate functional maturation and plastic rearrangements in the visual cortex.

## THE G.I.S.N. RESEARCH GROUPS

### A PIONEER OF ITALIAN NEUROSCIENCE: THE ACADEMIC LIFE OF RUGGERO BORTOLAMI

P. Clavenzani<sup>1</sup>, R. Chiochetti<sup>1</sup>, A. Grandis<sup>1</sup>, C. Bombardi<sup>1</sup>,  
M. Mazzoni<sup>1</sup>, N. De Sordi<sup>1</sup>, F. Rambaldi<sup>1</sup>, R. De Giorgio<sup>2</sup>

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Ruggero Bortolami, *emeritus* professor of Systematic and Comparative Veterinary Anatomy of the University of Bologna, passed away in July 2014 after a long life spent loving from the bottom of his hearth anatomy and neuroscience in most of its aspects. He was born in Padua on June 30, 1926 and in 1949 he graduated with honors in Veterinary Medicine at the University of Bologna. In 1959 he became full professor of Veterinary Anatomy at the University of Sassari and in 1968 and then he moved to the University of Bologna as professor of Systematic and Comparative Veterinary Anatomy, a position he held until his retirement in 1999. During his long and prestigious career Prof. Bortolami studied several topics including proprioception of the eye muscles; the morphological and functional relationships between the trigeminal nerve and the motor nerves of the eye and the synapses of primary afferents neurons, *via* the ventral roots of the spinal cord. His activity resulted in about 350 papers mainly in international peer-reviewed journals. He was the Editor of the Italian version of the Robert Barone treatise of veterinary anatomy and co-Author of the volume dedicated to the central nervous system. He funded the Italian Association of Veterinarian Morphologists and became member of several national and international scientific societies, *e.g.*, the World Association of Veterinary Anatomists, of which he was Vice President from 1971 to 1973. We owe him a lot for launching and co-founding our beloved Italian Group for the Study of Neuromorphology (G.I.S.N.) that he continued to support in any circumstances, with his high reputation and strong intellectual energy. The GISN will always remember Prof. Bortolami forever for his outstanding scientific achievements and devoted passion for science to innovation and progression of comparative anatomy and neuroscience. We are proud to be in his scientific legacy.

## NEW ACQUISITIONS IN NEUROMORPHOLOGY

### A NEW APPROACH FOR BRAIN CLEARING: CLARITY METHOD

I. Costantini<sup>1</sup>, A.P. Di Giovanna<sup>1</sup>, A.L.A. Mascarò<sup>1</sup>,  
L. Silvestri<sup>1</sup>, M.C. Muellenbroich<sup>1</sup>, L. Sacconi<sup>1,2</sup>, F.S. Pavone<sup>1,2,3</sup>

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Light scattering inside biological tissue is a limitation for large volumes imaging with microscopic resolution. Based on refractive index matching, different approaches have been developed to reduce scattering in fixed tissue. High refractive index organic solvents and water-based optical clearing agents, such as Sca/e, SeeDB and CUBIC have been used for optical clearing of entire mouse brain. Although these methods guarantee high transparency and preservation of the fluorescence, though present other non-negligible limitations. CLARITY is a new method for tissue transformation that allows high transparency, whole brain immunolabelling and structural and molecular preservation. This method however requires a highly expensive refractive index matching solution limiting practical applicability to large volumes. In this work we investigate the effectiveness of a water-soluble clearing agent, to clear mouse and human brain. This agent does not quench the fluorescence signal, is compatible with immunostaining and does not introduce any deformation at sub-cellular level. Moreover it is a suitable agent to perform brain slicing during serial two-photon (STP) tomography thanks to his not viscous nature. In fact, by improving penetration depth it reduces tissue slicing, decreasing the acquisition time and cutting artefacts. The potential of this method has been explored by imaging blocks of dysplastic human brain transformed with CLARITY, immunostained and cleared. This clearing approach significantly expands the application of single and two-photon imaging, providing a new useful method for quantitative morphological analysis of structure in mouse and human brain.

## SESSION I SYSTEMATIC AND DEVELOPMENTAL NEUROMORPHOLOGY

### EXPRESSION OF THE CATION-CHLORIDE COTRANSPORTERS KCC2 AND NKCC1 IN CEREBRAL CORTEX AND THALAMUS DURING MURINE POSTNATAL DEVELOPMENT

A. Amadeo<sup>1</sup>, P. Aracri<sup>2</sup>, M. Ferrari<sup>1</sup>, M. Seghezzi<sup>1</sup>, M.E. Pasini<sup>1</sup>, S. Smondelli<sup>1</sup>, A. Becchetti<sup>2</sup>

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In the adult central nervous system GABA mediates fast inhibitory transmission, whereas during early development it is an excitatory transmitter. This functional shift occurs as a result of a precise balance between the expression of NKCC1, which mediates chloride influx, and that of the KCC2 cotransporter, the major chloride extruder in mature neurons. This mechanism concurs in controlling morphogenesis and in shaping neural circuits. In several pathological conditions associated with hyperexcitability, such as epilepsy, suppression of KCC2 may contribute to alter the balance of excitation and inhibition especially during development. We studied the expression of NKCC1 and KCC2 in two representative areas of neocortex, somatosensory and prefrontal (PFC), and in the dorsal thalamus, at different postnatal stages by western blot and immunocytochemistry. In the first two postnatal weeks, we observed a conspicuous and increasing expression of NKCC1 and KCC2 in the two cortical areas, whereas a modest progressive decrease of the expression only of KCC2 was observed in the whole thalamus. By immunocytochemistry, the neuropil of thalamic nuclei (mainly the sensory ones) appeared intensely labelled for both cotransporters, except for KCC2 in the reticular nucleus. Lower expression was observed in cortical areas, with PFC displaying the lowest signal at birth. NKCC1 was distributed both in the different compartments of neuronal cells and in astrocytic glia. In the first postnatal week, KCC2 was mainly localized in the cell bodies of cortical GABAergic neurons and pyramidal cells. After P7, its expression gradually shifted to the membranes of the somatodendritic compartment, becoming prevalent in the neuronal neuropil by P14. Overall, our results suggest a complex spatiotemporal pattern of chloride transporters' expression in the murine prosencephalon, which may be related not only with the beginning of inhibitory transmission but also with the different arrangements of neuronal circuits in cortical and thalamic subregions.

### AUGMENTER OF LIVER REGENERATION (ALR) IMMUNOREACTIVITY IN THE HUMAN CEREBELLAR CORTEX

L. Lorusso, P. Flace, G. Ciccimarra, A. Rizzi, G. Ambrosi, V. Benagiano

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Augmenter of liver regeneration (ALR) has been described in cytosol, endoplasmic reticulum, mitochondria and nucleus of various cell types (e.g. hepatocytes, muscle fibres, neurons) and has been considered to be involved in various functions, including regulation of cell cycle, mitochondrial oxidative phosphorylation, intracellular redox state, immune responses and metal homeosta-

sis<sup>1,2,3</sup>. Biochemical and morphological analyses have revealed high expression of ALR in the rodent cerebellum<sup>4,5</sup>. On the contrary, data on the expression of ALR in the human cerebellum are still absent in literature. Fragments of human cerebellum were taken at autopsy 24-36h after death, fixed in an aldehyde-picric acid solution, embedded in paraffin, cut into 5µm sections and subjected to light microscopic immunohistochemistry for ALR, using a rabbit polyclonal anti-ALR antibody. *Results*. ALR immunoreactivity was observed in all the layers of the cerebellar cortex and the white matter. In the molecular layer, the immunoreactivity was observed in a subpopulation of stellate and basket neurons, dendrites of Purkinje neurons, and in terminals scattered in the neuropil. In the Purkinje neuron layer, the immunoreactivity was observed in the perikaryon of some Purkinje neurons, in their primary dendrites and in terminals surrounding their deep pole. In the granular layer, the immunoreactivity was observed in subpopulations of granules and large neurons and interstices among them. Finally, the immunoreactivity was observed in some nerve fibres in the white matter. The present immunohistochemical study confirms the presence of a wide distribution of ALR in the human cerebellar cortex and suggests a role of this substance in the regulation of function played by the different neuron-types of cerebellar cortex.

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2. Francavilla *et al.*, Hepatology, 1994.
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### DEVELOPMENTAL EXPRESSION OF THE TWO TYROSINE HYDROXYLASE TRANSCRIPTS TH1/TH2 IN CHICKEN

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Tyrosine hydroxylase (TH) catalyses the first, rate limiting step in catecholamine biosynthesis. The catecholamines dopamine, noradrenaline and adrenaline play in vertebrates important roles in many physiological functions in the central and peripheral nervous systems as well as in the endocrine system. Recently it has been shown that TH-related expression is due to two non-allelic genes, called TH1 and TH2, reported in almost all vertebrates except placental mammalian, which have lost TH2 gene during evolution. The development of dopaminergic and noradrenergic neurons has received much attention based on their modulatory effect on many behavioral circuits and their involvement in neurodegenerative diseases. Considering the importance of the TH expression during embryonic development and the presence in many species of multiple TH genes and TH protein/mRNA forms led us to study the enzyme expression during chick embryo brain development. First, by real time RT-PCR assays, we assessed that TH1 and TH2 mRNAs show progressive increase during embryo development with differential trends. Moreover, substantially different regulatory switch of expression was shown for the two genes: based on our comparative results, TH1 mRNA expression in chicken brain increases gradually during development reaching significantly high post-embryonic levels, whereas the TH2 mRNA seems to be more specifically linked to embryogenesis of vertebrate brain stem. We also carried out *in situ* hybridization with TH1 and TH2 specific probes in order to elucidate differential tissue distribution of the two transcripts. Preliminary results showed



that TH1 probe localization largely corresponds to immunolabeling with commercially available anti-TH antibodies; on the other hand, TH2 probe shows only limited colocalisation with TH1 and/or antibody staining. These results showing different TH1/TH2 expression patterns suggest different mechanism of transcriptional regulation related to potentially differing roles during development.

### **KISSPEPTIN AND FOOD INTAKE: NEUROENDOCRINE RELATIONSHIPS AT THE LEVEL OF THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS**

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The energy homeostasis of the organism is very important for both the regular course of puberty and for fertility. Several studies have suggested a relationship among kisspeptin system and neural circuits controlling food intake and energy metabolism. The most important center for the regulation of these two physiological activities is the hypothalamic paraventricular nucleus (PVN). The kisspeptin system is clustered in two main groups of cell bodies (in anterior ventral periventricular region and in arcuate nucleus) that send fibers mainly at the GnRH neurons and in a few other locations, including the PVN. In this study we analyzed the distribution of kisspeptin-positive fibers in PVN of adult CD1 female mice, in estrus or diestrus, and the innervation in postnatal development (PND12 to PND90). Immunohistochemistry for kisspeptin was performed by using a rabbit antibody (AC#566, gift from Franceschini, Tours, France) and the extension of immunoreactivity was measured with Image J software (Fractional Area). Our results indicate the presence of significant differences among the medial and the lateral portion of the PVN, as well as a profound effect of estrous cycle on the immunoreactivity. In particular, kisspeptin signal was higher in the medial part of the PVN and in the estrus. Kisspeptin immunoreactivity varies during development showing a significant increase up to PND18, stable up PND30, after that we observed a significant decrease. In parallel, there are changes in circulating LH (measured by Tena-Sempere, Cordoba, Spain) with a significant peak at PND21. In conclusion, these data demonstrate a heterogeneity in the innervation of the PVN by the kisspeptin, with changes during the estrous cycle and the development. The presence of a higher amount of fibers within the medial PVN suggest that kisspeptin could directly innervate parvocellular elements related to the control of energy metabolism and food intake.

### **DIET AND BEHAVIOR: EFFECTS OF PHYTOESTROGENS IN THE DEVELOPMENT OF THE HYPOTHALAMUS**

G. Ponti<sup>1,2</sup>, A. Rodriguez-Gomez<sup>2,3</sup>, A. Farinetti<sup>2,3</sup>, F. Filice<sup>2,3</sup>, B. Foglio<sup>2,3</sup>, G.C. Panzica<sup>2,3</sup>, S. Gotti<sup>2,3</sup>

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Phytoestrogens (e.g. genistein) are nutraceuticals that are highly present in edible plants such as soy, widely present in human and animal diet. They are able to exert both estrogenic and anti-estrogenic activities, which may have a beneficial or detrimental effect according to the administration paradigm, sex and age of exposure. Their administration during developmental critical periods may interfere with the formation of specific neuronal circuits, leading to irreversible behavioral and morphological alterations in adults

even at a much lower dose than the one considered non-toxic by law. In particular hypothalamic systems are sensible to estrogen and phytoestrogen regulation and their disruption may affect sexual behavior and anxiety. We observed that early postnatal administration of genistein, at doses similar to that of infant formulas, affect fertility in both males and females through different mechanisms. In females estrous cycles are disrupted and the ratios between uterus weight and total body weight is impaired. In males prostate/body weight ratio is decreased. Moreover, preference T maze-test indicates that males had a minor interest in the sexual cue than the food stimulus, while females have a significant delay in the time of vaginal opening (puberty landmark). Behavioral tests showed that genistein early administration during postnatal development have a dichotomic effect on anxiety: it have an anxiolytic effect on females and an anxiogenic effect on males. Interestingly, a significant increase of number of nNOS cells in the PaAP subdivision of PVN in males parallels the increase in anxiety behavior. These results are important for both human health and animal welfare, and may have relevant economical consequences. In particular, soy based supplements are largely used for farm animals like in pigs. Meanwhile hypo fertility is a common problem in those animals and the soy phytoestrogens could be one of possible causes.

### **DISTRIBUTION OF CALRETININ IMMUNOREACTIVITY IN THE LATERAL NUCLEUS OF THE DOLPHIN AMYGDALA**

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The amygdala is an anatomically heterogeneous structure involved in complex behavioral processes, such as generation of appropriate autonomic, endocrine and motor responses to emotionally sensory stimuli, emotional learning and modulation of the formation of memories for emotionally arousing events. Cortical and thalamic sensory information enters the amygdala mainly via the lateral nucleus, which provides the most extensive intramygdaloid projections. Calretinin has proved to be useful marker for labeling the subpopulations of neurons in the lateral nucleus of the rat and human amygdala. In the present study, we investigated the distribution of calretinin-immunoreactive (IR) neurons in the lateral amygdalar nucleus of the Bottlenose dolphin (*Tursiops truncatus*). From three brains, sections through the whole rostrocaudal extent of the amygdala were stained immunohistochemically with a monoclonal antibody raised against calretinin. Based on the shape and size of the soma and on the morphology of the dendrites, we divided the calretinin-IR neurons into two major categories: pyramidal and non-pyramidal. Pyramidal cells had large (mean area of 271,1  $\mu\text{m}^2 \pm 69,3$ ), lightly stained, pyramidal somata. Only the proximal dendrites were stained. Non-pyramidal neurons were divided into three morphologic types: spheroidal, polygonal and fusiform. Spheroidal neurons had a spherical soma and 3 to 5 dendrites, which were about equal thickness. The somal size of these neurons were 80,6  $\mu\text{m}^2 \pm 17,2$ . Polygonal neurons had an angular somata (mean area of 186,6  $\mu\text{m}^2 \pm 72,9$ ) and 3 to 5 primary dendrites of variable thickness. Fusiform neurons had a spindle-shaped somata (mean area of 112,6  $\mu\text{m}^2 \pm 39,9$ ) that emanated primary dendrites from the opposite pole of the cell body. Morphologically the majority of calretinin-IR cells resemble inhibitory neurons. This result suggest that calretinin containing neurons may have an important role in the inhibitory network of the lateral nucleus of the dolphin amygdala.

## SESSION II ANIMAL MODELS OF NEUROPATHOLOGIES

### SELECTIVE VULNERABILITY OF SPINAL AND CORTICAL MOTOR NEURON SUBPOPULATIONS IN DELTA7 SMA MICE

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Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease, representing the most common inherited cause of infant mortality. Loss of the survival motor neuron gene (SMN1) leads to motor impairment, weakness, atrophy, and premature death caused by motor neuron degeneration. Even though SMA etiology is known and is traditionally considered as related to spinal motor neuron loss, discrepancies still exist on number/subgroups of lower motor neurons affected during disease progression. Furthermore, analysis of brain morphology and evaluation of a possible vulnerability of the upper motor system have not been carried out in the murine model. In order to clarify such alterations, we employed the murine model delta7 SMA (representing the intermediate form of SMA), with a lifespan of about 2 weeks and early motor behavior impairment correlated with motor neuron loss. We collected brains and spinal cords from delta7 SMA and WT mice at embryonic day 19, postnatal day 4 (P4) and P13 for neuron counts and immunohistochemistry. Using stereological quantification methods, we investigated the cervical spinal cord and cerebral motor cortex of mice during development, to verify extent and selectivity of motor neuron loss. We found progressive post-natal loss of spinal motor neurons, already at pre-symptomatic stages, and a higher vulnerability of motor neurons innervating proximal and axial muscles. We also observed a selective reduction of layer V pyramidal neurons associated with layer V gliosis in the cerebral motor cortex. Our data indicate that in the delta7 SMA model SMN loss is critical for the spinal cord, particularly for specific motor neuron pools. Additionally, neuronal loss is not selective for lower motor neurons. These data further suggest that SMA pathogenesis is likely more complex than previously anticipated. Understanding properly the nature and progression of the anatomopathological manifestations of the disease can give useful suggestions for more efficient therapeutic interventions.

### POSSIBLE ROLES OF NG2-BEARING OLIGODENDROCYTE PRECURSORS ON BLOOD-BRAIN BARRIER LEAKAGE IN EXPERIMENTAL ENCEPHALOMYELITIS

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Previous studies on cerebral cortex demyelination, carried out in mouse models of experimental autoimmune encephalomyelitis (EAE), have demonstrated that oligodendrocyte precursor cells (OPCs) are increased in the chronic phase of the disease and that blood-brain barrier (BBB) damage is to be independent of

the presence of classical inflammatory infiltrates<sup>1,2</sup>. In these conditions, a subset of the hypertrophic/hyperplastic OPCs scattered throughout the cortex appears oriented toward a 'glia-limits'-like role, contributing with astrocytes to the pial and perivascular boundaries. The association of OPCs with BBB microvessels during EAE and the newly established relations with the components of the neurovascular unit (NVU) were more closely analysed utilizing OPC-specific markers, NG2 proteoglycan and PDGF receptor- $\alpha$ , and an IHC/confocal and morphometric approach. Chronic EAE was induced in C57Bl/6 mice, NG2 wild type (WT) and NG2 null (NG2-KO). Compared with healthy mice, both EAE WT and EAE NG2-KO reveal NVU alterations primarily affecting perivascular cell composition. To determine these changes the following parameters were analysed: (i) extension of microvascular network, (ii) pericyte/endothelial cell ratio, (iii) perivascular/parenchyma OPC ratio, (iv) incidence of vascular OPC proliferation. According to this analysis, during EAE the increased OPCs population forms a distinct subset of cells located at perivascular/juxtavascular sites, where they proliferate and extend processes toward the vessel wall, thus introducing a new component among the elements of the NVU. Overall, the results suggest that during EAE OPCs play a role in BBB endothelial tight junction dismantling and that their activity may depend on NG2 expression, thus indirectly demonstrating a role for the proteoglycan in basement membrane/tight junction relations.

1. Girolamo *et al.* Neurobiol Dis (2011).

2. Errede *et al.* J Neuropathol Exp Neurol (2012).

### ROLE OF A DIETARY ESSENTIAL OIL COMPONENT IN PREVENTING THE HYPOPERFUSION/REPERFUSION-INDUCED TISSUE DAMAGE IN THE RAT CEREBRAL CORTEX

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The cerebral bilateral common carotid artery occlusion followed by reperfusion (BCCAO/R) is a valid experimental model for mimicking the early steps of hypoperfusion/reperfusion-induced tissue damage in the frontal cerebral cortex. By extending our previous observations on the beneficial effect of dietary *Pistacia lentiscus* L. essential oil in reversing and/or modulating the BCCAO/R-induced changes in tissue and plasma levels of polyunsaturated fatty acids (PUFAs) and endocannabinoids (eCBs), here we evaluate the activity of one of its major components, beta-caryophyllene (BCP), already known to possess peculiar biological activities, in Wistar rat cerebral cortex. Cerebral hypoperfusion was produced by a 30 min bilateral common carotid artery occlusion followed by 60 min reperfusion. Animals were starved for 12 hours before surgery and, 6 hours prior to hypoperfusion, BCP (40 mg/kg/0,45 ml of sunflower oil as vehicle) was administered via gavage. Biological samples of brain tissue, plasma and cerebrospinal fluid (CSF) were examined by HPLC, western blot, gel zymography and immunohistochemistry and analyzed for fatty acids, expression of the enzyme cyclooxygenase-2 (COX-2), CB receptors for eCBs, peroxisome proliferator-activated receptor (PPAR)- $\alpha$  and enzymatic activity of matrix-metalloprotease-9 (MMP9). Data obtained indicate that BCP appears to influence the outcome of BCCAO/R cerebral injury by modulating changes in levels of PUFAs, biosynthesis of eCBs and eCB congeners, expression of CB1 and CB2 receptors, COX-2 protein levels and enzy-

matic activity of MMP9. Brain tissue response to the hypoperfusion/reperfusion-induced cerebral insult appears to be modulated by dietary administration of BCP, thus allowing to suggest the possible use of this molecule as nutritional treatment in neuroprotection.

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### **ALTERED WIRING OF PEPTIDERGIC HYPOTALAMIC NEURONS IN A MODEL OF CHRONIC NEUROINFLAMMATION**

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The balance between excitatory and inhibitory inputs to orexin-A (OX-A) neurons and melanin-concentrating hormone (MCH) neurons, which are intermingled in the lateral hypothalamus, is critical in regulating several central functions, including the sleep-wake cycle. OX-A neurons receive predominantly excitatory inputs, promoting and sustaining wakefulness; MCH neurons, which are sleep-active, inhibit OX-A neurons during sleep. We here investigated the wiring of these peptidergic neurons in a model of sleeping sickness (African trypanosomiasis). This severe chronic inflammatory disease is caused by the extracellular parasite *Trypanosoma brucei*. In both humans and experimental rodents, after a first systemic (hemolymphatic) stage, the disease evolves in a meningoencephalitic stage characterized by neurological disorders. These include a disruption of the sleep/wake cycle and sleep fragmentation. The pathogenetic mechanisms leading to these functional alterations are not completely understood. Our aim was to investigate whether sleep/wake alterations during trypanosome infection are associated with synaptic impairment of sleep/wake-regulatory peptidergic neurons of the hypothalamus. In mice at an advanced meningoencephalitic stage, maintained under a 12h/12h light/dark cycle and sacrificed during the light phase, we analyzed quantitatively in confocal microscopy the synaptic contacts apposed to OX-A and MCH somata. These were identified by triple immunofluorescence, labeling OX-A or MCH cell bodies, and using synaptophysin and V-Glut or V-GAT for the identification of glutamatergic and GABAergic synaptic contacts, respectively. The results showed that in the infected mice the synaptic boutons density apposed to OX-A or MCH cell bodies was not altered. However, interestingly, a switch from a prevalence of GABAergic inputs to a prevalence of glutamatergic inputs was found on both the analyzed cell populations. The findings reveal for the first time the susceptibility of the synaptic wiring of OX-A and MCH neurons to inflammatory signaling. Further studies are ongoing to unravel structural and functional correlates of this remarkable synaptic plasticity process.

### **SLEEP CHANGES AND SLEEP/WAKE NEURONS IN A MURINE MODEL OF INFLUENZA A VIRAL INFECTION**

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The sleep disorder narcolepsy has been strongly linked to spe-

cific human leukocyte antigen (HLA)-DQ haplotype, suggesting a possible autoimmune mechanism behind the reduction in the number of orexin-expressing neurons, which are located in the lateral hypothalamus, in the brain of narcoleptics. In recent years, influenza virus infections have been considered as a risk factor for narcolepsy. In order to study whether influenza virus *per se* can cause narcoleptic-like changes in the sleep pattern, we infected Rag1<sup>-/-</sup> mice (lacking B and T cells and, therefore, adaptive immunity) with a mouse-neuroadapted influenza A virus (A/WSN/33). The virus was administered intranasally. Electroencephalogram (EEG), electromyogram and actimetry were recorded using a NeuroLogger® device which allows the mouse to move freely in its cage. Infected and control mice were sacrificed at a matched time point which marked the onset of sickness signs in the infected ones. Immunohistochemistry for antigens of A/WSN/33 virus showed hypothalamic and upper brainstem viral localization, including neurons involved in sleep-wakefulness regulation. Furthermore, EEG analyses showed narcoleptic-like changes, including a fragmented sleep-wake pattern in the infected mice, especially during light phase in which sleep-state predominates in nocturnal states. Levels of viral RNA and inflammatory markers were analyzed by RT-PCR. Interestingly, presence of transcripts encoding the non-structural viral protein (NS1) persisted in the brain 3-4 weeks post-infection. Up-regulation of transcripts of *tumor necrosis factor-α*, *interleukin-1β* and *interferon-β* was seen in the brain of infected mice. Altogether the data indicate that *i*) sleep pattern changes during influenza infection independently from the adaptive immune response; *ii*) different subsets of sleep/wake neurons may be involved in this dysregulation. This murine model and approach may help understanding the role of the virus and the immune response in CNS changes following influenza infections.

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### **CEREBRAL ALTERATION IN OBESE ZUCKER LEPR<sup>(fa/fa)</sup> RATS AS A MODEL OF METABOLIC SYNDROME**

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Metabolic syndrome (MetS), also known as dysmetabolic or X-syndrome is characterized by obesity, insulin resistance, hypertension and dyslipidemia. Clinical studies have considered MetS and its components as a cause of increased cerebrovascular disease, coronary heart disease, and all-cause mortality. The obese Zucker rats (OZR), with a mutation in leptin receptors represent a model of obesity related to type-2 diabetes mellitus. They present a moderate degree of arterial hypertension and increased oxidative stress. This study has investigated brain microanatomy of OZR compared with their non-obese cohort lean Zucker rats (LZR) to assess possible relationships between MetS and brain injury. Male OZR and LZRs of 12, 16 and 20 weeks of age were used. Body weight, blood pressure and blood chemistry parameters were checked every two weeks and before killing. The brain was processed for immunohistochemical and immunohistochemical analysis of neurons identified by neuronal specific nuclear protein (Neu-N) and axons identified by neurofilament (NF) immunohistochemistry. Glial-fibrillary acid protein (GFAP) immunoreactive astrocytes were also investigated. Behavioural tests (open-field and passive-avoidance) were performed to identify possible cognitive changes.

OZR of different ages, showed higher body weight, systolic pressure, glycemia and insulin resistance, higher triglycerides and cholesterol levels in comparison with LZRs. An age-dependent increase of these parameters was found in OZR. In frontal cortex and hippocampus, morphological and immunohistochemical analysis revealed a decrease of Neu-N immunoreactive neurons not related to apoptosis in older OZR group compared to age-matched LZRs. In OZR of different age, a decrease of NF immunoreaction and an increase of GFAP immunoreactive astrocytes was observed compared to LZRs. OZR showed a reduction of retention latency time in the emotional learning task. These findings suggest that OZR may represent a model for assessing the influence of metabolic syndrome on brain and may represent the first step for characterized neurological changes potentially occurring in obesity.

### III SESSION MNGIE: 'FROM BENCH TO BEDSIDE APPROACH' (the ITA-MNGIE Research Progress)

#### CONVENTIONAL AND ADVANCED BRAIN MRI IN MNGIE

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Typically conventional brain MR of MNGIE patients shows in the white matter a widespread symmetrical and confluent signal intensity changes, increased on T2- and reduced on T1- weighted images, with sparing of U-fibres. The clinical correlates of leukoencephalopathy and its underlying pathomechanism has yet to be defined. In order to characterize the pathophysiology of the white matter changes on conventional MRI we optimized a protocol including advanced and non-invasive MR techniques. Diffusion-tensor imaging, DTI, is used to assess the integrity of white matter microstructure allowing the quantification of several water diffusion metrics, that depend on the interactions between water molecules and the structural barriers at cellular and subcellular levels. Proton MR spectroscopy, <sup>1</sup>H-MRS, allows the spatially resolved measurement of brain metabolites content, including N-acetyl-aspartate (NAA), marker of neuronal density or viability, choline (Cho), a complex of cell membrane phospholipids constituents, creatine (Cr), marker of brain energy metabolism and mio-Inositol (mI), glial cell marker. Thanks to the diagnostic-therapeutic work-up dedicated to patients with chronic idiopathic intestinal pseudo-obstruction (CIPO) activated at the Policlinico S. Orsola-Malpighi, University of Bologna, we evaluated seven MNGIE patients. In all patients, compared to a population of sex and age-matched healthy controls, the MR white matter investigation showed a reduction of [NAA], [Cr], and [Cho] content (p<0.05) and an increase of mean water molecules diffusivity parameter (MD) (p<0.05). In the post-mortem study of one of our patient brain axonal/neuronal loss, demyelination, and gliosis were absent. The reduction in all <sup>1</sup>H -MRS metabolites in the cerebral white matter of MINGIE patients associated with increase water diffusivity can be explained by a dilution effect due to increased brain water content, consistent with the functional alteration of the blood brain barrier supporting previous post-mortem findings.

## **SKELETAL MUSCLE PATHOLOGY IN MINGIE PATIENTS: BLOOD VESSELS DEPLETION**

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder characterized by ptosis and progressive external ophthalmoplegia, peripheral neuropathy, severe gastrointestinal dysmotility, cachexia, leukoencephalopathy and mitochondrial DNA depletion, multiple deletions, or both. This disorder is caused by loss-of-function mutations in the gene encoding thymidine phosphorylase (TP) a cytosolic enzyme that catalyzes phosphorolysis of thymidine to thymine and deoxyribose 1-phosphate. In MNGIE patients, TP activity is very low or absent resulting in dramatically elevated levels of plasma thymidine and deoxyuridine. TP is expressed in most human tissues but is not expressed in skeletal muscle usually affected in MNGIE suggesting that TP deficiency causes the disease through a toxic intermediate. In addition, TP is associated with angiogenesis and high concentrations of thymidine inhibit microvessels formation. In our preliminary study vessels number between two MINGIE patients and eleven controls was compared. Histologic slides were stained with Alkaline Phosphatase and ratio between blood vessels and fibres number was calculated for each sample. Even if cases and controls numbers are low and they have to be increased, a significative difference between MINGIE and control patients was revealed suggesting that angiogenesis inhibition could be involved in MINGIE pathogenesis.

## **HEMATOPOIETIC STEM CELL TRANSPLANTATION**

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Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) has been performed as treatment for patients with Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE) because offers the possibility of prolonged and sustained correction of thymidine phosphorylase (TP) deficiency. Twenty patients with MNGIE have been treated with HSCT and in all evaluable cases has been shown to restore TP activity, lowering thymidine levels and improving the gastrointestinal dysmotility, but rarely neurological symptoms. Although HSCT improves gastrointestinal assessment with the correction of the biochemical abnormalities, the procedure can be risky and the majority of patients were reported to die in the post-transplant period with 5-year mortality rate of about 70%. To date, clinical benefit from allogeneic HSCT for patients with MNGIE has not yet been demonstrated and there are several challenges in administering this treatment. Patients with MNGIE are generally in poor medical condition with limited capacity to tolerate transplant-related complications and conditioning and immunosuppressive regimens. The gastrointestinal function is disturbed with potential impairment of absorption and a need for prolonged parenteral administration of drugs. Although drugs with possible mitochondrial toxicity must be avoided, for many drugs used in HSCT their effects on mitochondrial function are not known. As transplant-related mortality increases with the progression of the disease, it has been suggested that individuals with MNGIE should be referred to HSCT when they are still relatively healthy in order to minimize the

complications of the procedure, but new transplant approaches have been proposed (orthotopic liver transplantation) with potential less toxic problems.

## **LIVER TISSUE: A PROOF-OF-CONCEPT STUDY FOR OLT IN MNGIE PATIENTS**

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an extremely rare autosomal recessive mitochondrial disease due to mutations to the nuclear TYMP gene. Thus, the mechanism which takes place in this condition leads to thymidine phosphorylase (TP) enzyme inactivity (either partial or complete - *i.e.*, virtually lacking) resulting in toxic accumulation of thymidine and therefore altered mitochondrial DNA. MNGIE is characterized by severe gastrointestinal dysmotility, neurological impairment, poor quality of life and reduced life expectancy. Currently, therapeutic options for MNGIE are still limited. In order to restore TP activity, allogeneic hematopoietic stem cell transplantation has been used as cellular source of TP although with modest results, the 5-year mortality rate being about 70%. This research tested the hypothesis that the liver can be an alternative source of TP. Eleven patients (7M; 35-55 years) who underwent hepatic resection for focal disorders were included. Margins of normal liver tissue were obtained during surgery and processed to identify, quantify and localize the TP protein by an array of techniques (Western Blot, ELISA, and immunohistochemistry) and to evaluate TYMP mRNA expression by qPCR. Western Blot identified TP in the liver with a TP/GAPDH ratio of  $0.9 \pm 0.5$ . ELISA estimated TP content as  $0.5 \pm 0.07$  ng/mg of total protein. TP was identified in both nuclei and cytoplasm of hepatocytes and sinusoidal lining cells. TYMP mRNA was expressed in the liver. In this study, we provided evidence that the liver is an important source of TP. Our experiments represent a proof-of-concept that orthotopic liver transplantation (OLT) can be indicated as possible therapeutic alternative for MNGIE patients.

## IV SESSION NEURODEGENERATION AND NEUROREGENERATION

### ULTRASTRUCTURAL DISSECTION OF CELL-TO-CELL COMMUNICATION IN NEURODEGENERATION

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Cells communicate by sending and receiving signals. Signals may come from the environment, or they may come from other cells or from the cell itself. In order to trigger a response, these signals must be transmitted across the cell membrane. These signals may propagate in different ways: (i) being secreted quantally and non-quantally to interact with trans-membrane receptors proteins, (ii) being transported by endosome-derived exosomes; (iii) being placed on fragments of the cellular plasma membrane that can be exchanged between two cells (trogoctosis); (iv) being transported along intercellular tunnels. These phenomena occur physiologically in normal conditions. In neurodegenerative disorders and abnormal cell proliferations these events may spread the cell pathology to neighbors including and degeneration and abnormal proliferation. Thus in these context abnormal cell-to-cell communication may represent a critical step in disease progression. In the present study we analyzed the ultrastructural correlates of cell-to-cell communication during specific cell pathologies. We studied specific proteins, which once misfolded may be particularly prone to be propagated to other cells thus being the seed for disease spreading. This is the case of alpha synuclein and the prion protein.

### GELATIN FIBRE DIAMETER INFLUENCES SCHWANN CELL BEHAVIOUR AND AXONAL OUTGROWTH

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For tissue engineering application, the distribution and growth of cells on a scaffold are key requirements. A number of studies demonstrated that micro-to-nano-scale topography plays an important role in controlling cell adhesion, proliferation and survival. Schwann cells (SC), forming bands of Büngner, support and promote axonal outgrowth leading to target reinnervation during nerve regeneration. In order to enhance SC adhesion, proliferation, migration and axonal outgrowth, a number of bio-mimetic materials were studied. Appropriate fibrous substrates, functioning as a temporary extracellular-matrix, can be easily prepared by electrospinning technique, which allows the obtainment of fibrous matrices suitable as internal filler for nerve guidance channels to be

applied in peripheral nerve repair. Gelatin micro or nano-fibres were prepared by electrospinning technique by tuning gelatin concentration and solution flow rate. The influence of gelatine fibre diameter on cell adhesion and proliferation, was tested *in vitro* by using SC and Dorsal Root Ganglia (DRG) explant cultures. Results demonstrated that gelatin fibres tested were bio-compatible. Cell adhesion was evaluated by quantifying cell spreading area, actin cytoskeleton organization and focal adhesion complex formation. Fibre diameter influences SC behaviour and morphology. Nano-fibres have been shown to promote cell spreading and actin cytoskeleton organization, resulting in higher cellular adhesion and proliferation rate in comparison to micro-fibres. Cell migration and motility were quantified by transwell and time lapse assays respectively. Cells cultured on micro-fibres displayed higher motility and migration rate in comparison to nano-fibres. Finally, axonal outgrowth evaluated by culturing DRG explants on the different fibres resulted in higher axonal outgrowth on micro-fibres in comparison to nano-fibres. These data provide a better understanding about glial cell and neuritis viability and organization on gelatin electrospun nano- and micro-fibres suggesting that micro-fibres can be a better filler to be used in the design of new devices for peripheral nerve repair applications.

### CHANGES OF THE NRG1/ERBB SYSTEM AFTER NERVE INJURY


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It is well documented that Schwann cells play a key role in peripheral nerve regeneration. One of the most important trophic factors for Schwann cell activity is Neuregulin1 (NRG1), which exerts its effects by binding the ErbB2/ErbB3 heterodimer receptor. In this study we focused our attention on the expression of the NRG1/ErbB system during nerve degeneration and regeneration. In order to clarify the relationship between the expression changes of the NRG1/ErbB system and the different phases of nerve degeneration and regeneration, two types of experimental lesions were performed: *axotomy* (crush lesion), that is a complete interruption of axon continuity without discontinuing the nerve, characterized by a fast regeneration, and *Wallerian degeneration*, where the nerve is cut without being repaired. mRNA and protein were extracted from adult rat median nerves at different time points from the injury (1,3,7,14, days and 4 weeks). Quantitative real-time-PCR and western blot analysis for the different NRG1 isoforms, the NRG1 tyrosine kinase receptors (ErbB2-ErbB3) and myelin basic protein (MBP) were performed. In parallel, to better understand and explain mRNA and protein expression results, we investigated the ultrastructure changes in nerve morphology by electron microscopy analysis. Results showed intense and significant changes in gene and protein expression, especially shortly after injury. Genes and proteins were modulated with different time courses following the different phases of nerve degeneration and regeneration. This analysis shows that the different NRG1 isoforms and ErbB receptors display a highly specific expression patterns, suggesting a precise role after peripheral nerve injury.

## MANIPULATING THE NRG1/ERBB SYSTEM IN PERIPHERAL NERVES: AN *IN VITRO* AND *IN VIVO* LABORATORY INVESTIGATION

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Peripheral nerve trauma or injuries may lead to sensory or motor function deficits if not properly treated. For this reason, in the last years nerve repair surgical techniques and the regenerative properties of the peripheral nervous system were the focus of many studies. With the aim of improving peripheral nerve regeneration, surgical approaches have matched with new biomedical strategies such as production of new devices and induction of specific factors to enhance the endogenous mechanisms of recovery. The Neuregulin/ErbB system plays an important role in peripheral nervous system both in normal and pathological conditions. In our study we explored the possibility to manipulate the system, *in vitro* and *in vivo*, in order to increase Schwann cell migration and thus improve injured peripheral nerve regeneration. To interfere with the Neuregulin1/ErbB system soluble ecto-ErbB4, a protein fragment endogenously released by cells expressing the cleavable isoform of the NRG1 receptor ErbB4, was expressed *in vitro* in Neuregulin1 expressing glial cells. A strong increase in cell motility was observed. Experiments suggest that activation of a back signaling, mediated by the transmembrane Neuregulin1 isoform, plays a crucial role in the migratory activity induced by fragment expression. Nevertheless, *in vivo* manipulation of the Neuregulin1/ErbB system through local delivery of soluble ecto-ErbB4 by gene transfer in the muscle-vein-combined nerve guide, did not result in strong motor functional recovery or improved nerve fiber regeneration. These results indicate that ecto-ErbB4 could be used *in vivo* as a tool to manipulate the Neuregulin1/ErbB system, but further studies are required to design an effective delivery strategy for an *in vivo* Neuregulin1/ErbB system manipulation, that can strongly promote post-traumatic peripheral nerve regeneration.

## METHODOLOGICAL PITTFALLS IN PERIPHERAL NERVE REGENERATION RESEARCH

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The combination of morphological and mRNA analysis is very important for the in-depth investigation of regenerated nerve fibers. In order to compare results of different treatments on nerve regeneration, quantitative data should be obtained. This raises several methodological problems. As regards quantitative morphology the main problem is the identification of nerve fibers in light microscopy. To address this issue, we used a design-based stereological method to evaluate the regenerative process in two experimental paradigms: crush injury and autograft repair, using both light and electron microscopy. Results show a significant underestimation of myelinated fiber number quantified with light microscopy compared to electron microscopy, due to the large number of very small axons. The

analysis of the size parameters also shows a higher number of small fibers in electron microscopy analysis, especially in regenerated nerves. As regards mRNA analysis, the main problem is the fact that nerve injury induces dramatic changes in terms of cellular composition that are reflected on RNA quality and quantity, making messenger RNA expression analysis very complex. To deal with this problem, we have developed a new method to identify new stable housekeeping genes based on publicly available microarray data on normal and injured nerves. Four new candidate stable genes were identified and validated by quantitative real-time PCR analysis on nerves during the different phases after nerve injury: nerve degeneration, regeneration and remyelination. The stability measure of these genes was calculated with both NormFinder and geNorm algorithms and compared with six commonly used housekeeping genes. This procedure allowed us to identify two new and highly stable genes that can be employed for normalizing injured peripheral nerve data: ANKRD27 and RICTOR. It is expected that these results can support researchers in ensuring unbiased analysis of peripheral nerve fibers and reliable interpretation of the results.

## NANOVESICLES FROM MESENCHYMAL STEM CELLS: EXPERIMENTAL ASSESSMENT OF AN INNOVATIVE THERAPEUTIC APPROACH FOR ALS

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Therapeutic strategies for the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) have not yet done satisfactory results. Stem cells are becoming increasingly important in the treatment of neurodegenerative diseases and their benefits seem to be due to a paracrine effect via the release of nanovesicles (NV). NV contain a broad range of molecules, primarily RNAs, lipids and protein, and they are the main mediator of cell-cell communication. Here we want to assess the efficacy of a novel non-cell therapeutic strategies based on the use of NV derived from adipose-derived mesenchymal stem cells (ADSC; ADSC-NV). To evaluate the neuroprotective effect of ADSC-NV *in vitro*, on motoneuronal cell culture, we first set up the protocol of reproducible isolation of ADSC-NV, identified the optimal dose of ADSC-NV which protect neural cells from apoptosis, the cell-plated density and the optimal concentration of H<sub>2</sub>O<sub>2</sub> used as pathological insult. These parameters were assessed on neuroblastoma cell line SH-SY5Y, primary culture of hippocampal neurons and on motor neuron-like cell line NSC-34. Interestingly, on naïve NSC-34 cells and on NSC-34 cells transiently transfected with human mutant SOD1(G93A) gene, the administration of ADSC-NV in the culture medium protected cells from oxidative damage (H<sub>2</sub>O<sub>2</sub>), with a 30% increase of cell viability. In conclusion, our results on motor neuron-like cell line NSC-34, naïve or transiently transfected, point out the neuroprotective role of ADSC-NV from oxidative damage. ADSC-NV could be a substitute for cell-based therapy and represents a promising approach in neurodegenerative disorders.

## V SESSION THE PERIPHERAL AND ENTERIC NERVOUS SYSTEM

### ENTERIC NEUROPROTECTION IN HUMAN NEURONS: EFFECTS MEDIATED BY PRUCALOPRIDE, A SEROTONINERGIC FULL 5-HT<sub>4</sub> SELECTIVE AGONIST

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Serotonin (5-hydroxytryptamine, 5-HT) and related transporters and receptors are involved in a wide array of digestive functions and disorders. 5-HT<sub>4</sub> receptors play a major role in intestinal peristalsis and among agonists, prucalopride (a full 5-HT<sub>4</sub> agonist) is an effective enterokinetic agent in the treatment of chronic constipation. In addition, 5-HT<sub>4</sub> receptor agonists may evoke enteric neuroprotection. We tested whether prucalopride exerts protective effects on enteric neuron cell cultures exposed to damaging factors, *i.e.* oxidative agents (H<sub>2</sub>O<sub>2</sub>). We aimed to: i) evaluate the expression and selective identification of 5-HT<sub>4</sub> receptors in human enteric neurons; and ii) define the 5-HT<sub>4</sub> receptor-mediated neuroprotection in human cell cultures by assessing the anti-apoptotic effect exerted by different doses of prucalopride. Human enteric neurospheres were generated from human gut tissue; Western blotting (WB) analysis were performed using different primary antibodies. SulfoRhodamine B (SRB) assay was used to determine the neuronal survival of SHSY5Y cells following oxidative stress. GR113808 was applied to SHSY5Y cells to reverse the protective effect of prucalopride. WB analysis demonstrated that all cell lines as well as cells from human neurospheres expressed the 5HT<sub>4</sub> receptor. SRB assay showed that SHSY5Y cells previously exposed to prucalopride at different concentrations were protected by the noxious effect induced by H<sub>2</sub>O<sub>2</sub>. Specifically, prucalopride at 1 nM concentration exhibited the best neuroprotective effect compared to neurons exposed to H<sub>2</sub>O<sub>2</sub> only (>76.5±0.1% of neuronal survival vs. 33.3±0.1%, respectively). Prucalopride concentrations applied alone to SHSY5Y neurons did not show any toxicity and resulted in 91±0.1% of neuronal survival. In contrast, the neuroprotective effect of prucalopride was reversed by the 5-HT<sub>4</sub> antagonists GR113808. Prucalopride, a 5-HT<sub>4</sub> receptor full agonist, mediated significant neuroprotection against oxidative-mediated proapoptotic effects. These results may pave the way to novel application of 5-HT<sub>4</sub> agonists as neuroprotective agents in enteric neuropathies.

1. Metzger *et al.*; Gastroenterology 2009.

### INFLUENCE OF DIABETES ON THE DOG ENTERIC NERVOUS SYSTEM

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The diabetes mellitus (DM) is a worldwide endocrine emerging disease affecting human but also domestic mammals, such as dogs and cats. The pathogenesis seems to be correlated to several factors such as the diet, genetic, behaviour, and reduced motility. The hyperglycaemia is responsible of severe clinical complications such as retinopathy, vascular damages, generalized neuropathies, and also gastrointestinal motility disorders (*i.e.* vomiting, constipation, diarrhea and fecal incontinence), which seem derive from enteric neuropathy. The DM seems to reduce the number of neurons in different tracts of the digestive system, as demonstrated in streptozotocin- and spontaneous type I DM rodents; particularly, in the early stages of DM, inhibitory nitrenergic neurons are affected whereas the cholinergic neurons seem to be altered only in the late DM stage. In the present research we quantified the percentages of nitrenergic neurons in the myenteric plexus (MP) of the stomach (antrum) and ileum of healthy (control) dogs (n=6) and, successively, investigated the effects of hyperglycemia on the nitrenergic subpopulations in DM-affected dogs (n=5). All myenteric neurons were immunohistochemically identified with the antibody anti-HuC/HuD (Hu), while nitrenergic neuronal cells were characterized with the antibody anti-neuronal nitric oxide synthase (nNOS) enzyme. The results showed different patterns of immunolabeling: in the stomach, the percentages of nitrenergic neurons observed in control and DM-affected dogs were similar, 28±6% and 25±2%, respectively. In the ileum of diabetic subjects, many ganglia were severely altered and Hu- and nNOS-immunoreactive neurons often uncountable. Anyway, in three of diabetic dogs there were some apparently "normal" ganglia and in those cases the percentage of nNOS-IR neurons were reduced respect to controls (19±5% vs. 30±4%). These findings indicate that, in the dog, DM could have a severe impact on the ileal motility; therefore clinicians have to focus also on the effects of DM on the canine gastrointestinal functions.

### BITTER TASTE RECEPTOR T2R38 EXPRESSION IN THE COLON OF OVERWEIGHT/OBESE AND LEAN SUBJECTS

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The sense of taste is important to evaluate the quality of nutrients and distinguish between safe and dangerous food prior to ingestion. In particular, bitter taste has evolved as a warning mechanism against toxic or harmful chemicals. Transcripts for bitter taste receptors (T2Rs) and their signaling molecules are distributed to the gastrointestinal tract mucosa. Intraluminal bitter tastants activate vagal afferent neurons, induce avoidance and affect feeding behavior and gastric emptying. Also, bitter tastants induce release of peptides involved in GI chemosensing. Our aim was to test whether T2R38 expression is altered in the mucosa of overweight or obese subjects compared to lean sub-



jects and to characterize the cell types expressing T2R38 in human colonic mucosa. Colonic mucosal biopsies were obtained during screening sigmoidoscopy from 30 volunteers: 15 overweight to obese (OW/OB) (8M, 7F; 20-55 yrs; mean BMI  $32\pm 0.7\text{kg/m}^2$ ) and 15 normal weight (NW) (7M, 8F; 22-55 yrs; mean BMI  $20\pm 0.5$ ) subjects. Biopsies were processed for hT2R38 gene expression assays and immunohistochemistry using antibodies anti-T2R38, anti-CgA, anti-GLP-1, anti-CCK and anti-PYY. hT2R38 mRNA in OW/OB subjects was markedly increased compared to NW subjects ( $4.20\pm 0.9$  vs.  $1.68\pm 0.5$ , respectively,  $P<0.05$ ). T2R38 immunoreactivity (-IR) was localized to enteroendocrine cells (CgA-IR cells). T2R38-IR cells coexpressed CCK-, GLP1- or PYY-IR. The number of T2R38/CgA cells in the OW/OB group was increased compared to lean controls ( $124.5\pm 15.9$  vs.  $55.88\pm 8.0$  in  $3.36\text{ mm}^2$ , respectively) ( $P<0.006$ ). There was an increase in T2R38/GLP1 and T2R38/CCK cells in OW/OB vs. NW subjects ( $51\pm 14.2$  vs.  $24.6\pm 3.9$ , and  $34.0\pm 6.4$  vs.  $19.6\pm 6.5$ , respectively); there was no difference in the number of T2R38/PYY cells in OW/OB vs. NW subjects ( $8.6\pm 2.1$  vs.  $9.2\pm 4.8$ ). In conclusion, T2R38 upregulation in OW/OB subjects might be due to changes in luminal content including alteration of microbiome, associated with obesity. This is consistent with the proposal that T2R38 activation initiate a protective response, which could involve the release of gut hormones.

## NEURONAL ANTIBODIES TO CENTRAL AND ENTERIC NERVOUS SYSTEM IN CELIAC DISEASE

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Neuronal antibodies (NA) have been recently described in celiac disease (CD). Our aims were to assess NA prevalence and their correlation with neurological disorders and bowel habits in CD. NA to central (CNS) and enteric nervous system (ENS) were investigated in 106 CD patients and in 60 controls with autoimmune disorders by indirect immunofluorescence on rat/primate cerebellar cortex and intestinal section. IgG NA to CNS (titer 1:50 – 1:400) were positive in 23 celiacs (21%), being more frequently detected in those with neurological disorders than in those without neurological dysfunction (49% vs. 8%,  $P<0.001$ ). Of the 26 celiacs (24%) with IgG NA to ENS, 11 out of the 12 had an antibody titer  $> 1:200$  and showed Roma III criteria based severe constipation. Only one patient with cerebellar ataxia and intestinal sub-occlusion was positive for NA directed to both CNS and ENS. NA to CNS and ENS were found in 7% and 5% of controls, respectively. In conclusion, in CD the positivity of NA to CNS can be considered a marker of neurological involvement, whereas NA to ENS are strictly related to severe constipation. The detection of NA to ENS suggests that humoral autoimmunity contributes to the pathogenesis of constipation in patients with CD likely via an impairment of the ENS function.

## HISTOPATHOLOGICAL CHANGES OF THE ENTERIC NEUROMUSCULAR COMPARTMENT IN EXPERIMENTAL COLITIS: AN INTEGRATED VIEW

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**Background.** Bowel inflammatory fibrosis has been largely investigated, but an integrated assessment of remodelling in inflamed colon is lacking. This study evaluated tissue and cellular changes occurring in colonic wall upon induction of colitis, with a focus on neuromuscular compartment. **Methods.** Colitis was elicited in rats by 2,4-dinitrobenzenesulfonic acid (DNBS). After 6 and 21 days, the following parameters were assessed on colonic samples by enzymatic assay, immunoblotting, histology, histochemistry, immunohistochemistry and confocal microscopy: tissue myeloperoxidase; tissue injury and inflammatory infiltration; collagen and elastic fibers; type I collagen content; HuC/D, glial fibrillar acidic protein (GFAP), proliferating cell nuclear antigen (PCNA), nestin, substance P (SP), von Willebrand factor, c-Kit and transmembrane 16A/Anoctamin1 (TMEM16A/ANO1). TMEM16A/ANO1 was also examined in isolated colonic smooth muscle cells (ICSMCs). **Results.** On day 6, inflammatory alterations and fibrosis were present in DNBS-treated rats; colonic wall thickening and fibrotic remodelling was evident on day 21. Colitis was associated with both an increase in collagen fibers and a decrease in elastic fibers. Moreover, the neuromuscular compartment of inflamed colon displayed a significant decrease in neuron density and increase in GFAP/PCNA-positive glia of myenteric ganglia, enhanced expression of neural SP, blood vessel remodelling and activation, reduced c-Kit- and TMEM16A/ANO1-positive interstitial cells of Cajal (ICCs), as well as an increase in TMEM16A/ANO1 expression in muscle tissues and ICSMCs. **Conclusions.** The variety of markers and cells examined in the present study provide an integrated view of the impact of inflammatory and fibrotic processes on colonic neuromuscular compartment. According to our findings, the rat model of DNBS-induced colitis displays significant processes of colonic remodelling, consisting not only in collagen deposition and wall thickening, but also in specific cellular alterations of the neuromuscular units, such as myenteric neurons, glial cells, ICCs, SMCs and vessels.

## SKIN BIOPSY REVEALS SMALL FIBER NEUROPATHY IN PATIENTS WITH CHRONIC INTESTINAL PSEUDO-OBSTRUCTION

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**Objective.** Chronic intestinal pseudo-obstruction (CIPO) is a failure of gut motility leading to recurrent episodes of intestinal sub-occlusion with no demonstrable mechanical reason. We tested whether CIPO patients have a small fiber neuropathy

(SFN), *i.e.* small size autonomic and/or somatic fiber alterations, using skin biopsy as investigational tool. *Materials and Methods.* Twenty-seven CIPO patients (M: 7, F: 20) entered the study. Each patient was evaluated with laboratory tests (*i.e.*, autoimmunity profile; 21/27 ganglionic acetylcholine receptor antibody, gACh-R), neurophysiological assessment and punch skin biopsy. Tissue sections were processed to establish somatic and autonomic skin innervation. Biochemical and molecular tests were also performed to verify the presence of known diseases sustaining SFN in histopathologically proven SFN-positive CIPO cases. *Results.* Nine patients had unspecific autoantibodies; 1 extra patient tested positive for anti-Scl-antibodies and Sjogren syndrome. None had elevated gACh-R antibody titer. Three patients showed polyneuropathy. Fourteen patients, including those with polyneuropathy and Sjogren disease had SFN. Thirteen patients had no SFN. Genetic tests (*e.g.*, transtiretin gene 6/14, alpha-galactosidase 2/14) were

negative. *Discussion.* Severe gastrointestinal symptoms and SFN have been previously described in amyloidosis and Fabry disease. This is the first study to detail the association between sporadic forms of CIPO and SFN. Demographic and disease-related variables, *e.g.* BMI and disease duration, are comparable between groups suggesting no influence of malnutrition or disease duration. The presence of symptomatic cases (patients with polyneuropathy) also suggests that cutaneous small fiber damage reflects nerve involvement in the gastrointestinal tract. The mechanism through which SFN may affect gut function, including motor coordination, is likely via autonomic abnormality. *Conclusions.* Based on the present results, skin biopsy is indicated to identify SFN in patients with severe gut dysmotility, *i.e.* CIPO. Whether these patients should undergo a combined skin and gut (full thickness) biopsy for a thorough characterization is a matter of extensive ongoing research.

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