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Proceedings of the 31st National Conference of the Italian Group for the Study of Neuromorphology "Gruppo Italiano per lo Studio della Neuromorfologia" G.I.S.N.

MAIN LECTURES

A FUNCTIONAL SINGLE CELL BIOLOGY PIPELINE TO CAPTURE AND IDENTIFY SIGNALLING NETWORK DYNAMICS AND BEHAVIOURS ACROSS NEURONAL CIRCUITS

Li L.L.[1,2], Ramos B.,[2] Courtney M.J.[1,2]

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Single cell heterogeneity, like temporal dynamics, is increasingly understood to be important for physiology, pathology and responses to therapeutic intervention. Biological outcomes often arise from rare events, yet current understanding of intracellular signalling networks remains largely based on end-point population averages, with kinetics and different pathways measured in independent samples. As spatiotemporal heterogeneity imposes a requirement that multiple pathways are measured longitudinally from each cell of a population, we applied single cell biology algorithms to multiplexed quantification of signaling pathway dynamics. Using synaptically-connected neuronal circuits as test case, we show that distinct and even rare response phenotypes can be easily distinguished. Mapping the emergence and convergence of single cell behaviors over time indicates the stability of the distinct response identities while guiding refinement and higher throughput of sample measurement. Finally, when applied to clinically used drugs, we identify unexpected indirect actions and subpopulation-specific responses. We conclude this analysis of functional single-cell pathway dynamics can reveal valuable information about cellular heterogeneity supporting better understanding of response diversity and the undesirable off-target actions of drugs.

THE RETINA AS A WINDOW ON ALZHEIMER'S BRAIN

Pediconi N.[1], Pizzarelli R.[1], Grimaldi A.[1], Rosito M.[1,2], Gigante Y.[3], Mautone L.[1], Scaringi G.[1,2], Ghirga S.[1], Soloperto A.[1], Di Angelantonio S.[1,2]

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Alzheimer's disease (AD) is a neurodegenerative disorder responsible for more than 80% of cases of senile dementia. Despite the presence of extracellular β-amyloid (A) and intracellular tau protein aggregates is an established hallmark of the AD pathology, the limited scientific evidence on the timing of the molecular cascade leading to AD-related neurodegeneration has made difficult the development of both early diagnostic strategies and effective therapies. Indeed, no solutions have been found to date that hinders disease progression thus improving the life quality of patients and caregivers. Moreover, most of the drugs developed in rodent AD models failed in reducing disease burden in clinical trials, likely due to the lack of globally recognized diagnostic criteria in pre-symptomatic phases of AD, and to the physiological and evolutionary species-specific differences that hamper translating to humans the results obtained in rodents. Thus, the actual challenges are: i) to define new biomarkers and non-invasive technologies to measure neuropathological changes *in vivo* at pre-symptomatic stages, and ii) to develop humanized AD models to better understand the molecular cascade and thus accelerate the screening process for diagnostic and therapeutic candidate molecules. Recent evidences obtained from human samples and mouse models indicate the possibility to detect protein aggregates and other pathological features in the retina, paving the road for non-invasive rapid detection of AD biomarkers. Considering the retina as window on the brain may provide a unique platform to study diseases of the nervous system. We report in AD mouse and autoptic human retina the presence of amyloid beta plaques, tau tangles, neurodegeneration and detrimental astrocyte and microglia activation according to a disease associated microglia phenotype (DAM). Thus, we propose to use retinal biomarkers *in vivo* as an additional diagnostic tool to overcome the need for invasive tests and histological specimens, and to longitudinally monitor individuals over time. Moreover, using human-induced Pluripotent Stem Cells (hiPSCs) we developed 2D and 3D models of human retina *in vitro* that may help to understand the causal and temporal relationships between molecular and cellular events, and, in a future perspective, to test patient's specific targeted therapies.

SESSION I
NEUROANATOMY: NEW TECHNIQUES AND FINDINGS

STRUCTURAL, FUNCTIONAL AND HYBRID NETWORK CONNECTIVITY REVEAL THE TOPOLOGICAL ORGANIZATION OF THE HUMAN CEREBELLUM

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Studying the brain as a network of interconnected nodes and the recent developments of network theory contributed to unveil the key structural principles underlying the topology of the healthy human brain connectome at macroscale level. However, despite the increasing advances in connectomics and network neuroscience, the way different cerebellar compartments connects to each other at the system level has been poorly investigated. Indeed, unlike the simplicity of elementary cerebellar circuit, disentangling comprehensively its structure and function at the macroscale level is still challenging. Herein, we aim at characterizing the cerebellar network topology, mapping the hubs of the cerebellum and their mutual relationship, as well as the cerebellar modular community structure. Whole-cerebellum structural and functional networks of 200 unrelated healthy subjects were reconstructed from diffusion magnetic resonance imaging (MRI) and resting-state functional MRI data (fMRI). In addition, we explore for the first time the cerebellar networks derived from track-weighted dynamic functional connectivity (tw-dFC), which has been recently proposed as a method to achieve a joint analysis of structural and dynamic functional connectivity in this framework. We show that the topology of structural, functional and hybrid (structural-dynamic functional connectivity) cerebellum network clusters into spatially and functionally coherent modules with high efficiency and short path length, thus reflecting an intrinsic small-world architecture, functionally segregated (local clustering) and integrated (global efficiency). In addition, the cerebellum seems to exhibit a rich-club organization, with highly connected and central nodes, being located predominantly in integrative cerebellar regions, having a strong tendency to be mutually interconnected, thus constituting a focal point for whole-cerebellum communication and information transfer. In future work, mapping cerebellum networks at the macroscale level could be useful for identifying novel biomarkers of cerebellar disease, characterizing individual variation and mapping the architecture of highly resolved neural circuits.

EFFECTS OF PERINATAL EXPOSURE TO BISPHENOL A OR S ON SEXUAL BEHAVIOR AND KISSPEPTIN SYSTEM IN MICE

Bonaldo B.[1,2], Casile A.[1], Bettarelli M.[1], Gotti S.[1,2], Marraudino M.[1], Panzica G.[1,2]

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Hypothalamic kisspeptin system is highly sensitive to the action of estrogens and of xenoestrogen molecules. In order to compare the effects of two different bisphenols (BPA and BPS) on brain circuits, we tested, in mice of both sexes, the effects of perinatal exposure to these compounds on the kisspeptin neurons and on reproductive behavior. Bisphenols, found in several plastics and epoxy resins, are well-known EDCs (Endocrine Disrupting Chemicals) and exposure to them is especially dangerous during specific "critical periods" of life when they can originate permanent effects. In the present experiment, we orally treated C57BL/6 dams with: a) a dose of 4 µg/kg body weight/day (*i.e.* TDI dose for BPA) of BPA or b) a similar dose of BPS, or c) vehicle (corn oil) only, from mating until the weaning of the offspring. We monitored the development of the offspring, evaluating their body weight (daily), food intake (weekly), puberty onset and estrous cycle (in females) until the PND90 when we analyzed the reproductive behavior (two-bedding T-Maze test and sexual behavior). BPA and BPS alter the onset of the puberty: BPA- or BPS-exposed males show anticipation of the puberty, while BPA-exposed females present a delay, while the estrous cycle of BPA- or BPS-exposed females was altered. BPA-exposed males have a decreased interest towards females (less time spent in the arm with the female bedding and a decreased number of mounts and intromission), while BPS-exposed males show an increased number of mounts, intromissions, and anogenital sniffing. Control males show a decreased number of mounts and intromissions towards BPS-exposed females. Finally, the immunohistochemical analysis of the hypothalamic kisspeptin system (rostral periventricular area of the third ventricle, paraventricular nucleus and arcuate nucleus) confirmed the sexual dimorphism in all the analyzed nuclei, but the system was altered in BPA or BPS-treated mice, with a significant increase of immunoreactivity in males. These results support the idea that the kisspeptin system is a not only a target for BPA, but also for BPS and that its alterations are probably linked to modifications on both physiological and behavioral parameters.

COMPARISON OF DECELLULARIZATION PROTOCOLS TO GENERATE PERIPHERAL NERVE GRAFTS: A STUDY ON RAT SCIATIC NERVES

El Soury M.[1,2,3,†], García-García Ó.D.[3,4,†], Moretti M.[5,6], Perroteau I[1], Raimondo S[1,2,*] Lovati A.B.[5,‡] Carriel V.[3,4‡]

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[†]These authors have contributed equally to this work and share first authorship. [‡]These authors share senior authorship.

In critical nerve gaps where direct tensionless repair cannot be applicable, an additional nerve graft or conduit are needed to fill the gap and connect the two transected nerve stumps. Decellularized nerve allografts are considered one of the promising tissue engineering strategies that can provide superior regeneration results compared to nerve conduits. The superiority of the decellularized nerves over nerve conduits is owed to the availability of natural well-conserved extracellular matrix component that has proven to play an important role in supporting axonal guiding and peripheral nerve regeneration. Up to now, the known decellularized techniques are time and effort consuming. In the present work performed on rat sciatic nerves, we aimed to investigate a novel nerve decellularization protocol able to combine an effective decellularization in short time with a good preservation of the extracellular matrix component. To this aim, a decellular-

ization protocol that has proven to be efficient for tendons (DN-P1) was compared with a decellularization protocol specifically developed for nerves (DN-P2). The outcomes of both the decellularization protocols were assessed by a series of *in vitro* evaluations, including qualitative and quantitative histological and immunohistochemical analyses, DNA quantification, SEM and TEM ultrastructural analyses, mechanical testing, and viability assay. The overall results showed that DN-P1 could provide promising results if tested *in vivo*, as the *in vitro* characterization demonstrated that DN-P1 conserved a better ultrastructure and ECM components compared to DN-P2. Most importantly, DN-P1 was shown to be highly biocompatible, supporting a greater number of viable metabolically active cells.

PHYLOGENETIC VARIATION OF "IMMATURE" NEURONS IN SUBCORTICAL REGIONS OF MAMMALS: PRELIMINARY RESULTS

Ghibaudi M.[1,2], Amrein I.[3], La Rosa C.[1], Bonfanti L.[1,2]

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In mammals, brain plasticity can vary depending on animal species. The genesis of new neurons (adult neurogenesis) is spatially restricted to stem cell niches and reduced from mice to humans. A population of prenatally generated (non-dividing) "immature" neurons (INs), which retain expression of typical markers of immaturity, is known to occur in the layer II of rodent paleocortex. We recently demonstrated that these cells are particularly abundant in the neocortex of gyrencephalic brains, also extending in subcortical regions. Here, we analysed claustrum and amygdala of six mammalian species characterized by different brain size, gyrencephaly and cortical IN density. Three young-adult animals/each species were analysed. Whole brain hemisphere, amygdala and claustrum volumes were evaluated on histologically stained serial coronal sections (40 μm thick) scanned with Axioscan. To study INs, doublecortin (DCX) was employed as a marker and quantitative/qualitative analyses were carried out on 480 nm-interval sections covering the whole extension of both subcortical regions. The marker for cell division Ki-67 antigen was used to check the nature of either dividing or "immature" neurons for the DCX+ cells. Direct cell counting was performed to obtain a quadratic/volume cell density (cells/mm^2-mm^3) using Neurolucida Software. Populations of DCX+ cells were found in both claustrum and amygdala of cat, rabbit, marmoset, while no immunoreactive elements were detected in mouse and naked mole rat. Different morphological cell types were identified, spanning from bipolar to multipolar neurons (3 types in amygdala, 2 in claustrum). Quantitative analyses revealed interspecies heterogeneity of IN occurrence, with prevalence in non-rodent mammals. This study confirms that non-rodent mammals, generally characterized by reduction in stem cell-driven adult neurogenesis, can rely on populations of young neurons within brain regions underlying the most important cognitive functions.

EFFECT OF 3D SYNTHETIC MICROSCAFFOLD NICHOID ON THE MORPHOLOGY OF HIPPOCAMPAL NEURONS

Musi C.A.[1,2], Colnaghi L.[2], Giani A.[2], Tomaselli G.[1,2], Marchini G.[2], Conci C.[4], Tironi M.[2], Raimondi M.T.[4], Remuzzi A.[3] Borsello T.[1,2*]

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The human brain is the most complex organ in biology. This complexity is due to the number and the intricate connections of brain cells, and has so far limited the development of *in vitro* models for basic and applied brain research. We decided to create a new, reliable, and cost-effective *in vitro* system of hippocampal neurons and astrocytes co-cultured based on the Nichoid, a 3D microscaffold microfabricated by two photon laser polymerization technology. After 21 days in culture, we morphologically characterized the 3D spatial organization of the hippocampal astrocytes and neurons within the microscaffold and we compared our observations to those made using the classical 2D co-culture system. We found that the co-cultured cells colonized the entire volume of the 3D devices. Using confocal microscopy, we observed that within this time period the different cell types had well differentiated. This was further elaborated with the use of Drebrin and PSD-95 antibodies as markers for mature and differentiated dendritic spines. Drebrin and PDS95 labelled the majority of neurons both in the 2D as well as in the 3D co-cultures. Using scanning electron microscopy, we found that neurons in the 3D co-culture displayed a significantly larger amount of dendritic protrusions compared to neurons in the 2D co-culture. This latter observation indicates that neurons growing in a 3D environment may be more prone to connections than those co-cultured in a 2D condition. Our results show that the Nichoid can act as a 3D device that can be used to investigate structure and morphology of neurons and astrocytes in a 3D volume. In the future, this model can be used as a tool to determine the factors at the basis of different human brain diseases, by plating cells derived directly from patients. This system may potentially further be used for drug screening in various brain diseases.

GENISTEIN: SEXUAL DIMORPHIC EFFECTS ON SEROTONERGIC SYSTEM IN MICE

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Phytoestrogens can act as endocrine disruptors (EDC), producing estrogenic or non-estrogenic effects, and can be dangerous during development, in specific critical periods. Among phytoestrogen, genistein (GEN), an isoflavone naturally present in the plant kingdom with estrogen-like activity, is particularly present in high quantities in the soy plant (*Glycine max*). Several studies have analysed the effects of GEN administration, in the mouse, during development, showing that it can alter brain neural cir-

cuits as well as behaviours, especially anxious behaviour, fertility and energy metabolism. Serotonergic system is deeply involved in almost all the above-mentioned behaviours. Therefore, we hypothesized that GEN exposure may alter the development of this system in sexual dimorphic way. Given that the treatment mimics the effects of raising newborns with soy-based preparations, this study may help to clarify possible caveats in the use of those formulas. Therefore, we analysed the effects of an early postnatal treatment with a dose of GEN comparable to the exposure level in babies fed with soy-based formulas, on serotonin (5-HT) brain circuits and anxious behaviour. We treated male (N=24) and female (N=24) CD1 mice with GEN (50 mg/kg body weight dissolved in sesame oil) or with the vehicle (control, CON). At PND60 six animals per group were tested with Open Field (OF) and Elevated Plus Maze (EPM). At the same time, we collected also feces to measure the fecal corticosterone in order to evaluate the anxious behaviour. The other mice were sacrificed for the immunohistochemical (IHC) analysis of 5-HT. In the OF test, treated mice have a reversal of anxiety-like behaviour: GEN induces an anxiolytic effect in male and an anxiogenic effect in female. The OF results are confirmed by the corticosterone levels, higher in treated male than in untreated male. Rather, the EPM showed only a sexual dimorphism in CON mice, with male more anxious than female, but, unlike OF, not an inversion of behaviour in treated mice. These findings are confirmed by the results of the IHC study (male, N=8; female, N= 8), indicating that GEN induced a reversal of serotonin neuron content in raphe nuclei, dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN). Specifically, GEN influenced 5-HT neuronal populations in a sexually dimorphic manner, notably in DRN: treated female mice showed a decrease of 5-HT neurons, whereas treated male mice showed an increase, compared with control.

SESSION II
NEURODEGENERATION AND NEUROPROTECTION

NEURODYNAMIC EFFECTS ON NERVE REGENERATION AND PAIN

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Nerve injuries and diseases affecting nerves are a rising problem leading to disability due to sensory and motor impairments often associated with neuropathic pain and in particular mechanical allodynia and hyperalgesia. The neurodynamic treatment (NDT) consisting of selective uniaxial nerve repeated tension protocols has been described to effectively reduce mechanical allodynia and hyperalgesia in neuropathic pain patients. Nevertheless, even if some studies on *in vivo* and *in vitro* models reported the ability of NDT to promote nerve regeneration and pain modulation the most of the biological effects involved are still unknown. Moreover, no standardized protocols are available. Since mechanical allodynia and hyperalgesia are linked to processes detected in the dorsal root ganglia (DRG), we aimed to define *in vivo* and *ex vivo* whether NDT protocols could induce selective biological effects promoting nerve regeneration and mechanical allodynia suppression. A model of rat median and ulnar nerve crush injury was adopted to define the effects of NDT on motor and sensory nerve recovery and pain modulation. A DRG ex vivo model was adopted to confirm the NDT effects on sensory recovery and in pain modulation to be selective on sensory neurons and not to be dependent on other non-neural tissue mediated responses. The obtained results show that NDT induced significant sensory and motor recovery preventing intraneural fibrosis. The regulation of TACAN and PIEZO1 gene expression in the DRG ipsilateral to the nerve injury was observed. No protective effect of NDT on nerve injury-related pain and loss of functions were detected. Ex vivo results confirmed that NDT promoted a dose-dependent neurite outgrowth and significantly downregulates the expression of TACAN and PIEZO1. Also, NDT does not promote pro-apoptotic effects in the DRG. Notably, the analyzed genes involved in mechanical allodynia are expressed in murine and human DRGs and coding for a high threshold mechanosensitive channel induced by inflammation (TACAN) and a low threshold mechanosensitive ion channel and are related to mechanical pain modulation. No significant effects are induced on other genes involved in neuroinflammation and myelin-activated mechanical allodynia. These results show that NDT activates selective anti-allodynic mechanisms related to the regulation of the mechanosensitive channel helping to understand its efficacy in pain reduction and nerve regeneration promotion.

ADMINISTRATION OF METHAMPHETAMINE TOGETHER WITH AUTOPHAGY INHIBITORS *IN VIVO* PRODUCES LOSS OF MESENCEPHALIC CELL BODIES

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The widely abused psychostimulant methamphetamine (METH) leads to neurotoxicity for nigrostriatal dopamine (DA) terminals in rodents and primates, including humans. *In vitro* studies indicate that METH toxicity is modulated by autophagy. In detail, when autophagy is inhibited METH toxicity increases, while stimulating autophagy prevents METH-induced toxicity in cell cultures. According to the hypothesis that ongoing autophagy prevents METH-induced DA toxicity, in the present study, we tested whether systemic injection of autophagy inhibitors worsens METH toxicity. In the whole brain, METH alone destroys meso-striatal DA axon terminals, while fairly sparing DA cell bodies within substantia nigra pars compacta (SNpc). No damage to either cell bodies or axons from ventral tegmental area (VTA) is currently documented. METH (5 mg/Kg × 4, 2 h apart) administered to C57Bl/6 mice following systemic injection of autophagy inhibitors such as asparagine (ASN, 1000 mg/Kg) or glutamine (GLN, 1000 mg/Kg), a frank loss of cell bodies takes place within SNpc and a loss of both axons and cell bodies of VTA neurons is documented. These data indicate that autophagy inhibition extends METH toxicity to DA cell bodies, within both SNpc and VTA, where autophagy was found to be inhibited, while ongoing autophagy protects DA neurons and determines the refractoriness of cell bodies to METH-induced toxicity.

ACETYLATED α-TUBULIN AND STRESS GRANULES: NOVEL INSIGHTS INTO LEWY BODY FORMATION

Giampietro F.[1], Mazzetti S.[1,2], Isilgan B.[1], Amadeo A.[1], Calogero A.M.[1], Giaccone G.[3], Bramerio M.[4], Pezzoli G.[2,5], Rolando C.[1], Cappelletti G.[1,6]

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Lewy Bodies (LBs) are the main pathological hallmark of Parkinson's disease (PD). LBs are proteinaceous and lipid-rich inclusions that accumulate in neuronal cytoplasm of PD patients and highly aggregated α-Synuclein (α-Syn) is the main component. α-Syn fibrils localized in LBs are abundantly phosphorylated at the level of Serine 129 (pS129). Very recently, Moors and colleagues (2021)[1] identified an onion skin-type architecture in mature nigral LBs showing cytoskeletal components (*e.g.* neurofilaments and β-tubulin) and pS129 α-Syn at the periphery and DAPI staining in the inner core. They also identified six different stages of LB morphogenesis using pS129 α-Syn as a marker. We aimed at going more deeply into LBs formation and composition and investigated: 1) the presence of different stages of LBs in our *post-mortem* brain tissues using total α-Syn and α-Syn oligomers that allow us to identify *pale bodies*, the precursors of LBs characterized by less aggregated α-Syn; 2) the involvement of cytoskeleton, focusing on a post-translational modification of α-Tubulin, the acetylation of Lysine 40, which is known to be unbalanced in cellular and animal models of PD; 3) the potential role of stress granules studying two of their components, G3BP and TIAR. We analysed *post-mortem* human brain tissues obtained from both PD patients fulfilling clinical and neuropathological diagnostic criteria for PD and control subjects, in whom the absence of neurodegenerative pathologies was assessed. We used an immunohistochemical approach to detect total α-Syn, acetylated α-tubulin and stress granules proteins, and Proximity Ligation Assay (PLA) to detect α-Syn oligomers. First, we identified four stages of LB morphogenesis in the *substantia nigra* of our tissues taking advantage of confocal microscopy and high resolution (spinning disk microscopy). Then we found that acetylated α-Tubulin is present and accumulated at the peripheral region of the *pale bodies,* but it is not present inside mature LBs indicating its involvement in the early stages. Furthermore, G3BP and TIAR are localized both at the peripheral and central region of the *pale bodies*; in addition, they are still present in mature LBs colocalizing with total α-Syn. Together, our data strongly indicate that both acetylated α-Tubulin and stress granule components contribute to α-Syn aggregation, suggesting novel insights in pathogenesis of PD and LB morphogenesis.

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COLOCALIZATION AND INTERACTION STUDY OF NEURONAL JNK3, JIP1, β-ARRESTIN2 TOGETHER WITH PSD95

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The c-Jun-N-terminal kinases (JNKs) are a family of stress-activated serine threonine protein kinases belonging to the Mitogen-Activated Protein Kinases (MAPKs). Mammals express three different JNK isoforms: JNK1, JNK2 and JNK3. While JNK1 and JNK2 are widely expressed in all body tissues, JNK3 is selectively expressed in the central nervous system, cardiac smooth muscle and testis. JNK3 is also the JNK isoform most responsive to stress stimuli in the brain and is involved in synaptic dysfunction and general neurodegenerative processes. JNK3 pathway is organized, as other MAPKs, in a cascade of signaling amplification in which the signal transduction occurs by a phosphorylation mechanism. Since different MAPKs shared common upstream activators, the pathway specificity is guaranteed by scaffold proteins. In this contest, JIP1 and β-arrestins2 are two of the most important regulators of the JNK signalling and they are highly expressed in the brain. Due to the strong involvement of JNK3 in synaptic dysfunction, we investigated whether these interactions occur in the whole brain homogenate as well as at the level of the dendritic spines performing hippocampal in-vitro culture and isolating the post-synaptic enriched protein fraction from brain lysate. We biochemically studied the interaction of JNK3 with JIP1 and/or β-arrestin2 in C57bl6/J mice total brain homogenates and in the post-synaptic compartment. We found by immunoprecipitation that the proteins can be found interacting together with PSD95, a post synaptic density marker. We next took advantage of super-resolution microscopy to demonstrate the co-localization between JNK3-PSD95-JIP1 and JNK3-PSD95-β-arrestin2 in cultured hippocampal neurons. Targeting JNK3, taking advantage of its interaction with scaffold proteins

could represent a therapeutical tool against many brain diseases, since it mediates stress-pathways, neuronal death and synaptic injury.

VITAMIN C ADMINISTRATION REDUCES NEUROINFLAMMATION IN A MOUSE MODEL OF NEURODEGENERATION

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Vitamin C (Vit C) is vitamin known anti-oxidant and anti-inflammatory properties. In this research we investigated the neuroprotective effects of vitamin C in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced animal model of PD and its role in the modulation of neuroinflammation. Vit C significantly reduced the MPTP-induced loss of tyrosine hydroxylase (TH)-positive dopaminergic neuronal cells in the substantia nigra, as well as microglial cells activation and astrogliosis. In addition, gait and spontaneous locomotor activity, evaluated by an automated treadmill and the Open Field test respectively, were partially ameliorated by Vit C treatment in MPTP-intoxicated animals. Taken together, these results showed that the reduction of some of the motor symptoms of our model of Parkinson's like disease was accompanied by the diminution of the activation of the cells involved in neuroinflammatory processes. In relation to neuroinflammation, results show that Vit C reduced the protein and mRNA expression of pro-inflammatory cytokines such as IL-6, TLR4, TNF-α, iNOS, CD40 while anti-inflammatory proteins such as IL-10, CD163, TGF-β and IL-4 resulted increased. Interestingly, we have demonstrated for the first time that Vit C reduces neuroinflammation by modulating microglial polarization and astrocyte activation. In conclusion, our study has evidenced that Vit C may represents a new promising diet supplement for the prevention and alleviating the inflammatory cascade in PD, contributing to neuroprotection.

EFFECTS OF CHOLINE-ALPHOSCERATE AND THIOCTIC ACID ON THE BRAIN OF HYPERTENSIVE RATS

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Hypertension, which is caused by an elevation of blood pressure and increased arterial wall thickness, represents a risk factor for the development of cerebrovascular disease and cognitive impairment. Evidence suggests that hypertension leads to neuroinflammation, which significantly contributes to the physiopathology of cerebrovascular alterations due to the increasing production of reactive oxygen species and cholinergic pathways dysfunction. A cholinergic precursors drug, choline alphoscerate or alpha-glycerylphosphorylcholine (α-GPC), causes an increase in acetylcholine levels improving the cholinergic system countering cognitive impairment, associated with cerebrovascular damage, and could targeting neuroinflammation. (+)-Thioctic acid [(+)-TIO] is the naturally occurring eutomer that have been shown to anti-inflammatory and antioxidant effects in the brain.

The study was designed to investigate if treatment with the two compounds, alone or in association, could induce neuroprotection in the brain of spontaneously hypertensive rats (SHR) used as an animal model of cerebrovascular alterations. 24-weeks old SHR were treated with α-GPC (150 mg/kg/day) and (+)-TIO (125 μmol/kg/day), alone or in combination, for 4 weeks. Age-matched normotensive Wistar Kyoto (WKY) rats were used as normotensive control. The frontal cortex and the hippocampus were collected for western blot and immunohistochemistry investigations of neuronal and neuroinflammatory markers. Blood pressure (BP) was higher in SHR rats compared to normotensive WKY. After 4 weeks of treatment with α-GPC and (+)-TIO, alone or in association, they reduced systolic BP while only their association reduced the diastolic BP. The immunochemical and immunohistochemical results showed that α-GPC alone restored the levels of neuronal nuclei proteins. The two compounds, alone or in association, did not prevent the downregulation of synaptophysin and microtubule-associated protein-2. α-GPC and (+)-TIO counteracted the astrogliosis, microglial activation, and decreased the level of tumor necrosis factor-alpha. Our results indicate that treating hypertensive rats with α-GPC and (+)-TIO reduced neuronal damage and glial response in the two brain areas, providing neuroprotection. The administration of the two compounds could represent a new perspective strategy to prevent hypertension-associated brain alterations. Further investigations may allow evaluating the effects on clinical trials in hypertensive patients.

BDNF AND TRKB IN THE ROMAN RAT BRAIN AFTER ACUTE MILD STRESS

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The Roman Low Avoidance (RLA) and Roman High Avoidance (RHA) rats are two divergent phenotypes displaying respectively reactive and proactive coping styles in face of aversive environmental conditions. Thus, when exposed to stressors, RLA rats display depression- and anxiety-related behaviors, while RHA rats are resistant to stress-induced depression. Different forms of stress-induced depression-like symptoms impair the signalling of brain neurotrophins like the Brain Derived Neurotrophic Factor (BDNF) and induce alterations of synaptic plasticity. We have previously reported behavioral and immunochemical data in the hippocampus (HC) of Roman rats exposed to the forced swimming (FS) as acute robust stressor. Here we extend the characterization of Roman rat brain by investigating the effects of tail pinch (TP), a mild stressor, on BDNF/trkB neuronal signalling in two brain areas: the HC, which plays a role in the control of emotions and in the mnemonic consolidation, and the prefrontal cortex (PFC), involved in the process of decision-making, by using western blot and immunohistochemistry assays. Behavioural testing showed that after 40 min of TP, the RLA rats exhibited a reactive coping activity characterized by freezing, grooming, and tail licking, while RHA rats behaved proactively, spending a longer time biting the clamp and trying to remove it from their tails. Immunochemistry showed that in the dorsal and ventral HC (dHC and vHC) of RLA rats the TP induced an increase of the basal BDNF- and trkB-like immunoreactivity (LI) in the dHC, whereas in the vHC TP caused a decrease in trkB-LI but did not modify the BDNF-LI. As for the RHA rats, TP increased the

basal level of BDNF- and trkB-LI in the dHC, and produced a small decrease of the basal BDNF-LI in the vHC. The expression of BDNF and trkB appears to be differentially affected by TP also in the PFC subregions of RHA vs. RLA rats. As for the PFC subregions, the TP stress elicited a significant increase in BDNF-LI in the prelimbic/infralimbic (where TP also induced an increase, albeit not significant, of trkB-LI) and the anterior cingulate cortices of both lines though the effect was more marked in RHA rats. The results demonstrate that TP stress interferes with the baseline BDNF signalling, eliciting different changes in the brain cortex and between the two rat lines. Interestingly, in RHA rats the TP stress appears to be associated with alterations in BDNF signalling that are not present during FS.

ROLE OF MESENCHYMAL STEM CELL DERIVED EXOSOMES IN NEURONAL SURVIVAL

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Mesenchymal stem cells (MSCs) are multipotent progenitor cells able to differentiate into a variety of cell types, to migrate to injured sites, to regenerate tissues and to promote neuronal survival, thus, showing a huge potential as cell-based therapy[1]. During the last decade, MSCs have also shown promising results for therapy of neurodegenerative diseases. It is believed that their positive effect on neuronal survival is not due to its differential capacity but due to trophic effects[2]. Among the existing hypotheses on MSCs mechanism of action, different studies report the paracrine effects of MSC secreted vesicles for tissue repair[3], however, their composition and role remain to be established. The aim of this work was to analyze the effect of MSCs derived vesicles on neuronal cells' survival. Extracellular vesicles (EV) were purified from medium of MSCs cultured alone or in co-culture with sensory primary neuronal cells. After the staining with lipophilic membrane dye PKH26 EV were added once a week to primary neuronal cell cultures for 6 weeks. Neurons were followed up for 6 weeks to analyze stained EV intracellular localization and neuronal survival. Confocal microscope analysis demonstrated that EV were able to enter into neurons and to localize in cytoplasm of both cell body and neuronal processes. Moreover, EV showed a perfect co-localization with exosomal marker Cd9. Our results also showed that neurons that received MSC derived EV, both from MSC alone or from the co-culture, were able to survive longer. In conclusion, this work demonstrates that MSC could support the sensory neurons survival through exosome release. Such a mechanism could be therefore exploited to design a cell free therapy to support neuronal survival. We are now investigating the putative molecules mediating such an effect.

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SYNAPTOPATHY SIGNALING PATHWAY IN 5XFAD ACROSS DIFFERENT AGES

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Alzheimer disease (AD) is the first leading cause of dementia worldwide and no treatments are available to cure or slow down the pathology. One of the key features of AD is the synaptic dysfunction, the first event in the neurodegenerative process. Identify modulators of this process is of particular significance in the treatment of AD. Several evidence in literature suggest that the c-Jun N-terminal Kinases (JNKs) have a pivotal role in AD pathogenesis. To study JNKs function in the AD synaptopathy we analyzed 4, 6, and 10 months old male and female 5xFAD mice, an established mouse model of AD, evaluating the progression of cognitive deficits with the 6-Arms-Water Maze and the synaptic dysfunction correlated to the biochemical changes at the post-synaptic element (TIF fraction). Our findings showed a clear progression of the memory deficit in transgenic (tg) mice, in the three time-points considered, coupled with increased activation of JNK signaling pathway in the total homogenate of cortex and hippocampus, the two main areas impaired in AD. In addition, the biochemical evaluation of the main biochemical markers of the excitatory synapses, *i.e.* AMPA and NMDA glutamate receptors, and scaffold proteins like PSD95, Shank3 and Drebrin in the PSD-region (TIF) reveals a general disorganization of the dendritic spines in tg vs wt mice. These alterations correlate with JNKs activation, in this subcellular compartment, that increased during time and strongly correlate with cognitive deficit. Lastly, at six and ten months of age, when mice displayed the stronger memory deficit, the JNK3 isoform levels, the most responsive isoform to stress, in the TIF fraction were sharply increased in tg vs wt animals. These results confirm JNKs as a crucial actor in the AD neurodegenerative process in 5xFAD mice and propose JNK3 as a potential target to tackle AD synaptopathy.

EFFECTS OF ASC-EXOSOMES ON ALS AND SMA MURINE MODELS

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The use of stem cells represents a possible treatment for neurodegenerative disorders, like Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy (SMA). In particular, their beneficial action seems to be due to the paracrine release of exosomes, main mediators of intercellular communication. Indeed, through the release of their content (proteins, miRNA and nucleic acids) they are able to promote neurogenesis, inhibit apoptosis, enhance immunomodulation in different pathophysiological contexts, recapitulating the effect of origin cells. Despite ALS and SMA are two distinct neurodegenerative diseases caused by different pathogenic mechanisms, they share several pathogenic

mechanisms. For these reason, our aim is to investigate the protective influence of exosomes in two different *in vivo* models. We therefore isolated and characterized exosomes from the culture media of murine adipose-derived stem cells (ASC-exosomes) and delivered them in the SOD1(G93A) mice, the murine model of ALS, *via* intranasal injections; this could represent a non-invasive procedure and an efficient approach to deliver exosomes to the central nervous system. Moreover, we delivered ASC-exosomes with intracerebro-ventricular administration in the SMN 7 murine model, the most widely used one to study SMA. The results showed that ASC-exosomes could improve the motor performance of animals, both in treated SOD1(G93A) and SMN 7 mice. They could also protect lumbar spinal cord motor neurons from neurodegeneration. Furthermore, in the peripheral tissues we could observe a higher number of innervated neuromuscular junctions and an attenuated skeletal muscle atrophy in the treated SMN 7 group. These outcomes could allow to better understand the effects of ASC-exosomes and to target them as a possible approach in the treatment of neurodegenerative disorders.

SESSION III
EMERGING ROLES OF GLIAL CELLS

DEX AND GFS ROLE ON GFAF AND ERK SINTHESYS IN ASTROCYTES

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Steroid hormones and neurotrophic factors regulate astroglial cell survival, proliferation, and differentiation in culture. The present study examines the interaction between glucocorticoids and growth factors (GFs) on cytoskeletal proteins and extracellular signal-regulated kinase 2 (ERK2) expression in stressed astroglial cultures at 25 days *in vitro*, according to the following experimental condition. Glial Fibrillary Acid Protein (GFAP) localization was found in the cytoplasm and in subcellular compartments, and a typical polygonal flat shape of astrocyte was also ascertained. The pre-treatment with bFGF alone or in combination with the subsequent treatment with 10^{-9} M Desamethasone (DEX) for 48 h induced an enhancement of GFAP expression with respect to untreated controls. The treatment with progression GFs EGF, Insulin (INS), and IGF-1 alone in the last 12 h significantly increased GFAP expression with respect to untreated controls and also with respect to Serum supplemented medium (SSM) cultures. No significant modification in GFAP expression with respect to untreated controls was observed in the other treatments in which the GFs were coadded. In addition, GFAP expression was significantly decreased after coaddition of EGF + INS and EGF + IGF-1 with respect to bFGF 24-h/DEX 48-h pre-treated cultures. It is easy to observe a marked enhancement of vimentin expression for all the treatments with progression GFs added alone or in combination compared with untreated controls. This enhancement was particularly evident in the treatment with INS alone in the last 12 h. All the treatments (EGF, INS, and IGF-1 alone or in combination) induced a highly significant increase of the ERK2 expression in comparison with the untreated controls and also with SSM cultures. The present study shows that glucocorticoids may cooperate with GFs or may abrogate their effects, depending on the experimental culture conditions used as well as the exposure time and the types of GFs added. Because increasing evidence suggests that these well-known neuroactive molecules exert significant neuroprotective effects against a variety of neurodegenerative pathologies. Our findings provide evidence of interactive dialogue between GFs and neurosteroids in cultured astrocytes. This may have implications in the therapeutic approach to neurologic disorders associated with astrogliosis.

ZINC PREVENTS CADMIUM-INDUCED GLIAL STRESS AND ACTIVATION

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The air pollution is known to have serious health effects. However, even if a decrease of air pollutant emissions is carrying on, the risk of morbidity and mortality is increasing. Among the environmental pollutants, the heavy metal cadmium (Cd) has a pivotal role as it is present in pesticides, plastics, smoke cigarette, colours and many other sources. Due to its long half-life, ranging between 15 and 20 years, Cd toxicity has many health effects affecting different organs, including the central nervous system (CNS). As recently reported, Cd is able to affect the blood-brain barrier tight junction integrity thus leading to its impairment. Once inside the CNS, Cd can interact with other cell types belonging to the neurovascular unit (NVU). Among all the cells that constitute the NVU architecture, astrocytes and microglia, through their relationship, have a pivotal role in detecting the needs of neuronal supply and triggering necessary responses for such demands.The toxicity of this metal seems mainly due to a Cd-dependent increase in oxidative stress. On the other hand, it has been reported that zinc (Zn) can counteract this toxic effect, by both competing for the same pathways and preventing the Cd-induced oxidative stress. The aim of this study was to evaluate the role of Zn in ameliorating the Cd toxic effects on astrocyte and microglial cell cultures. The DITNC1 (astrocyte) and BV-2 (microglial cells) cell lines were used in their appropriate growth medium. The stimuli used were cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) both at 1 μM concentration for different time of exposure (4-48 h).
Cell viability assay, immunofluorescent staining and western blotting analyses were performed in order to evaluate the Zn protection against Cd-induced toxicity. Our results demonstrated that Zn is able to counteract the reactive oxygen species (ROS) overproduction during Cd treatment along with a decrease in GRP78 protein expression levels, a well-known ER stress marker. Also, Zn is able to prevent cytochrome C spillage from mitochondria caused by the Cd treatment Most importantly, the presence of Zn counteracts the Cd-dependent increase of glial activation markers, such as GFAP and S100β for astrocytes, and Iba1 and CD86 for microglial cells. Overall, these data demonstrated the protective role of Zn against Cd toxic effects by mitigating both oxidative and ER stress.

ASTAXANTHIN EFFECT ON TISSUE TRANSGLUTAMINASE EXPRESSION IN OLFACTORY GLIA EXPOSED TO AMYLOID-β

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Alzheimer Disease (AD), one of the most common neurodegenerative diseases, is characterized by progressive neuronal loss and accumulation of proteins, including Amyloid-beta (Aβ), a neurotoxic protein. It is known that tissue transglutaminase (TG2), an ubiquitary calcium-dependent protein, is involved in protein aggregation in AD. Previous our studies showed that Aβ(1-42) and its fragments Aβ(25-35) and Aβ(35-25) induced an overexpression of TG2 and its isoforms on olfactory ensheathing cells (OECs), a glial population of the olfactory system expressing neural stem cell markers, including nestin. In the last years growing attention rose on neuronutraceutics and their effect on mental health, as many compounds are characterized by antioxidant and anti-inflammatory activities, these properties could have beneficial effects in counteracting the multifactorial onset and progression of AD. Among these molecules, we focus on Astaxanthin, a natural compound predominantly found in marine organisms, the green microalgae *Haematococcus pluvialis* and *Chlorella zofingiensis*; it can be transported into the brain through the blood–brain barrier and exhibits powerful antioxidant activity. In addition, Astaxanthin can effectively scavenge intracellular free radicals and destroy peroxide chain reactions, protecting cell and biological membranes from oxidative damage. Therefore, it exhibits numerous health benefits, such as anti-inflammatory actions, anti-tumor effects, hepatoprotective effects, and immunomodulatory activity. In this study, the effect of Astaxanthin pretreatment on TG2 and its isoform expression exposed to Aβ(1–42) or by Aβ(25–35) or Aβ(35–25) on OECs was assessed. Moreover, we evaluated Astaxanthin effect on the expression of some cytoskeletal proteins, vimentin, glial fibrillary acid protein (GFAP), nestin and caspase-3. Astaxanthin pretreatment reduced TG2 overexpression, modulating the level of TG2 isoforms and reduced ROS production, GFAP and vimentin expression, inhibiting apoptotic pathway activation and induced an increase in the nestin levels. Our data demonstrated that Astaxanthin pre-treatment stimulated OECs self-renewal through the reparative activity played by TG2. Therefore, pretreatment Astaxanthin might represent an innovative mechanism to contrast TG2 overexpression in AD.

INVESTIGATING THE INVOLVEMENT OF SATELLITE GLIAL CELLS IN PACLITAXEL-INDUCED PERIPHERAL NEUROTOXICITY

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Paclitaxel (PTX) is a common chemotherapeutic agent used to treat several cancers however inducing a peripheral neurotoxicity with paraesthesia, dysesthesia, tingling, and burning pain in a glove-and-stocking distribution. Peripheral nerves and dorsal root ganglia (DRG) represents a susceptible target for PTX neurotoxicity due to the absence of the blood brain barrier. Satellite glial cells (SGCs) in DRG are in close contact with the adjacent sensory neurons and essential for sustaining their metabolic requirements. SGCs can be activated, increased in number and morphologically modified after nerve injuries of different origins. Upregulation of glial fibrillary acidic protein (GFAP) as well as an increment in the communicative connection between adjacent SGCs were detected after neuronal injury or inflammation. Gap junctions are supposed to regulate SGCs coupling and are composed of different proteins (connexins, Cxs). Among them, connexins 36 (Cx36) and 43 (Cx43) have been identified in the perineuronal SGCs of DRG. In this work, we investigated whether SGCs can undergo modifications after chronic administration of PTX in rat. Twenty-four rats were injected intravenously with PTX 10 mg/Kg once a week for 4 weeks. In order to assess peripheral neurotoxicity and pain, neurophysiological analysis and behavioural tests were performed at baseline and at the end of PTX treatment. DRG were collected for light microscopy observations, immunohistochemical (IHC), immunofluorescence (IF) and western blot (WB) analyses. Protein expression and localization of GFAP, Cx36 and Cx43 were evaluated. Altered neurophysiology, mechanical allodynia and mild thermal hyperalgesia were evidenced after 4 weeks. At microscopy level, a trend of enlargement of satellite cells morphology and an evident reduction of interstitial space between neurons were observed in

DRG of treated animals. IHC-qualitative and quantitative analysis showed an increased GFAP-positivity in rat DRG treated with PTX, which demonstrated SGC activation and hyperproliferation. In addition, perineuronal spot distribution of Cxs was evident at IF analysis in PTX-treated rats with a higher Cx43 staining signal. WB supported this increment. Therefore, the investigation of the interactions SGC-SGC and SGCs-neurons could be important in the identification of new molecular mechanisms underlying PTX-induced peripheral neurotoxicity.

SESSION IV
BRAIN TUMORS

ACTIVITY-DEPENDENT NEUROPROTECTIVE PROTEIN (ADNP) INVOLVEMENT IN GBM

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Glioblastoma multiforme (GBM) is the most common primary brain cancer affecting adults with poor prognosis. It is a solid tumor characterized by high cellular heterogeneity as well as epigenetic and genetic alterations responsible of tumor malignancy. The core of tumoral mass is constituted by hypoxic niches overexpressing hypoxia-inducible factors, such as HIF1α and HIF2α, which in turn induce the activation of vascular endothelial growth factor (VEGF), responsible of neoangiogenesis and tumor progression. The standard therapy for GBM treatment consists in multimodal approach including surgery, radiotherapy and chemotherapy to improve survival and quality of life. Recent studies have demonstrated the anti-invasive effect of pituitary adenylyl cyclase-activating peptide (PACAP) in GBM. It is known that some of its effects are mediated through the activity-dependent neuroprotective protein (ADNP). This latter is involved in brain development as well as in various type of tumors where it exerts controversial role. To date, no evidence exists regarding ADNP role in GBM. In this work, we have investigated its involvement in GBM malignancy by analysing its expression in a human glioblastoma sample. By immunofluorescence analysis, we have demonstrated that ADNP is expressed in most of glial cells, predominantly in hypoxic areas overexpressing HIF-1α. To investigate the role of ADNP on HIF-VEGF axis in GBM, we have exposed U87MG glioblastoma cell line to a hypoxic mimetic agent, DFX, and treated with NAP, the smallest active element of ADNP. Results have demonstrated that ADNP modulate hypoxic-angiogenic pathway by reducing VEGF secretion and cell migration. Even if further investigation are needed, the present study suggested that PACAP effect in GBM could be due to ADNP induction.

THE ROLE OF COLLAGEN TYPE VI AND PROTEOGLYCAN NG2 IN BRAIN AND GLIOBLASTOMA VASCULARIZATION

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Glioblastoma (GBM) is the brain neoplasia with the worst prognosis and is known to be the most vascularized tumor in humans, even though the molecular factors involved in the process of neovessel formation have not been completely established. Growing evidence is available about the underestimated role of extracellular matrix proteins in GBM vascularization. In particular, collagen type VI (COL6) shows an increased expression in many tumors, including glioblastomas, where it is a component of the tumor vessel basal lamina (BL). Among the numerous cell recep-

tors for COL6, the part-time proteoglycan neuron/glia antigen 2 (NG2) gains interest due to its overexpression, both in tumor cells and pericytes. The putative role of the COL6/NG2 axis in vessel growth, along with its links to glioma vessel neo-formation, remains an open field of investigation. In this study, we have analysed COL6/NG2 immunolocalization and relations within the neurovascular unit of developing brain and GBM samples. Observations carried out during normal brain vascularization confirm that COL6 is a component of the BL and demonstrate that it prevails in the parenchymal layer, where it appears tightly related to NG2-expressing pericytes, on the penetrating radial vessels and on their growing collaterals as well. In glioblastomas, a remarkable presence of COL6, that forms the multi-layered BL of hyperplastic vessels, parallels the overwhelming presence of NG2-expressing tumoral pericytes, identifies distinctive niches of pericyte proliferation, and characterizes tumor vessel sprouting. The possible role of the COL6/NG2 axis in GBM vascularization has been further investigated by applying the dual IHC/ISH RNAscope assay to detect COL6 and cell-specific markers together with *VEGF/Flk-1* ligand/receptor system expression in hyperplastic microvessels. The results show an unexpected expression of the receptor Flk-1 by vascular niche pericytes, which suggests these proliferating cells as a cellular reservoir for the unconventional pericyte-initiated tumor vessel growth, a modality that could represent a new therapeutic target for counteracting GBM neovascularization.

THE EFFECTS OF RAPAMYCIN ON THE OCCURRENCE OF PRIONOIDS IN GLIOBLASTOMA MULTIFORME

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Recently, increasing evidence indicates a role for cellular prion protein (PrPc) in fostering stemness and invasion of glioblastoma multiforme (GBM). Besides being implicated in the pathogenesis and transmission of prion diseases, PrPc is emerging as a key in maintaining glioblastoma cancer stem cells (GSCs) phenotype, thereby strongly affecting GBM infiltration and relapse. This might apply to other proteins, which share a prion-like structure and biology. Thus, suppressing the expression of these proteins and occluding their ability to spread from cell-to-cell may be useful to provide novel treatments along with an in-depth knowledge of GBM neurobiology. Since these proteins appear to be regulated by the autophagy pathway, in the present study, we administered the gold standard mTOR inhibitor/autophagy inducer, rapamycin, to analyze the effects on GBM cell cultures concerning the expression of specific prionoids. Rapamycin dose-dependently suppresses the expression of prion-like proteins, while producing a marked differentiation of GBM cells. These effects are remarkable when transwell co-cultures of normal human astrocytes (NHA) are seeded in close contact with GBM cells separated by a semi-permeable membrane. In these experimental conditions, a transformation of astrocytes occurs along with increased expression of prionoids, which depends on the spreading of specific molecules through the membrane. Rapamycin administration occludes cell-to-cell spreading, while bringing back NHA to their previous phenotype.

DIFFERENTIAL ROLE OF MICROGLIA, MACROPHAGES AND ASTROCYTES DURING GLIOBLASTOMA PROGRESSION

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The invasive behaviour of the glioblastoma (GBM) is permitted by a complex interaction with glial cells, that work as the brain innate immune system. Microglia, astrocytes, and resident macrophages undergo (epi-)genetic, molecular, and morphological changes to acquire a tumor-supporting phenotype. These modifications of the brain elements are due to the intrinsic high plasticity of the nervous system and occur gradually. Therefore, the identification of time-related molecular targets may help in understanding the pathways through which GBM affects the glial cells. GL261 glioma cells were injected in the right striatum of immuno-competent C57Bl6J mice, and animals were sacrificed after 7, 14, and 21 days (7D, 14D, 21D). The tumor development was assessed through 3D reconstruction of tomographic imaging and brains were processed by immunohistochemistry, immunofluorescence, and Western blotting. In the early stage, the proliferating tumor (ki67$^+$) appeared with a spotted distribution and triggered astrocytes (GFAP$^+$, glial fibrillary acidic protein) reaction. Microglia and macrophages (Iba1$^+$, Ionized calcium-binding adaptor molecule 1) were scarcely represented at the site of the tumor injection, and the chemokine-CCL-2-dependent recruitment of inflammatory monocytes appeared to be reduced. The tumor bulk became established at 14D and was surrounded by a dense scar of reactive astrocytes, paralleled by an increase of the phagocytic cells in the peritumoral area (CD68$^+$;Iba1$^+$;GFAP$^+$), which may remove tissue debris and contribute to the extracellular matrix (ECM) remodelling. Accordingly, the ECM modifier metalloproteinase 9 (MMP9), and tenascin C (TnC) protein levels peaked at this stage, allowing CD133$^+$ glioma stem cells to migrate out of the primary bulk, which appears inflamed, necrotic and infiltrated by microglia/macrophages at 21D, as indicated by the rise in the level of CCL2 and Iba1. However, the protein expression of the specific microglial activation marker TMEM (transmembrane protein 119) was downregulated, suggesting differential activities for tumor-associated-resident microglia and peripheral macrophages. Our results showed the dynamic of the tumor progression along with the response of astrocytes, microglia, and macrophages, shedding light on potential time-related targets to consider for further studies.

SESSION V
INSIGHTS INTO PERIPHERAL NERVOUS SYSTEM

MORPHOFUNCTIONAL CHARACTERIZATION OF CIPN

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Chemotherapy-induced peripheral neurotoxicity (CIPN) is a long lasting/permanent toxicity of most common anticancer drugs; it causes sensory loss and neuropathic pain at limb extremities. Different drug classes have a different pattern of damage. Preclinical studies allow morphological analyses to characterize the damage and allow pathogenetic investigations. This is not feasible at clinical level in this specific population. However, some functional testing can be applied to predict anatomical damage: nerve excitability testing (NET), a far more advanced technique than the standard nerve conduction studies (NCS), allows to test axonal properties and explore precocious signs of damage. We characterized the morphological and functional pattern of damage of 2 different drugs: paclitaxel (PTX) which determines a severe sensory-motor polyneuropathy in patients and Oxaliplatin (OHP) which causes a mild, only sensory, neuronopathy. Female Wistar rats were chronically treated with PTX in experiment 1 and with OHP in experiment 2 and compared to control animals (n=8/group). NET was assessed at 24, 48, 72 h after 1st administration and at end of treatment. NCS and dynamic test were assessed at baseline and at end of treatment. At end of treatment, harvesting of caudal nerves and skin biopsy, for intraepidermal nerve fiber density (IENFD) assessment, were performed. NET findings after 1st injection showed alterations compatible with a transient alteration of kinetics in sodium-voltage operated channels in OHP group; PTX group, instead, showed precocious signs of ongoing axonal dysfunction. At end of treatment, NCS showed a mild sensory neuropathy in OHP animals and a relevant sensory-motor neuropathy in PTX animals. Both groups showed painful behavior. Neuropathological analysis confirmed these patterns. NET recordings, after the 1st administration, showed a different pattern in PTX and OHP animals and this mirrors the different observations at morphological analyses. NET monitoring gave precious information on nerve damage after 1st administration. NET was first applied in clinical setting and then transferred to preclinical setting. Therefore, it can be the ideal link between morphological analyses – mainly feasible at bench side – and clinical testing at bed side. Ideally, it can be proposed to transfer, to clinical trials, preclinical morphological evidence of efficacy of new potential neuroprotectants.

BIALLELIC VARIANTS IN *LIG3* CAUSE A NOVEL MITOCHONDRIAL NEUROGASTROINTESTINAL ENCEPHALOMYOPATHY

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Mitochondrial encephalomyopathies can be characterized by leukoencephalopathy and severe gut dysmotility, such as chronic intestinal pseudo-obstruction, an impairment of gut propulsion mimicking a mechanical obstruction without detectable anatomical causes. Mitochondrial neurogastrointestinal encephalopathies (MNGIE or 'MNGIE-like' phenotypes) are caused by mutations in TYMP or POLG, although some cases remain unresolved. We aimed to identify the genetic defects in seven patients from three independent families whose affected members (2 female and 5 male; age range: 18.1±15.5 years) showed severe gut dysmotility and neurological abnormalities, including leukoencephalopathy, epilepsy, migraine, stroke-like episodes, and neurogenic bladder. None of the patients carried mutations in TYMP, POLG or mitochondrial DNA (mtDNA). Whole exome sequencing analysis was performed on peripheral blood-extracted DNA obtained from each affected family member. Dermal fibroblasts were obtained from patients' and controls' skin biopsies and grown in standard culture media. Functional lig3 ablation in the zebrafish model was performed *via* morpholino analysis and/or CRISPR/Cas9 gene editing. We identified compound heterozygous variants in a new disease gene, named *LIG3*, in all patients. All variants were predicted to have a damaging effect on the protein. The *LIG3* gene encodes the only DNA ligase and plays a pivotal role in mtDNA repair and replication. *In vitro* assays in patient-derived cells showed a decrease in *LIG3* protein levels and ligase activity. We demonstrated that the *LIG3* gene defects affect mtDNA maintenance leading to mtDNA depletion. A decreased number of myenteric neurons, and increased fibrosis and elastin levels were the most prominent changes in the gut. In the skeletal muscle, we showed a marked decrease of cytochrome c-oxidase (COX) staining. Disruption of lig3 in zebrafish reproduced the brain alterations and impaired gut transit *in vivo*, and was rescued by the wild-type human *LIG3* isoform, but not by the mutant one. We identified biallelic variants in the *LIG3* gene that result in a novel mitochondrial phenotype characterized by prominent neuro-myopathic gastrointestinal dysmotility, leukoencephalopathy, and neuromuscular abnormalities.

A NEW PROTEIN TO IMPROVE MOTOR PERFORMANCE WITH A LIMITED HYPERTROPHIC EFFECT IN YOUNG AND OLD MICE

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Several diseases (*e.g.* neuromuscular diseases and sarcopenia) determine skeletal muscle atrophy and weakness, representing an important clinical problem. Current efforts to improve muscle performance are focused on muscle trophism *via* inhibition of the myostatin pathway. The activin receptor (ActR-Fc), a myostatin inhibitor, can induce a remarkable gain in body weight: however, such muscular hypertrophy per se is not sufficient to ensure a prolonged muscle performance. We speculate that increasing muscle mass without providing adequate incremental innervation could be counterproductive for sustaining long-term efforts. We

synthetized a novel protein (ActR-Fc-nLG3) by combining the C-terminal agrin nLG3 fragment (a key molecule in development/maintenance of NMJs) to the soluble activin receptor, to support both the myogenic and neurogenic component of muscle performance. Nine-week-old (young) and 22-month-old (aged) C57BL/6j male mice received subcutaneously 10 mg/kg ActR-Fc-nLG3; other age-matched animals received ActR-Fc or vehicle (control). Body weight and motor performances were monitored by rotarod or treadmill. After sacrifice, gastrocnemius, quadriceps and triceps muscles were collected and histologically analyzed. ActR-Fc-nLG3 administration determined a moderate but significant body and muscle weight increase compared to control (although inferior to ActR-Fc). In the young animals, ActR-Fc-nLG3 ensured a remarkable increased performance in the rotarod test. Moreover, concerning the old mice undergone treadmill exercise, during weeks, control and ActR-Fc groups started decreasing the distance run which at study end was significantly shorter (18.2% and 14.4% less, respectively) than at the beginning, whereas ActR-Fc-nLG3 group showed an increase of 2.8% from baseline. Finally, histology demonstrated that, in both treated groups, ActR-Fc-nLG3 administration increased the enfolding of junctional folds of the motor endplates, amplifying the surface area for acetylcholine receptors. Our results support the hypothesis that improving nerve-muscle interaction is an important factor for sustaining long term muscle activity: our work raises the hope that a possible therapy may be developed not only for sarcopenia, but also for many other neuromuscular disorders.

SPINAL MALADAPTIVE PLASTICITY FOLLOWING PERIPHERAL NERVE INJURY IS NOT A GFAP-DEPENDENT PROCESS

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Peripheral nerve injury has been used as a model to induce maladaptive changes in the central nervous system (CNS). The primers of this cascade-like process are the glial cells (namely astrocytes and microglia) that rapidly undergo to morphological and functional changes, leading to a reactive state (reactive gliosis). Although reactive gliosis is well-documented, the role of intermediate filaments in the reactive gliosis and maladaptive plasticity process is yet to be established. Glial acid fibrillary protein (GFAP) is the hallmark of reactive astrocytes and is significantly upregulated in reactive astrogliosis. To verify the role of GFAP in establishing spinal maladaptive plasticity, we used a GFAP-KO mice model of sciatic spared nerve injury (SNI) and compared it to wild-type (WT) counterparts. Behavioural tests (von Frey and plantar test) were performed and later spinal cord sections were studied with immunohistochemistry and western blot (WB) for astrocytic (vimentin) and microglial (Iba1) markers. Glial and neuronal markers of the glutamate/GABA system (GLAST, GLT1, vGLUT, vGAT, GAD) were also analysed. The astrocytic marker vimentin was strongly increased in KO-animals after SNI. In both KO- and WT-mice after SNI, we observed 1) a strong increase of Iba1 levels, suggesting an activation of microglial cells, 2) a significant reduction of the glial glutamate transporters GLAST and GLT1, 3) an upregulation of the glutamate neuronal transporter EAAC1, 4) an increase of the GABAergic system markers vGAT and GAD, and 5) an enduring neuropathic behavior. vGLUT expression was unchanged at baseline and after SNI in WT animals but was significantly upregulated in KO mice. Our results demonstrated a GFAP-independent reaction following SNI that was able to perturb synaptic homeostasis and induce complex morpho-functional changes in the spinal cord that also sustain neuropathic behaviour. These results, therefore, prompt to hypothesize alternative molecular pathways linking intermediate filaments, reactive gliosis and maladaptive plasticity.

POSTERS SESSION

INVESTIGATING THE WHITE MATTER SUBSTRATES OF FUNCTIONAL CONNECTIVITY DYNAMICS

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The comprehensive characterization of brain connectivity is one of the main aims of modern neurosciences. Along with structural and functional connectivity, dynamic connectivity, which quantifies the fluctuations in functional connectivity between brain areas in a given time window, has gained increasing importance in recent years as a promising tool to investigate brain activity. While several studies have focused on the relationship between structural and functional connectivity, the contribution of structural connectivity to functional connectivity dynamics is still far from being fully elucidated. Track-weighted dynamic functional connectivity (tw-dFC) has been recently proposed as a method to achieve a joint analysis of structural and dynamic functional connectivity: in this framework, information deriving from resting-state fMRI is windowed and functional connectivity from each time window is mapped on subject-specific priors derived from tractography. In the present work, we applied this framework on 210 healthy subject's high spatial and temporal resolution DWI and resting state fMRI (rs-fMRI) data from the Human Connectome Project (HCP) repository. The tw-dFC maps were analyzed using an independent component analysis (ICA) approach, aiming at identifying consistent, spatially independent white matter components which support dynamic changes in functional connectivity. Spatial ICA of tw-dFC data resulted in a series of well-recognizable, anatomically meaningful patterns of white matter connectivity. Each component consisted of a white matter spatial map, which represents the spatial distribution of white matter bundles, which show consistent fluctuations in functional connectivity at their endpoints, and a time course representative of the functional connectivity fluctuations occurring along these tracts. White matter spatial maps showed striking similarity to known functional networks derived from rs-fMRI, while their time courses showed specific patterns of correlation between components, revealing functionally meaningful clusters with tightly related activity. Along with providing an unsupervised, functional classification of the brain white matter, our results suggest that dynamic fluctuations in functional connectivity are supported by specific, anatomically defined white matter bundles, and shed new light on the organization of brain connectivity at both structural and functional level.

PERINATAL EXPOSURE TO BISPHENOL A OR S ALTERS ANXIETY-RELATED BEHAVIORS AND SEROTONINERGIC SYSTEM IN MICE

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Bisphenols (BPs), organic synthetic compound used in the production of plastics, are an extremely abundant class of Endocrine Disrupting Chemicals, *i.e.*, exogenous chemicals, or mixture of chemicals, that can interfere with any aspect of hormone action. Exposure to BPs can led to a wide range of effects and it is especially dangerous if it occurs during specific *critical periods* of life. Focusing on the effects of perinatal exposure to BPA or to its largely used substitute BPS, we treated C57BL/6 dams orally with a dose of 4 µg/kg body weight/day (*i.e.*, EFSA Tolerable Daily Intake dose) of BPA or BPS dissolved in corn oil or with vehicle alone, starting with mating and continuing until the weaning of the offspring. In adulthood (PND90), the offspring of both sexes performed the elevated plus maze (EPM) and the open field (OF) tests. During the EPM test, BPA-treated males showed a significant increase in the time spent in the open arms compared to controls and a decrease of the latency of the first enter in the open arms that was also displayed by BPS-treated males, while BPA-treated females showed a significant decrease in time spent in open arms compared to the controls. During the OF test, BPA- and BPS-treated males spent more time in the center of the arena and less time in the border compared to control males, while BPA- and BPS-treated females spent less time in the center and more time in arena compared to the control female. These behavioral alterations suggested different effects of the BPs exposure on anxiety-related behavior in males (anxiolytic) and females (anxiogenic), Therefore, we analyzed the serotoninergic system in Raphe nucleus, which is highly involved in the control of anxiety-related behavior. We performed an immunohistochemical analysis of the serotonin immunoreactivity (5-HT-ir), both in terms of number of cells and fractional area covered by the immunopositive elements, in the dorsal raphe (DR), distinguishing its dorsal (DRD) and ventral (DRV) component, and in the median raphe (MnR). In control mice, we detected sex dimorphism of the system in the DR only, with control females showing higher values of 5-HT-ir when compared to control males. BPA-treated males displayed a significant increase of 5-HT-ir in all analyzed nuclei, whereas BPS-treated males showed an increase in DRV only. In females, both BPA- and BPS-treated groups showed a significant increase of 5-HT-ir in DRD compared to the controls, and BPA-treated females also showed a significant increase in MnR. In conclusion, BPs exposure during early phases of life is altering, in sexually differentiated way, both anxiety-related behaviour and the Raphe population of serotonin, which is involved in the control of this behaviour.

USING A 3D APPROACH TO DESCRIBE CELL POPULATIONS IN THE RAT DRG

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Dorsal root ganglia (DRG) sensitive neurons represent the connection between the peripheral sensorial receptors and the central nervous system. These neurons are enwrapped individually by the satellite glial cells (SCGs) from which they receive metabolic support. Together, neuron and SCGs, become a functional unit that, in absence of the blood brain barrier, is easily exposed to external stress and damage insults. This intimate connection/relationship, both morphological and functional, can be partially pictured and studied following traditional slicing 2D histopathological techniques. Indeed, morphological cellular and

subcellular alterations and changes in protein expression and/or distribution can be observed using classical techniques. However, a whole-3D approach avoids the serial sectioning required for quantitative results plus is able to show the cyto-architecture of the organ and a more complete picture of the anatomical relationship between cell populations close to physiological conditions. Here we use a 3D imaging technique to show the cyto-architecture of the DRG after "colouring" by immunofluorescence the different DRG cell populations and to assess alterations in DRG of neuropathic rats. CGRP, IB4 and MAP2 markers were useful to study the different neuronal populations. The IB4-MAP2 combination was able to label all neurons while the CGRP-IB4 couple could not but still both settings showed a small subpopulation of neurons where the proteins were co-expressed. Moreover, GFAP, ATF3 and connexin 43 were used as markers of damage in the DRG from neuropathic animals.

MICROGLIAL CCL2 RELEASE AND AMELIORATIVE EFFECTS OF MESENCHYMAL STEM CELLS (MSCS) TREATMENT IN EAE MOUSE NEOCORTEX

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Experimental autoimmune encephalomyelitis (EAE) mouse neocortex reveals inflammation, demyelination and blood-brain barrier (BBB) dysfunction. The treatment with mesenchymal stem cells (MSCs) has been reported in literature to induce a significant reduction of neuroinflammation and astrogliosis in EAE. In this study, we investigated the cellular sources of CCL2, a chemokine involved in BBB disruption and leukocytes recruitment, and the effects of MSC treatment in EAE mouse neocortex. We analyzed, by immunohistochemistry (IHC) and dual RNAscope IHC/*in situ* hybridization, macrophage/microglia markers and microglia-specific markers, *i.e.* TMEM119 and SALL1, combined with CCL2. The results revealed that hypertrophic microglia represents the main source of CCL2 release in EAE mouse neocortex. The MSC treatment reduced microglial CCL2 expression and release and restored BBB structure and functionality. The findings of our study furtherly investigated the pathogenesis of neuroinflammation and BBB damage in EAE mouse neocortex and demonstrated remedial effects of MSC treatment, raising possible therapeutic implications for neuroinflammatory diseases.

THE BLOOD BRAIN BARRIER AND THE PERIVASCULAR NEURON TYPE

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The blood brain barrier (BBB) is the morphofunctional structure involved in the control of selectively molecular transport mechanisms between the blood and the central nervous system (CNS), and protect it from hurtful agents and modulates the passage of pharmacological agents. Moreover, a structural damage of the BBB in neurologic and psychiatric disorders, has been demonstrated. Now, the BBB is structurally composed by a layer of endothelial cells, incompletely covered by pericytes surrounded by the extracellular matrix sheathed by end-feet astrocyte processes. The end-feet astrocytes are the mainly target of neuronal processes. Data on the exact neuronal role in control on the BBB and the composition of the neurovascular unit it is not completely know. Studies demonstrated the presence of perivascular neuronal processes involved in the modulation of the BBB and presently, only few studies evidenced in several regions of the CNS the presence of neuronal cell bodies in close relationship with the wall of vessels. The aim of this immunohistochemical study is to investigate on the presence of monoaminergic and peptidergic perivascular neuronal elements in the human cerebellum. The study was carried out on autoptic fragments of human cerebellum fixed in an aldehyde picric acid solution, embedded in paraffin, cut into 5 μm sections and subjected to light microscopic immunohistochemistry with rabbit polyclonal antibodies for serotonin (5-HT), dopamine transporter (DAT), dopamine type 2 receptor (DRD_2), neurotensin (NT), neurotensin receptor type 1 (NTR_1). The immunoreaction revealed in the molecular layer, in the three zones of the granular layer of the cerebellar cortex, in the dentate nucleus the presence immunoreactive for 5-HT, DAT, DRD_2, NT and NTR_1 neuronal cell bodies and processes in close relationship with the wall of microvessel. Although, this data provides further insights, we suggest that the perivascular neuron may be considered a new specific neuron type of the neurovascular unit involved in the permeability control mechanisms of the BBB.

BLOOD VESSELS: THE HIGHWAYS USED BY SCHWANN CELLS TO DRIVE REGENERATION WITHIN NERVE CONDUITS

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The repair of a severe nerve injury requires the use of an autograft or a nerve guidance conduit to bridge the gap and avoid axon dispersion and off target reinnervation. When a severe injury occurs, the gold standard technique to repair the gap is the autograft. Nevertheless, tubular structures can be used to link the two ends of injured nerves, thus providing a protected environment in which the regenerating nerves can grow, but their efficacy is comparable with autografts only for short gaps. Understanding the nerve regeneration within hollow conduits might improve their design and their luminal enrichment to reach a good efficiency also for longer gaps. Indeed, the environment inside a nerve guide is completely different from that of the auto-

graft, since conduits need to be colonized, while in the autograft most of the players involved in the nerve regeneration are already on site. It has been previously shown by others that within the tissue spontaneously formed to reconnect injured nerves, called "nerve bridge", Schwann cells use endothelial cells as a path. To better understand what happens during regeneration inside a conduit, we investigated if Schwann cells behave the same way when they migrate within a conduit. To this aim, adult female rat median nerves were injured and repaired with a 10 mm chitosan conduit and the nerve portion regenerated within the conduit was analysed at different time points (7, 14, 21 and 28 days) by means of confocal immunofluorescence analysis of sequential thick slices. As hypothesized, our data show that migrating Schwann cells use newly regenerated blood vessels as a substrate for migration within the conduit. These results confirmed that, in this experimental paradigm, angiogenesis within nerve conduits plays a key role, not only to sustain cell survival, but also to provide a path for migrating repair Schwann cells, thus suggesting that factors promoting vascularization might be used to promote nerve regeneration within longer conduits.

BENZO[a]PYRENE ALTERS ELECTROPHYSIOLOGICAL PROPERTIES AND GnRH RELEASE IN hfHYPO CELLS

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Benzo[a]pyrene (BaP) is a widespread pollutant that can act as endocrine disrupting chemical (EDC) and interfere with reproductive function and embryo development. To date, the study of BaP effects on human reproductive axis at central level is lacking. The central regulatory network of the reproductive system is mediated by gonadotropin-releasing hormone (GnRH) neurons, which release in a pulsatile manner the GnRH into the hypothalamic-hypophyseal portal circulation and maintain the reproductive function. Here, we investigated the effects of BaP on GnRH neuron function taking advantage of a primary culture isolated from the human fetal hypothalamus (hfHypo). hfHypo cells express the enzymes cytochrome P450 (CYP1A1 and CYP1B1), required for metabolic activation of BaP and that expression was strongly induced by Bap exposure (10 μM, 24 h). From a functional point of view, BaP exposure significantly reduced the mRNA level of the kisspeptin receptor (KISS1R), the main physiological regulator of GnRH neuron function. Interestingly, BaP increased phospho-ERK1/2 signaling which is a known intracellular mechanism associated with KISS1R by Kisspeptin activation. Moreover, BaP induced changes in electrophysiological membrane properties causing a significant depolarizing effect and significantly increased GnRH secretion, with both effects being not changed by the addiction of kisspeptin. In conclusion, our findings demonstrate that BaP may affect GnRH neuron function by altering electrophysiological properties and interfering with KISS1R signalling and GnRH secretion, suggesting a possible EDCs-related mechanism at central level underlying reproductive function alterations.

NOVEL FINDINGS ON GENETICALLY DRIVEN ENTERIC NEUROPATHY: THE RAD21 KNOCK-IN MOUSE MODEL

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RAD21 is a double-strand-break repair protein of the cohesin complex and a regulator of transcription processes, which plays key roles in the maintenance and survival of various cell types including neurons. In a consanguineous family with a clinical phenotype of neurogenic chronic intestinal pseudo-obstruction, *i.e.* the worst expression of gut dysmotility, our group identified a novel causative RAD21 (Ala622Thr) missense mutation. By immunohistochemistry, we showed Rad21 immunoreactivity (IR) in a subset of neurons of the mouse enteric nervous system. Furthermore, we developed a genetically re-constructed Rad21 conditional knock-in (Rad21KI) mouse carrying the Ala626Thr mutation (mouse homolog of human mutation) to understand how the RAD21 mutation impairs gut motility. The aim of this study was to perform a qualitative and quantitative characterization of myenteric neurons in the small intestine (duodenum, jejunum and ileum) and colon of Rad21KI *vs.* wild type (WT) mice. Immunohistochemical analysis was performed in whole mount myenteric plexus preparations using the pan-neuronal marker HuC/D, choline acetyltransferase (ChAT, a cholinergic marker for excitatory motor neurons) and neuronal nitric oxide synthase (nNOS, a nitrergic marker for inhibitory motor neurons). The total number of HuC/D myenteric neurons did not significantly change in Rad21KI *vs.* WT in the small intestine and colon. However, in the small intestine, we showed that Rad21KI HuC/D/ChAT-IR myenteric neurons/field were 18.43±1.6 *vs.* 31.18±2.4 of WT mice ($P \leq 0.005$); HuC/D/nNOS-IR myenteric neurons/field were 11.87±1 in Rad21KI *vs.*14.83±0.6 in WT ($P \leq 0.005$). HuC/D/ChAT-IR myenteric neurons/field in the mouse colon were 19.88±2.1 in Rad21KI *vs.* 32.15±3.8 in WT mice ($P \leq 0.005$). There were no significant changes to HuC/D/nNOS-IR myenteric neurons in the colon of Rad21KI and WT mice.

In conclusion, the total number of HuC/D neurons showed no changes in both Rad21KI and WT mice. However, the small intestine of Rad21KI mice showed a significant decrease of cholinergic and nitrergic neurons. Conversely, in the colon, only cholinergic neurons were reduced. Our findings provide an accurate neurochemical basis to understand the neuropathic features of the RAD21-related CIPO patients. Further analyses are needed to decipher the mechanisms through which individual subsets of nitrergic and cholinergic myenteric neurons are affected in distinct gut segments of the Rad21KI mouse.

MODULATION OF CORTICAL IMMATURE NEURONS BY EXTERNAL CUES

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The ability of the brain to make structural changes depending on a challenging environment (structural plasticity) is vital for adaptation and brain repair. It is generally categorized into synaptic plasticity (synapse formation/elimination) and adult neurogenesis (regionally restricted genesis of new neurons driven by stem cells). Recently, a novel population of "immature" neurons was found in the cortical layer II of the adult brain. These cortical "immature" neurons (cINs) are generated prenatally, then remain immature through adulthood in a "dormant" status before incorporation into neural circuits. Our lab showed that high amount of cINs are present in the widely extended neocortex of gyrencephalic mammals, suggesting that they might represent a reservoir of "young" neurons. Here we investigated the possible modulation of cINs in 15 sheep (*Ovis aries*; hosting these cells in the entire neocortex) that were kept in different environmental conditions (enriched environment, isolation, and control). Until now, we analyzed three animals for each group. Histologically stained coronal sections of each brain (one hemisphere cut serially at 40 μm thickness) were used to define 4 anterior-to-posterior levels, as previously established. Immunohistochemistry was performed for the cytoskeletal protein Doublecortin (DCX), used as a marker for immaturity depicting the whole cell shape of the cINs. We quantitatively analyzed the total number of DCX^+ cells in paleo- and neo-cortex (twelve coronal sections/hemisphere; three sections/level) in order to obtain a linear density (number of cells/mm of layer II perimeter). In addition, after identification of type 1 (small, bipolar, more immature) and type 2 (large, ramified, less immature) immature neurons, we counted their relative proportion in cortical layer II. Cell countings were carried out using Neurolucida software. Preliminary results show a difference in DCX^+ cell density between group C (14.08 ± 5.87) and groups A (4.38 ± 2.71) and B (2.64 ± 0.65). Statistical analysis of density differences shows a trend towards significance. The analysis of the still missing subjects (the fourth and fifth animal), increasing the sample size, will help to make the result more robust and reliable. The present results indicate that possible changes can occur in cINs when animals undergo different behavioral tasks, suggesting the cINs might represent a population of undifferentiated cells, which may remain adaptable.

CHANGES OF VGF PEPTIDES IN AN ANIMAL MODEL OF PARKINSON'S DISEASE

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We have previously shown that proVGF-derived peptides are reduced in the GABAergic axon terminals of the rat substantia nigra (SN) with unilateral 6-hydroxydopamine lesions as well as in the blood of Parkinson's disease (PD) patients, particularly at the initial disease phase. To better investigate whether VGF peptides might be affected in the early stage of nigrostriatal neurodegeneration, we assessed their changes in an animal model of PD based on the overexpression of human α-synuclein (α-syn) gene, characterized by α-syn-rich inclusions and progressive loss of dopaminergic neurons, mimicking more closely the human pathology. Rats received unilateral injection of adeno-associated virus vectors expressing the human α-syn (group 1; n=13) or the green fluorescent protein genes (group 2; n=13, as control) into the SN, while the group 3 (n=6, healthy control) did not receive any treatment. After eight weeks, rats of the groups 1 and 2 (n=6, each) were perfused and their brains were collected for immunohistochemistry (IHC) analysis. ELISA was performed with blood samples collected from the remaining rats of group 1 and 2 (n=7, each) and the entire group 3. Brain morphological analysis were performed by using antibodies anti tyrosine hydroxylase, proVGF C-terminus and phosphorylated α-syn. To correlate brain morphological alterations with plasma changes, concentrations of five proVGF-derived peptides (AQEE, NAPP, TLQP, C-terminal, NERP-1) were measured. In the SN, inclusions of α-syn were observed in the injection sites only, in parallel with a loss of dopaminergic cell bodies (~30%; $p<0.05$; stereological analysis), along with a decreased expression of VGF within the neuron fibres (~25%; $p<0,05$; densitometric analysis). Although present in the ipsilateral striatum, α-syn inclusions were less pronounced than those observed in the SN. In agreement, no significant changes of TH and VGF immunostaining were observed through a densitometric analysis in the striatum. In parallel to the reduced brain expression of VGF, significant reductions in the plasma levels of the TLQP, NAPP and VGF C-terminus peptides were found among rats of group 1 compared to group 3. Overall, given the low level of dopaminergic degeneration seen with α-syn overexpression, our data support the idea that VGF reduction (in both SN and plasma) represents an early event in PD, supporting our hypothesis that VGF C-terminal peptides can be potential biomarkers for the diagnosis of PD.

IDENTIFICATION OF NOVEL BIOMARKER FOR ALZHEIMER'S DISEASE EARLY-STAGE DIAGNOSIS

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Alzheimer's disease (AD) is the most common neurodegenerative disorder and the first cause of dementia in the elderly. To date, no treatments are available to cure or slow down the pathology. Synaptic Dysfunction has been identified as the first neurodegenerative event in AD, therefore it represents a mandatory target to the development of pharmacological strategies. One of the key players in this process is c-Jun N-terminal kinase (JNK). JNK3 the brain specific JNK isoform is the most responsive to stress-stimuli in animal models. Therefore, the main aim of this project is to characterize synaptopathy and JNK activation in AD human samples. We found that AD patients shown JNK activation in the frontal cortex compared to control patients, in line with increased phosphorylation of JNK elective target c-Jun. This brain area also showed increased levels of phosphorylated APP in T668, JNK main phosphorylation site. In the post-synaptic enriched fraction (TIF) JNK was also highly activated in AD compared to controls. Furthermore, Drebrin, a marker for mature dendritic spines, and NMDA receptors levels were severely reduced in AD as expected, indicating spine pathology. Now we

are currently measuring JNK3 levels in CSF and olfactory mucosa (OM) exploiting its potentiality as a new biomarker. Preliminary results indicates that JNK3 levels increased in AD patients compared to controls. and confirmed JNK3 as a potential diagnostic biomarker.

EFFECTS OF MET IN A MULTIPLE SCLEROSIS MURINE MODEL

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Multiple sclerosis (MS) is an autoimmune disease of central nervous system (CNS) characterized by immune-mediated inflammation, demyelination, and axonal damage. Among the new investigated pathological mechanisms involved in MS, glutamate excitotoxicity exerts crucial role. MS patients show increased glutamate levels in brain and cerebrospinal fluid in comparison to healthy controls and upregulation of glutamate receptor (NMDAR) in brain lesions. Interestingly, the inhibition of NMDAR in the murine model of MS, represented by the experimental autoimmune encephalomyelitis (EAE), ameliorates the neuropathological signs characteristic of the model. Recently, the Hepatocyte Growth Factor (HGF) and its receptor MET, have emerged as critical molecules able to modulate the glutamatergic synapse. It is known that the overexpression of HGF in neurons induces reduction of infiltrating cells in the CNS and protective action through a pro-tolerogenic response in the EAE model. Evidence suggests also that HGF/MET axis promotes survival of oligodendrocytes and neurons, but the mechanisms are yet unknown. Our unpublished data indicate that HGF and MET activating monoclonal antibody (METamAb) mitigate the NMDAR-induced calcium influx and induce resistance against neuronal death *in vitro*. Further studies are needed to understand the possible interaction between Met and NMDAR. Since HGF binds and is sequestered by the extracellular matrix, its use as a therapeutic molecule is difficult. Thus, here we evaluated whether METamAb alters the EAE disease course in term of disability and neurological damage. EAE mice were intravenously injected with METamAb or vehicle starting before onset (about 6 day post immunization, dpi). Doses were decided starting from pharmacokinetics. Interestingly, we observed delayed EAE onset and reduced cumulative clinical disability score in the group of METamAb treated EAE mice in comparison to vehicle group. Accordingly, the neuropathological analysis at 21 dpi revealed a mild reduction in the number of inflammatory infiltrates and microgliosis. No differences in demyelinated areas and astrogliosis were highlighted. We provides new insights about the function of MET receptor in development of EAE and bases for the development of new drugs targeting MET or NMDAR to counteract MS.

AUTOPHAGY-BASED EFFECTS OF CURCUMIN IN METH-INDUCED TOXICITY

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In recent years, the beneficial effects of curcumin (CUR) have been widely demonstrated in experimental and clinical studies. This natural polyphenol protects against toxic agents acting on the human body, including the nervous system. In particular, CUR has been investigated for its multiple biological effects mostly focusing on autophagy activation, which is considered particularly relevant to counteract various toxicants and disease conditions. In detail, the present study, we specifically challenged the protective efficacy of CUR on METH-induced toxicity based on the molecular events triggered by METH, which consist in profound alterations in the autophagy machinery. These effects were investigated in a catecholamine-containing cell line, namely the rat pheochromocytoma PC12 cell line. This latter was chosen as unbiased, low variable *in vitro* model to test the pro-autophagic activity of CUR. In such a system, a strong protection was exerted by CUR against METH toxicity. This was associated with increased autophagy flux, merging of autophagy and lysosomal proteins and re-allocation of LC3 inside the autophagy vacuoles, which instead is dispersed by METH. This is expected to enable the autophagy machinery. Under the effects of CUR, LC3 increases within autophagy vacuoles to commit them to cell clearance and promotes the autophagy flux. This is in line with the evidence that in METH-treated PC12 cells α-synuclein accumulates within the cytosol, whereas CUR promotes the clearance of this autophagy substrate. The present data provide evidence that CUR induces neuroprotection against METH toxicity by promoting the autophagy pathway.

ROLE OF NLRP3 IN OBESITY-RELATED INTESTINAL SYMPTOMS

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Gut dysbiosis, impairments of intestinal epithelial barrier and enteric inflammation contribute to intestinal disturbances in obesity. NLRP3 inflammasome acts as a key immune sensor involved in the pathogenesis of immune/inflammatory responses. This study examined alteration of intestinal epithelial barrier, NLRP3-induced immune/inflammatory responses and colonic motility in mice with high-fat diet (HFD)-induced obesity. Wild-type C57BL/6J and NLRP3-KO (NLRP3$^{-/-}$) mice were fed with HFD or standard diet for 8 weeks. *In vivo* colonic transit was assessed (n.pellet/h). At sacrifice, blood samples were collected to evaluate circulating lipopolysaccharide (LPS). The activation of inflammasome pathway [ASC, caspase-1 and IL-1β] in

colonic tissues from obese mice was assessed by RT-PCR and ELISA. The alterations of intestinal barrier (epithelial tight junctions) and the activation of gut resident macrophages were assessed by confocal immunofluorescence. The role of NLRP3 in *in vitro* colonic tachykininergic contractile activity was evaluated. The effect of substance P (SP) on NLRP3 pathway was tested in macrophages. HFD mice displayed increased plasma LPS as well as colonic IL-1β levels and ASC and caspase-1 mRNA expression. HFD animals were characterized by a decreased claudin expression in epithelial cells along with an increased ASC immunopositivity in F4/80-positive macrophages in colonic wall. *In vivo* colonic transit were decreased while *in vitro* colonic tachykininergic contractions were increased in HFD mice. NLRP3 gene deletion in HFD mice was associated with lower increase in systemic and bowel inflammation. The NLRP3 gene deletion was associated with a recovery of colonic transit and a normalization of of tachykinergic neuromuscular contractions were normalized. In macrophage cell lines, SP induced IL-1β release. Such an effect was abrogated in the presence of caspase-1 inhibitor or NK_1 receptor antagonist and was not observed in $ASC^{-/-}$ cells. In the setting of obesity, the activation of NLRP3 inflammasome in tissue-resident macrophages contributes to enteric motor disorders. Thus, the NLRP3 inflammasome may represent a suitable molecular target for the development of novel pharmacological approaches for the treatment of digestive symptoms associated with obesity.

NERVE INVOLVEMENT OF THE E51G TRANTHYRETIN VARIANT

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The hereditary transthyretin amyloidosis (hATTR) is the most common hereditary systemic amyloidosis. hATTR is a multiorgan disease affecting the heart, the gastrointestinal tract, the kidneys, the eyes and the nervous system. hATTR generally presents with the typical length-dependent small fiber neuropathy, also named familiar amyloidotic polyneuropathy (FAP) type I. But, alterative presentations are common, including the FAP type 2 in which mixed axonal and demyelinating neuropathy is the typical of late onset cases with cardiac involvement. Cardiac involvement without neuropathy, also name familiar amyloidotic cardiomyopathy, is also well defined form of hATTR. Specific TTR gene mutations were associated with neurologic and cardiac presentations respectively. The underlying mechanisms of these genotype phenotype correlations are still poorly understood. To describe the nerve involvement of the rare, cardiac E51G (p.Glu71Gly) TTR variant in a Piedmontese family with heterozygous and homozygous carries. A clinical, neurophysiological, molecular and morphological study was carried out. Four siblings (1M and 3 F) were investigated. The age of onset ranged from 53 and 63 with median age of onset 58. Insidious lower limb polyneuropathy was the presentation in three subjects, while pure cardiac presentation was the onset of the fourth patient. Neurophysiological findings revealed mixed axonal and demyelinating polyneuropathy in two subjects. Sensitive skin innervation study and intraepidermal nerve fiber density identified a small fiber neuropathy in a third subject, while they were normal in the fourth sibling. Autonomic involvement was demonstrated in all patients with neuropathy. The TTR gene sequencing disclosed the p.Glu71Gly mutation coding the E51G variant. Two subjects were heterozygous for this mutation while two siblings were homozygous. Homozygous condition anticipated the onset but did not correlate with a more severe disease. This is the first report of nerve involvement of the E51G TTR variant, which was previously associated with pure cardiac phenotype. Our report extend the spectrum of multi-organ involvement of E51G variant, supports the hypothesis of nerve involvement also in cardiac TTR mutations and underlines the importance of searching for polyneuropathy in all amyloidogenic TTR variants, as disease modifying therapies are available only for hATTR with neuropathy.

ENDOCANNABINOID SYSTEM IN BORTEZOMIB NEUROTOXICITY

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Chemotherapy-induced peripheral neurotoxicity (CIPN) is a common complication in the successful treatment of cancer. This side effect is dose-limiting and clinically reflects in an axonal peripheral neuropathy with sensory loss, combined often with neuropathic pain. These symptoms may be disabling, adversely affecting the quality of life of patients. Here we focussed on bortezomib (BTZ), a first-in-class proteasome inhibitor used for the treatment of multiple myeloma, which is associated with a relatively high incidence of CIPN. The pathogenesis of CIPN has not been completely understood, and there are no effective strategies or drugs to prevent or treat this side effect. Among possible pharmacological treatments of CIPN, modulation of the endocannabinoid system might be particularly promising. To investigate this hypothesis, we performed electrophysiological, behavioral and pathological analyses in a rat model of painful CIPN induced by BTZ-treatment. Animals were intravenously injected with BTZ 0.20 mg/kg, 3 times a week for 8 weeks. Moreover, localization of CB1R/CB2R-like immunoreactivity (LI) and protein quantification for CB1R/CB2R were performed in dorsal root ganglia (DRG) and in the spinal cord. In addition, cd68-LI macrophages in the peripheral nerve as well as resident Iba-1 positive cells, macrophages or microglia, were also evaluated in the DRG and spinal cord dorsal horn (DH), respectively. BTZ induced alterations in rat electrophysiological endpoints and behavioral studies of pain associated with a reduction in intraepidermal nerve fiber density if compared to control rats. Moreover, huge M1 proinflammatory infiltrating cells in caudal nerves and increased Iba-1 positive cells in DRG and microglia in the DH of rats after 8 weeks of treatment were also observed. In addition, BTZ induced an increase in the number of CB1R- and CB2R-LI DRG neurons, as well as an increase in CB1R and CB2R protein expression in DRG. The densitometric analysis on BTZ-treated DH showed an increase in CB1R-LI. In conclusion, the results suggest that the alteration of the endocannabinoid levels in peripheral and central nervous tissues appears involved in the development and progression of CIPN. Therefore, improved understanding of the pathophysiology of BTZ-induced neurotoxicity will inevitably assist in the development of effective pharmacological intervention on the cannabinoid system as potential therapy for CIPN.

BRAIN IRON DEPOSITS DURING AGING: ACTIVATION OF THE HEPC/FPN1 PATHWAY

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During aging, iron accumulates in brain regions which are particularly vulnerable to neurodegeneration: the cerebral cortex and the hippocampus. However, the mechanism of iron regulation in the brain remains scarce and more data is needed on the age trend of iron concentrations and on its the cellular distribution within neuronal tissue. Here, we demonstrated for the first time the activation of the Hepcidin/Ferroportin1 pathway in brain iron metabolism during aging in the C57BL/6 wild-type mice brain. Hepcidin, a peptide produced mainly by hepatocytes, is the main regulator of iron content and availability; it is known that Hepcidin interacts with the iron exporter Ferroportin1, causing its degradation and iron retention in the cell. In old mice, we observed an age-dependent alteration of the Blood Brain Barrier (BBB) integrity that could be responsible for the increase of iron flux from systemic circulation to the brain, leading to iron accumulation. As consequence, the increase of iron in the brain triggers the neuroinflammatory and antioxidative stress response to iron transition from the systemic circulation to the brain. Indeed, the two main markers of neuroinflammation and oxidative stress SAA1 and Nrf2 are expressed in aged brains together with reactive GFAP-positive astrocytes. Interestingly, we found that brain iron overload drives also Hepcidin upregulation and, consequently, the inhibition of the iron exporter Ferroportin1. Moreover, both in the cerebral cortex and hippocampus Ferroportin1 colocalizes with astrocytes, while the iron storage protein ferritin light-chain with neurons. This differential distribution suggests that astrocytes mediate iron shuttling and neurons are unable to metabolize it. Furthermore, we observed NCOA4-dependent ferritinophagy of ferritin heavy-chain isoforms determining the increase of light-chain enriched ferritin heteropolymers that are more efficient as iron chelators. Altogether, these data highlight the involvement of the Hepcidin/Ferroportin1 axis and NCOA4 during mice aging as a response to a higher iron influx to the brain and represent the starting point to clarify the basis of iron handling in the brain on which one could act to avoid brain iron overload, a typical pathological feature of aging and several neurodegenerative disorders.

NEW BIOACTIVE DEVICES FOR PERIPHERAL NERVE INJURIES RECOVERY: AN *IN VIVO* STUDY

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The surgical treatment of peripheral nerve injuries (PNI) with severe substance loss (≥5 mm) still represents a challenge in clinical practice. Interposition of autologous nerve grafts is the current gold standard; however, risk of donor-site comorbidities and possible size-mismatch are a significant limit. The availability of on-the-bench effective nerve conduits (NCs) is an interesting option, but intense efforts are still required for the identification of the ideal device. In this study, different NCs based on the new oxidized polyvinyl alcohol (OxPVA) were implanted in a rat model of sciatic nerve transection (gap=5mm). Animals (n=18) were randomized to 6 groups implanted with: Reverse Autograft (RA, control); Reaxon® (made of chitosan); OxPVA; OxPVA+EAK peptide; OxPVA+EAK-YIGSR peptide; OxPVA+Nerve Growth Factor (NGF) (end point: 6 weeks). The specific outcomes were evaluated by functional tests (*i.e.*, gait analysis, sciatic functional index) and histological (toluidine blue staining) and immunohistochemical (CD3, F4/80, S100, β-tubulin) analyses. Second Harmonic Generation (SHG) microscopy was also adopted for identification of fibroconnective infiltrate. All the NCs promoted nerve regeneration, as showed by functional studies; despite no significant difference was detected among groups, OxPVA+NGF distinguished for better functional outcomes. Upon explant, all grafts were clearly recognizable, without severe adherences at the surgery site; n=1 neuroma was observed in Reaxon®. Histological/immunohistochemical analyses proved presence of regenerated nerve fibers in the central portion of all grafts (S100, β-tubulin) without inflammation (CD3, F4/80). Moreover, the morphometric study showed a total axons number that followed this descending order: OxPVA+EAK-YIGSR>RA>OxPVA>Reaxon®>OxPVA+NGF>OxPVA+EAK (central portion); and RA>OxPVA>OxPVA+EAK-YIGSR>OxPVA+NGF>OxPVA+EAK>Reaxon® (distal portion). SHG microscopy highlighted a less intense signal for: Reaxon®<OxPVA+EAK<OxPVA+NGF<RA<EAK-YIGSR<OxPVA<OxPVA; Coherency analysis (collagen fibers orientation) showed highly isotropic areas for Reaxon® (0.02 AU) and comparable values for the other groups (range: 0.07-0.10 AU). According to the study results, the new polymer oxidized polyvinyl alcohol may be a promising and versatile material for next-generation NCs, supporting morpho-functional recovery from severe PNI.