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Nervous system development and circuitry

ASTROGLIAL HETEROGENEITY IN THE CEREBELLUM RESULTS FROM DISTINCT EMBRYONIC AND POSTNATAL PROGENITORS WITH DIFFERENT PROLIFERATIVE BEHAVIORS

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The cerebellum displays an extraordinary heterogeneity of neuronal and astroglial phenotypes, with distinct morphological and spatial features. While the mechanisms of neuronal diversification have been partially clarified, astrogliogenesis remains poorly explored. Here, we investigated the genesis and development of astroglial phenotypes in the cerebellum. Firstly, in order to study their lineage relationships, we performed *in vivo* clonal analysis of embryonic ventricular progenitors using Star Track plasmids. We found clones containing both cortical and white matter (WM) astrocytes, separately or as members of the same family. The clone composition indicated the existence of four major embryonic progenitor types producing either granular layer (GL) or WM astrocytes, or a mixed progeny of Bergmann glia (BG) and GL astrocytes. In parallel, analysis in Confetti mice revealed that, postnatally, radial progenitors in the purkinje cell layer (PCL) divide in situ to generate both BG and GL astroglia. Moreover, early in embryonic development, another progenitor type produces big heterogeneous clones comprising WM, GL and BG astrocytes, whereas afterwards this capability is significantly reduced. Notably, this reduction parallels the increase of homogeneous clones, namely those in the WM, suggesting a progressive fate restriction as development proceeds. Furthermore, in WM-GL-BG clones, astrocytes in the WM are overall fewer when compared to their cortical counterparts. Finally, double-thymidine and birthdating analyses showed that astroglial progenitors settled in distinct layers amplify following different dynamics.

In conclusion, our study demonstrates that cerebellar astrogliogenesis occurs from distinct embryonic progenitors, following a progressive shift from multipotency to fate-restriction that occurs according to a well-defined spatiotemporal pattern. Notably, here we highlight the existence of a secondary progenitor pool besides the ventricular zone, represented by radial cells in the PCL capable of generating, postnatally, both BG and GL phenotypes.

INHERENT HETEROGENEITY IN DORSAL AND VENTRAL OPC OF THE MOUSE CNS UNVEILED BY CITRON-KINASE DELETION

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Oligodendrocyte progenitor cells (OPCs) comprise the main cycling cell population of the CNS parenchyma during the early postnatal period and adult stages. However, the molecular mechanisms governing the OPC division are still by and large obscure. Additionally, despite the heterogeneous embryonic sources of OPC having been identified, whether the corresponding lineages differ in molecular or functional features is not understood.

By studying a mutant model of microcephaly, we found that the germinal ablation of Citron-kinase (Cit-k KO), a crucial regulator of cytokinesis, triggers distinct responses in postnatal dorsal and ventral telencephalic OPCs. We observed that dorsally generated OPCs often failed to undergo cytokinesis, as indicated by multinucleated cells, and underwent depletion by apoptosis within the second week after birth. In contrast, ventral OPCs of the striatum and hypothalamus were mostly uninucleated and overall preserved, despite decreased in density. Interestingly, preservation of ventral OPCs was associated with the upregulation of kinases that may possibly compensate Cit-k functions during mitosis. Notably, these cells did not progress along oligodendrogenesis, as shown by lack of pre-myelinating or myelinating cells in the entire Cit-k KO forebrain, indicating additional differentiation defects. Upregulation of markers associated with senescence (ecrg4, p21, p16) was observed in the ventral OPCs in Cit-k KO animals, suggesting a metabolic shift contributing to both their persistence and stalling. In conclusion, our results demonstrate that, when challenged by Cit-k deletion, dorsal and ventral OPCs display distinct behaviors (i.e. fail to undergo cytokinesis and die by apoptosis, or compensate Cit-k ablation and become senescent-like). Hereby, we provide the first evidence of the molecular and functional heterogeneity in postnatal OPC subsets.

UNIQUE TUNNELLING NANOTUBES CONNECT ANGIOGENIC PERICYTES AND SPROUTING ENDOTHELIAL CELLS DURING HUMAN BRAIN VASCULARIZATION

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During human cerebral cortex vascularization, nascent vascular sprouts send pericytes in advance to trace the pathway and bridge the gap between anastomosing microvessels. The pericyte associated to the leading front of the sprouting vessel, here referred to as the 'leader' pericyte, extends a tunnelling nanotube-like structure (TNT-like) and lays down a matrix scaffold, which includes collagens type IV and VI, and cleaved fragments of proteoglycan NG2. According to this mode of vessel sprouting, the perforating, radial vessels increase the density of the cortex vascular network in concert with the progressively greater metabolic needs of developing neurons, following a geometrically precise pattern of distribution. Details on this process of radial vessel collateralization have been analysed by high resolution confocal microscopy, applied to the detection of endothelial-, pericyte-, and ECM-specific markers, revealed on z-series of single optical planes at a distance of 250 nm for a total of 20-30 μm. This approach allows the recognition of tip endothelial cells, their filopodial processes and associated microvesicles (MVs), together with pericyte's TNT-like structures. TNTs could be involved in cell-to-cell communication during the described process of vessel growth, working as conveyors for pericyte and endothelial signalling molecules, eventually supporting migration of endothelial tip cells. Accordingly, new details and an original sequence of events are proposed, that pinpoint the activity of a 'leader' TNT-bearing pericyte involved in the angiogenic process of vascular branching and anastomosis during cerebral cortex vascularization.

ROLE OF S100B IN THE DEVELOPING ENTERIC NERVOUS SYSTEM

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S100B is a diffusible Ca^{2+} binding protein expressed by glial cells. Mostly studied in central and enteric nervous system, S100B has emerged as a crucial modulator of many physiological and pathophysiological processes. However, its role during the enteric nervous system (ENS) development remains poorly elucidated. In this study, we investigated the development of enteric glia and the effect of inhibiting the onset of S100B expression in the development of the ENS using the *Wnt1-CRE;R26R-GCaMP* transgenic reporter mouse line. Immunohistochemistry was performed on embryonic (E14.5, E16.5) and postnatal day (P) 0 mice using anti-S100B to label glial cells. To examine the effect of inhibiting S100B expression we cultured intact explants of embryonic gut from E13.5 mice, the day before the first expression of S100B in the gut, in the presence of arundic acid (300μM), an inhibitor of S100B synthesis. Changes in the numbers of HuC/D-immunoreactive enteric neurons and Sox10-immunoreactive neural precursors were analyzed. "Rescue" experiments were conducted with the addition of exogenous S100B (5nM and 500nM) with arundic acid. The earliest S100B-immunoreactive positive cells were detected at E14.5 day where they made up about 10% of the total number of the GFP+ cells in the rostral small intestine. The number and proportion of S100B+ cells increased through embryonic development to 50% of GFP+ cells at P0. Exposure to arundic acid caused: 1) a reduction in the proportion of Sox10+ cells; 2) a decrease in the density of ENS precursors due to a decrease in their proliferation; and 3) a novel co-expression of Sox10 and HuC/D in a subpopulation of cells. Addition of exogenous S100B did not restore the change in Sox10+ cells numbers. Hence, cell-intrinsic intracellular S100B appears to be important for maintaining Sox10+ lineages, thereby supporting its role in neuronal precursor proliferation during ENS development.

CLINICAL ANATOMY OF CEREBROCEREBELLAR CIRCUIT

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The cerebrocerebellar circuit is a feedback nervous circuit (loop) that connects bidirectionally cerebral cortex and cerebellum. The cerebrocerebellar circuit is classically considered as a nervous circuit through which the neocerebellum regulates the somatic motor areas of neocortex. In recent years, studies carried out in experimental animals by tract tracing techniques, and in humans by resonance magnetic imaging, have indicated that the connections between neocortex and neocerebellum, mediated by the cerebrocerebellar circuit, concern not only the motor areas of neocortex, but also other areas of it, perhaps all. The anatomical basis of the regulatory function of neocerebellum would be represented by the organization of the cerebrocerebellar circuit in a number of parallel loops. Each loop runs separately from its origin, represented by a specific area of neocortex, to termination, represented by the same neocortex area from which the loop had originated.

Using techniques of tractography, we obtained data on the existence of a compartmentation extending throughout the cerebrocerebellar loop. Both the descending limb (neocorticopontoneocerebellar limb) and the ascending limb

(neocerebellothalamoneocortical limb) of the loop appeared organized in distinct fasciculi, which run in parallel and connect specifically discrete regions of all the nervous centres intercalated in the loop.
Through separate loops, the different areas of neocortex may be regulated by related regions of neocerebellum. The demonstration of different cerebrocerebellar loops constitutes the prerequisite to ascribe new functional roles to the neocerebellum, concerning in particular the regulation of non-motor areas of neocortex, including those involved in cognitive and affective functions.

Technical advancements

CORRELATIVE LABEL-FREE TWO-PHOTON FLUORESCENCE MICROSCOPY AND POLARIZED LIGHT IMAGING FOR 3D RECONSTRUCTION OF MYELINATED FIBERS ORIENTATION

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Connectomics aims at identifying the interactions of brain regions through the reconstruction of the neuronal fiber network. 3D-Polarized light imaging (3D-PLI) allows to assess fiber orientation and inclination angles in histological brain sections based on measurements of the birefringent properties of the myelin sheaths. This optical method enables a fast analysis of whole human brains without any exogenous labeling. A single 3D (fiber) orientation vector is obtained for each voxel and reflects the net effect of all comprised fibers. In this work, we employ an integrated dual approach that combines 3D-PLI with two-photon fluorescence microscopy (TPFM) to study the mixture of various fiber orientations within the sample (and voxel) of interest. We exploit the higher axial and radial resolution of TPFM optical sectioning in combination with myelin autofluorescence to perform the 3D reconstruction of fiber orientation within each brain section. The correlation of data obtained by the two methods permits to reconstruct areas of the brain at high resolution, below the one-micrometer level. Such approach provides a novel tool for 3D reconstruction of nerve fiber orientations in postmortem tissue. The integration of different techniques opens new perspectives of an in-depth analysis of brain connectomics, linking the submillimeter-organisation of fibers to large tracts.

METHODS FOR QUANTITATIVE ANALYSIS OF BRAIN MICRO-ARCHTECTURE FROM CLARIFIED TISSUES

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One of the grand challenges of digital imaging in the field of neuroanatomy is the ability to extensively quantify anatomical structures and thus investigate the brain's structure-function relationship in great detail.
In the light of this challenge, this work aims to investigate the brain's micro-structure to obtain faithful and reproducible information on neuron morphology within their native three-dimensional arrangement.
A rigorous workflow was designed, that integrates delipidation methods, advanced imaging techniques and image processing algorithms to better understand neural micro-structure and its contribution to neural function. In particular, the workflow provides i) the optimization and standardization, through the quantification of non-invasive and macroscopic indices, of the CLARITY protocol, a tissue clarification method which eliminates lipids and reduces tissue scattering from thick brain slices, ii) the development of a Smart Region Growing (SmRG) algorithm for single neuron tracing from confocal three-dimensional datasets representing densely packed neurons within the brain, and iii) the implementation of N3MO, an open-source tool for quantitative morphometric extraction and multivariate analysis of neurons.

Key-note Lecture

HOW SUPER-RESOLUTION IMAGING ALLOW TO REVISIT SYNAPTIC TRANSMISSION

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AMPA-type glutamate receptors (AMPAR) are responsible for fast synaptic transmission. Due to their relatively low affinity for glutamate, the spatiotemporal nano-organization of these receptors in postsynaptic membrane is fundamental to understand synaptic transmission and information processing by the brain. With the recent developments in live and fixed super-resolution microscopy that we coupled to electrophysiology, we demonstrated that, in basal conditions, AMPARs are organized in few nanodomains of ~70 nm which are mostly stable for up to 1 hour at synapses. That AMPAR lateral mobility tunes the ability of synapse to follow frequency stimulations, and we found that pre-postsynaptic machinery alignment is a key determinant of synaptic transmission efficacy. All these discoveries allow a deep insight into the understanding of basal synaptic transmission properties.

Neuroplasticity in the normal brain and in nerve regeneration

THE TIMING OF BEHAVIORAL STATES: OSCILLATION OF THE EXCITATORY / INHIBITORY INPUT TO OREXINERGIC NEURONS AND AGING-RELATED CHANGES

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Neurons containing the orexin (OX)/hypocretin peptides reside in the lateral hypothalamus, and project widely to several targets in the neuraxis, acting as main regulators of arousal, wakefulness stability, energy homeostasis, motivated behaviors. Electrophysiological recordings have shown that orexinergic neurons discharge during wakefulness and are silent during sleep. We here tested whether the structural correlate, hitherto unknown, of such neuronal firing rhythmicity could be represented by synaptic plasticity phenomena, and could be modified by aging which frequently leads to altered regulation of vigilance states. Adult (3-6 month-old) and aged (20 month-old) C57BL/6J mice were sacrificed at either of two time points: daytime, in the period of sleep predominance in nocturnal rodents, or at night, during wake predominance. Sleep and wake states were verified with electroencephalographic recordings in matched mice. Triple immunofluorescence was used to visualize cell bodies containing OX-A, synaptophysin (Syn) as presynaptic marker, and the vesicular glutamate transporter (VGluT)2 as marker of glutamatergic elements or the vesicular GABA transporter (VGAT) as marker of GABAergic elements. Quantitative analyses of varicosities in contact with $OX\text{-}A^+$ somata were pursued in epifluorescence and is currently ongoing using confocal microscopy. The results obtained up to now showed that in the group of adult mice glutamatergic ($Syn^+/VGluT2^+$) varicosities in apposition to OX-A+ perikarya were significantly more numerous at night, whereas the subset of GABAergic ($Syn^+/VGAT^+$) varicosities prevailed significantly at daytime, in the absence of significant day/night difference in the combined total number of these varicosities. In the group of aged mice, a trend was found toward such diurnal changes of excitatory and inhibitory wiring of OX-A+ neurons, but without a significant day/night oscillation. The findings point to a daily reorganization of the excitatory/inhibitory input to orexinergic neurons, which could represent a key mechanism of plasticity in relation to the animal's behavioral state. The findings also indicate that such plasticity mechanisms are dampened during normal aging.

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HETEROGENEITY OF STRUCTURAL PLASTICITY IN THE BRAIN PARENCHYMA OF THREE MAMMALIAN ORDERS

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Brain structural plasticity is essential for adaptation to the changing environment and potentially useful for brain repair. New neurons functionally integrate in the neural circuits through adult neurogenesis coming from stem cell niches. Yet, such process dramatically decreases in large-brained, long-living species (e.g., dolphins and humans) and does not provide reparative outcomes. Other, "parenchymal" brain regions have been found to host populations of non-newly generated, immature cells. They express Doublecortin (DCX; marker of structural plasticity) and appear to vary in mammals. Current knowledge about these cells is largely incomplete, most studies having been conducted in small-brained, short-living species (laboratory rodents), which host the immature, non-newly generated cells only in the paleocortex. We are systematically investigating a wide range of mammals in order to clarify the possible occurrence of parenchymal DCX+ cells in species endowed with different neuroanatomy, brain size, lifespan, ecological

niche. Here, eigth mammalian species belonging to the orders carnivora, primates and chiroptera have been analysed immunohistochemically. Preliminary results indicate remarkable and widespread presence of DCX+ cell populations in all species, with respect to laboratory rodents. Extra-cortical regions (white matter and pericapsular structures) are also involved. Carnivora host DCX+ cells in more brain areas with respect to chiroptera and primates. These cells have different spatial organization and morphologies and they can be found in different areas: single or scattered bipolar or multipolar cells are distributed in amygdala or tightly-packed clusters of DCX+ cells are distributed in external capsule; the cerebral cortex contains DCX+, neuron-like cells organized into a layer.
Hence, brain structural plasticity appears to be heterogeneous in mammals, in terms of spatial organization and neuroanatomical location. These results support the hypothesis that non-neurogenic plasticity might be maintained/increased in non-rodent mammals, in spite of a reduction in neurogenic plasticity.

NON-NEUROGENIC SVZ-LIKE NICHE IN AQUATIC MAMMALS DEVOID OF OLFACTION

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Adult neurogenesis has been implicated in brain plasticity and, possibly, brain repair. In mammals, it is mostly restricted to specific brain regions and, likely, to specific physiological functions. The function and evolutionary history of mammalian adult neurogenesis remain elusive. The largest neurogenic site in mammals (subventricular zone, SVZ) generates neurons destined to the olfactory bulb. The SVZ neurogenic activity appears related to the importance of olfaction since it occurs at high rates throughout life in animals strongly dependent on this function for their survival, whereas it dramatically decreases in humans, who do not depend so much on it. Here, the question was asked whether the SVZ neurogenic site does exist in mammals devoid of olfaction and olfactory brain structures, such as dolphins. We show that a small SVZ-like region persists in these aquatic mammals, yet having lost its neurogenic activity since neonatal stages. Instead, some SVZ cells already show features of mature neurons. Since cetaceans evolved from terrestrial ancestors, non-neurogenic SVZ indicates extinction of adult neurogenesis in the absence of olfactory function, with retention of an anatomical region either vestigial or of still unknown role.

STUDY OF PERIPHERAL NERVE REGENERATION THROUGH IN VITRO MODELS: AN OVERVIEW

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The study of peripheral nerve repair and regeneration is particularly relevant for the high clinical incidence of nerve lesions. However, the clinical outcome after nerve injury is often far from satisfactory and functional recovery is almost never complete. In light of this, several approaches are under study, the local delivery of trophic factors and other bioactive molecules, biomaterials and complex nerve implants. Although the translation of the new therapeutic approaches to the patients always requires a final pre-clinical step using in vivo animal models. The need to limit as much as possible animal use in biomedical research, however, makes the preliminary use of in vitro models mandatory from an ethical point of view.
Here we will propose an overview on in vitro models that can be used for the study of nerve regeneration.
A *three-step stair model* based on their increasing ethical impact will be adopted: 1) cell line-based models, that raise no ethical concern; 2) primary cell-based models, that have low ethical impact since animal use, though necessary, is limited; 3) organotypic ex vivo-based models, that raise moderate ethical concerns since the use of laboratory animals is required though with much lower impact on animal wellbeing in comparison to in vivo models of peripheral nerve regeneration.
This work aims to help researchers in selecting the best experimental approach for their scientific goals being driven by the ‘‘Three Rs’’ rules (Replacement, Reduction or Refinement of animal use in research) for scientific research.

Neural disorders

MICROTUBULE DYSFUNCTION IN PARKINSON'S DISEASE: FROM PURE PROTEINS TO HUMAN BRAIN

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Looking at the multiple hit hypotheses in Parkinson's diseases (PD), the concept that microtubule dysfunctions might play a role in the disease progression is emerging. We previously reported that microtubule destabilization is an early event specifically associated to dopaminergic neuron degeneration in mice treated with the parkinsonism-inducing neurotoxin MPTP and that microtubule stabilization is neuroprotective (Cartelli *et al*., 2013). We are currently studying the impact of mutations in PD-linked genes, *SNCA* and *PARK2*, on microtubules. Furthermore, we are investigating the distribution of post-translational modifications of tubulin in brain from PD patients. *SNCA* encodes for α-synuclein, the first protein associated to familial PD. We showed that wild-type α-synuclein regulates microtubule dynamics both in purified systems and in neuronal cells, whereas α-synuclein PD-linked mutations impair this property and trigger microtubule aggregation (Cartelli *et al*., 2016). Next, our unpublished data demonstrate that wild-type and mutated α-synuclein differently impact on microtubule ultrastructure. *PARK2* gene, linked to the Autosomal Recessive Juvenile Parkinsonism, encodes for parkin, an E3 ligase, whose interaction with microtubules has been poorly investigated. In parkin-silenced cells, we observed that parkin deficiency makes microtubules overly dynamics, and triggers the impairment of mitochondria transport, which, interestingly, is rescued by the treatment with the microtubule stabilizer Taxol. Next, we analysed tubulin post-translational modifications related to microtubules with different stability in *Parkin* knockout and *Parkin* heterozygous mice. We found that the unbalancing in tubulin modification pattern occurs very early in the nigrostriatal pathway, accumulates over time, and precedes defects in axonal transport. Finally, we are investigating acetylated-α-tubulin, being related to microtubule stability, in *post-mortem* brains from PD patients. Our preliminary results reveal its differential distribution in brain tissues from PD patients compared to controls. Collectively, our data converge on the view that microtubule dysfunction could play a central role in the pathogenic mechanisms in PD neurodegeneration.

EXPRESSION OF THE K+/CL- COTRANSPORTER KCC2 IN A CONDITIONAL MURINE MODEL OF AUTOSOMAL DOMINANT NOCTURNAL FRONTAL LOBE EPILEPSY

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The K+/Cl- cotransporter KCC2 is the main chloride extruder in neurons and it exerts an essential role in determining the polarity of GABAA receptor-mediated chloride currents. The increase of KCC2 expression after birth is responsible of the "switch of GABA" effect from excitatory to inhibitory. KCC2 also covers a critical role in dendritic spine morphogenesis and in maintenance of glutamatergic synapses. Alteration in its expression could lead to an imbalance between excitation and inhibition and to the pathogenesis of diseases such as epilepsy. We studied KCC2 expression and distribution in a murine model of Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE), conditionally expressing the ADNFLE-linked nicotinic β2-V287L mutation (TG), for comparison with control mice (CTRL) in prefrontal cortex (PFC), somatosensory cortex (SS) and thalamus (TH). By immunocytochemical approaches, we carried out densitometric analyses of KCC2 expression at different postnatal ages (P8, P21, P60) and we observed a significant increase of KCC2 expression in PFC layer V and a decrease of this cotransporter in the thalamic reticular nucleus (RT) in TG P60 mice compared to age-matched controls. Further analyses of some GABAergic markers showed no substantial alterations. Next, we estimated GABAA reversal potential (EGABA) by perforated patch-clamp recordings on acute dissociated neurons from TG and CTRL mice during postnatal development. EGABA progressively hyperpolarized up to the second postnatal week, but no difference was found between TG and CTRL mice. On the other hand, in mature (older than P28) cortical slices, a potentiation of both EPSCs and IPSCs stimulation was detected in response to nicotine in TG compared to control neurons and, especially in the case of EPSCs, leading towards excitation. Our results suggest that KCC2 and the GABAergic system could be implicated in the pathogenesis of ADNFLE and that β2-V287L mutation could alter the excitatory/inhibitory balance in murine forebrain.

NG2-POSITIVE PERIVASCULAR CELLS INVOLVED IN NEUROVASCULAR UNIT DYSFUNCTION DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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The concept of neurovascular unit (NVU) emphasizes the critical role of cell-to-cell interaction and communication between glial, neuronal, and vascular cell components during blood-brain barrier (BBB) development, and in adult normal and pathological conditions. In this study we have analysed the involvement of the nerve/glial antigen 2, NG2, a chondroitin sulphate proteoglycan, highly expressed in developing and adult CNS, in cell cross-talk within the NVU. During CNS development NG2 is expressed by activated pericyte and appears downregulated as these cells undergo terminal differentiation. NG2 has also been identified on the surface of oligodendrocyte precursor cells, OPCs, evenly distributed throughout the CNS already by the end of the first postnatal week in mice and throughout adulthood. With the aim of understanding if reactivation of pericytes occur and if a specific subset of NG2-bearing OPCs specifically contributes to the cell composition of the NVU during EAE, we have explored, by morphometric analyses applied to laser confocal microscopy, pericyte and OPCs distribution and their reciprocate relationships in the cerebral cortex of WT controls and naïve NG2KO and in EAE WT and EAE NG2KO mice, at both early (20 dpi) and late (40 dpi) disease stages. In EAE WT mice, activated pericytes, juxtavascular (JV) and perivascular (PV) OPCs were identified in a higher number compared to healthy mice. On the contrary, absence of NG2 in EAE NG2 KO mice seemed to affect the proliferative response of these NG2-positive cells, specifically inhibiting the emergence of the JV and PV OPC subsets. The results indicate that in WT mice during EAE, the NVU microenvironment, classically formed by perivascular astrocytes, receives the insertion of activated NG2-positive pericyte together with OPCs, as an additional component of the NVU, and suggest NG2 as one of the key molecule involved in the observed NVU dysfunction.

MICROVASCULAR NG2-POSITIVE PERICYTES INVOLVEMENT IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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In the CNS, pericytes are microvessel wall-encircling cells that, together with endothelial cells, perivascular glial endfeet and basement membrane, form the blood-brain barrier (BBB). Dysfunction of the BBB, migration of autoreactive T lymphocytes and monocytes and microglia activation into the CNS are histopathological hallmarks of both Multiple Sclerosis (MS), a chronic demyelinating disease, and experimental autoimmune encephalomyelitis (EAE), a widely used MS animal model. The proteoglycan NG2, which has been described to accumulate within MS plaques and at spinal cord (SC) injury sites, is a primary component of activated, angiogenic pericytes, engaged in pericyte/endothelial cell interaction, proliferation and migration. To explore the role of NG2-expressing pericytes during neuroinflammation and BBB dysfunction of cerebral cortex and SC, pericyte coverage (pericyte number/vessel length) and density (pericyte number/tissue volume), pericyte/endothelial cell ratio, and angiogenic pericyte percentage were studied by immunohistochemistry and confocal microscopy morphometry using specific pericyte markers, NG2, PDGFRβ, TEM1, and CD13. The observations were made in mice affected by MOG-induced chronic EAE with two different genetic C57BL/6 backgrounds: wild type (WT) and homozygous NG2 null ($NG2^{-/-}$). In literature, $NG2^{-/-}$ mice did not exhibit gross phenotypic or vascular alterations, whereas our results demonstrated an unaltered pericyte density associated with slightly decreased pericyte coverage index and pericyte/endothelial cell ratio. These observations were confirmed in $NG2^{-/-}$ EAE-affected mice, which showed an attenuated disease severity and demyelination, and a milder BBB leakage, leukocyte infiltration, and angiogenic phenotype as compared with EAE WT. Taken together these results lend support to the idea of a direct involvement of NG2 proteoglycan in pericyte-endothelial cell interactions responsible for the BBB dysfunction observed during WT EAE.

EFFECT OF ACUTE STRESS ON THE HIPPOCAMPAL EXPRESSION OF BDNF AND TRKB IN A GENETIC MODEL OF STRESS-INDUCED DEPRESSION-LIKE BEHAVIOUR: THE ROMAN HIGH- AND LOW-AVOIDANCE RATS

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The outbred Roman high- (RHA) and low-Avoidance (RLA) rats were psychogenetically selected for respectively rapid vs. poor acquisition of active avoidance in a shuttlebox, and differ in many behavioural traits: RHA rats are impulsive, novelty seekers, and proactive copers, whereas RLA rats display behavioural traits that resemble some of the cardinal symptoms of depression (1). Beyond the monoamine hypothesis, compelling evidence suggests that mood disorders are characterized by reduced neuronal plasticity. Thus, it has been shown that exposure to stress and antidepressant treatments modulate the expression of neurotrophic factors, and that these changes show an anatomical specificity (2). To characterize the molecular and neuronal systems involved in both the pathogenesis of stress-induced depression and the mechanism of action of antidepressant treatments, we used western blot

(WB) and immunohistochemistry techniques to investigate the intensity of expression and localization of the brain-derived neurotrophic factor (BDNF) and its high affinitiy receptor trkB in the hippocampus of RHA and RLA rats upon acute exposure to the Forced Swim Test (FST) for 15 min. WB analyses showed that, under basal conditions, the relative levels of BDNF were lower in RLA vs. RHA rats, whereas, after FST, the relative levels of BDNF were unexpectedly higher in the hippocampus of RLA vs. RHA rats. On the other hand, no line-related changes were observed for trkB. In brain tissue sections, BDNF- and trkB-like immunoreactive material labeled neuronal cell bodies, proximal processes and varicose nerve fibers. Densitometric analysis showed the occurrence, under basal conditions and upon FST, of regional differences in density of immunolabelling; thus, in the case of BDNF they were located mostly to the CA3 and CA2 sectors of the hippocampus proper, whilst in the case of trkB they were located in the dentate gyrus (DG). These results are at variance with previous studies showing that the expression of BDNF in the hippocampus is reduced in depressed patients and in rats exposed to stressors. In conclusion, the present study provides morphological evidence for an unexpected differential regional expression of BDNF in the hippocampus of RLA vs. RHA rats upon acute stress and supports the view that stressors modulate neuronal plasticity in genetic animal models of depression. *This work was supported by grants from L.R. 07/2007, RAS project 2012*.

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EVIDENCE OF OBESITY-RELATED BRAIN INJURY IN DIET-INDUCED OBESITY (DIO) RATS: MACRO- AND MICRO-ANATOMICAL ANALYSIS.

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Obesity is an excessive or abnormal accumulation of adipose tissue due to a high-caloric diet and/or reduced physical activity. Statistical analysis revealed that more than 2 billion people worldwide are overweight and of these over 600 million are obese. Obesity is now considered more and more a medical challenge. Furthermore obesity may involve the development of chronic diseases such as cerebrovascular diseases promoting a cognitive decline.

Caloric-dense diet-induced obesity (DIO), represents an interesting animal model showing several common features with human obesity. DIO rats of 7 weeks of age exposed to high fat diet (45%) at libitum after 5 weeks develop the obese phenotype.

For explaining the relationship between obesity and nervous system changes, DIO rats were studied after 5 weeks and 17 weeks of high fat diet. CHOW rats were used for comparison. During and at the end of treatment, memory performance was assessed with different cognitive tests. Ultrasonographic (US) and computer tomography (CT) techniques were used to detect adipose tissue accumulation. Magnetic Resonance Imaging (MRI) was used to highlight brain morphological changes. Frontal cortex and hippocampus microanatomical changes were assessed by immunohistochemical techniques.

Our results confirmed the development of obesity after 5 weeks of fat diet. After long-term (17 weeks) high fat diet exposure, body weight values were remarkably increased compared to the control group and younger DIO rats. In DIO rats a reduction of a retention latency time in the emotional learning task was noticeable. US and CT analysis revealed an increase of deposition of both visceral and subcutaneous adipose tissue and a decrease of hepatic attenuation in older DIO rats. MRI did not show significant morphological and vascular brain changes. Immunohistochemical and immunochemical analysis showed an increased expression of glial-fibrillary acid protein (GFAP) in the frontal cortex and hippocampus of older DIO rats compared to age-matched CHOW rats. A decrease of neuronal specific nuclear protein (NEU-N) was found in 17-week-old DIO rats compared to control CHOW primarily in the hippocampus.

These findings show that the development of obesity, although not accompanied by macroscopic brain changes, is accompanied to a brain injury characterized by astrogliosis and neurodegeneration. The discovery of the above changes in the brain of DIO rats represents the first step to better clarify obesity-related brain injury and may contribute to identify therapeutic and nutritional strategies to prevent target organ damage.

CHOLINERGIC SYSTEM AND SINAPTIC VESICLES ALTERATIONS IN BRAIN AREAS OF AN ANIMAL MODEL OF METABOLIC SYNDROME

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The metabolic syndrome (MetS) refers to the concomitant presence of obesity, dyslipidaemia, insulin resistance and hypertension, that induces Type-2 diabetes mellitus (T2DM), cardiovascular and cerebrovascular diseases. Moreover, obesity and MetS are recognised as risk factors in the development of cognitive impairment such as Alzheimer's disease (AD) and vascular dementia (VaD). These neurodegenerative diseases affect the cholinergic system with a decrease of acetylcholine (ACh) levels and activity of biosynthetic enzyme choline acetyltransferase (ChAT).

This study has investigated cholinergic system of obese Zucker rats (OZRs) compared with non -obese cohort lean Zucker rats (LZRs) to demonstrate a relationship between obesity and brain disorders. The OZRs, with a mutation of leptin receptor, represents a model of obesity related to T2DM. Male OZRs and the littermate LZRs of 12, 16 and 20 weeks of age were used. The rats were monitored for body weight, food intake, blood pressure and blood levels of triglycerides, cholesterol and glucose. The OZRs were hyperphagic, hyperlipidemic, hyperglycaemic and exhibiting a moderate degree of arterial hypertension respect to controls LZRs. Behavioural tests are performed and revealed that in OZRs there are no changes in anxiety and emotional learning tasks.

In the brain, immunochemical and immunohistochemical analysis were performed for vesicular acetylcholine transporter (VAChT), ChAT, acetylcholinesterase (AChE), nicotinic (nAChRα7) receptor, muscarinic (mAChR) receptor subtypes (mAChR1, mAChR3, and mAChR5) and isoforms A, B and C of synaptic vesicle 2 proteins (SV2). SV2 are integral proteins localized on the surface of synaptic vesicles in different neurons, and are involved in exocytosis and neurotransmitter release. SV2B has been reported to control synaptic vesicle release dynamics. A decrease of this protein was found in hippocampus and cortex of AD patients. Colocalization of SV2C and ChAT was found in different brain areas.

Our results confirm the presence of VAChT in the cerebral areas involved in cognitive functions, such as the frontal cortex and hippocampus, with its reduction in 20-weeks-old OZRs. Furthermore, a lower expression of AChE in OZR at the same age suggests a possible physiological compensatory mechanism. A reduction in the expression of nAChRα7 was found, and this could be involved in the modulation of the inflammatory response at the central level. Different mAChRs expressions were detected in two investigated areas with a decrease of m1AChR subtype in hippocampus in 20 weeks OZRs. It could be related to the induction of brain damage linked to obesity. Moreover a decrease expression of SV2B in frontal cortex and hippocampus of 20 weeks-old OZRs was found and this could represent an alteration of synaptic vesicle release.

The results demonstrate that modulation of the cholinergic system and synaptic vesicle release in obese rats, are extremely complex phenomena. They could be related both to inflammation and processes of cognitive impairment induced by obesity.

Neurotoxicity

OXALIPLATIN-DEPENDENT IMPAIRMENT OF THE BLOOD BRAIN BARRIER IN AN *IN VITRO* MODEL OF RAT BRAIN MICROVASCULAR ENDOTHELIAL CELLS

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Oxaliplatin is a chemotherapeutic organic compound widely used for solid cancer, especially for metastatic colorectal cancer. Despite its beneficial effects in tumor reduction, it is well known to contribute to the pathogenesis of neuropathic pain, which is the main reason for dose reduction and for the therapy discontinuation. While neurons and glial cells are well known to be implicated in these functional changes, very little is known whether the blood–brain barrier (BBB) is also involved. BBB is a crucial structure that share dynamic interactions with neurons and glial cells, so forming the Neuro-Vascular Unit (NVU).

In this study we demonstrate, for the very first time, that oxaliplatin affect BBB permeability triggering caspase 3 activation, leading to tight junctions impairment and affecting cytoskeleton organization as well.

To better resembling a BBB *in vitro* model, RBE4 (rat brain endothelial cell line) were used to test the effects of oxaliplatin, at different concentrations and exposure time. Cell viability assay, caspase 3 activation, extracellular ATP concentration (an indirect measure of Pannexin-1 - Panx-1- activation), ZO-1 and F-actin immunofluorescent staining were performed.

Our results show that at oxaliplatin sub-cytotoxic concentration (10 μM), an increase in caspase 3 activation was induced, followed by an extracellular ATP increase. Furthermore, tight junction dislocation and cytoskeleton F-actin disorganization were observed.

Summarizing, these data clearly show the oxaliplatin capability to indirectly act on cytoskeleton and tight junction organization, suggesting a possible mechanism for the oxaliplatin-dependent BBB impairment.

THE SEXUALLY DIMORPHIC OBESOGENIC EFFECT OF EARLY POSTNATAL GENISTEIN ADMINISTRATION ON CD1 MICE

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Genistein (GEN), a phytoestrogen contained in soy and other legumes, may interfere with the endocrine system in multiple ways. In particular, GEN can act as 'obesogen' and increase the risk of developing metabolic disorders such as diabetes or obesity. The obesogenes increase body weight in mammals by acting on the fat tissue. Recent studies demonstrated that some of them (in particular the tributyltin) may induce permanent morphological alterations of estrogen sensitive circuits in adults as orexigenic and anorexigenic systems that influence food intake and energy expenditure. We analyzed the effects on adult CD1 mice of both sexes (age 2-months) of an early postnatal treatment (from PND1 to PND8) with GEN (50 mg/kg body weight dissolved in sesame oil) or with the vehicle (control, CON). In particular, we examined the expression of the POMC neuronal system within different hypothalamic nuclei [Paraventricular Nucleus (PVN), Arcuate Nucleus (Arc) and Dorsomedial Nucleus (DM)] and the orexin system in the lateral hypothalamic area (LHA). Early postnatal exposure to GEN, in a dose comparable to the exposure level in babies fed with soy-based formulas, induced sexually dimorphic effects. GEN treatment induced a significant increased body weight in adult GEN female ($P<0,001$), but there was no difference on food intake and daily feed efficiency. POMC immunoreactivity (measured as fractional area covered by the immunoreaction, FA) was significantly reduced in adult GEN females compared to CON females only in PVN (FA, $P<0,001$), while we have not observed any significant difference in DM and ARC, and in males. In addition, we observed an increase of the positive cell number in the inner part of Arc in GEN-treated females ($P<0,01$), whereas no changes were observed in males. The orexin system in the LHA is sexually dimorphic in CON mice (having males more cells than females), and this dimorphism was totally reverted in GEN mice: the cell number increased in GEN female ($P<0,05$) and decreased in GEN male ($P<0,041$). In conclusion, the early postnatal exposure of CD1 mice to GEN determines long-term sex specific organizational effects on neural circuits controlling food intake and energy metabolism. The increase of weight on GEN female but not of food intake as well as the morphological alterations on the two circuits expressing orexin and POMC suggest that the effect on body weight is due to only alteration of metabolic regulation.

PERMANENT EFFECTS OF EARLY POSTNATAL GENISTEIN ADMINISTRATION ON TH POSITIVE CATECHOLAMINERGIC NEURONS IN MOUSE

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Genistein (GEN) is a phytoestrogen present in high concentration in edible plants such as soy, largely present in human and animal diet. It has been reported that phytoestrogens are able to exert both estrogenic and anti-estrogenic activities, which may have a beneficial or detrimental effect according to the administration paradigm, sex and age of exposure. GEN administration during developmental critical periods may interfere with the formation of specific steroid-sensitive neuronal circuits, leading to irreversible behavioral and morphological alterations in adults even at a much lower dose than the one considered non-toxic by law.

We have previously shown that early postnatal administration of GEN (at doses similar to that of infant formulas) may affect the expression of neuronal specific nitric oxide synthase in specific hypothalamic circuits. Nitrergic system is involved in the control of many behaviors by interactions with other neurotransmitters such as catecholamines, for this reason, we investigated whether GEN treatment may affect tyrosine hydroxylase (TH, a specific marker for catecholaminergic neurons) expression in selected hypothalamic and mesencephalic populations.

GEN treatment affected only hypothalamic TH+ neurons and had no effect on mesencephalic neurons. Similarly to the effect previously observed for nNOS and AVP, this effect is sexually dimorphic, but, it was not mimicked by E_2 treatment.

Present and past results indicate that GEN exposure in early postnatal life may result in permanent alteration of several widely diffused neurotransmitters' systems, which control reproduction, anxiety behavior, energetic metabolism and many other behaviors. These results are important for both human health and animal welfare, and may have relevant economic consequences. In particular, soy based supplements are largely used for farm animals like in pigs that are commonly affected by hypo-fertility: the soy phytoestrogens could be one of possible causes.

SEXUALLY DIMORPHIC EFFECT OF CHRONIC TREATMENT WITH TRIBUTYLTIN IN THE ORGANIZATION OF BRAIN CIRCUITS INVOLVED IN THE *FOOD INTAKE* BEHAVIOR IN ADULT MICE

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Tributyltin (TBT), an antifouling agent of boat paints, is a common contaminant of marine and freshwater ecosystems. TBT is rapidly absorbed by organic materials and accumulated in fish, water birds, and mammals. Human exposure may be mediated by ingestion of contaminated food or by indirect exposure from household items containing organotin compounds. TBT is defined as an endocrine disruptor (EDCs) because it may bind to androgen receptors (ARs), it is also included in the list of obesogenic compounds. The brain is a target of TBT, as demonstrated by TBT acute administration inducing the expression of *C-fos* in the arcuate nucleus (ARC), a hypothalamic nucleus involved in the regulation of the *food intake*. Recent studies demonstrated that TBT interferes with the *food intake* system in mice, producing a strong decrease of circulating level of leptin and a reduction of NPY expression and its receptor (YR-1) in hypothalamic nuclei with sexually dimorphic effects. In this experiment we investigated the effect of a chronic treatment with TBT on the mouse anorexic system in both sexes. We investigated the POMC expression in PVN, DMH, VMH and ARC and the activation of leptin receptor, studying the leptin-induced P-STAT3 activation, in ARC. Our results show a sexually dimorphic effect of TBT on both systems studied: the TBT produced a significant decrease of POMC positive-structures in female mice in DMH, ARC and PVN, but not in male. Furthermore, in male mice, the TBT-treatment produced a decrease of PSTAT3 positive cells in ARC, while in female there is no effect on the number of activated leptin receptors. These results may help to better understand the obesogenic effect of TBT on the brain: it interferes with the anorexinergic system in hypothalamic areas involved in *food intake*, inhibiting the POMC expression in female but not in male mice. At the same time, this EDC affects the distribution of the activated leptin-receptors in ARC in male but not in female. In conclusion, the obesogenic effect of TBT is due to sexually differentiated effects on both orexigenic and anorexigenic systems, Y1 receptor, circulating leptin and its receptors.

Neuroprotection

VITAMIN D ADMINISTRATION IN A 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP)-INDUCED ANIMAL MODEL OF PARKINSONISM REDUCES NIGROSTRIATAL NEURODEGENERATION

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In the last decades it have been clearly shown that neuroinflammation plays a role in the pathogenesis and progression of various neurodegenerative diseases, such as Parkinson's Disease (PD). Following the activation of glial cells, mainly microglia and astrocytes, inflammatory mediators, reactive oxygen species, proinflammatory cytokines, and chemokines are released and they can exacerbate neuronal loss. In particular, overactivation of microglia in the brain of both PD patients and PD animal models has been demonstrated, suggesting its involvement in neuronal damage. Vitamin D is a steroid hormone known for its crucial role in the regulation of plasma calcium concentrations, and has also potent immunomodulatory activities in both innate and adaptive immunity. Clinical studies suggest that vitamin D insufficiency is associated with an increased risk of developing brain diseases, such as Alzheimer's disease, PD and brain ischemia, but the mechanisms underlying the role of vitamin D in neuroinflammation remains unclear.

In this study, the effect of 25(OH)D3 administration has been investigated in an in vivo MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced mouse model of PD. The results showed that Vitamin D significantly reduced the loss of tyrosine hydroxylase-positive neuronal cells, microglial cell activation and the expression of some markers typical of the proinflammatory microglia phenotype. Additionally, Vitamin D was able to induce the up-regulation of the anti-inflammatory cytokines interleukin (IL)-10, IL-4 and tumor growth factor (TGF)-beta.

These results provide novel support to the potential role of vitamin D in slowing down neurodegeneration and the consequent progression of PD and, potentially, other degenerative diseases in which neuroinflammation is involved.

PHARMACOLOGICAL JNK-PATHWAY INHIBITION REDUCES SEVERITY OF SPINAL MUSCULAR ATROPHY DISEASE IN A MOUSE MODEL OF SMAII

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Spinal muscular atrophy (SMA) is a severe neurodegenerative disorder that occurs in early childhood. The disease is caused by the deletion/mutation of the survival motor neuron 1 (SMN1) gene resulting in progressive skeletal muscle atrophy – particularly of the proximal muscles- and paralysis, due to the degeneration of spinal motor neurons (MNs). Currently, no treatment is available to prevent neuronal degeneration, and the cellular and molecular mechanisms underlying MN death are mostly unknown. Recently, it has been shown that the JNK-signalling pathway might be involved in the SMA pathogenesis.

Here we tested a synthetic JNK-inhibitor peptide (DJNKI) on a SMAII mouse model. A chronic treatment of DJNKI-peptide was administered to SMA mice and their wild-type (WT) littermates from P1 to P10. Control mice (both SMA and WT) received PBS. Using a multiple set of motor tests specific for pups, we evaluated motor function in treated and untreated mice. Then, at P12, spinal cord and quadriceps muscle were histologically analysed. Our results show that JNK inhibition has a positive effect on SMA mice behaviour, improving motor performances and hind limb muscular tones. Indeed, we found that DJNKI administration improves the trophism of SMA muscular fibers and the size of the neuromuscular junctions, compared to untreated SMA pups, leading to a better innervation of the muscles. Moreover, in the spinal cord the inhibition of JNK-pathway delays MN death and decreases astrogliosis. Finally, administration of DJNKI significantly increases lifespan in SMA mice, compared to the controls. Notably the inhibition of JNK does not affect the development of WT mice.

Together, our findings identify JNK inhibition as a way to reduce progressive skeletal muscle denervation and atrophy, and provide insight into the role of JNK-pathway for developing alternative pharmacological strategies for the treatment of SMA.

NEUROPROTECTIVE EFFECT OF COMBINED EPIGENETIC DRUGS IN AMYOTROPHIC LATERAL SCLEROSIS MURINE MODEL

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that causes degeneration of motor neurons in the central nervous system, and for which no effective therapy exists.

Defects in histone homeostasis have been recently implicated in the pathogenesis of neurodegenerative diseases, including ALS. Histone acetyltransferases (HATs) and Histone deacetylases (HDACs) catalyze the acetylation and deacetylation, respectively, of histone proteins. HATs and HDACs use as substrates also transcription factors, such as nuclear factor (NF) kB. Transcriptional dysregulation occurs in human sporadic ALS and in the SOD1(G93A) mouse model. Five DNA-binding proteins can compose the NFkB complex. The NFkB dimer p50/RelA has a dual, neuroprotective or neurotoxic effect depending on its acetylation state.

Our aim was to test if the treatment with epigenetic drugs modulates the acetylation of RelA in the spinal cord of SOD1(G93A) mice, slowing down ALS disease progression. In order to promote a proper acetylation of NFkB, a combination of the HDAC 1-3 (class I) inhibitor MS-275 and the sirtuin 1 (class III) activator Resveratrol were administered intraperitoneally every day in SOD1(G93A) mice starting from the age of 50 days and until the death of the animals.

Behavioral tests showed a significant improvement of motor performance in the treated group *versus* the control group. Furthermore a significant delay of clinical disease onset and a significant increase of the survival rate were documented in the treated group compared to the untreated once. The epigenetic treatment also showed a significant neuroprotective effect on the survival of lumbar spinal cord motor neurons in the treated group compared to the control group.

Our study reveals that the combined epigenetic drugs delay the neurodegenerative process which occurs in ALS, representing a future promising therapy for this disease.

IDENTIFICATION OF PECULIAR PHENOTYPIC AND METABOLIC CHARACTERISTICS IN OLFACTORY ENSHEATHING CELLS: A PROMISING TOOL FOR CELL THERAPY

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Olfactory Ensheathing Cells (OECs) show a peculiar plasticity as well as exhibit antigenic and morphological characteristics of both astrocytes and Schwann Cells. *In vitro*, OECs promote axonal growth and *in vivo* they can form myelin, promoting remyelination of damaged axons. Therefore, OECs have emerged as possible supportive cells for regeneration and functional recovery in neurodegenerative disorders. We previously demonstrated the functional characterizations and expression/modulation of some markers, such as Vimentin, S-100β, Nestin, Glial Fibrillary Acidic Protein, on OECs grown in different culture conditions: standard or serum-free media with/without Growth Factors (GFs). We observed a change of OEC usual morphology, reduction of cell viability and marker expression in serum-free medium. In addition, a positive influence of GFs on both viability and marker expression was observed. Since cell metabolism is a key determinant factor for the pluripotency and fate commitment of stem/progenitor cells during development, ageing, pathological onset and progression, we also described for the very first time the metabolic fingerprint of OECs. To identify and quantify metabolites, OEC lysates were analysed by proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy coupled to multivariate analysis. This approach revealed that OECs contain high levels of Lactate (39.9%), Acetate (22.3%) and Alanine (15.5%). These latter two metabolites are generally related to stem features whereas highest Lactate levels in OECs may be a reservoir for neuronal energy supply, essential to contrast imbalanced cell metabolism during neurodegeneration. Therefore, we demonstrate that the precise metabolites' content defines the function and identity, thus allowing an integrated bioengineering approach for univocal biologic fingerprints able to dissect the cell molecular specificities.
In conclusion, our detailed characterization demonstrates the plasticity and specific metabolic peculiarities of OECs, which can be exploited in the treatment of neurodegenerative diseases.

CURCUMIN PROTECS OLFACTORY ENSHEATHING CELLS EXPOSED TO HYPOXIA

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Curcumin is a natural polyphenolic compound which is extracted from the rhizomes of *Curcuma longa* and it has been used in traditional Chinese and Indian medicine for centuries. Recently, a wide range of pharmacological activities have been widely recognized, such as anti-inflammatory, anti-tumor, anti-depression and anti-oxidant activity; in addition curcumin is able to cross the blood-brain barrier because of its low molecular weight, therefore it has been used as a therapeutic agent since it confers protection in different neurodegenerative diseases, cerebral ischemia and excitotoxicity. Olfactory Ensheathing Cells (OECs), glial cells of the olfactory system, are able to secrete several neurotrophic growth factors, promoting axonal growth and supporting remyelination of damaged axons. OEC transplantation has emerged as a possible experimental therapy to induce repair of spinal cord injury (SCI), even if the functional recovery is still limited. Since hypoxia is a secondary effect in SCI, this *in vitro* study investigates on the protective effect of curcumin in OECs exposed to hypoxia. Primary OECs were obtained from neonatal rat olfactory bulbs and placed both in normal and hypoxic conditions. Furthermore, some cells were grown with basic Fibroblast Growth Factor (bFGF) and/or curcumin at different concentration and times. The results were analyzed by immunocytochemical procedures to assess morphological features and by MTT test to evaluate cell viability. We showed that curcumin stimulated cell viability in OECs grown in normal and hypoxic conditions and the synergistic effect of curcumin and bFGF was the most effective exerting protection on OECs. Since SCI is often accompanied by secondary insults, such as ischemia or hypoxia, our results suggest that curcumin in combination with bFGF might be considered a possible approach for restoration in injuries.

MUSCLE-ENRICHED NERVE GUIDES FOR PERIPHERAL NERVE REGENERATION

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Recent studies demonstrated that the chitosan guides used for peripheral nerve repair showed results similar to those obtained using autologous nerve grafts after immediate repair of rat sciatic nerve gaps. These promising pre-clinical results, led to approval of the chitosan tubes for clinical use in 2014 as Reaxon® Nerve Guide (Medovent GmbH, Mainz, Germany).
In this study we show a strategy to further improve the performance of the chitosan tube by the introduction of longitudinal skeletal muscle fibers. As previously demonstrated, muscle fibers used to fill a vein ("muscle-in-vein" conduit) improve peripheral nerve regeneration when used to bridge a nerve defect up to 2cm.
The rat median nerve was repaired by means two different conduits: (i) 10 mm hollow chitosan tube; (ii) 10 mm chitosan tube enhanced by the introduction of skeletal muscle fibers (a longitudinal piece of the pectoralis major muscle was introduced inside the tube, muscle-in-tube). 10 mm autologous nerve graft was used as a positive control. Samples were harvested at both early (7, 14, 28 days after nerve repair) and late time points (3 months), and functional, morphological, stereological and biomolecular analysis were carried out.

The biomolecular analysis carried out on early time points shows that the muscle inside the tube produces and releases neuregulin1, a key factor for the survival and activity of Schwann cells usually released following nerve injury. Moreover, the morphological analysis showed that only in the muscle-in-tube few fibers were already present after 14 days from nerve repair.
These preliminary results are very promising, because they combine the simplicity and rapidity of the use of the chitosan tube, with the effectiveness of the muscle fibers to promote axon regeneration.

Autophagy and mitochondrial dysfunction

INHIBITION OF AUTOPHAGY BY 3-MA IMPROVES MOTOR NEURON SURVIVAL AND LIFE SPAN IN A MOUSE MODEL OF SPINAL MUSCULAR ATROPHY

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Spinal Muscular Atrophy (SMA) is a recessive autosomal neuromuscular disease, caused by homozygous loss or mutation of the Survival Motor Neuron 1 (SMN1) gene. SMA is characterized by motor neuron (MN) degeneration, leading to motor impairment, muscle atrophy, and premature death. Emerging evidence suggests that autophagy dysregulation can contribute to MN degeneration. We investigated the role of autophagy in the SMNdelta7 mice (SMA II murine model), which present motor deficits by postnatal day 5 (P5) and die by P14.
Lumbar spinal cords from wild type (WT) and SMA pups were collected for histological, immunohistochemical, ultrastructural, and molecular analyses. Motor behavior was assessed daily with different tests. We showed by WB that Beclin 1 and LC3-II expression levels increased in the lumbar spinal cord of SMA pups. Electron microscopy and immunofluorescence studies confirmed that autophagic markers are enhanced in the ventral horns of SMA mice: indeed at P10 most MN cell bodies displayed shrinkage and vacuolization of cytoplasm, swelling of the endoplasmic reticulum and the perinuclear membrane, convoluted nuclei associated with few chromatin condensation and increased autophagic features (autophagosomes and autolysosomes).
To clarify the role of autophagy, we administered ICV (at P3) either an autophagy inhibitor, 3-methyladenine (3-MA) or an autophagy inducer (rapamycin) in SMA pups. 3-MA significantly improved motor performance, extended the lifespan and delayed MN death in lumbar spinal cord compared to control group. Inhibition of autophagy by 3-MA suppressed autophagosome formation, and reduced apoptotic activation (cleaved caspase-3 and Bcl2) and the appearance of TUNEL-positive neurons, underlining that apoptosis and autophagy pathways are intricately intertwined. On the contrary, rapamycin-treatment accelerated MN degeneration and shortened the lifespan in comparison to the control group.
Therefore, autophagy is likely involved in MN death in SMA II mice, representing a promising target for delaying the disease progression in humans as well.

A REAPPRISAL OF CELL-CLEARING MECHANISMS: THE AUTOPHAGOPROTEASOME HOSTING AUTOPHAGY AND UBIQUITIN PROTEASOME.

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Autophagy (ATG) and ubiquitin proteasome system (UPS) are two quintessential clearing mechanisms widely involved in eukaryotic cell biology. The dysfunction of either pathway is associated with systemic diseases, tumor progression and neurodegeneration. Classic ATG occurs within a double-layered cytosolic vacuole named autophagosome, gifted with a rich enzymatic apparatus aimed at clearing various cell cargoes and waste compounds. In contrast, the classic UP pathway does not imply a well-defined cell organelle and it occurs within dispersed cytosolic domains. Here, UP subunits interact to recognize altered ubiquitinated substrates and provide proteolytic clearance. Although these pathways have been long considered as biochemically and morphologically independent, recent data suggest a functional interplay and reciprocal compensation between ATG and UPS.
In this study, we demonstrated that ATG and UP components are hosted in the same organelle that we named autophagoproteasome. This novel organelle was characterized in a human glioblastoma U87MG cell line, in baseline conditions displaying defective ATG signaling, as well as following mTOR inhibition aimed at achieving a fine-tune modulation of both ATG and UPS. The quantitation carried out by confocal microscopy and ultrastructural morphometry shows that mTOR inhibition remarkably increases autophagoproteasomes. Nonetheless, within the autophagoproteasome, the relative amount for ATG compared with UP depends on the stimuli provided upon different experimental conditions. Noteworthy, upon strong m-TOR inhibition, the rich ATG compartment remains partly independent, while almost all cytosolic UP structures are sequestered within either early or late ATG vesicles, yielding a novel powerful clearing apparatus gifted with enriched catalytic pattern. Most notably, the occurrence of their co-immunoprecipitation strengthens the evidence for a reciprocal binding and a functional interplay in a newly integrated cell-clearing

scenario, which is still under our scrutiny even in other experimental models. Our findings suggest that this ultimate organelle may be a specific target for drugs against neuronal degeneration.

RAPAMYCIN-INDUCED RESCUE OF AUTOPHAGY DRIVES GLIOBLASTOMA STEM/PROGENITOR CELLS DIFFERENTIATION

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Glioblastoma (GBM; WHO grade IV glioma) is the most common, aggressive and malignant primary brain tumor. Recently, evidence indicates that GBM proliferation and tissue invasion are supported by the presence of cancer stem/progenitor cells niches, named GSPCs (Glioblastoma Stem/Progenitor Cells). In these niches, cells share the biological properties of normal stem cells, such as self-renewal, pluripotency and marked proliferation; they are key in tumor initiation, relapse and resistance to standard treatments. At molecular level, GSPCs are characterized by marked up-regulation of the mammalian Target Of Rapamycin (mTOR), a master regulator of cell growth and metabolism. In particular, mTOR acts as a negative modulator of autophagy. Although autophagy suppression in GBM is well established, its significance remains controversial, whereas the increase in autophagy may lead either to cell differentiation or to cell death, depending on the experimental conditions and the dose of autophagy inducers.

Therefore in the present study we analyzed the efficacy of the mTOR inhibitor and autophagy activator, rapamycin, on GBM cell proliferation and cell differentiation. We found that rapamycin induced a marked reduction of the stemness marker Nestin, along with an increase for the early and late neuronal marker, beta-III tubulin and NeuN, respectively. No effects were noticed for the glial marker GFAP.

These effects, together with evidence of a massive tumor volume reduction in the absence of any cell death, generate the working hypothesis that rapamycin acts on GBM both reducing cell proliferation and promoting GSPCs phenotypic switch towards neurons.

MITOCHONDRIAL METABOLIC BIOMARKERS LINKED TO THE G94A MUTATION OF SOD1 PROTEIN

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Amyotrophic Lateral Sclerosis (ALS) is neurodegenerative disorder with fatal outcome characterized by the progressive degeneration of motor neurons. Several studies suggest that mitochondrial dysfunction may play a pivotal role in early stages of the disease. The NSC34 mouse motor neuron-like cell line provides an experimental model suited to investigate neurodegenerative diseases. We used NSC34 cells stably transfected with a vector for the inducible expression of the gene coding for the human SOD1 bearing the G93A mutation associated to a form of familial ALS. To investigate the effect of the SOD1G93A mutation on the mitochondrial metabolic profile of these cells by high resolution respirometry (O2k HRR), we have optimized a SIUT (Substrate Uncoupler Inhibitor Titration) protocol. The NSC34 cells expressing the mutant SOD1G93A protein showed a significantly reduced mitochondrial oxidative capacity (ETS). The expression of the mutant dismutase also resulted in a significant reduction of complex I-linked phosphorylation and a significantly reduced efficiency of the electron transport system, with higher rate of dissipative respiration.

This study adds important information on the bioenergetic defects associated with the SOD1G93A mutation in a cell model for the study of ALS, paving the path for a “pharmacological” approach to phenotypic recovery.