



Obestatin inhibits high glucose-induced reactive oxygen species production and apoptosis in bovine aortic endothelial cells**Arnoletti Elisa**

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In the first year, my doctoral work has dealt with the biological actions of obestatin, a peptide produced by the ghrelin gene, on the survival of endothelial cells. Endothelial dysfunction is thought to be a major cause of vascular complications in diabetes (1). Our research shows that obestatin, a 23-aminoacid amidated peptide recently identified as a product of the ghrelin gene (2), inhibited high glucose-induced apoptosis in cultured bovine aortic endothelial cells (BAEC). Exposure to high glucose concentration (30 mM) for 72 h caused a significant increase in apoptosis, as evaluated by Hoechst staining, but co-treatment of rat with obestatin (from 10 pM to 100 nM) eliminated in a dose-dependent manner high glucose-induced apoptosis in BAEC. Obestatin also protected endothelial cells from high glucose by reducing reactive oxygen species (ROS) production. Blockade of adenylyl cyclase and cAMP-dependent protein kinase A signaling prevented the inhibitory effect of obestatin on ROS production. Obestatin also activated phosphatidyl inositol 3-kinase (PI3K/Akt) and ERK1/2 pathway, whereas PI3K and ERK inhibitors counteracted the obestatin anti-apoptotic effect. Finally, saturation binding studies with radioiodinated [¹²⁵I]-obestatin recognized high-affinity ($K_d=0.5$ nM) specific binding sites in the BAEC cell line, suggesting that these sites may be involved in the cytoprotective effect of the peptide. In conclusion, the results of our study demonstrate, for the first time, that obestatin inhibits both high glucose-induced apoptosis and ROS production in endothelial cells and suggest that this peptide may have potential in preventing vascular complications in diabetic patients.

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Sonic hedgehog induces proliferation of neural progenitor cells isolated from rat postnatal cerebellum**Barbierato Massimo**

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Sonic hedgehog (Shh) is a secreted morphogenic and mitogenic protein named for the bristle phenotype observed in *Drosophila* embryos that lack the corresponding gene. In vertebrates Shh is essential for the development of multiple organ systems, including the nervous system. Moreover, Shh regulates stem cell production and activation during tissue repair after injury, and appears to drive proliferation in a variety of tumours, including those arising in the brain, foregut, lung, breast, pancreas, stomach, and prostate (1). In cerebellum Shh controls the proliferation of granular neural precursor cells (GNP) (2,3). Cellular responses in the hedgehog pathway are controlled through the Patched1 (Ptch1) and Smoothened (Smo) membrane proteins. In the absence of Shh, Ptch1 maintains Smo in an inactive state, thus silencing intracellular signalling. With the binding of Shh, Ptch1 inhibition of Smo is released and the signal is transduced through Gli transcription factor family (Gli1–3) (1). Gli family members can either promote activation or silencing of gene transcription: Gli1 is described as the major activating transcription factor and is regulated by Shh; on the contrary, Gli2 and Gli3 act to downregulate mRNA transcription. We have previously described the isolation and characterization of cerebellar neural precursor cells (CNPC) from postnatal day rat (4). To investigate the role of Shh in CNPC proliferation, we treated cells with recombinant rat Shh. Isolated CNPC expressed mRNAs for the Shh receptor Ptch1, and for Gli2 and Gli3, together with very low levels of Gli1 protein. Treatment of CNPC at day 10 *in vitro* with Shh (1-300) nM for 24 h concentration-dependently increased cell proliferation, evaluated as [³H]thymidine incorporation, up to 84.9±3.4%. Furthermore, initial results show that Shh increased mRNA levels for Gli1 but not the cell cycle protein cyclin D1, while Erk1/2 phosphorylation increased within one h of Shh addition to the cultures. Although still preliminary, our results demonstrate that rat postnatal cerebellum contains neural precursor cells capable of proliferating in response to Shh.

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Morphological characterization of mast cells in neuropathic mice after a repeated treatment with palmitoylethanolamide**Bettoni Isabella**

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Recent evidences suggested that mast cell activation and degranulation have a key role in the beginning and maintenance of a persistent pain, such as neuropathic one. Particularly mast cells are known to release NGF (1) and to express trkA (2) receptors and thus NGF binding may cause mast cell degranulation, leading to a further release of the neurotrophic factor NGF and many other pronociceptive and proinflammatory mediators. In a previous study we established that the endogenous lipid palmitoylethanolamide (PEA) was efficacy *in vivo* against the neuropathic pain. PEA, administered i.p. at the dose of 10 mg/kg for one week starting the day after the lesion, evoked a relief of both thermal hyperalgesia and mechanical allodynia (3). In addition, PEA significantly reduced the NGF levels in the sciatic nerve of neuropathic mice (3). Starting by this assumption we wanted to characterize mast cell degranulation in the same animal model; therefore, mice were treated daily with the same dose of PEA. Three and 8 days after the nerve ligation, 24 h after the last administration, mice were sacrificed and the sciatic nerve removed and fixed, processed, and embedded in paraffin wax or Epon to obtain thin longitudinal 5- μ m or semi thin transversal 1- μ m sections, respectively. Both longitudinal and transversal sections were stained with hematoxylin eosin and toluidine blue, respectively, in order to examine the morphological structure of nerves. In control (Sham) mouse (48 h and 7 days), hematoxylin eosin staining highlighted arranged and consistent nerve morphology and we observed a homogeneous localization of nuclei Schwann cells. These data have been confirmed by the transversal sections since there were no histological abnormalities. In chronic constriction injury of the sciatic (CCI) nerves of 48 h group there were a lot of myelinated fibers that underwent a Wallerian-like degeneration, suggested by a dense and flocculent axoplasmic matrix and 7 days after the lesion, this scenario had a worsening. Finally, even in longitudinal nerve section of mice treated with PEA for 48 h we observed a loss of compactness of fibers and this result was partially confirmed in transversal sections. The thin longitudinal sections were stained with toluidine blue and a limited number of intact and degranulated mast cells were present in the sciatic nerve of control (sham) mice, indicative of physiological resident mast cells. In CCI mice mast cells were more than in sham mice: particularly, intact mast cells were missing near the damage site and were increased in the distal part of the nerve. The degranulated mast cells acted in an opposite way: they were more abundant near the damage site and less in the central part of nerve. Furthermore, in neuropathic mice sacrificed the third day after the surgery, the degranulated mast cells were localized near the damage site while in the other group of CCI mice they moved also in the central part of the nerve. These results confirm that degranulation of mast cells plays a key role in the initiation of neuropathic pain, with a strong recruitment of mast cells in the damaged site. In neuropathic mice treated with PEA for 48 h there was a significant mast cell recruitment and a higher number of intact mast cells as compared to CCI mice. These data suggest that PEA acts on both modulation of mast cell degranulation and on their recruitment in the damage site. Mast cells were also characterized immunohistochemically to evaluate the presence of secretory markers: the results obtained are comparable to previous data. The findings presented herein strongly suggest that PEA may prevent mast cell degranulation through the already described ALIA (autacoid local inflammation antagonism) mechanism and that this effect accounts for the antinociceptive property of PEA.

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Hypoxia regulates apoptosis and inflammation at all stages of human atherosclerotic plaque**Bitto Alessandra**

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Apoptosis and inflammation are important features of atherosclerotic plaques. We investigated whether this two apparently separate pathway can be triggered by a common signal molecule. Hypoxia inducible factor (HIF-1 α) is known to participate in atherosclerosis (1) and also to stimulate apoptosis signal-regulating kinase 1 (ASK-1), one of the mitogen-activated protein kinase kinases, which is activated by various extracellular stimuli and involved in a variety of cellular function (2). We tested carotid artery specimens from 55 subjects who underwent angioplasty for both Western blot and histological analysis. ASK-1 was detected in plaques of any composition from lipidic to calcific and this expression increased with the stage of the plaque and with the expression of inflammatory (p-ERK, RANK-L) and apoptotic molecules (caspase 9 and JNK). The hypoxic status was investigated by means of nitrite and by HIF-1 α immunostaining in carotid specimens. Our data suggest that hypoxia is the key regulating factor which triggers inflammation as well as apoptosis in the human atherosclerotic plaque.

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Isolation and characterization of mouse lung endothelial cells from mice genetically modified for the expression of EP receptors**Bocci Elena**

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During inflammation the endothelium, inflammatory cells, and stromal cells produce prostanoids, molecules that are rapidly released into the extracellular space where they exert autocrine or paracrine effects. Prostaglandin E2 (PGE2) is the principal prostanoid formed from arachidonic acid by cyclooxygenase and has been demonstrated to contribute to angiogenesis and tumour progression. PGE2 exerts its effects through cell surface receptors, namely four G protein coupled receptors EP1, EP2, EP3, and EP4 (1). Genetically modified mice for each of the four receptors have been developed by Ono Pharmaceuticals (Japan) and are available in our lab. The use of tissues and cells from mice lacking one of the receptors can help to understand the role played by each receptor in PGE2-mediated angiogenesis. The aim of this study was to isolate and characterize murine lung endothelial cells (MLEC) from mouse strains genetically depleted for one of the EP receptors. The lung endothelial cells were isolated by lung tissue mincing and enzyme digestion (dispase) (2). Endothelial cells were recovered by the use of magnetic beads covered with anti CD144 (VE-Cadherin) antibodies and cultured on fibronectin coated dishes. For lung isolation, we started from 2 mice and we obtained cells to be seeded in two wells of a 12-well plate. When the monolayer arrived to confluence, cells were trypsinized and propagated in a well of a 6-well plate; after that, cells were further propagated in plates of 6 cm of diameter. Cell culture medium was D-MEM/F12, containing 20% FCS and endothelial cell growth supplement (ECGS-H, 8 µl/ml). At microscopic examination, the primary cultures contained small amounts of other contaminating cell types, and therefore they need to be purified at each splitting. We repeated the purification for up to three passages and at the third passage we characterized the endothelial cells culture. Endothelial cell characterization was assessed by immunofluorescence analysis of endothelial marker (CD31). Smooth muscle cell contamination on primary cultures was assessed through immunofluorescence analysis using α -smooth muscle actin antibody, and the positive cells were less than 1%. The proliferative rate was very slow, the cells arrived to confluence in about 4-5 days and we recovered about 1 million cells in the final plate of 6 cm. Endothelial cells maintained their characteristics until passage 10-11. In conclusion, methods and characterization for endothelial isolation from genetically modified mice were set up and validated.

Acknowledgement: We thank Ono Pharmaceuticals (Japan) for mice KO EP receptors.

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High mobility group box-1 expression correlates with outcome in critical ill patients**Bonaiuto Antonio**

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High mobility group box-1 (HMGB-1) is a late mediator of severe sepsis that has been associated with mortality in experimental conditions (1). We studied the change in HMGB-1 mRNA expression in patients with myocardial infarction and stroke. Furthermore we also investigated the expression of tumor necrosis factor receptor (TNFR)-I and II, and CD40 and its ligand, to understand the relationship between HMGB-1 and apoptosis (2) in this peculiar clinical conditions. Patients referring to the Emergency Department with clinical signs of myocardial ischemic infarction or ischemic stroke were enrolled in the study after obtaining informed consent. Blood samples were obtained at the time of hospital admission and after 24 h to monitor the effectiveness of therapy. Total mRNA was extracted by blood monocytes following routine procedures and subsequently mRNA was reverse transcribed and tested by Real Time PCR for HMGB-1, TNFR-I, TNFR-II, CD40, and CD40-L. Elevated HMGB1 levels were associated with augmented apoptosis and adverse clinical outcomes in patients with both myocardial infarction and ischemic stroke. Our present results suggest HMGB-1 as a useful biomarker to evaluate patients' outcome.

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Colourful fruit and vegetable-based anticancer strategy: insights into drug metabolizing enzymes, antioxidant defences and oxidative stress**Bonamassa Barbara**

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A reduced cancer risk associated with fruit and vegetable phytochemicals initially dictated chemopreventive approaches focused on specific green variety consumption or even single nutrient supplementations. However, these strategies not only failed to provide any health benefits but gave rise to detrimental effects. In parallel, public-health chemoprevention programmes were developed in the USA and Europe to increase whole vegetable consumption. Among these, the National Cancer Institute (NCI) sponsored plan “5 to 9 a day for a better health” was one of the most popular. This campaign promoted wide food choice through the consumption of at least 5 to 9 servings a day of colourful fruits and vegetables. In this study the effects of the diet suggested by NCI on transcription, translation, and catalytic activity of both xenobiotic metabolizing (XME) and antioxidant enzymes were studied in an animal model. In fact, the boost of both antioxidant defences and “good” phase-II together with down-regulation of “bad” phase-I XMEs is still considered one of the most widely-used strategies of cancer control. Six male Sprague Dawley rats for each treatment group were used. According to the Italian Society of Human Nutrition, a serving of fruit, vegetables, and leafy greens corresponds to 150, 250, and 50 g, respectively, in a 70 kg man. Proportionally, rats received 1 or 5 servings of lyophilized onion, tomato, peach, black grape or lettuce - for white, red, yellow, violet or green diet, respectively - or 5 servings of each green (“5 a day” diet) by oral gavage daily for 10 consecutive days. Liver subcellular fractions were tested for various cytochrome P450 (CYP) linked-monooxygenases, phase-II supported XMEs such as glutathione S-transferase (GST) and UDP-glucuronosyl transferase (UDPGT) as well as for some antioxidant enzymes. Hepatic transcriptional and translational effects were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. dROMs test was used to measure plasmatic oxidative stress. Routine haematochemical parameters were also monitored. While the 5 servings administration did not significantly vary XME catalytic activity, the lower dose caused a complex pattern of CYP inactivation with lettuce exerting particularly strong effects (a loss of up to 43% and 45% for CYP content and CYP2B1/2-linked XME, respectively; $P < 0.01$). “5 a day” supplementation produced the most pronounced modulations (a loss of up to 60% for CYP2E1-linked XME and a reduction of CYP content of 54%; $P < 0.01$). Testosterone hydroxylase activity confirmed these results. RT-PCR and Western blot analysis revealed that the “5 a day” diet XMEs inactivation was the result of both a transcriptional and a translational effect while lettuce did not exert such effects. All administrations brought out none or fewer modulation of phase-II supported XMEs. Apart from “5 a day” supplementation and the single serving of lettuce, which strongly induced DT-diaphorase (an increase of up to 141 and 171%, respectively; $P < 0.01$), antioxidant enzymes were not significantly changed. RT-PCR analysis confirmed DT-diaphorase induction brought about by the administration of both “5 a day” diet and a single serving of lettuce. Furthermore, it unmasked a similar result for heme-oxygenase. dROMs test provided insight into a condition of high systemic oxidative stress as a consequence of animal diet supplementation with “5 a day” diet and a single serving of lettuce (an increase of up to ~600% and 900%, respectively; $P < 0.01$). Haematochemical parameters were mildly affected by such dietary manipulations. According to the classical chemopreventive theory, these results could be of particular relevance. In fact, even if antioxidant enzymes were only mildly affected, the phase-I inactivating ability of these vegetables would be a worthy strategy to cancer control. However, the recorded systemic considerable amount of reactive oxygen species and the complexity of these enzymes and their functions suggest caution in the widespread use of vegan/vegetarian diets as human chemopreventive strategies. In fact, recent literature rather suggests that only diets rich in fruits and vegetables and poor in certain types of fat, together with moderate caloric intake, could be associated with reduced cancer risk.

Beneficial effects of intravenous mesenchymal stem cells administration to mutant SOD1/G93A(+) mice**Bonifacino Tiziana**

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Amyotrophic lateral sclerosis (ALS) is a chronic neuromuscular disorder, clinically characterized by muscle wasting, weakness and spasticity reflecting a progressive degeneration of upper and lower motor neurons (1). To date riluzole, the only approved pharmacological treatment, inadequately prolong survival in ALS patients (2). Development of more effective neuroprotective therapies is hampered by lack of understanding of the mechanisms of neuronal death. ALS occurs both in sporadic and familial (FALS) forms. In about 25% of familial cases, the disease is caused by mutation in the gene encoding cytosolic copper-zinc superoxide dismutase (SOD1) (3). Consistently, transgenic mice over-expressing FALS-associated SOD1 mutants (G93A, G37R, and G85R) develop a progressive neuromuscular disease resembling human ALS (1,4,5). Mesenchymal stem cells (MSC), a subset of adult stem cells derived from the bone marrow stroma, represent a possible cell source for tissue repair including the nervous system. MSC can modulate immune responses, exert an anti-apoptotic effect and migrate into the central nervous system when i.v. injected (6). We studied here the effects of MSC administration in mice expressing human SOD1 with a G93A substitution [SOD1/G93A(+)] (4). MSC (10^6 cells/animal, i.v.) were administered at day 90 to SOD1/G93A(+) mice, well after the onset of the first disease symptoms. The MSC-treated mice exhibited a significant prolonged survival time compared to saline injected SOD1/G93A(+) controls (136.0 ± 3.9 days vs 117.0 ± 3.4 days; $P < 0.005$). Such effect was associated with a significant amelioration of the motorial skills. Using the Rota-Rod paradigm (from 4 to 40 r.p.m. in 5 min) we found a significant improvement of the motor performance in the MSC-treated SOD1/G93A(+). Deambulation and posterior limb posture were assessed on the basis of 12 level score scale. Also in this case, a significant improvement of the score was observed in MSC-treated mice. We have recently found that neuronally-originated glutamate release, induced by depolarizing or non-depolarizing stimuli, was enhanced in the spinal cord of SOD1/G93A(+) mice, with respect to controls, and that this is a very precocious, pre-symptomatic, effect (unpublished observation, 7). Interestingly, MSC treatment almost abolished this extra-release of the excitatory amino acid. Upon i.v. injection, only a few luciferase-labelled MSCs were detected inside mice spinal cord sections. Nevertheless, histochemical analysis showed a decreased number of perikarial ubiquitin aggregates in spinal neurons of treated mice as well as a significant amelioration of astrogliosis and of microgliosis. A rabbit anti-chAT polyclonal antibody was used to identify motoneurons and it was found that lower motor neuron cell bodies were significantly more numerous in MSC-treated mice. We can conclude that the i.v. administration of MSCs to SOD1/G93A(+) mice presents a beneficial impact on animal survival and disease progression as well as on a number of biochemical and histological parameters, and may therefore be considered as a promising therapeutic opportunity to be explored for ALS.

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Selective fatty acid amide hydrolase inhibition differentially affects ethanol relapse behaviour in Wistar and in genetically selected alcohol preferring rats**Braconi Simone**

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The endocannabinoid system is involved in the regulation of various aspects of alcohol related behaviour. For instance, treatment with selective CB1 receptor antagonists reduces ethanol intake and conditioned reinstatement of ethanol seeking, whereas CB1 receptor agonists facilitate alcohol drinking. URB597 is a potent, selective, and systemically active inhibitor of fatty acid amide hydrolase (FAAH) that, following peripheral administration, increases anandamide levels in the brain and potentiates anandamides' effects enhancing CB1 receptor activation. Here, we studied the effect of URB597 on alcohol self-administration, on stress- and cue-induced reinstatement of alcohol-seeking in Wistar and in genetically selected alcohol preferring Marchigian Sardinian (msP) rats. Rats were trained to operantly self-administer 10% ethanol until stable baseline of responding was established. At this point, rats were treated with URB597 (0, 0.1, 0.3, 1.0 mg/kg) 30 min prior to the self-administration session. Results showed no effect of drug on both msP and Wistar rats. In a subsequent series of experiments, the ability of URB597 to modulate reinstatement of alcohol seeking induced by environmental conditioning factors, or by yohimbine (an anxiogenic drug), was tested. For conditioned reinstatement, rats were trained to self-administer 10% ethanol or water in 30 min daily session on a fixed ratio 1. Ethanol availability was signalled by orange odour (S+) that served as a discriminative stimulus and the activation of the house light for 1 s (CS+) contingent to each lever pressing. For water, anise odour (S-) and 1 s white noise (CS-) were used. Discrimination was followed by an extinction phase during which lever presses did not result in the delivery of ethanol, water, or presentation of the corresponding cues. After 15 extinction sessions, animal responding was below 10. Re-exposure to the alcohol cues (S+/CS+) but not water cues (S-/CS-) reinstated responding ($P < 0.01$). Treatment with URB597 did not modify cue effects either in msP or in Wistar rats. For stress-induced relapse, after acquisition of ethanol self-administration animals were subjected to an extinction phase (15 days) and then injected with yohimbine (1.25 mg/kg). As expected, yohimbine given under extinction condition elicited a significant reinstatement of responding that was significantly reduced by URB597 but only in msP rats. These data demonstrates that, contrary to the effect observed following direct activation of CB1 receptors with agonists, increase in CB1 receptor activity consequent to FAAH inhibition does not facilitate alcohol abuse vulnerability. In addition, data provide evidence that FAAH inhibition may selectively reduce relapse associated to stress in animals with innate vulnerability to abuse alcohol.

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Effect of a combined treatment with prednisolone and taurine on *in vivo* and *ex vivo* markers of dystrophic progression in mdx mice**Capogrosso Roberta Francesca**

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X-linked mutations of the dystrophin gene lead to severe Duchenne muscular dystrophy (DMD) in humans and to dystrophic conditions in animals, as the mdx mouse (1). Dystrophin-deficient muscles cannot withstand the contractile stress and undergo a progressive myofiber death and fibrosis, as a consequence of a complex cascade of events (1). Nowadays the sole drugs of clinical interest for DMD are glucocorticoids, although their use is restricted by the severe side effects. Several preclinical studies on mdx mice are ongoing to identify strategies to improve the profile of glucocorticoids as well as safer effective drugs. In the frame of a large screening of drugs on the more severe model of treadmill-exercised mdx mouse, we found that α -methyl-prednisolone (PDN), clinically used in DMD, and taurine, a safe aminoacid used as food integrator, were independently able to ameliorate some of the parameters altered by the disease (2). In the present study we wanted to test whether a combined treatment with PDN (1 mg/kg day i.p.) and taurine (1 g/kg day *per os*) on exercised mdx mice may exert a synergistic effect. *In vivo*, the effects of the combined treatment of PDN-aurine were evaluated on fore limb strength measured by a grip meter. For each mouse, the normalized fore limb strength (fore limb/body weight) at the beginning (time 0) and at the end of 4 weeks of exercise (time 4) and the relative increment were measured. First, we confirmed the ability of both PDN and taurine, singularly administered, to significantly contrast the exercise-induced weakness. In addition, the association of PDN-aurine counteracted the deleterious effect of exercise on mdx mouse force better than the two drugs alone. Indeed, the increase in normalized strength was 2.75 ± 0.28 (n=6) in PDN-aurine treated exercised mdx mice, a value significantly greater with respect to that of untreated exercised mdx mice (0.59 ± 0.42 ; n=4, $P < 0.001$ by Student *t*-test). The increase in normalized strength, observed in PDN-aurine treated mdx mice was even greater than that observed in wild-type mice (0.98 ± 0.17 ; n=7). HPLC determination of taurine concentration in different tissues showed a significant increase of its levels in *tibialis anterior* and brain of PDN-aurine treated mice with respect to untreated counterparts, supporting the ability of exogenous taurine to reach the target organs. *Ex vivo*, we evaluated by point voltage clamp recordings, the mechanical threshold, an electrophysiological index of calcium homeostasis, in *extensor digitorum longus* (EDL) muscle fibers. Taurine>PDN significantly ameliorated the negative threshold voltage values of the exercised mdx EDL fibers (rheobase voltage -74.1 ± 0.4 mV) (2). The muscles from mice treated with the combination PDN-aurine showed an almost full restoration of the mechanical threshold toward the values of wild-type myofibers (rheobase voltage -68.9 ± 0.5 mV vs -66.4 ± 0.4 mV, respectively). We did not observe any significant reduction of creatine kinase and lactate dehydrogenase levels in plasma of mdx animals treated with PDN and taurine, either alone or in combination. However, PDN, either alone or in association with taurine, exerted a mild amelioration of the histology profile of *gastrocnemius* muscle, with a significant 60-70% decrease in degenerating area with respect to untreated counterparts. In conclusion, the combined treatment of PDN with taurine may have a synergistic effect on the altered functional parameters of dystrophic animals such as mouse strength and muscle excitation-contraction coupling, likely in relation to the ability of both compounds to positively modulate the calcium homeostasis.

(Supported by Telethon-Italy grant number GGP05130)

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Oxytocin receptor involvement in autism**Capurro Valeria**

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There is growing interest in the neuropeptide oxytocin (OT) and its receptor (OTR) in the pathogenesis of autism. OT controls the ability to remember individuals previously encountered, a form of social recognition that is essential for the establishment of all complex relationships (1), suggesting that the OT system is involved in the normal processing of socially relevant cues. The crucial role of OT in regulating the social brain has been recently confirmed in knockout $OTR^{-/-}$ mice, an animal model characterized by marked defects in maternal and social behaviour (2). Aim of the present study was to examine the behavioural phenotype of heterozygous $OTR^{+/-}$ and knock-out $OTR^{-/-}$ mice in comparison to wild type $OTR^{+/+}$ mice with particular attention for more specialized behavioural tasks relevant for autism such as: the social approach to stranger mouse, in which mice were given a choice between spending time in the proximity of another mouse or staying alone; the preference for social novelty, where mice had to choose between spending time in the proximity of an unfamiliar mouse or an already investigated mouse. Furthermore, reversal learning was evaluated in the T-maze task which consisted in training mice to obtain food placed at the end of the same arm of the maze until criterion was reached and then switching the reinforcer position to the opposite arm, registering the number of days needed to reach the criterion again (3). Our results indicate that both $OTR^{+/-}$ and knock-out $OTR^{-/-}$ mice had a good general health and normal neurological reflexes. However, they were severely impaired in: social recognition, as they preferred staying alone rather than investigating a novel mouse, social memory, as they showed no interest in investigating an unfamiliar mouse, and in reversal learning in the T-maze task, as they needed more days to re-reach the criterion when the reinforcer position was switched. Social impairments were completely reversed by oxytocin i.c.v. treatment (0.5 ng/mouse) in both heterozygous and knock-out mice. These findings demonstrate that the OTR may play a role in the core symptoms characterizing autistic disorders. Moreover, the deficits found in the heterozygous $OTR^{+/-}$ mice make these animals an interesting model for the *in vivo* screening of new pharmacological compounds targeting the OT/OTR system.

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Effects of ethanol exposure on nociceptin system-related gene expression in the rat brain**Caputi Francesca Felicia**

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Alcohol is the most socially-accepted addictive drug and alcoholism is the leading cause of disability and deaths. Many evidences now indicate that alcohol interacts with endogenous opioid systems to produce some of its effects (1). Most of the studies regarding opioid biosynthesis in response to ethanol (EtOH) exposure have been performed in inbred strains of rodents that consume high amounts of alcohol (2) and/or exhibit a high preference for the drug (3). Moreover, it is not known whether opioid biosynthesis is a critical event related to the reinforcing properties of EtOH and in maintaining a high alcohol drinking behaviour. Propensity to self-administer EtOH may be connected to baseline expression levels of endogenous opioid peptides such as dynorphin and nociceptin. The aim of the study is to investigate the effects of low, clearly not toxic (0.75 g/kg), and high (3 g/kg) EtOH doses, at different time points after single and repeated oral drug administration, on the regulation of the endogenous opioid system in different brain regions. So far, our data show that intragastric administration of a single acute dose of EtOH (3 g/kg) induces a significant increase in pronociceptin mRNA in the amygdala 30 min after the administration. No substantial changes in nociceptin gene expression were observed after 2 h in this brain region and in the prefrontal cortex at both time points. Moreover, there were no alterations in the gene expression of the NOP receptor in both the brain regions investigated at all the time points. These preliminary findings indicate that acute EtOH exposure affects nociceptin biosynthesis in non-selected rodents in the amygdala complex, in which molecular and cellular adaptations related to the reinforcing effects of alcohol have been previously observed, and thus suggesting that this effect could represent an important event in the neuroadaptive responses to the drug.

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Biological properties of *Pistacia lentiscus* essential oil**Casarin Elisabetta**

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Pistacia shrubs of the *Anacardiaceae* family includes various species such as *Pistacia Vera*, edible, *Pistacia terebinthus*, and *Pistacia lentiscus*. Since ancient times, the phytocomplex from *Pistacia lentiscus*, an essential oil extracted from leaves, fruits, and trunk exudate, is known to be effective in the treatment of gastralgia, diarrhoea, dyspepsia, peptic ulcer, and bacterial and fungal infections. More recently, it has been observed also a protective activity against stomach cancer. In this study we investigated the antitumor properties of the volatile oil from *Pistacia lentiscus* twigs and leaves using human cell lines from ovaric (2008 and C13*) and colon (LoVo) carcinoma, and human stable fibroblast line (HFFF2) as *in vitro* models. Cell viability was determined using the MTT test. The results showed that after 3 h treatment, the phytocomplex was able to inhibit the growth of each cell line at similar concentrations (about 150 µg/ml). After 24 h treatment, the IC₅₀ on 2008 and LoVo cells resulted about 3 folds lower, while, on C13* cells no appreciable differences were evidenced between 3 and 24 h treatment. The effect of treatment with the oil upon proliferation in HFFF2 was investigated by the MTT test after 24, 48, and 72 h of exposition. In this cell line, the oil resulted active only after 72 h with an IC₅₀ of about 78 µg/ml. Western blot analysis confirmed the volatile oil's capability to reduce, in carcinoma cell lines tested, the expression of p-ERK, MAPKs induced by mitogenic stimuli, which are associated with pro-survival activity. By optical microscope we observed morphological changes in treated cells: vacuolization, decrease cellular size and brightness which are directly proportional to cells' vitality reduction. We also observed, using both Annexin V combined with propidium iodide (PI) and the analysis of reduced mitochondrial membrane potential with Rhodamine 123, that oil from *Pistacia lentiscus* is able to stimulate apoptosis. In particular, late apoptosis increased in a dose-dependent manner. The uptake of PI in treated but not permeated cells, investigated by microscopy, displayed a cellular membrane permeability alteration. Following these findings, we considered in detail the mechanisms by which the oil is able to induce programmed cell death. Mitochondria play a key role in apoptosis by releasing cytochrome c once alterations in their membrane potential occurred. Subsequently, cytochrome c stimulates the activation of a highly conserved family of aspartic acid-specific cysteine proteases, the caspases. Among these, caspase 3 plays a central role in the apoptosis and its activation seems to be the convergence point of different apoptotic signalling pathways. By Western blot analysis we confirmed that caspases are involved in the apoptotic response induced by the essential oil, and our data indicate that *Pistacia lentiscus* oil might cause programmed cell death via a caspase 3-dependent apoptotic pathway. We also performed a cell cycle distribution analysis using flow cytometry, observing that the phytocomplex is also able to induce G₂M arrest in a dose-dependent manner.

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GSK-3 β AND PPAR- γ AS NEW PHARMACOLOGICAL TARGETS *IN VIVO* MODELS OF CEREBRAL ISCHEMIA/REPERFUSION INJURY AND DIET-INDUCED INSULIN RESISTANCE**Castiglia Sara**

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Brain ischemia/reperfusion (I/R) injury is a leading cause of long-term disability and death in the Western countries and susceptibility is significantly increased in diabetic patients. Insulin may reduce brain injury evoked by I/R (1). Insulin is a well-known inhibitor of glycogen synthase kinase-3 β (GSK-3 β) that has emerged as a key element in the modulation of neurodegeneration and inflammation (2). During my PhD, I have investigated the role of GSK-3 β on the organ injury associated with cerebral I/R. The selective GSK-3 β inhibitor TDZD-8 was administered to rats subjected to ischemia (30 min) followed by reperfusion (1-24 h). Rats that underwent 24 h reperfusion showed an infarct volume (IV) of 24% of total brain and TDZD-8 administration reduced IV (3). When I/R injury was evaluated in the brain of streptozotocin (STZ)-induced diabetic rats, the degree of I/R-induced damage was significantly increased when compared with non-diabetic littermates and IV was almost 35% of total brain. Interestingly, when insulin was administered to diabetic rats, a significant improvement in infarct size was recorded and a reduction in neutrophil infiltration was observed. To better define the role of GSK-3 β in the protective effects exerted by insulin, TDZD-8, was used as a comparative pharmacological tool. In STZ-induced diabetic rats, TDZD-8 evoked a reduction in both IV and neutrophil infiltration similar to that recorded with insulin. Overall, these results demonstrate that insulin protective effects are mediated, at least in part, by inhibition of GSK-3 β activity (4). Insulin resistance is a pathological condition widely spread in Western countries and is associated to high consumption of fat and simple sugar, typical components of the unhealthy Western diet (5). On these bases, I recently contributed to develop an animal model of diet-induced insulin resistance obtained by feeding rats with a high-fat diet and 10% fructose solution (HCF) for 15 weeks. At the end of the dietary regiment, HCF rats exhibited hyperlipidemia, hyperinsulinemia, impaired glucose tolerance, and insulin resistance. During the last month of dietary manipulation, a group of rats received daily oral administration of pioglitazone, an agonist of PPAR- γ , belonging to TZD class of anti-diabetic drugs. TDZs are known for their lipid-lowering and insulin-sensitizing actions, although the molecular mechanisms of their beneficial effects are not yet fully understood (6). In rats fed HCF diet, pioglitazone administration evoked a significant improvement in lipid metabolism and insulin sensitivity (5). Diet-induced PPAR- γ expression was unaffected by pioglitazone treatment, whereas it markedly reduced hepatic expression of the suppressor of cytokine signalling (SOCS)-3, a key signalling pathway involved in both inflammation and insulin resistance (7). Besides, pioglitazone reduced the neutrophil infiltration and the expression of inflammatory markers. Taken together, these data indicate that feeding rats with HCF-diet may represent a valid model of non-genetic diet-induced insulin resistance and corroborate the potential of chronic pioglitazone administration in the treatment of insulin resistance associated with excess dietary intake. Besides, the present study shed also more light on the mechanism of action of pioglitazone in the liver, suggesting a key role for SOCS-3.

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L-aspartate as an amino acid neurotransmitter: mechanisms of the depolarization-induced release from cerebrocortical synaptosomes**Cavallero Anna**

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The question of whether aspartic acid can be classified as a classical neurotransmitter of the central nervous system has been a matter of great controversy. One point of major debate in the literature regards the characteristics of the neuronal depolarization-induced release of L-aspartate. It has been suggested that the Ca^{2+} -dependency of its release might be only apparent and just reflects carrier-mediated heteroexchange of intracellular L-aspartate for extracellular L-glutamate released by Ca^{2+} -dependent exocytosis, especially in intact tissues (1). Opposing this view is the observation of a robust Ca^{2+} -dependent component of the depolarization-evoked L-aspartate release from synaptosomes in superfusion (2-4), an experimental condition known to minimize reuptake of released neurotransmitters (5) that, therefore, should exclude heteroexchange phenomena. Further support for a true Ca^{2+} -dependent release comes from a recent work by Bradford and Nadler (6) who found that blockade of the aspartate/glutamate carrier was not able to decrease the Ca^{2+} -dependent release of endogenous L-aspartate elicited by different depolarizing stimuli from hippocampal synaptosomes. Our study characterized the main release mechanisms of endogenous aspartate from cerebrocortical synaptosomes in superfusion and compared them with those of the well-know excitatory neurotransmitter glutamate. In addition, [^3H]D-aspartate and [^3H]L-glutamate release mechanisms have been investigated and compared with those of the corresponding endogenous amino acids. High KCl and 4-aminopyridine were used as depolarizing agents. At 15 mM KCl, the overflows of both transmitters (L-aspartate 120 ± 38 pmol/mg protein; L-glutamate 638 ± 114) were almost completely dependent on external Ca^{2+} . At higher KCl concentrations (35 and 50 mM), the overflows of L-aspartate (886 ± 82 and 1439 ± 223 pmol/mg protein, respectively), but not those of L-glutamate (2801 ± 354 and 3853 ± 899 pmol/mg protein, respectively), became sensitive to DL-threo- β -benzyloxyaspartic acid (DL-TBOA), an excitatory amino acid transporter (EAAT) inhibitor. At 50 mM KCl, the EAAT blocker was able to reduce L-aspartate release by 42%. In addition, in the presence of DL-TBOA, Ca^{2+} deprivation was still able to reduce the 50 mM KCl-induced release of aspartate by 56%. The DL-TBOA insensitive, external Ca^{2+} -independent component of the 50 mM KCl-evoked overflows of L-aspartate and L-glutamate was significantly decreased by the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger blocker CGP 37157 (-85%). The Ca^{2+} -dependent, KCl-evoked overflows of L-aspartate and L-glutamate were diminished by botulinum neurotoxin C, although to a significantly different extent. The 100 and 300 μM 4-aminopyridine-induced L-aspartate and L-glutamate release was completely external Ca^{2+} -dependent and never affected by DL-TBOA. Superimposable results have been obtained by pre-labelling synaptosomes with [^3H]D-aspartate and [^3H]L-glutamate. Therefore, our data showing that L-aspartate is released from nerve terminals by Ca^{2+} -dependent, exocytotic mechanisms support the neurotransmitter role of this amino acid.

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Effects of MDMA on N/OFQ-NOP system in MPP⁺ induced model of Parkinson's disease**Cavina Chiara**Dipartimento di Farmacologia, Università degli Studi di Bologna, via Irnerio 48, 40126
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We have recently shown that dopamine (DA) modulates N/OFQ-NOP system gene expression in *caudate putamen* and *substantia nigra* (SN), strengthening the hypothesis that this neuropeptidergic system could be involved in the mechanisms underlying Parkinson's disease (1). It has been suggested that MDMA (3,4-methylenedioxymethamphetamine; ecstasy), belonging to a novel class of drugs named entactogens, has a good anti-parkinsonian effect. Comparing the effects of the MDMA enantiomers with the racemic drug gives evidence that the mechanism of the anti-parkinsonian actions of MDMA could be mediated neither by an indirect DA agonism nor by agonism at the 5-HT₂ receptor: racemic MDMA is much more potent than each of the MDMA-derivatives. The poor effect of the enantiomers on catalepsy indicates that there must be a new unknown component in the MDMA effects which manifests itself only in the synergism of both enantiomers R- and S-MDMA (2). MDMA was shown to prolong the action of L-DOPA while suppressing dyskinesia in a single patient with Parkinson's disease. The clinical basis of this effect is unknown (3). In order to clarify if MDMA has an anti-parkinsonian effect, we investigated whether MDMA produces alteration of N/OFQ-NOP gene expression in selective rat brain areas. Animals were divided into 8 groups (n=5 for each group). The animals belonging to the first, second, third, and fourth groups were treated with saline (first group) or MPP⁺ (40 mg in 5 µl of saline, second, third, and fourth groups) by i.c.v. administration. After 10 days, the third group received acute MDMA (8 mg/kg, i.p.) and the fourth group chronic MDMA (8 mg/kg, i.p. twice daily for 7 days). The other groups were treated only by i.p. administrations: acute saline (2 ml/kg, fifth group), chronic saline (2 ml/kg twice daily for 7 days, sixth group), acute MDMA (8 mg/kg, seventh group), chronic MDMA (8 mg/kg twice daily for 7 days, eighth group). The effects on N/OFQ-NOP mRNA levels were investigated using real time PCR. In the brainstem, we observed a significant increase in N/OFQ and NOP gene expression (120.9±7.4% and 126.0±6.6% of controls, respectively) after acute MDMA and in NOP mRNA levels (126.5±8.6% of MPP⁺) after MPP⁺/chronic MDMA. In the ventral tegmental area (VTA), real time-PCR analysis showed a significant increase in N/OFQ gene expression after acute and chronic MDMA, and after MPP⁺ administrations (127.4±5.5%, 134.0±6.8%, and 127.8±10.7% of controls, respectively). About NOP receptor gene expression, we observed an increase after chronic MDMA (130.5±12.2% of controls) and a decrease after MPP⁺ (88.2±8.7% of controls). In the substantia nigra, N/OFQ gene expression increases significantly only after treatment with MPP⁺ (201.8±27.6% of controls) whereas NOP gene expression decreases significantly in rats treated with MPP⁺ (69.8±7.1% of controls) and in rats treated with both MPP⁺ and MDMA administration (acute and chronic) compared to the group treated only with the neurotoxin (170.8±11.2% and 148.9±9.9% of MPP⁺). In the *nucleus accumbens* (NA), we observed a significant increase in NOP gene expression after MPP⁺ administration (174.8±29.5% of controls) whereas the treatment with acute MDMA after the neurotoxin injection reinstates NOP mRNA physiological levels (60.5±9.6% of MPP⁺). These results show that MDMA has different effects on N/OFQ-NOP gene expression depending upon the brain areas. They confirm that this neuropeptidergic system could be implicated in the mechanisms underlying Parkinson's disease and, more importantly, provide evidence that DA exerts an inhibitory tone on N/OFQ gene expression levels in SN and VTA, whereas exerts a stimulatory tone on NOP gene expression in the same areas. Furthermore, DA exerts an inhibitory tone on NOP mRNA levels in NA. In conclusion, MDMA has a positive effect on N/OFQ-NOP system in MPP⁺-induced model of Parkinson's disease and, particularly, is able to reinstate NOP gene expression at physiological levels in SN and NA.

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Effect of amyloid β peptide on zebrafish embryos (*Danio rerio*)**Cetti Elisa**

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Gene function conservation between zebrafish (*Danio rerio*) and human makes zebrafish an extremely attractive model system for the study of human diseases and for high-throughput drug screening (1). In fact, as an animal model, zebrafish offers several advantages over rodent models: short generation time, external development, allowing easy embryo manipulation, and optical transparency of embryos. Zebrafish has been used as a model for neurodegenerative disease including Parkinson and Alzheimer's disease (AD) (2,3). A method for designing disease models in zebrafish involves the use of different molecules to induce disease-like states, and small molecules, like amyloid beta ($A\beta$) peptides, can be added directly to the fish media and diffuse into embryos, inducing *in vivo* observable effects (4). Over the past 5 years, our lab has been interested in studying the effects of $A\beta$ peptide on angiogenesis and endothelial cell functions since deposition of $A\beta$ in brain capillaries is correlated with AD progression. The aim of this study was to investigate the effects of repeated $A\beta$ peptide ($A\beta_{1-40}$) treatment on zebrafish embryos, evaluating toxicity and occurrence of developmental anomalies. $A\beta_{1-40}$ peptide diluted in fish water (1, 2.5, and 5 μ M), was administered at 15 h post fertilization (hpf) until 72 hpf and dose-range studies were performed. We observed an increase of embryos mortality at the highest concentrations (2.5 and 5 μ M), whereas the lowest concentration of $A\beta$ peptide (1 μ M) was not toxic. In treated zebrafish embryos we also observed morphological changes of the vascular bed and increase of heart frequency. Mortality and severity of phenotypes were dose dependent. In conclusion, the administration of human $A\beta$ peptide alters zebrafish development. Particularly the highest concentrations promoted general toxicity, decreasing survival embryos. In addition, toxic effects of $A\beta$ treatment seemed to be more pronounced on vascular system development.

Acknowledgments: Telethon Foundation (research Project n° GGP06148)

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Purified human TSC2^{-/-} cells cause lymphangioleiomyomatosis-like lung destruction in nude mice: effects of anti-EGFR antibody and rapamycin treatments**Chiaramonte Eloisa**

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Tuberous sclerosis complex (TSC) is an autosomal dominant disease characterized by the development of hamartomas in multiple organs, most commonly affecting the kidney, brain, lung, and heart (1). TSC is caused by mutations in *TSC1* or *TSC2* genes encoding for hamartin and tuberlin, respectively (2,3). TSC is related to lymphangioleiomyomatosis (LAM), a rare disease characterized by widespread proliferation of abnormal smooth muscle-like cells that leads to cystic destruction of the lung parenchyma, obstruction of airways and lymphatics, and loss of pulmonary function (4). LAM affects almost exclusively young women and the mechanisms causing the disease are not yet clarified. LAM cells can migrate or metastasize to other organs; in fact, cells with *TSC2* mutation, have been found in angiomyolipomas (AMLs) and lung lesions of LAM patients (5,6). In our laboratory human TSC2^{-/-} α -actin smooth muscle cells (ASM) have been isolated from an AML of a TSC2 patient (7). These cells are characterized by hyperphosphorylation of S6 kinase and its substrate S6 and positivity to HMB45 antibody, a marker of TSC and LAM cells (7). *In vitro*, cell growth and proliferation depend on epidermal growth factor (EGF) and antibody raised against EGF receptor (anti-EGFR), by blocking EGFR extracellular domain, causes progressive cell death (7). Rapamycin, a specific mTOR kinase inhibitor, reduces proliferation rate but it does not affect significantly the TSC2^{-/-} ASM cell survival (7). To develop an animal model to study TSC and LAM pathogenesis, we endonasally administered TSC2^{-/-} ASM cells (2×10^5), previously labelled with red dye PKH26-GL, in 5 weeks old Hsd:ATHimic nude-nu/nu mice. Effects of endonasal administration were evaluated after 60 and 220 days. We previously showed that TSC2^{-/-} ASM cells were massively infiltrated into pulmonary alveolar walls and lymph nodes. In lymph nodes and lung parenchyma a diffuse reactivity to HMB45 antibody and a strong S6 phosphorylation were detected. TSC2^{-/-} ASM cells grow and proliferate in lung parenchyma and lymph nodes, as showed by Ki-67 and S6-phosphorylation double staining. Pharmacological treatments blocked cellular proliferation, with anti-EGFR antibody being more effective than rapamycin. The average number of TSC2^{-/-} ASM cells in lung parenchyma was reduced from $197,00 \pm 58,27/\text{mm}^3$ to $32,17 \pm 12,74/\text{mm}^3$ by anti-EGFR antibody whereas only to $134,18 \pm 53,94/\text{mm}^3$ by rapamycin. TSC2^{-/-} ASM cells caused progressive destruction of the lung parenchyma with an emphysematous-like picture that was reversed by anti-EGFR treatment while rapamycin was less effective and caused hemoptysis. TSC2^{-/-} ASM cells promoted a significant increase of LYVE-1 reactivity in lungs suggesting a possible correlation between TSC2^{-/-} ASM cells and lymphangiogenesis. LYVE-1 reactivity decreased following anti-EGFR antibody and rapamycin treatments but, while anti-EGFR antibody suppressed the excessive lymphatic vessel, rapamycin caused their collapse. Moreover, murine VEGF levels in homogenate of lungs, measured by ELISA assay, were increased following TSC2^{-/-} ASM cells endonasal administration and were reduced following anti-EGFR antibody and rapamycin treatments. In conclusion, TSC2^{-/-} ASM cells can migrate, proliferate and invade lymph nodes and lungs causing LAM-like lesions. Anti-EGFR antibody is more effective than rapamycin in promoting lung regeneration, blocking proliferation, and reducing lymphangiogenesis. These data suggest a new therapeutic approach for the treatment of TSC and LAM disease.

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HYDROGEN SULPHIDE INDUCES MOUSE HIND PAW OEDEMA VIA PLA₂ ACTIVATION**Coletta Ciro**

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Hydrogen sulphide (H₂S) is a naturally occurring gas, involved in cardiovascular homeostasis. H₂S is formed in mammalian cells largely (but not exclusively) from L-cysteine catabolism by the activity of two pyridoxal phosphate dependent enzymes: cystathionine-γ lyase (CSE) and cystathionine-β synthetase (CBS) (1). The expression of both enzymes has been detected in many human and other mammalian cells in a tissue specific manner (2). CBS is mostly located at the level of the central nervous system (3), whereas CSE is predominant in vascular tissues such as aorta, mesenteric artery, portal vein (2,4). More recently a pro-inflammatory effect of H₂S has been also reported. In fact, an increase in plasma H₂S concentration as well as in liver and kidney homogenates has been shown in animal models of endotoxic or septic shock (5). Interestingly, plasma H₂S concentrations were also markedly increased in patients with septic shock (5). Moreover, a significant increase in H₂S-synthesizing activity was detected in rat carrageenan-inflamed hind paws; pretreatment with DL-propargylglycine, an inhibitor of CSE, significantly reduced carrageenan-induced hind paw oedema in a dose-dependent manner (6). Thus, in this study we attempted to assess the involvement of H₂S pathway in mouse oedema as a model of acute inflammation, evidencing the underlining mechanism of action. Male mice (CD-1, Harlan, Italy, 40 g) were divided into groups (n=6/group) and were used throughout. Mice received subplantar injection of 100, 300 or 500 μg of NaHS, a stable donor of H₂S, in the left hind paw, diluted in 30 μl of potassium phosphate buffer (PPB, pH 7.4). The volume of the hind paw of each animal was measured by using a hydroplethysmometer specially modified for small volumes (Ugo Basile, Italy) before subplantar injection of the inflammatory challenge (time 0), each 15 min during the first h, and at different time intervals thereafter for up to 72 h. In order to assess the involvement of phospholipase A₂ (PLA₂) in H₂S pathway we used dexamethasone (Dex, a PLA₂ inhibitor, 1 mg/kg per os), indomethacin (a cyclooxygenase inhibitor, 10 mg/kg per os), or 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA, a specific PLA₂ inhibitor, 0.1, 0.3 or 1 mg/kg per os) 2 h before the challenge. Results were expressed as the increase in paw volume (μl). Injection of NaHS into the mouse hind footpad produced a significant dose dependent increase in paw volume compared to the control group (P<0.001). The peak oedematogenic response occurred 30 min after NaHS injection at all doses tested and decreased gradually thereafter, but was still elevated after 24 h. Oral administration of Dex or indomethacin resulted in a significantly reduction of oedema formation (P<0.001). In order to ascertain the involvement of PLA₂ in NaHS induced paw oedema we used OBAA. Oral administration of OBAA resulted in a concentration dependent reduction of oedematogenic response to NaHS (P<0.001). In conclusion, since the role of H₂S in inflammation is still far from clear, we confirmed the proinflammatory H₂S effect suggesting that it could represent an inducer of the inflammation and not only a mediator. In fact, we hypothesized that H₂S may activate the PLA₂ responsible of the oedematogenic effect.

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Effects of GABA and Taurine on glutamate/MPP⁺ mediated cell death of rat cultured primary cortical and cerebellar granule neurons**Contartese Antonella**

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A loss of balance between excitatory and inhibitory signalling leads to excitotoxicity, and contributes to ischemic cell death (1). Reduced synaptic inhibition as a result of dysfunction of the ionotropic GABA_A receptor has been suggested as one of the major causes for this imbalance, although the underlying mechanisms remain poorly understood (2). Therefore, GABA and GABA-mimetic drugs are expected to protect the brain from glutamate-mediated cell death (3-4; Ricci L et al. unpublished observation). Aim of the present work was to assess neuroprotection afforded by GABA and taurine (an inhibitory neurotransmitter) on excitotoxic-mediated cell death of rat cultured primary neurons. In particular, rat cortical neurons (CN) exposed to glutamate, to mimic a direct excitotoxic insult, and rat cerebellar granule neurons (CGNs) treated with the mitochondrial poison MPP⁺, to mimic an indirect excitotoxic insult, were used. Neuronal damage and neuroprotection were assessed by monitoring cell viability with MTT tetrazolium salt assay. In CN, glutamate (50 μ M for 24 h) caused a significant reduction in cell viability by about 54%. This effect was completely prevented by the non-competitive NMDA-receptor antagonist MK801 (10 μ M added 30 min before glutamate). GABA (0.01-1 μ M), when added 30 min or 2 h before the toxic insult, partially antagonised glutamate-induced cell death. The maximal effect was observed at 0.1 μ M, with a recovery of cell viability to about 70%. Higher GABA concentrations (up to 100 μ M) were ineffective as well as the addition of taurine (10 μ M-1 mM) 30 min or 2 h before the toxic insult. In rat CGNs, MPP⁺ (50 μ M for 24 h) caused a significant increase in cell death by about 30%. GABA (0.01-1000 μ M), when applied 30 min or 2 h before the toxin, reverted MPP⁺-induced cell death. This effect was concentration-dependent, the maximum of 26.5% in cell recovery being achieved with 1000 μ M or 100 μ M GABA added 30 min or 2 h before the toxic insult, respectively. On the contrary, taurine prevented cell death only at the highest concentration tested (1 mM) with a recovery in cell viability of about 20 %. In conclusion, the activation of GABAergic system might represent a promising approach to counteract ischemia/reperfusion-like injuries as already observed *in vitro* in brain slices (3-4; Ricci L et al. unpublished observation) and *in vivo* (5). Finally, the molecular mechanisms involved in GABA- and taurine-mediated protection, with particular concern to apoptosis signalling, will be performed during the second year of PhD.

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S-Glutathionylation, thiol competition thiol exchange and mechanism of protein thiolation/dethiolation in rat

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S-glutathionylation, the formation of glutathione-protein mixed disulphide (GSSP), is a reversible post-translational modification with critical roles in sulphhydryl homeostasis, signal transduction and detoxification mechanism. S-Protein glutathionylation increases during oxidative stress (such as that associated with cardiac ischemia-reperfusion (1), and more selectively in the presence of reactive oxygen species produced during physiological signalling [such as by growth factors or angiotensin II (2,3)]. Protein disulphides (PSSP) can also occur during oxidative stress when competition among thiols of high (namely protein SH groups, PSH), and low-molecular weight (RSH) thiols is won by PSH. Thiol competition may be considered an important device to modulate the biological response in living organisms. Here we report data that explain the importance of thiol pKa in the regulation of protein thiolation/dethiolation phenomena, using diamide (DIA) to explore thiol competitions evoked by oxidative stress. DIA is an electrophilic agent reacting with thiols by a two step mechanism. For example, if PSH has good exposure to the milieu and lower pKa than GSH, DIA reacts primarily with PSH rather than GSH, forming PS-DIA. Afterwards PS-DIA or GS-DIA reacting with residual thiols may form symmetrical or asymmetrical disulphides (GSSP, PSSP, GSSR) depending on thiol pKa (and concentration). pKa-Dependent rules are also important during dethiolation, that is detachment of RSH from RSSP with regeneration of PSH

$\text{PSSR} + \text{R}'\text{SH} \Rightarrow \text{RSSR}' + \text{PSH}$ [1]

For example the rate of GSSP dethiolation by thiol, in the absence of deglutathionylation enzyme (glutaredoxin, GRX), is higher when PSH has a lower pKa than GSH. Reaction rates of rat plasma albumin [pKa=5-7 (4-6)] and GSH (pKa=8) were analyzed in order to better understand GSH/PSH competition and GSSP generation during DIA exposure. Moreover, the reaction mechanism of DIA with thiols, originally described by Kosower and Kosower (7), is proposed in more detail. DIA-pretreated rat RBC are characterized by massive GSSP formation, high GSH depletion and unchanged GSSG, so GSSP dethiolation can not occur by GRX because the cells lack GRX substrate (GSH). On the contrary, *in vivo* rapid RBC dethiolation is observed by plasma CSH generated by SH/SS exchange reactions between GSH, exported from the liver into plasma and CSSC:

$\text{GSH} + \text{CSSC} \Rightarrow \text{GSSC} + \text{CSH}$ [2]

$\text{GSSC} + \text{GSH} \Rightarrow \text{GSSG} + \text{CSH}$ [3]

CSH, uptaken by RBC, is able to dethiolate GSSP by the following reactions:

$\text{CSH} + \text{GSSP} \Rightarrow \text{GSSC} + \text{PSH}$ [4]

$\text{GSSC} + \text{CSH} \Rightarrow \text{CSSC} + \text{GSH}$ [5]

$\text{CSSC} + \text{PSH} \Rightarrow \text{CSSP} + \text{CSH}$ [6]

Therefore CSH is not only a nucleophilic group that blocks DIA, but also a factor that reduces GSSP, forming CSSP by multiple SH/SS exchange reactions. This GSSP dethiolation with formation of CSSP is called dethiolation by protein substitution, already described by Summa et al. (8). S-thiolation by protein substitution is not only a way to salvage GSH but probably prolongs a persistent state of S-thiolation. This possibility may be of physiological relevance for the modulation of essential PSH that are S-thiolated during oxidative stress.

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Selective adenosine A_{2A} receptor antagonism reduces JNK MAPK activation in oligodendrocytes after cerebral ischemia**Corti Francesca**

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Adenosine is a potent biological mediator which increases dramatically in concentration following brain ischemia (1). During ischemia adenosine is in a concentration range (μM) that stimulates all four adenosine receptor subtypes (A₁, A_{2A}, A_{2B}, A₃). In recent years, evidence indicates that the A_{2A} receptor subtype is of critical importance in stroke (2). Genetic deletion of A_{2A} receptors reduces ischemic and functional damage in a mouse model where focal ischemia is induced by medial cerebral artery occlusion (MCAo) (3). Evidence suggests a noxious role of adenosine A_{2A} receptors in brain ischemia. The mechanisms by which A_{2A} receptors are noxious during ischemia still remain elusive. It was previously shown that 24 h after MCAo, A_{2A} receptors up-regulate on neurons and microglia of ischemic striatum and cortex (4) and that sub-chronically administered adenosine A_{2A} receptor antagonists protect against brain damage and neurological deficit and reduce activation of p38 mitogen-activated protein kinase (MAPK) in microglial cells (5). Activation of p38 and JNK MAPKs is well documented in focal cerebral ischemia models (5,6). Modification of phospho-ERK1/2 MAPK has been reported at different times after focal cerebral ischemia models both in neurons and glial cells (6). MAPKs are essential in regulating cell growth, survival, differentiation, and death (7). The cell type in which MAPKs are activated is relevant to understanding of death or survival mechanisms in ischemia. The objective of this study was to investigate whether the adenosine A_{2A} antagonist 7-(2-fenilettil)-5-amino-2-(2-furil)-pirazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) affects JNK and MEK1/ERK MAPK activation. A further aim was to investigate cell types expressing activated JNK and MEK1/ERK MAPK after ischemia. We hereby report that the selective adenosine A_{2A} receptor antagonist, SCH58261, subchronically administered (0.01 mg/kg i.p) 5 min, 6 h and 20 h after MCAo in male Wistar rats reduces JNK MAPK activation (immunoblot analysis: phospho-JNK54 isoform by 81% and phospho-JNK46 isoform by 60%) in the ischemic striatum, 24 h after MCAo. Twenty-four h after MCAo, the Olig2 transcription factor of oligodendroglial progenitor cells and mature oligodendrocytes is highly expressed in cell bodies in the ischemic striatum. Immunofluorescence staining shows that JNK MAPK is maximally expressed in Olig2-stained oligodendrocytes and in a few NeuN stained neurons. The adenosine A_{2A} receptor antagonist reduces activation of JNK MAPK mostly in oligodendrocytes. The A_{2A} antagonist reduced (immunoblot analysis: by 55%) striatal Olig 2 transcription factor and prevented myelin disorganization, assessed by myelin associated glycoprotein (MAG) staining. Twenty-four h after MCAo, ERK1/2 MAPK was highly activated in the ischemic striatum mostly in microglia while it was reduced in the ischemic cortex. The A_{2A} antagonist did not affect activation of the ERK1/2 pathway. The efficacy of A_{2A} receptor antagonism in reducing activation of JNK MAPK in oligodendrocytes suggests a mechanism of protection consisting of scarring oligodendrocyte inhibitory molecules that can hinder myelin reconstitution and neuron functionality.

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Application of cybrid methodology for basic mitochondria and human disease studies**De Nardo Vera**

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The past two decades have witnessed an evolving understanding of the mitochondrial genome's (mtDNA) role in basic biology and disease. From the recognition that mutations in mtDNA can be responsible for human disease to recent efforts showing that mtDNA mutations accumulate over time and may be responsible for some phenotypes of aging, the field of mitochondrial genetics has greatly benefited from the creation of cell and animal models of mtDNA mutation. Cytoplasmic hybrid (cybrid) cells are one of the mainstays of mitochondrial research. They are created when cytoplasmic contents of two different cells are rendered coexistent within a single plasma membrane boundary. Specifically, the approach is designed so that mtDNA residing in cytosolic mitochondria of one cell type becomes perpetually incorporated within the cytoplasm of the other cell type. The cybrid methodology is also applied to studies of mtDNA mutation in Leber's hereditary optic neuropathy (LHON). LHON is a maternally inherited mitochondrial disorder, leading to a selective loss of retinal ganglion cells (RGC) and degeneration of the optic nerve, which results in severe visual impairment or even blindness. The primary causes are point mutations (G11778A) of the mitochondrial DNA (mtDNA), associated with aminoacid exchanges in complex I of the electron transport chain (ETC), which are thought to disturb oxidative ATP generation in the mitochondria. The aim of this study is to characterize the amounts of glutathione (GSH) and oxidized glutathione (GSSG) and the oxygen consumption in control and LHON cybrids.

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Pharmacological countermeasures on disuse-induced skeletal muscle function impairment**Digennaro Claudio**

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Rodent hind limb unloading (HU) is a widely accepted model for muscle disuse (1). In this model, the postural slow-twitch *soleus* (Sol) muscle goes towards a progressive and severe atrophy measurable as soon as after 3 days of HU, while a partial slow-to-fast phenotype transition, characterized by an increased expression of fast myosin heavy chain (MHC), initiates on the fourth day of HU (2). After 15 days of HU, the proportion of fast MHC-positive fibres may reach about 40% of total Sol muscle fibres compared to <15% in control animals (3). These effects are slowly reversed after several weeks of reloading (3). The resting ClC-1 chloride conductance (gCl) of sarcolemma, which controls muscle excitability, and resting cytosolic calcium (restCa) are sensitive indices of HU-induced impairment, although the alteration of these parameters does not affect each muscle phenotype. Indeed restCa, measured by a cytofluorimetric technique using FURA-2, decreased and gCl, measured by 2-intracellular-microelectrode current-clamp technique, increased in Sol after 3-14 days HU towards typical values of fast-twitch muscle, before initiation of MHC transition. Also gCl increased as a result of the reduction of protein kinase C (PKC) activity (4). We also found a change of the mechanical threshold (MT), an integrative index of excitation-contraction coupling that allows the calculation of minimum voltage able to elicit contraction upon long-duration stimulation, towards that observed in the fast phenotype. To prevent all these alterations, we tested drugs able to affect those parameters modified by HU. The amino acid taurine is a sulfonic amino acid ubiquitously and abundantly distributed in skeletal muscle tissue that controls many physiological processes such as osmoregulation, calcium mobilization, and reactive oxygen species (ROS) production. Taurine is essential for normal development and proper function of the excitable tissues of mammals (5). As far as skeletal muscle is concerned, taurine plays a fundamental role in the electrical stabilization of cell membrane. A taurine-enriched diet significantly prevented changes in gCl, restCa, and MHC expression, but not muscle atrophy. Because of the strong correlation between atrophy and oxidative stress (6), we performed a treatment of HU-mice with the potent antioxidant trolox, a water-soluble derivative of vitamin E that penetrates biomembranes and protects mammalian cells from oxidative damage. In fact, trolox treatment fully prevented fluorimetric measured lipoperoxidation, and gCl increase, partially counteracted MHC isoform transition, but again had no effect on atrophy. Furthermore, the growth hormone secretagogue ghrelin, which has been found to improve lean body mass in catabolic states (7) and to reduce gCl through PKC activation (8), has been tested. Preliminary experiments suggest that ghrelin improved gCl and fiber diameter in Sol muscle of 3-14-days HU mice, arguing for possible beneficial effects of the hormone/growth hormone secretagogues in prevention of HU-induced muscle impairment.

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Obestatin effects on feeding and body weight in the rat**Di Michele Pierpaolo**

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Obestatin is a recently identified 23 aminoacid peptide that is derived from proteolytic cleavage of the same polypeptide precursor, preproghrelin, which originates ghrelin. Opposite to ghrelin, which plays a well established role in food intake stimulation, obestatin has shown anorectic effects in a number of experimental paradigms, after both peripheral and central administration (1,2). However, several researchers were unable to reproduce these findings (3,4), casting a shadow on the role played by obestatin in feeding and body weight regulation. Since all previous experiments have been performed in animals fed a standard laboratory diet, in order to further elucidate the role of obestatin in food preferences, we aimed to study obestatin effects in rats fed both a standard laboratory chow diet and a highly palatable cafeteria-style diet, which is well known to increase daily caloric intake and weight gain. Male adult Wistar rats (200-250 g) were housed in plexiglas cages (40 cm×25 cm×15 cm), one rat per cage, in climatized colony rooms (22±1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00-19:00), with free access to tap water and food. Housing conditions and experimentation procedures were strictly in accordance with the European Community ethical regulations on the care of animals for scientific research. After 1 week acclimatization, rats (n=80) were randomized to standard (STD) diet (n=40) or cafeteria (CAF) diet (n=40) for 12 days. STD diet rats were fed laboratory chow in pellets (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). CAF diet rats were given, in addition to the standard chow as above, cafeteria-style food (into separate troughs), which included chips of parmigiano-reggiano cheese, potato chips, roasted hazelnuts, cookies, curls of salt butter, and bits of torrone chocolate. The average composition of this diet was: 30% fat, 56% carbohydrate, and 14% protein, 4.20 kcal/g. Both STD and CAF diet rats had free access to food and water 24 h/day throughout the study, with no fasting periods. The millboard was changed every 12 h. Both STD and CAF diet fed rats were divided into 4 groups of 10 animals each and injected daily for 12 days intraperitoneally, at 9.00 am, during the light phase, as follows: i) vehicle (0.2 ml saline); ii) obestatin, 10 nmol/kg; iii) obestatin, 50 nmol/kg; iv) obestatin, 100 nmol/kg. Twenty-four h after each daily treatment food and water consumption and body weight were recorded. Statistical analysis was performed using GraphPad Prism version 5.00. Results are expressed as means±s.e.mean and were analyzed by one-way ANOVA, followed by Student-Newman-Keuls multiple comparison test. P<0.05 was considered statistically significant. After 1 week acclimatization, the weight of rats was not statistically different between the 8 groups of rats. In STD diet rats, which were fed laboratory chow in pellets, we found that daily intraperitoneal injections of obestatin for 12 days decreased daily caloric intake and body weight gain compared to vehicle-treated rats. The anorectic and weight reducing effects of obestatin treatment were evidenced since day 6 and day 8 of treatment, respectively, and were consistent through the end of treatment. On the other hand, in CAF diet rats, where animals were given the choice between standard and cafeteria food, obestatin treatment did not modify either daily caloric intake or body weight gain compared to vehicle-treated rats. However, in CAF diet rats, even if daily caloric intake and body weight gain was not affected by obestatin treatment compared to vehicle, the percentage intake from standard food was decreased, balanced by an increase in cafeteria food intake, but only in the groups of rats treated with the higher doses of obestatin (50-100 nmol/kg). Obestatin treatment neither affected water consumption nor the intake of any specific food within the cafeteria diet.

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“Lifestyles: state of psycho-physical health of women”. An educational process to transfer research in the daily practice of pharmacist**D’Incau Paola**

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The project entitled “Lifestyles: state of psycho-physical health of women”, an epidemiological research, was developed following a request of the Equal Opportunities Commission of Veneto (an Italian Region) to carry out a research to investigate women's discomfort. The project was drawn as a continuing medical education (CME) activity in the “field training” involving Veneto's community pharmacists (1). The objectives of the “Lifestyles” project were two-fold: i) epidemiological: to describe women's psycho-social-physical health problems, to assess whether there is a correlation between these health problems and the psychotropic medication used and whether this relationship is predictive of women's mental health disorders; ii) learning: to promote knowledge, skills, attitudes and abilities of community pharmacists regarding the "malaise" of women, so as to maintain their proficiency, to provide a better service and to respond to patients' needs (2). One of the key issues of this project was the so-called “field training”, which gave importance to the role of pharmacists' duties and to scientific and research activities in terms of education and professional development. The union between epidemiological research and a continuing medical education activity is a process to transfer know-how in the daily practice of pharmacists (3). This abstract reflects upon the learning method adopted to improve pharmacists' knowledge, skills, and competencies in following patients in general and women with mood disorders in particular. The educational activities were carried out during 2008 in three phases: initial meeting, epidemiological observational study, and reflection. Three outcomes, pharmacists' ability, learning needs, characteristics and assessment on training model effectiveness were gathered from a series of interviews. A high participation rate (n=249, 88%) was achieved. A large number of women were recruited (n=11358) during the observational study. Participating pharmacists endorsed a positive attitude toward epidemiological research and learning program activities. They perceived symptoms and life events related to depression and anxiety disorders, but felt uncomfortable discussing about them. Pharmacists often reported two types interaction barriers: lack of privacy and lack of a relaxing atmosphere. Other three barriers emerged: lack of undergraduate training in mental health, lack of awareness of the meaning of counseling, lack of communication between pharmacist and general practitioner or other health practitioners. Community pharmacists improved their knowledge and sensitivity to women mood disorders. They “dispensed” advice and information and also showed an interest in listening to women's expressed needs. Pharmacists potentially added value to patient care improvement of clinical outcomes and enhancement of patient compliance. It is worth developing strategies that improve understanding of modern communication models. A future pharmacist practice model for patient counseling and treatment monitoring should enhance the partnership between pharmacists and patients.

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Anti-inflammatory effects of NCX-4040, a nitric oxide-donating aspirin, by inhibiting I κ B- α degradation**Dovizio Melania**

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NCX-4016 and NCX-4040 are NO donor-aspirin which contain NO-donating moiety in *meta* and *para* position, respectively. The two isomers differ in their metabolism *in vivo* that likely correlate with their differential effects on cancer cell growth (1). The aim of this study was to compare the antiinflammatory properties of NCX-4040 vs NCX-4016 and aspirin (ASA) *in vitro*. Thus, I assessed their impact on cyclooxygenase (COX)-2 activity [by measuring prostaglandin (PG)E₂ levels] and protein expression and cytokine generation [interleukin (IL)-1 β , IL-18, TNF- α , and IL-10] in human whole blood (HWB) and isolated human monocytes stimulated with bacterial endotoxin (LPS). The effect on COX-1 activity was assessed by measuring serum thromboxane (TX)B₂ levels in HWB. Then, I addressed whether NCX-4040, NCX-4016 and ASA affected NF- κ B activation which plays a central role in the inflammatory responses to LPS. It occurs via phosphorylation of the inhibitory I κ B α protein followed by proteasome-mediated degradation. Thus, in human monocytes and monocytic cell line (THP1) stimulated with LPS, I studied the effects of the 2 NO donor-ASA, ASA, and the proteasome inhibitor MG-132 on COX-2, I κ B α and phospho-I κ B α levels. In HWB, ASA was a potent inhibitor of COX-1 activity [IC₅₀ (95% Confidence Intervals, CI): 7.9 (4.4-14) μ M] without significantly affecting COX-2 activity up to 5 mM. NCX-4016 was >10-fold less potent than ASA in inhibiting COX-1 activity [IC₅₀:102 (65-159) μ M] and similarly to ASA did not affect inducible PGE₂ generation. The presence of a NO donating moiety in *para* position in NCX-4040 caused a reversion of COX selectivity. In fact, it inhibited LPS-induced PGE₂ generation with an IC₅₀ value of 0.41 (0.23-0.73) μ M associated with only 67% inhibition of COX-1, even at 5 mM. NCX-4040, but not ASA and NCX-4016, inhibited the release of IL-10, IL-1 β , and TNF- α with IC₅₀ values of 0.16 (0.08-0.32), 1.8 (0.8-4.2), and 1.2 (0.7-1.9) μ M, respectively. These inhibitory effects were not reverted by the guanylate cyclase inhibitor ODQ. MG-132 affected PGE₂ and cytokine generation. Comparable results were obtained in human monocytes stimulated with LPS. NCX-4040 and MG-132 down-regulated COX-2 expression. Interestingly, inhibition of COX-2 expression by NCX-4040 ranged in parallel to the inhibition of PGE₂ generation (maximal inhibition at 100 μ M). In THP1 cells stimulated with LPS for 1 h, similarly to MG-132, NCX-4040 caused a concentration-dependent accumulation of I κ B α and its phosphorylated form at concentrations which down-regulated COX-2 expression, PGE₂, and cytokine generation. ODQ did not reverse NCX-4040-dependent I κ B α accumulation. In contrast, ASA and NCX-4016 up to 100 μ M did not cause the accumulation of I κ B α . In summary, NCX-4040 affected COX-2 and cytokine production in LPS-stimulated HWB and isolated monocytes. This effect was due to inhibition of NF- κ B activation through stabilization of I κ B α . This mechanism did not involve NO-dependent cGMP generation, but presumably it was due to an inhibitory effect on proteasome function by NCX-4040 metabolite(s). In conclusion, NCX-4040 is a novel compound with improved antiinflammatory properties vs the parent drug due to an inhibitory effect on NF- κ B activation. The compound is of interest to assess the role of inflammation in different pathological conditions.

Supported by grants from NICOX and EICOSANOX (LSMH-CT-2004-005033 to Paola Patrignani).

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Spinal cord injury: new potential therapeutic targets**Esposito Emanuela**

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Spinal cord injury (SCI) is a highly debilitating pathology. Although innovative medical care has improved patient outcome, advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration have been limited. The complex pathophysiology of SCI may explain the difficulty in finding a suitable therapy. The primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered and regenerated. The events that characterize this successive phase to mechanical injury are called "secondary damage". Secondary damage is determined by a large number of cellular, molecular, and biochemical cascades. The presence of a local inflammatory response amplifies secondary damage. Resident microglia and macrophages originating from blood are two key cell types related to the occurrence of neuronal degeneration in CNS after traumatic injury. In particular, these cells can release various neurotrophic peptides such as brain-derived neurotrophic factor. Concomitantly, different proinflammatory mediators, such as proinflammatory cytokines (1) and reactive oxygen species (2), are also produced. In addition, NO is closely involved in the development of pathological processes in post-traumatic spinal cord (3). Several studies have implicated peroxynitrite, generated when NO and superoxide combine, in the secondary neuronal damage of SCI (4). Much of the damage that occurs in the spinal cord after traumatic injury is due to the secondary effects of glutamate excitotoxicity, Ca^{2+} overload, and oxidative stress that take part in a spiralling interactive cascade ending in neuronal dysfunction and death. It is believed that inflammatory and immune responses are the major component of secondary injury and play a central role in regulating the pathogenesis of acute and chronic SCI (5). Primary injury to the adult spinal cord is irreversible, whereas secondary degeneration is delayed and therefore amenable to intervention. Accordingly, several studies have shown that therapies targeting various factors involved in the secondary degeneration cascade lead to tissue sparing and improved behavioural outcomes in spinal cord-injured animals (6). One of the reasons why most of the treatments utilized in preclinical studies have had limited success in clinical trials is because of the complexity of the secondary degenerative response. In fact, many treatments affect only one aspect of this response, and a successful treatment will probably have to target several of these mechanisms. A number of experimental animal models have been developed to simulate the pathophysiology of acute clinical spinal cord compression in humans. The most commonly used model is the compression model induced by applying an aneurysm clip to the spinal cord. This experimental procedure is closer to the human situation, since it replicates the persistence of cord compression. Modern neuroscience techniques have led investigators to a better understanding of the importance of both primary (passive) and secondary (active) events in causing progressive loss of neural tissue. There are three stages of SCI where pharmacotherapy may have a therapeutic role. In the acute stage, treatments aimed towards diminishing the immune or inflammatory response, excitotoxicity, and lipid peroxidation may limit secondary mechanisms of injury. In the subacute stage, initiation of neurotrophic therapies may help to reconstitute the damaged tissue. Interventions in the chronic stage of SCI will more than likely involve neurotrophic substances in combination with tissue or mesenchymal stem cell transplantation. Nevertheless, only limited therapeutic measures are currently available for the treatment of SCI and only an extensive knowledge of the molecular and cellular mechanisms involved in the etiopathogenesis of SCI will lead to a crucial therapeutic intervention.

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Sigma-1 (σ_1) receptor modulation of the dopamine transporter activity in a cell model**Fazio Nicola**

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σ receptors are well established as a non-opioid, non-phencyclidine, and haloperidol-sensitive receptor family with its own binding profile and a characteristic distribution in the central nervous system (CNS) as well as in endocrine, immune, and some peripheral tissues. Two σ receptors subtypes, termed σ_1 and σ_2 , have been pharmacologically characterized, but, to date, only the σ_1 has also been cloned. σ_1 antagonists have been investigated for the treatment of psychosis, neuropathic pain, depression, epilepsy, and psychostimulant abuse since they are able to block several cocaine and amphetamine mediated effects (1). These drugs are also able to directly bind the σ_1 receptors at μ M concentrations, achievable following their *in vivo* administration (1). In basal conditions, the σ_1 receptor is bound to a protein complex regulating the Ca^{2+} efflux from the endoplasmatic reticulum (2); following agonist's exposure, the σ_1 receptor would be able to detach from the complex, migrating in the cytoplasm and in the nucleus, and to modulate several cellular functions and ion channels activities. The σ_1 receptor mechanism of action seems to involve also the protein kinase C (PKC) family, in particular the PKC β subtype (3) which is known to strongly modulate the dopamine transporter (DAT) membrane recycling as well (4). Moreover, changes of intracellular ion concentrations are also known to affect or even revert DAT activity (5). Considering the common pathways, σ_1 could: i) modify the DAT recycling through the involvement of the PKC system; ii) modify the DAT basal activity via protein-protein interactions; iii) modulate the physiological DAT functions altering the intracellular ions concentrations. For these reasons, my studies have been focused in understanding whether and how the human σ_1 ($h\sigma_1$) receptor is able to directly modulate the human DAT (hDAT) activity in a cell model. First, HEK-293 cells were permanently transfected with the $h\sigma_1$ receptor. Subsequently, they were transfected with another plasmid for transiently expressing the hDAT. HEK-293 cells transiently transfected only with the hDAT were used as controls. In the first part of my study, the DAT activity was evaluated using the described [^3H]dopamine ([^3H]DA) uptake assay (5). Different controls were done in order to validate the assay efficiency. In the cells transfected with the hDAT, the σ_1 ligands (+)-pentazocine (PTZ) and 1,3-di-*o*-tolylguanidine (DTG) did not affect [^3H]DA uptake; haloperidol (HAL) and (-)-(3-hydroxyphenyl)-N-*n*-propylpiperidine (3-PPP) caused a 40% reduction compared to control; finally, carbetapentane (CBP) increased by 30% the [^3H]DA uptake. When assayed on the $h\sigma_1$ -hDAT co-transfected cells, these same compounds increased [^3H]DA uptake. HAL and 3-PPP restored the 100% uptake activity and PTZ showed an increase of approximately 40% of the uptake activity. DTG showed an uptake increase of about 25%. CBP-induced increase of [^3H]DA uptake did not change in the $h\sigma_1$ -hDAT transfected HEK-293 cells. Effects elicited by σ_1 ligands on [^3H]DA efflux will be also presented as well as data on the DAT membrane recycling. In these latter experiments, the distribution of an enhanced green fluorescent protein-hDAT (EGFP-hDAT) fusion protein inside the cell will be monitored using confocal microscopy techniques. Therefore, it will be possible to understand whether the observed $h\sigma_1$ -stimulated [^3H]DA uptake increase was due to an enhanced recycling towards the plasma membrane or a direct potentiation of the hDAT uptake activity. In conclusion, this is the first study aimed to demonstrate a putative cross talking between the $h\sigma_1$ receptors and the hDAT in a cell model. The potentiation of the hDAT mediated [^3H]DA uptake observed in the presence of agonists activating the σ_1 receptors would put a new light in understanding both the profile and the antipsychostimulant activity showed by several σ_1 ligands (1).

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Effects of chronic lithium treatment in ameliorating Alzheimer's disease-like pathology in the TgCRND8 mice**Fiorentini Anna**

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Senile plaques, made of fibrillary β -amyloid ($A\beta$) peptide deposits, and neurofibrillary tangles, made of hyperphosphorylated tau protein are the histopathological hallmarks of Alzheimer's disease (AD). Glycogen synthase kinase-3 β (GSK-3 β) is one of the most involved kinase in the pathogenesis of AD. An aberrant activation of this enzyme leads to tau hyperphosphorylation contributing to the progression of AD, thus the inhibition of GSK-3 β represents a promising drug target for therapeutic intervention in the disease. Lithium, a drug for bipolar disorder, is an unselective inhibitor of GSK-3 β . In this study we evaluated the effect of a chronic treatment with lithium on $A\beta$ burden, tau hyperphosphorylation and on adult neurogenesis in a mouse model of AD: the TgCRND8 mouse. Two- (n=12) and 6- (n=8) month old tg mice, representing early pre-symptomatic and symptomatic stage of AD, respectively, and aged matched wt mice were daily i.p. injected with either 0.6 M LiCl (10 μ l/g of body weight) or sterile NaCl (10 μ l/g of body weight) for a 5 period weeks. For evaluations of neurogenesis, every mouse received twice daily for 3 days i.p. injections of 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg), then animals were killed 24 h after the last BrdU injection. Immunohistochemical and Western blotting analyses were performed. A significant increase in phospho-GSK-3 β (ser9) expression, the inactive form of the kinase, was found both in the cortex and hippocampus of 3- and 7-month-old lithium treated tg mice as compared to saline treated tg mice while total GSK-3 β levels were not changed, confirming the effective GSK-3 β inactivation by lithium in the brain of tg mice. The lithium effects were accompanied, in 3-month old tg mice, with a significant reduction of $A\beta$ deposition and of glia activation, both in the cortex and hippocampus, and, in 7-month old tg mice, with a significant reduction in PHF-1 levels. In the youngest tg mice, moreover, lithium significantly improved working memory ($P<0.001$) and spatial learning abilities ($P<0.01$) evaluated by "Step Down" inhibitory avoidance test and Morris Water Maze task, respectively. Double labeling experiments with antibodies raised against BrdU (a proliferation marker) and doublecortin (DCX, a marker for immature neurons) revealed a significant increase in new born neurons in the subgranular zone of the hippocampus of lithium treated tg mice as compared to control tg mice. In order to assess how lithium is able to stimulate neurogenesis, in 3-month old tg mice we evaluated the expression of β -catenin, a down stream target of GSK-3 β and the key effector of Wnt signaling pathway. We found an increased immunoreactivity for β -catenin both in the cortex and in the *dentate gyrus* of the hippocampus of lithium treated tg mice respect to saline treated tg mice, suggesting that lithium might enhance neurogenesis through Wnt signaling activation. Altogether these data support that lithium effectively inhibits GSK-3 β in TgCRND8 mice and that this drug or other more selective GSK-3 β inhibitors could represent an efficacious approach to treat AD. Lithium effects on $A\beta$ loading and neurogenesis in the oldest group of animals are still under evaluation.

Gold nanoparticles toxicity in rat precision-cut liver slices: a preliminary study**Franco Giulia**

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Nanoparticles are particulated nanostructures that have “nano” dimension (<100 nm). The increasing exposure of people to nanoparticles is a matter of concern. Recent data support that nanosized particles are able to enter the systemic circulation in function to their size (1). The ability of nanoparticles to enter the cells is an important property since it suggests that they could be used to drive drugs and/or DNA into the cells target. However, it can not be excluded that the particles interfere with cellular functions (2) and promote toxic effects (3). The aim of the present study was to evaluate the possible toxic effects of gold nanoparticles in rat precision-cut liver slices, a useful experimental model to study xenobiotics, toxicity, and metabolism (4). Slices (200-300 µm of thickness) were individually incubated for 2-24 h in RPMI 1640 under 95% O₂ - 5% CO₂ atmosphere at 37°C in 12 well plates in the presence of different concentrations (5-500 µM) of 5-10 nm gold nanoparticles, suspended in polyvinylpyrrolidone (PVP) to maintain their chemical and physical properties. Liver slices viability was evaluated by LDH release and MTT reduction. For histochemical analysis, slices incubated for 2 and 24 h were embedded in paraffin and sections at 8 µm were prepared and stained with haematoxylin and eosin. The LDH release and MTT reduction were not statistically different in slices incubated in the presence of gold nanoparticles at all concentrations compared to PVP (control) incubation. Furthermore, histochemical analysis showed that gold nanoparticles at the highest concentration (500 µM) were able to penetrate inside slices incubated for 24 h. Silver-enhanced method showed the presence of gold particles in capillary endothelium and in few cells (Kupffer cells?). On the contrary, when liver slices were incubated for 2 h, nanoparticles were stored only in peripheral zone where damaged cells are present. In conclusion, these preliminary experiments indicate that gold nanoparticles did not affect the rat precision-cut liver slices viability. However, other studies will be necessary to assess the interaction of nanogold particles on liver functions such as albumin synthesis, metabolic capability, and Kupffer cells activation.

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Role of GITRL/GITR system in the inflammatory response in acute pancreatitis**Galuppo Maria**

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The acinar cell damage associated to the acute pancreatitis results in local activation of the immune system, including dendritic cells, macrophages, fibroblasts, T cells, and endothelial cells among others (1). GITR (glucocorticoid-induced TNFR family related gene), a costimulatory molecule for T lymphocytes, belonging to the TNFR superfamily (TNFRSF), increases CD4+CD25-effector T cell activation, while inhibiting suppressor activity of CD4+CD25+ T regulatory (Treg) cells (2). T cell mediated immunoregulation is one of the main mechanisms that are responsible for maintaining antigen-specific tolerance *in vivo* and for controlling T cell homeostasis (3). We analyzed the role of GITRL-GITR interaction in the regulation of the inflammatory response associated with cerulein-induced acute pancreatitis using GITR^{-/-} and GITR^{+/+} mice. GITRL/GITR system participates in the development of autoimmune diseases and potentiates response to infection and tumours (4). Recent evidences suggest that physiological or pharmacological triggering of GITR exacerbates acute and chronic inflammatory response not only due to T cell modulation but also to modulation of extravasation and innate immunity (5). Results indicate significantly less acute pancreatitis in GITR^{-/-} mice than in GITR^{+/+} mice, with marked differences in oedema, neutrophil infiltration, pancreas dysfunction and injury. The NFκB activation as well as the production of TNF-α, IL-1β, inducible NOS (iNOS), nitrotyrosine, poly-ADP-ribose (PAR), ICAM-1 and P-selectin were also lower in cerulein-treated GITR^{-/-} mice. Finally, co-treatment of GITR^{+/+} mice with cerulein and Fc-GITR fusion protein (6.25 µg/mouse, by mini-osmotic pump), a dimer of a fusion protein formed by the extracellular domain of GITR and the Fc portion of human IgG1, decreased the inflammatory response and tissue injury as compared to cerulein-treatment alone, confirming that GITR plays a role in the modulation of acute inflammatory response in the pancreas.

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The anti-apoptotic and anti-oxidant effect of phytochemical on UVB-irradiated normal human keratinocytes and melanocytes**Giudice Stefania**

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UV radiation, in particular its UVB component, is an important environmental factor in the pathogenesis of skin aging and cancer. One of the features of UVB-caused DNA damage is the formation of cyclobutane pyrimidine dimers and (6-4) photoproducts. Furthermore, indirect DNA damage is also caused by increase in the level of reactive oxygen species (ROS) (1) that cause oxidative damage reacting with DNA, proteins, fatty acids, and saccharides (2). Such injuries result in a number of harmful effects: disturbed cell metabolism, morphological and ultrastructural changes, attack on the regulation pathways, and alterations in the differentiation, proliferation, and apoptosis of skin cells (3). These processes can lead to photoaging and skin cancer development. In order to avoid UVB radiation damage, phytochemicals and antioxidants as photoprotectives has been considered (4). In recent years naturally occurring herbal compounds such as phenolic acids, flavonoids, and high molecular weight polyphenols have gained remarkable attention as strong protective agents (5,6). In the present study we have investigated the anti-oxidant and anti-apoptotic effect of 46 phytochemicals in UVB-irradiated normal human keratinocytes and melanocytes. In the first part of our study we assessed the activity on UVB-irradiated normal human keratinocytes and melanocytes. These substances were submitted to a primary *in vitro* screening by MTT test in order to evaluate proliferation rate. Subsequently, 2',7'-dichlorodihydrofluorescein diacetate (DCF) assay was performed to determine formation of intracellular ROS. The results showed that glabridin, 18 β -glycyrrhetic acid, kaempferol, quercetin, rutin, and butein presented interesting properties. In the second part of our study we investigated the antiapoptotic effects of these 6 compounds in UVB-irradiated normal human keratinocytes and melanocytes by Western blot analysis. The results showed that pre-treatment of human keratinocytes and melanocytes with these phytochemicals inhibited UVB mediated apoptosis through the activation of p53, down-regulation of bcl-2, and bid full-length, up-regulation of Bax and inhibition of PARP cleavage. For these purpose we proposed that some substances could be combined to evaluate their synergistic effects. To further elucidate the molecular mechanism of phytochemicals mediated apoptosis, we have decided to investigate cell cycle analysis and the extrinsic and intrinsic apoptotic pathways. Human skin is constantly exposed to the UV radiation present in sunlight. This may induce a number of photobiological cellular changes. The development of novel preventive and therapeutic strategies depends on our understanding of the molecular mechanism of UV-damage. Phytochemical that were identified may be candidates for prevention of adverse effects of UV radiation and melanoma on the skin and evaluation of there clinical efficacy is awaited.

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Toxicity assessment of Zearalenone in “precision cut slices” of rat intestine**Gori Maila**

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Fungi *Fusarium* species infestation of cereal crops occurs worldwide. *Fusarium* toxins, such as zearalenone (ZEN), have been shown to cause diverse toxic effects in animals and are also suspected to promote disease in humans. ZEN promotes different effects on cells such as protein synthesis inhibition, lipid peroxidation induction, and sphingolipid metabolism inhibition (1). The aim of the present study was to investigate the toxic effect of ZEN in small intestine precision-cut slices. Intestines were obtained from male Wistar rats (250-350 g) sacrificed after anaesthesia. Intestine slices were prepared from ileum 10 cm-long segments, subsequently ligated on one side, filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C and allowed to gel in 4°C ice-cold Krebs-Henseleit buffer. The cylinders obtained were used to prepare precision-cut slices at 400 µm of thickness using a Krumdieck tissue slicer (2). Slices were first incubated in RPMI1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 well-plates with continuous gentle shaking for 30 min. Then the medium was replaced with fresh one containing different concentrations of ZEN (40-240 µM), 3% v/v EtOH (final concentration). In another series of experiments, slices were incubated in the presence of both red wine (1:10) (rosso di Montalcino, Villa Banfi) and ZEN 240 µM. Intestine slices viability was assessed by measuring LDH release and MTT reduction. In the presence of 240 µM ZEN, LDH release was significantly increased after 6 h incubation and a significant decrease of MTT was observed after 24 h. When slices were incubated in the presence of both ZEN and wine a significant increase of the viability was observed. MTT assay results showed that red wine protected against 240 µM ZEN-induced damage in small intestine slices with a statistically significant difference after 2, 4, and 24 h of incubation. Lipid peroxidation and antioxidant enzymes activity were also affected by the presence of ZEN in the incubation medium. These results indicate that ZEA caused a toxic effect on gut; wine, and more in general food, could, in part, counteract this action.

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Modifications in the DARPP-32 phosphorylation pattern after repeated palatable food consumption undergo rapid habituation in the *nucleus accumbens* shell of food non-deprived rats

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In food non-deprived rats a palatable meal induces a transient increase in dopamine output in the prefrontal cortex, *nucleus accumbens* shell and core (1). Rapid habituation to a second palatable meal selectively develops in the shell. The development of this phenomenon is not observed in fasted animals (2). Consumption of a palatable food also induces time-dependent modifications in the dopaminergic signalling, in particular in the dopamine and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32) phosphorylation pattern (3). The modifications in the DARPP-32 phosphorylation pattern are prevented when SCH 23390, a selective dopamine D₁ receptor antagonist, is administered after the meal (3). This study aimed to investigate whether habituation in terms of dopaminergic output in the nucleus accumbens shell had a counterpart in DARPP-32 phosphorylation changes. In food non-deprived rats, two consecutive palatable meals were followed by similar sequences of modifications in DARPP-32 phosphorylation levels in the prefrontal cortex and *nucleus accumbens* core, while changes after the second meal were blunted in the *nucleus accumbens* shell. Moreover, in the shell of rats food-deprived for 18-20 h, consecutive consumptions of palatable food induced similar phosphorylation changes. Thus, in the *nucleus accumbens* shell, DARPP-32 phosphorylation changes matched the modifications in extraneuronal dopamine levels in response to repeated palatable meals. Finally, the administration of SCH 23390 to food non-deprived rats shortly after the first palatable meal did not affect the increase in extraneuronal dopamine levels in response to the first consumption of palatable food and prevented the development of habituation in response to the second meal. Moreover, SCH 23390 administration prevented the modifications in DARPP-32 phosphorylation pattern after the first meal, but after the second meal the modifications observed were similar to those observed in control rats after the first meal. Thus, a prevalent stimulation of dopamine D₁ receptor seems to underlie the development of rapid habituation.

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Protective effect of TAT-parkin on neuronal cells

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The major pathologic hallmarks of Parkinson's disease (PD) are the progressive and selective loss of dopamine neurons (DN) in the *substantia nigra pars compacta* with associated decreased levels of striatal dopamine (DA), and presence of cytoplasmic inclusions (Lewy bodies; LBs) in the surviving nigral DN, containing mainly aggregated α -synuclein (α -syn) and parkin (1). Motor symptoms become manifest when the striatal DN loss exceeds 70%. PD is always sporadic, except rare cases associated with genetic mutations responsible for early-onset PD, including: two missense mutations (α -synA53T and α -synA30P) in the α -syn gene and mutations in the ubiquitin C-terminal hydrolase L1 (UCH-L1) gene, causing autosomal dominant PD; mutations in the parkin gene, accounting for the majority of familial autosomal recessive and sporadic early-onset PD cases, typically lacking LBs. Research on the inherited forms of PD is pointing towards a common theme, that accumulation of intracellular normal or abnormal (mutated, misfolded, unassembled, or damaged) proteins and ubiquitin-proteasome system dysfunction can lead to the death of DN in PD (2). It is relevant that recent both *in vitro* (3) and *in vivo* (4) studies have attributed a protective role to parkin in DN survival, thus opening attractive prospects to explore parkin as a new target for innovative PD treatment strategies, based on endogenous neuroprotection. The present study intends to investigate the putative neuroprotective effect of parkin against α -synA30P-induced neurodegeneration by using an *in vitro* experimental models that consists of rat adrenal pheochromocytoma PC12 cells, either undifferentiated or induced to develop some of the phenotypic traits of dopaminergic neurons by exposure to neuronal growth factor (NGF). For purposes of comparison and model validation, the putative neuroprotective effect of parkin against toxicity induced by the dopaminergic toxin 6-OHDA will be also evaluated. In order to evaluate how TAT-parkin is able to prevent the toxicity by α -synuclein here we have cloned human parkin and human α -synA30P protein fused to a protein transduction domain derived from the human immunodeficiency TAT protein (TAT-parkin and TAT- α -synA30P) to ensure diffusion across cell membranes. Both proteins were purified to homogeneity using a Ni^{2+} -NTA column. We have found that high doses of TAT-parkin or TAT- α -synA30P are toxic to PC12 cells. To examine the oxidative stress, PC12 cells were treated with various doses of 6-OHDA (from 300 μM to 5 μM) and we observed a cell viability that decreases in a time- and concentration-dependent manner (40% at 0 h and 70% at 6 h). In this way we have estimated ED_{50} at 60 μM 6-OHDA. At this concentration, PC12 cells were preincubated with 100 μM TAT-parkin (no toxic concentration) for 24 h, showing a protective effect only at 0 h. So PC12 cells were preincubated with 100 μM TAT-parkin for 24 h, before treatment with 60 μM 6-OHDA, and then reincubated with 100 μM TAT-parkin: we have showed an increase of survival for all incubation times. Our result suggest that 6-OHDA significantly decreased PC12 cell viability in a concentration-dependent manner, but the presence of TAT-parkin protein is able to protect PC12 cells against cytotoxic treatment of 6-OHDA. In conclusion, we have obtained an expression plasmid for TAT-Parkin and have purified the recombinant protein. This TAT-protein could to be an important tool for study its role in oxidative stress response and neuronal maintenance.

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$^2\text{H}_3\text{-U}^{13}\text{C}$ disaturated phosphatidylcholine as tracer of surfactant to estimate its catabolism in preterm infants with respiratory distress syndrome**Isak Ilena**

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Pulmonary surfactant is a mixture of phospholipids, neutral lipid, and associated proteins. A specific phospholipid, disaturated phosphatidylcholine (DSPC), is predominantly responsible for the modulation of surface tension at the alveolar air-liquid interface. Surfactant deficiency could cause hyaline membrane disease, also called respiratory distress syndrome (RDS). The inability of preterm newborns to produce adequate quantities of surfactant due to pulmonary immaturity constitutes the primary aetiology of RDS that represents the most common respiratory disease of preterm infants. The insufficient surfactant production is one of the hallmarks of RDS, which is caused by reduced synthesis, increased catabolism, and surfactant inactivation. Systemic and pulmonary inflammation markers are involved in these pathways; in particular, phospholipase A₂ (sPLA₂) is responsible of the hydrolysis of the ester bonds at the sn-2 position of membrane phospholipids. In the lung this enzyme is produced by macrophages and granulocytes, secreted on the alveolar surface and activated by oxidative stress. Its main function is to catabolise surfactant phospholipids yielding to production of lysophospholipids and free fatty acid, which in turn induce further lung injury. In the early 1990s administration of exogenous surfactant was introduced as a treatment in preterm infants with RDS and it has greatly reduced morbidity and mortality (1). During the last decade our research group developed new methods based on stable isotope technology, suitable for the study of both endogenous and exogenous surfactant metabolism in humans (2-4). These techniques are safe and ethically acceptable: surfactant tracer labelled with stable, non radioactive isotope, was administrated and the tracer enrichment was measured from tracheal aspirates (TA). Our objective was to investigate the catabolism of $^2\text{H}_3\text{-U}^{13}\text{C}$ DSPC, a newly stable isotope tracer synthesized to discriminate different kinetic of the sn-1 and sn-2 palmitate moieties in preterm infant with RDS. During the last year our research group collected 23 DSPC studies in 18 preterm infants who required a single dose or multiple dose of 100 or 200 mg/kg of exogenous surfactant and prolonged mechanical ventilation for RDS. All infants received exogenous surfactant mixed with $^2\text{H}_3\text{-U}^{13}\text{C}$ DSPC to trace surfactant DSPC (2 mg/120 mg of surfactant). Sequential TA were collected and DSPC was extracted, isolated by thin layer chromatography, and its isotopic enrichment was measured by gas chromatography mass-spectrometry (GC/MS). $^2\text{H}_3$ -palmitate and U^{13}C -palmitate half-life (HL) were measured for each different moiety of palmitate labelled in the sn-1 ($^2\text{H}_3$) and sn-2 (U^{13}C) position of the tracer DSPC. $^2\text{H}_3$ -palmitate DSPC HL was 38 ± 44 h and U^{13}C -palmitate DSPC HL was 34 ± 39 h ($P=0.73$). To deeply investigate the different kinetic rate between the sn-1 and sn-2 position of the DSPC, HL ratios $\text{U}^{13}\text{C}/^2\text{H}_3$ were calculated for all infants. Preliminary comparisons indicate that there are two different groups: in 13 preterm infants the ratio was >1 , in 10 infants was <1 . In the second group the mean HL U^{13}C -palmitate was reduced due to an increased catabolism of the sn-2 position. Further studies are in progress to assess whether the accelerated kinetic of the sn-2 position is due to an increased inflammatory damage or an alteration of pulmonary surfactant caused by oxidative stress.

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Neuropeptide S-induced increase of cocaine-seeking behaviour is mediated by activation of the hypothalamic hypocretin system**Kallupi Marsida**

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The association of cocaine's reinforcing effects with specific environmental stimuli is thought to be a critical factor for relapse risk in addiction (1). This study examined, in rats, the effects of a newly deorphanized neuropeptide receptor and its cognate ligand, neuropeptide S (NPS) (2,3), on cocaine self-administration and reinstatement of cocaine-seeking by environmental cues previously associated with drug availability. To assess the intrinsic motivational properties of NPS, we have also investigated whether the peptide induces conditioned place preference. In addition, we studied whether NPS administered i.c.v. or into discrete brain areas, including the lateral hypothalamus (LH), the perifornical area of the hypothalamus (PeF), and the central amygdale (CeA), alters cocaine craving and affect relapse to cocaine-seeking (4). Finally we investigated at molecular and pharmacological level whether these NPS effects depends on interactions with the hypothalamic hypocretin/orexin (Hcrt-1/Ox-A) system. Results showed that central NPS injection potently increases cocaine craving elicited by stimuli previously associated with cocaine availability. This effect is specific for drugs of abuse because it does not generalize to natural rewards (i.e. food). In addition, we also demonstrated that despite NPS increases cocaine-related appetitive behaviors it is devoid of motivational properties *per se*. Brain microinjection studies showed that the LH, in part the PeF, but not the CeA, are the brain sites involved in the effect of NPS on craving and relapse. We also found that NPS receptors co-localize with hypocretin-A/Orexin-1 (Hcrt-1/Ox-A) immunoreactive neurons in the LH. Consistent with this observation, pharmacological studies demonstrated that block of the Hcrt-1/Ox-A receptor-by the selective antagonist SB334867 completely prevents NPS-induced facilitation o cocaine relapse (5). Overall, these data provide compelling support for the role of the NPS system in the regulation of cocaine craving and indicate that activation of the NPSR facilitates Hcrt-1/Ox-A system activity in the LH that, in turn, appears responsible for the marked augmentation of conditioned cocaine reinstatement observed here.

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AMP and adenosine-related inflammatory pathways in vascular smooth muscle cells from control and streptozotocin-diabetic rats**Malorgio Francesca**

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Adenosine formation is regulated by the enzymatic step catalyzed by ecto-5'-nucleotidase (CD73), which hydrolyzes AMP into adenosine (1). Both cellular uptake and metabolic degradation to inosine contribute to extracellular adenosine clearance. In the cardiovascular system, extracellular adenosine displays a number of protective actions including stimulation of NO release (2,3). Vascular inflammation is a central feature of diabetes and its complications. In this study, we investigated the pathways of extracellular adenosine production and elimination by vascular smooth muscle cells (VSMCs) from diabetic as compared with normoglycaemic rats. We also evaluated the influence of adenosine and related compounds on nitric oxide synthase (iNOS) expression in response to inflammatory mediators. Diabetes was induced in Sprague-Dawley rats by i.v. injection of streptozotocin (STZ) 4 weeks before sacrifice. Aortic VSMCs from control and diabetic rats were incubated for 24 h in the presence of 1 µg/ml LPS combined with a cytokine mixture comprising 10 ng/ml IL-1β, 25 ng/ml TNF-α, and 10 ng/ml INF-γ to mimic the *in vivo* environment of some vascular inflammatory events. VSMCs were exposed to exogenous adenosine or AMP (both 1 mM) in the presence or absence of the adenosine transport blocker S-(4-nitrobenzyl)-6-thioinosine (NBTI; 10 µM) or the CD73 inhibitor α,β methylene-ADP (AOPCP; 200 µM). Inhibitors were added to the medium 30 min before purines. An HPLC method (4) was used to quantify AMP, adenosine, and their metabolites in the culture medium. The levels of iNOS protein were assessed by Western blotting. After incubation (24 h) of VSMCs from normoglycaemic rats with exogenous AMP (1 mM) in the presence of LPS plus cytokines, the nucleotide was undetectable in the culture medium, being converted mainly to inosine (253±33 µM) and hypoxanthine (566±31 µM). Similar findings were obtained in the medium of diabetic VSMCs. Treatment with the CD73 inhibitor AOPCP in control VSMCs allowed the recovery of 400±14 µM AMP, while reducing inosine to 70±13 µM (-72%) and hypoxanthine to 229±9 µM (-60%). By contrast, residual AMP concentration in diabetic VSMCs' medium after AOPCP treatment was 59±12 µM. At the end of 24-h incubation with 1 mM adenosine, the exogenous nucleoside disappeared from the medium of control and diabetic VSMCs. The sum of adenosine metabolites (inosine and hypoxanthine) was comparable to the initial amount of the added nucleoside. Treatment with the adenosine transport blocker NBTI allowed 60% and 39% recovery of added adenosine in control and diabetic VSMCs, respectively (P<0.05). Cytokine stimulation for 24 h induced iNOS expression, which was undetectable in unstimulated VSMCs. Exogenous adenosine (1 mM) did not change iNOS levels in control VSMCs, but potentiated the response to cytokines in diabetic VSMCs. This effect was prevented by the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 1 µM) and further enhanced by NBTI. Treatment with the adenosine precursor AMP in the presence or absence of AOPCP increased iNOS levels in diabetic but not in control VSMCs. Diabetic VSMCs showed qualitative and quantitative alterations in exogenous AMP/adenosine metabolism and the pharmacological modulation thereof as compared with control VSMCs. These modifications were linked to different cytokine-induced iNOS formation in the two groups, suggesting that alterations in adenosine-related inflammatory pathways are involved in diabetic vascular dysfunction.

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Cocaine sensitization as a possible model of anhedonia in rats**Marchese Giovanna**

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Depression is a complex disorder and the availability of experimental models for its study would be of great relevance. However, neurobiological mechanisms underpinning depression are still unsolved and it is then difficult to model this disorder as a syndrome (1). Thus, numerous single symptom experimental models have been proposed, some of which attempt to reproduce anhedonia. Anhedonia, defined as inability to experience pleasure, is a core symptom of depression that also characterizes substance abuse-related mood disorders, in particular stimulant abuse. The aim of this study was to investigate the condition of cocaine sensitization in rats as a possible model of anhedonia. Food non-deprived cocaine-sensitized rats showed no interest in a palatable food, vanilla sugar (VS), emitted a scarce hedonic response to VS consumption, and failed to acquire instrumental appetitive behaviour, vanilla sugar-sustained appetitive behaviour (VAB) (2). Moreover, the dopaminergic response to VS administration, in terms of increases in extraneuronal dopamine levels in the nucleus accumbens shell and medial prefrontal cortex, was blunted in sensitized compared to control rats. Similar behavioural and neurochemical responses to palatable food exposure were observed in rats exposed to a chronic stress protocol (3,4). These results indicate that cocaine sensitization fulfils the face validity (reasonable analogy to the human symptom) and construct validity (reasonably similar causal mechanism) requirements for an experimental model (1). To further validate the condition of cocaine sensitization as an anhedonia model, cocaine-sensitized rats were treated with imipramine (5 mg/kg i.p. twice a day) during VAB training. Imipramine consistently reinstated the competence to acquire the appetitive behaviour in cocaine-sensitized rats. This finding adds to the model predictive validity that is the sensitivity of the modelled symptom to respond to classical antidepressant treatments.

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Role of cardiac progenitor cells in heart diseases**Marino Laura**

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For a long time the heart has been considered to be a post-mitotic organ, therefore the recent identification of distinct families of cardiac progenitor cells in adult heart begins to build a foundation for our better understanding of the development of human cardiovascular diseases. In several studies, stem cells with replicative and regenerative capacity were isolated from heart of adult animals, such as lineage negative, c-kit positive cell population (CPCs) (1), cardiac progenitor cells based on the expression of Sca1 (2) or ATP-binding cassette transporter (side population cells) (3). Similarly, further studies identified a heterogeneous population of cells called cardiospheres (4) and an endogenous cardiac c-kit positive cell population from human heart (5,6). On this basis, in the first part of my doctoral work, I tested the hypothesis that the cardiotoxic effect of doxorubicin (DOXO), the most powerful and widely used anthracycline (7), is primarily directed to the stem cell compartment partially ablating the reserve of functionally-competent CPCs. In the *in vitro* study, CPCs were treated with 0.1, 0.5, and 1 μ M of DOXO for 12, 24, and 48 h. CPCs viability was assessed by a colorimetric MTT assay, while apoptosis was measured by TdT assay, DNA laddering and caspase-3 activity. The impact of DOXO on CPCs growth was determined by BrdU and phospho-H3 labeling. Moreover, the molecular regulators of G1, G1/S transition (cyclin D1 and cdk4) and G2/M transition (cyclin B1 and phospho-cdc2) were measured. To determine whether the generation of reactive oxygen species (ROS) was responsible for DNA damage in CPCs, the presence of 8-OH-deoxyguanosine (8-OHdG) was measured. Moreover the expression and the activity of the antioxidant enzymes Mn SOD, Cu/Zn SOD, and catalase were assessed. Importantly, we evaluated the dysfunctional telomeres that trigger a cascade of events followed by activation of apoptosis or cellular senescence. In the current study, the expression of phosph-p53 at serine 15, ATM kinase, p21^{Cip1}, Bax, Bad, and p16^{INK4a} was assessed in CPCs. The inhibition of CPCs division in combination with the accumulation of oxidative DNA damage, growth arrest, cellular senescence, and apoptosis dramatically decreased the number of functionally-competent progenitors in the failing heart. Therefore, DOXO-induced cardiomyopathy can be viewed as a stem cell disease. In the last period of my doctorate, I compared human multipotent adult stem cells (MASCs) (8), obtained from end-stage heart failure (R-MASCs) and non-pathological donor atria (D-MASCs), focusing on biological and functional differences. Under permissive culture conditions, MASCs differentiated into endothelial cells and cardiomyocytes. Nevertheless, cell growth, migration capability, and differentiation potential were more prominent in D-MASCs than in R-MASCs. These data might explain the relation between MASCs senescence and the onset of heart diseases.

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Alterations of glutamate release in the spinal cord of mice affected by experimental autoimmune encephalomyelitis**Marte Antonella**

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Multiple sclerosis is a demyelinating, progressively degenerative disorder of the central nervous system, with an autoimmune based mechanism. It is now well established that alterations of glutamate neurotransmission are involved in several neurodegenerative diseases. The observation that antagonists of AMPA receptors are neuroprotective and useful in mice with experimental autoimmune encephalomyelitis (EAE) (1,2), a murine model of human multiple sclerosis (3), confirms the involvement of glutamate in the damage of spinal cord neurons, but its role in this pathology is still unclear. Therefore, we have investigated the release of this excitatory amino acid from EAE mice spinal cord slices at different time points after EAE induction with myelin oligodendrocyte glycoprotein (MOG) (4). After the incubation of the slices with [³H]D-aspartate (a non metabolizable marker of glutamatergic neurons), we have evaluated its basal and 35 mM KCl-evoked efflux in EAE (MOG+) and control (MOG-) mice. Our results show that at 13 days post immunization (p.i.), onset of the pathology, the KCl-evoked efflux in MOG+ slices was significantly lower than that measured in controls (30%; 0.905 ± 0.060 vs 1.309 ± 0.101 , respectively). The results are similar at 21 days p.i. (peak of the disease; 40% decrease; 0.911 ± 0.069 vs 1.530 ± 0.125 , respectively). In this case, also the basal release was diminished (20%; 0.856 ± 0.040 vs 1.077 ± 0.040). To understand whether this variation was due to changes in glutamate release from neurons or glial cells, we have measured the efflux of [³H]D-aspartate from purified spinal cord isolated nerve terminals (synaptosomes) or glial particles (gliosomes) in superfusion. Surprisingly, at 21 days p.i. we have observed a significant enhancement of the 15 mM KCl-evoked efflux of [³H]D-aspartate both in synaptosomes (34%; 1.047 ± 0.107 vs 1.400 ± 0.119) and gliosomes (20%; 1.518 ± 0.090 vs 1.826 ± 0.116) from EAE mice with respect to controls; the basal release instead was increased only in gliosomes (35%; 3.288 ± 0.141 vs 4.443 ± 0.290). On the contrary, at 13 days p.i. we did not find any significant difference. We have also found that the uptake of [³H]D-aspartate (1 and 10 μ M) in spinal cord synaptosomes of EAE mice was significantly increased with respect to controls (1 μ M, 5.378 ± 0.728 vs 3.566 ± 0.508 ; 10 μ M, 10.909 ± 1.596 vs 7.034 ± 0.687 nmol/2 min/mg prot) at 21 but not at 13 days p.i. These data indicate that, at the peak of the pathology, the amount of glutamate released at the synaptic level is increased, thus suggesting a possible excitotoxic role of this amino acid in the neurodegenerative processes characterizing multiple sclerosis. The increase of neuronal uptake of glutamate, probably responsible of the decrease of [³H]D-aspartate efflux observed in slices, may represent an adaptive response of glutamatergic nerve terminals to limit cell damage.

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Screening of antimetastatic metal-based drugs targeting cell adhesion molecules

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Distant metastases of solid tumours still remain a hurdle in cancer treatment and selective anti-metastatic agents are needed to face up with this aspect of cancer disease. Among metal compounds HIm[Ru(III)Cl₄Imdmsol] (NAMI-A), emerged for its ability to selectively combat the development of metastases in solid tumours. The first aim of this study was to investigate how variations on NAMI-A chemical structure could influence the anti-metastatic activity. For this purpose some representative complexes have been chosen: two heterocyclic compounds HIm[Ru(III)Cl₄(Im)₂] (KP418) and HInd[Ru(III)Cl₄(Ind)₂] (KP1019) presenting a different N-donor ligand, and three organometallic compounds, [(η⁶-biphenyl) Ru(II)Cl(ethylenediamine)]PF₆ (RM175), its osmium congener [(η⁶-biphenyl)Os(II)Cl(ethylenediamine)]BF₄ (AFAP51) and Ru(II)Cl₂(η⁶-C₇H₈)(PTA) (RAPTA-T) carrying a PTA ligand (1,3,5-triaza-7-phosphoadamantane) instead of ethylenediamine. Their interference with some steps of the metastatic progression was evaluated with appropriate *in vitro* tests (cell detachment, migration, invasion, adhesion to a new substrate) comparing the behaviour of MDA-MB-231 highly invasive breast cancer cells to that of HBL-100 non tumorigenic mammary epithelial cells. The *in vitro* effects were compared with the *in vivo* anti-metastatic activity studied in the MCa mammary carcinoma of the CBA mouse. Although some of the compounds are able to affect one or more of the metastatic steps mimicked *in vitro*, *in vivo* activity is shown by KP1019 and RM175: for both ruthenium compounds this effect can be ascribed to cytotoxicity at primary tumour level. On the contrary, a peculiar activity is shown by RAPTA-T which was able to act selectively on the highly invasive cell line, with only marginal effects on the non tumorigenic one and correspondingly to selectively affect metastasis development. This feature of RAPTA-T could be ascribed to the higher ruthenium content in the MDA-MB-231 cells, as detected from uptake experiments performed on cells after RAPTA-T treatment. Cell adhesion, migration, and invasion are directly related to actin assembly and disassembly and these phenomena are regulated by the RhoGTPases. To understand if the activity of RAPTA-T involves a modulation of Rho, the capability of cells (MDA-MB-231 and HBL-100) to resist to trypsin harvesting was evaluated by treating cells with RAPTA-T after a pre-treatment with a Rho inhibitor. Results show that the adhesion of cells grown on polylysine is influenced neither by the inhibitor nor by RAPTA-T. The inhibitor decreases the adhesion of cells on collagen IV, an effect that is completely reverted by 100 μM RAPTA-T. Differently from polylysine and collagen IV, which gave similar results with both cell lines, when cells are seeded on fibronectin the Rho inhibitor and the counteracting effect of RAPTA-T could be detected on the invasive MDA-MB-231 cells only. These evidences allow us to hypothesize a role of Rho modulation in the RAPTA-T mechanism of action. This is also partially confirmed by a study on the effects of RAPTA-T treatment on actin cytoskeleton and on focal contacts formation in MDA-MB 231 cells. Results show that on cells seeded on collagen IV RAPTA-T treatment leads to a reorganization of the actin cytoskeleton in stress fibers and to focal contacts formation, mainly at 100 μM concentration and these effects are even more evident on cells grown on fibronectin. On cells seeded on polylysine the effect is weaker and visible when cells are treated with 1 μM RAPTA-T. On these bases, the analysis of RhoA activation is in progress. RAPTA-T activity on cell cytoskeleton is more evident when cells are seeded on fibronectin or collagen IV, where cell adhesion is mediated by specific membrane molecules, while in the case of cells seeded on polylysine the effect is weaker or absent. These differences can not be explained with a different uptake of the compound by cells or by a preferential binding of the compound with the ECM substrates, as detected by atomic absorption spectroscopy experiments. A central role in cell-ECM interaction is covered by integrins: preliminary results showed that RAPTA-T is able to activate β1 integrin on MDA-MB-231 grown on fibronectin, suggesting this class of adhesion molecules is a likely target for RAPTA-T. This hypothesis is under study by a series of tests such as collagen gel contraction assay and binding of the compound with integrins in a cell-free system.

Evaluation of the neuroprotective effect of parkin against α -synuclein-induced neurotoxicity in a rat model of Parkinson's disease

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Parkinson's disease (PD) is one of the most common neurodegenerative disorders. PD is characterized by the progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), leading to a loss of dopamine in the target structure *striatum*, and development of motor symptoms such as bradykinesia, rigidity, and tremor (1). The aetiology of PD is still largely unknown. At least six different casual genes linked to rare familial forms of PD have been identified, while late-onset idiopathic PD likely resulting from the interplay between predisposing genes and environmental factors. In particular, two missense mutations (A30P and A53T) in the α -synuclein (α -syn) gene have received great attention with the discovery that abnormal metabolism and accumulation of α -syn in dopaminergic neurons lead to both sporadic and familial forms of PD (2,3). Parkin functions as an E3 ubiquitin ligase: loss of its activity seems to cause an autosomal recessive form of PD (4). We have recently described a hemi-parkinsonian rat model, based on the stereotaxic injection of TAT- α -syn-A30P in the SNpc of the right hemisphere (3). The TAT sequence allows diffusion of the fusion protein across the neuronal plasma membrane and results in a localized dopaminergic loss (3). The research project is designed to examine possible neuroprotective effects of TAT-parkin in this model. Rats were stereotaxically injected with TAT- α -syn-A30P, TAT-parkin, or both. At different times after injection, all animals will be subjected to behavioural testing to evaluate impairment in motor function. Then, dopaminergic cell loss extension will be evaluated with the apomorphine-induced rotation test and tyrosine hydroxylase immunohistochemistry. The α -syn-parkin-based model better reproduces the pathophysiology of PD and could be of utility to understand the mechanisms that lead to dopaminergic neurodegeneration. Moreover, it could help identify disease-modifying strategies as opposed to therapies which provide only symptomatic relief.

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A preclinical model of binge-eating in female rats: effect of sibutramine, fluoxetine, topiramate and midazolam**Micioni Maria Vittoria**

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Binge eating episodes are characterized by uncontrollable, distressing eating of a large amount of highly palatable food (HPF). These episodes represent a central feature of eating disorders, such as the binge-eating disorder and bulimia nervosa; they also occur in obese individuals, significantly contributing to their high caloric intake and overweight. Preclinical models are needed to investigate the neuro- and psycho-biology of binge eating, and to identify innovative pharmacotherapeutic strategies. According to the hypothesis that dieting and stress are key etiological determinants of binge eating, the model proposed by Boggiano (formerly Hagan) and co-workers (1) combines cycles of food restriction/refeeding and acute stress to evoke binge eating for sweet HPF. In this model, female rats are submitted to cyclic caloric restriction and stressed with electric foot-shock. An evolution of this model has been developed by our group to further increase its face validity and reliability and to evaluate its predictive value. In our study electric foot-shock was substituted with a stressful procedure characterized by exposure of female rats to HPF, but preventing them from access to it for 15 min, even though they were able to see it and to smell its odour. Therefore, stress was related to temporary lack of control over the environmental circumstances. This type of stress offers several advantages over the electric foot-shock stress procedure. Firstly, it is a mild stress that, unlike electric foot-shock, never induces fear and freezing, but at the same time it elicits a robust behavioural activation; secondly, the 15 min period of prevented access to HPF predisposes the rat to a loss of control over it, as soon as it becomes freely available; thirdly, the stressful experience is related to the relationship with a HPF, as usually occurs in humans, thus providing a further element of face validity. Four groups of female rats were considered: group 1 was normally fed and not stressed on the test day (25th); group 2 was maintained in similar conditions but was exposed to an acute stress on 25th day; groups 3 was exposed to three cycles (4 days 66% of the chow intake + 4 days food ad libitum) of yo-yo dieting but not stressed, and group 4 was exposed to cyclic yo-yo dieting and then stressed. All groups were familiarized to HPF for 2 h on day 5-6 and 13-14. The combination of cyclic food restriction and stressful exposure to food markedly and reliably increased HPF intake. Sibutramine and fluoxetine inhibited food intake independently to the experimental conditions. Topiramate selectively inhibited compulsive HPF intake in rats submitted to caloric restriction and stress. Midazolam increased HPF intake. In conclusion, the present study confirms that cyclic caloric-restriction and acute environmental stress in young female rats interact in a synergistic manner to produce a robust compulsive HPF intake. The stressful procedure adopted offers not only the advantage of generating a pronounced and highly reproducible binge eating for HPF, but increases also the face validity of the approach. Even though the stress is mild, nevertheless, it is sufficient to induce neuroendocrine changes typical of a stress response. Finally, the peculiar pharmacological profile of the drugs tested suggests that the model, in addition to construct and face validity as an isomorphic model of binge eating, is apparently endowed with good predictive validity. Thus, the present model may be useful to evaluate new pharmacological strategies for treatment of bingeing-related eating disorders.

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B7h triggering inhibits adhesion of umbilical vascular endothelial cells to colon carcinoma cell lines and polymorphonuclear cells**Minelli Rosalba**

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Colon cancer represents one of the most important causes of death in developed countries. At the moment the best therapeutic option for treating this tumour is surgical resection, but chemoprevention is very important in preventing metastasis and tumour spread. The process of metastatization is due to cell migration through blood vessels or lymphatic vessels. An important aspect of this process, consisting in the ability of cancer cells to adhere to the endothelium (this is fundamental for the passage through the monolayer), involves several adhesion molecules. These play a key role, as suggested by the observation that colon carcinomas cells, derived from patients, express increased level of the adhesion molecules ICAM-1 and VCAM-1 (1,2); moreover, several data have demonstrated that the ability of metastatization of colon cancer cell is directly linked to the ability of the cells to bind the CD62E (3). On the base of these observations we propose that drugs inhibiting the adhesive activity of cancer cells may be helpful tools in preventing metastasis formation. Our experiments have been performed on human umbilical vascular endothelial cells (HUVEC), providing a simplified model to mimic the tissue microvascular circulation involved in the interactions with tumours cells (1). Cell adhesion was quantified by computerized micro-imaging system. Some our recent experiments have shown that the fusion molecule human ICOS-mouse Ig, a stimulator of B7h receptor (membrane receptor belonging to the B7 family and expressed by activated T cell) is able to reduce the adhesion of human neutrophils to HUVEC. Endothelial cells and colon cancer cells express high level of B7h receptors (4,5). We demonstrated here the anti adhesive propriety of ICOS-Ig on different colon immortalized cell lines (HT29, DLD1, and HCT116). Treatment with ICOS-Ig (0.01-2 µg/ml) significantly inhibited adhesion of HT29 and, at a lesser extent, DLD1 in a concentration-dependent manner, whereas had no significant effect on HCT116. Maximal inhibition was 50±2% (IC₅₀=0.067 µg/ml) for HT29, and only 35±4% for DLD1, reached at 2 µg/ml of ICOS-Ig. The effect of ICOS-Ig in inhibiting cell adhesion is probably directed to both endothelial and tumour cells, because treatment of only HT29 or HUVEC with ICOS-Ig determines a relevant inhibition of cell adhesion. To investigate duration of the inhibitory effect, we performed the adhesion assay on HUVEC pre-treated for 30 min, 1, 2, and 24 h with ICOS-Ig using HT29 cells. Results showed that inhibition was less efficient at 60 than at 30 min, and became even ineffective at 120 min, but it increased again after 24 h, giving again a maximal inhibition of 50±3% with 2 µg/ml ICOS-Ig. Future experiments will be performed in order to clarify the mechanism of action by which ICOS-Ig causes these biological effects. Since ligands for CD62E are Sialyl Lewis x and a receptors (7), further experiments will be conducted in order to evaluate the expression of these adhesion molecules on our colon cancer cell lines. Thereafter we want to assess whether ICOS-Ig is able to modulate gene expression (evaluated by immunofluorescence and flow cytometry) and cytokine secretion (assessed in the culture supernatants using a cytokine protein array). Some data have reported an increased rate of phosphorylation of p38 MAP kinase after CD62E stimulation (3,6), so we shall verify whether ICOS-Ig modulates the MAP kinases pathway. Since the HUVEC are an artificial vascular endothelial model, the adhesion experiment and the protein analysis will be repeated on the commercially available microvascular endothelial model derived by tissues (for example lung and bowels).

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Effect of HIF-1 modulation on the response of two- and three-dimensional cultures of human colon cancer cells to 5-fluorouracil, oxaliplatin and doxorubicin**Molteni Roberta**

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Clinical and preclinical studies have firmly established that tumour hypoxia represents a major obstacle to the success of radiotherapy and chemotherapy, due in part to poor perfusion and restricted drug access to hypoxic areas (1). However, a major role is played by activation of a family of hypoxia-inducible transcription factors (HIFs), orchestrating a coordinated adaptive response (2,3). HIFs act as heterodimers, consisting of an oxygen-dependent α and a constitutively expressed β subunit. Regarding tumour response to drug treatment, HIF-1 upregulation has been shown to induce the expression of genes involved in the decrease of the effectiveness of a number of currently used anticancer agents. Based on these observations, in recent years considerable efforts have focused on HIF-1 modulation as a strategy to inhibit tumour growth and metastasis, as well as to sensitize tumours to chemotherapy. A number of small molecules and nucleotide-based agents inhibiting HIF-1, mostly by specifically targeting HIF-1 α synthesis and/or degradation, have been identified and developed (4,5). In the present study we have investigated the effects of HIF-1 modulation on the response of the human colon adenocarcinoma cell lines HCT116 and HT29 to 5-fluorouracil (5FU), oxaliplatin (OxPt), and doxorubicin (DOX). Preliminary results have shown that increasing HIF-1 activity, either by exposing cells to hypoxia (pO₂ 1%) or by growing cells as spheroids, which more closely reproduces the hypoxic conditions occurring in solid tumours, results in poor cell response (evaluated by cell count and percentage of apoptotic cells) to these chemotherapeutic agents. Different strategies have been used to modulate the expression of HIF-1 α and HIF1 activity: i) treatment with a thioredoxin-1 inhibitor, PMX290, known to decrease HIF-1 activity, without affecting HIF-1 α levels, in a number of different cancer cell lines (6); ii) transfection with EZN2968, a locked nucleic acid (LNA) targeting HIF-1 α mRNA. Our results show that PMX290 inhibits significantly HIF-1 activity and concomitantly sensitizes hypoxic cells to chemotherapeutic agents. These results were confirmed in HCT116 cells grown as three-dimensional spheroids. EZN2968 is also able to inhibit the expression of HIF-1 α in hypoxic condition and to restore cell sensitivity similar to normoxia. Taken together, the results of this study suggest that targeting cells presenting hypoxic areas or increased HIF-1 activity, by using quinol compounds or LNA strategy, could help to improve the success rate of current regimens used in the clinical management of colon cancer.

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Distribution of full-length and truncated interleukin-18 receptor alpha transcripts in the mouse brain**Montanari Claudia**

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Interleukin (IL)-18 is a potent pro-inflammatory cytokine belonging to IL-1 family that was originally isolated as an interferon-gamma (IFN- γ) inducing factor. Many data indicate that IL-18 is active in the central nervous system (CNS) where can function as an anorexigenic agent, modulate appetite and sleep, reduce the hippocampal long term potentiation, and regulate the hypothalamus-pituitary-adrenal axis activity (1-3). Hence, IL-18 has been proposed to be an important modulator of neuronal function even considering the increasing evidence of IL-18 participation in a number of pathological states of the CNS such as inflammation and neurodegenerative and/or neuropsychiatric diseases (4-7). However, the mechanisms of IL-18 action in CNS remain largely unknown. Peripherally, IL-18 actions are mediated by binding to its receptor complex closely resembling IL-1 receptor and consisting of an α subunit, to which IL-18 binds, and a β subunit which initiates signal transduction (8). By data mining, we have identified a canonical full length IL18R α and a truncated IL18R α that we have arbitrarily named type I and type II, respectively. The shorter IL18R α isoform was predicted to lack the Toll-like IL-1R (TIR) intracellular domain required to promote signal transduction. This evidence suggests that the two isoforms could have different functional roles and, additionally, that type II IL18R α could act as an endogenous decoy receptor for IL-18. In an effort to clarify the biological actions and significance of IL-18 in the CNS, we first determined the presence and then analyzed the expression patterns of these two isoforms in the C57BL6 mouse brain. We found that both IL18R α isoforms exist in the CNS where they are expressed throughout the brain with differences in their distribution. Type I IL18R α is expressed at high levels on neurons of several brain areas including cerebral cortex, hippocampus, olfactory bulbs, amygdala, epithalamus, thalamus, hypothalamus, and cerebellum. Conversely, the type II IL18R α signal was highly detected, mainly in certain cerebral regions and nuclei of epithalamus, hypothalamus, amygdala, hippocampus, and cerebral cortex. Considering the neuronal expression of both isoforms, these results suggest primarily that IL-18 can act directly in modulating CNS functions. Moreover, this isoform-specific distribution may help to explain some of the central actions observed for IL-18. Finally, the existence of a truncated IL18R α isoform possibly working as decoy receptor displays that IL-18 system seems to possess an elaborate control mechanism of its actions more complex than previously believed. Further analysis on the IL-18 system in the brain may lead to the identification of molecular targets for new and more effective drugs to treat variety of physiological conditions and diseases for which has been described an involvement of IL-18.

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Role of mature endothelial cells on the differentiation of stem cells in endothelial progenitor cells**Musilli Claudia**

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Emerging evidence suggests that circulating bone marrow-derived stem and progenitor cells play an important role in tissue regeneration throughout the cardiovascular system. Special attention has been directed to endothelial progenitor cells (EPCs), a subset of bone-marrow-derived progenitor cells, which have been shown to have the potential to proliferate and differentiate into mature endothelial cells (1). EPCs may play an important role in endothelium maintenance, being implicated in both re-endothelialization and neovascularization, then ameliorating the function of ischemic organs, possibly by either induction or modulation of vasculogenesis and angiogenesis (2). However, the mechanisms by which EPCs interact with mature vascular wall cells remain unclear. Based on the foregoing, the main purpose of the present study was to assess whether vascular mature endothelial cells were able to foster the differentiation of stem cells toward endothelial progenitor cells. For this purpose peripheral blood mononuclear cells (PBMCs), which are a source of circulating haematopoietic stem cells, were co-cultured with human coronary endothelial cells (HCAECs) and the degree of differentiation in EPC was evaluated by detection of endothelial markers. A co-culture model, in which the two cell types share the same culture medium in the absence of any exogenous angiogenic stimulus, was used. Moreover, since hypoxia may be involved in EPC recruitment and behaviour modulation, its effect on the co-culture model was also assessed, by pretreating HCAECs with 3% O₂ before co-culture setting. A 3-day co-culture period increased the expression of VEGF-R2, VE-cadherin, $\alpha_v\beta_3$ - and α_5 -integrin in both the adherent and suspended-PBMCs, assessed by cytofluorimetric analysis. The acquisition of an EPC phenotype was further confirmed by double staining of cells for UEA-1 binding and acLDL uptake. HCAECs also influenced PBMC adhesion, transendothelial migration, and cell organization on Matrigel. Hypoxia modulated either PBMC differentiation or their functional properties. These data strongly suggest that human mature endothelium may support the differentiation of PBMCs into EPCs.

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Inhibition by novel *N,N*-dicyclohexane-4-olamine aryl esters of Pgp-mediated 123 rhodamine efflux in L5178Y MDR-1 transfected mouse lymphoma cells

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Many tumour cells become resistant to commonly used cytotoxic drugs due to the overexpression of ATP-binding cassette (ABC) transporters. Pgp (MDR-1, ABCB1) and MRP-1 (ABCC1) have been demonstrated to pump a wide selection of the most commonly used cancer drugs (1). Several generations of pharmaceutical inhibitors of Pgp have been examined in preclinical and clinical studies. The main problems associated with the development of these drugs seem due to poor specificity, low potency and interference with physiological functions (2). A new series of Pgp-dependent MDR inhibitors having a *N,N*-bis(cyclohexanol)amine scaffold has been designed, on the basis of the frozen analogous approach (3). This scaffold (see Fig. 1) allows the synthesis of 4 geometrical isomers when the *N,N*-bis(cyclohexanol)amine moiety is esterified with two different aryl acids ($Ar_1=3,4,5$ -trimethoxy-benzoyloxy; $Ar_2=3$ -(3,4,5-trimethoxy-phenyl)-propionyloxy for single bond compounds, 3-(3,4,5-trimethoxy-phenyl)-propynoyloxy for double bond compounds, and 3-(3,4,5-trimethoxy-phenyl)-acryloyloxy for triple bond compounds). The nature of the bond between C_2 and C_3 of Ar_2 gives origin to three different groups of compounds characterized by single, double, and triple bond, respectively. The resulting 12 compounds have been evaluated for their Pgp inhibiting properties by measuring the efflux of Pgp specific fluorescent substrate rhodamine 123 (R123) in MDR1-gene transfected mouse T-lymphoma L5178 cells in presence of different concentrations of the selected compounds and quantified as fluorescence increase by flow cytometry (4). Their effects were compared to those of the well known Pgp inhibitors verapamil, 3,5-dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) (4), and cyclosporine A. Pgp blocking activity was described by α_{max} , which expresses the efficacy, and by IC_{50} , which measures the potency of the inhibitor. α_{max} varied between 0 (in the absence of the inhibitor) and 1 (when the amount of R123 found in L5178 MDR1 cells was equal to that determined in presence of 5 mM vanadate that fully inhibited R123 efflux). All the aforementioned compounds inhibited Pgp-mediated R123 efflux in a concentration-dependent manner although with different potency and efficacy. Isomers MC176 and MC259 were very potent and efficient inhibitors, with IC_{50} values in the nanomolar range and α_{max} values very close to 1. Furthermore, MC185 and MC260 exhibited two different sites for the inhibition of Pgp-mediated efflux of R123: one characterized by a low-affinity and the other by a high-affinity binding constant. In contrast, isomers MC247, MC250, MC251, MC252, MC257, MC258, DM407, and DM410 exhibited IC_{50} values two or three orders of magnitude higher. In conclusion, isomers of the cis-cis and trans-cis series with both double or triple bond appear useful probes for studying Pgp and sister proteins and very promising leads for the development of safe and efficient MDR reverters.

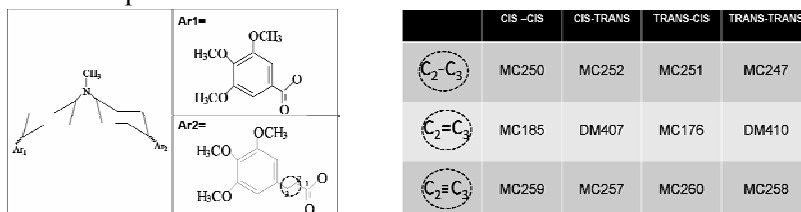


Figure 1: Structure of the compounds investigated

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Phenotypical characterization and transplantation of third trimester human amniotic fluid cells with potential for therapy in spinal cord injury**Nicora Emanuela**

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Pharmacological approaches aimed at preventing secondary degeneration in spinal cord injury have been recently flanked by cellular methods and stem cells have been studied as a new tool for spinal cord therapy (1,2). Amniotic fluid cells (AFCs), which contains differentiated and undifferentiated cells arising from all three germ layers, may be a novel source of stem cells for therapeutic use (3) since they constitute a large and accessible reservoir (3,4). The amniotic fluid is known to contain multiple cell types derived from the developing foetus. The outcome of this characterization is based upon morphological, biochemical, and proliferation assays. AFCs cells are classified in three main groups: epitheloid cells (from fetal skin and urine tract), amniotic fluid specific cells (from fetal membranes and trophoblast) and fibroblastic cells (from fibrous connective tissue and dermal fibroblasts). Some of these cells resulted to be multipotent cells that can differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic pathways. The aim of this study was to isolate cells from the third trimester amniotic fluid (instead that cells usually retrieved from amniocentesis) and test their therapeutic potential in a mouse model of spinal cord injury. Different populations of adherent cells were isolated [as described in (5)] from 11 human amniotic fluids; they were then characterized for *in vitro* proliferation and differentiation potential. The antigenic profile was performed both by immunocytochemistry and citofluorimetric analysis. In particular, 3 cultures were more thoroughly investigated by immunoistochemical analysis and all of them showed the expression of neural markers such as nestin, β tubulin III and GFAP. The citofluorimetric analysis showed the expression of adult mesenchymal markers (CD146-, CD73+, CD105+, CD90+) directed to the muscle-neural lineage (CD146-, NG2+, CD56+) (#3.5); one of them also expressed CD117 (#3.6). The third culture (#1.1), instead, showed a mesenchymal phenotype directed to the perivascular lineage (CD146+, CD90+, CD73+). These 3 populations were applied to spinal cord injured mice. After lesioning, the animals were intravenously injected with AFCs or PBS (controls), and hind limb motor recovery was evaluated for the following 35 days according to the Basso Mouse Scale (6). The animals transplanted with culture #3.5 and #3.6 showed a significantly improved motor recovery compared to PBS treated mice. Differently, we failed to observe any positive effects by applying culture #3.1. Furthermore, the histological analysis of the lesion site showed that animals transplanted with culture #3.6 had a better preserved ventral myelin, and the number of infiltrated inflammatory cells (macrophages and neutrophils) was also reduced. Transplanted cells were detected at the lesion site and 4 mm away in rostral position to the injured area. These results suggest that the administration of amniotic fluid derived cells with a muscle-neural phenotype can attenuate secondary degeneration following traumatic lesion to CNS by reducing the cellular component of neuroinflammatory reaction. This may represent a novel therapeutic avenue for spinal cord injury patients.

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PKC activity regulates the intracellular trafficking of the neuronal EAAC1 glutamate transporter**Padovano Valeria**

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Glutamate is the primary excitatory neurotransmitter in the central nervous system. Increased levels of extracellular glutamate induce excitoneurotoxicity and may contribute to neuronal damage in neurodegenerative diseases (1). Glutamate transporters play a fundamental role in maintaining extracellular glutamate below neurotoxic levels. Among the glutamate (or excitatory amino acid) transporters, EAAC1 (human EAAT3) is expressed in neuronal cells (2) and in several extraneuronal tissues (3). In renal tubule cells, EAAC1 is the main system involved in the re-uptake of glutamate and aspartate from urine and glutamate transporter EAAC1-deficient mice develop dicarboxylic aminoaciduria (3), a very rare human disorder. The activity of neurotransmitter transporters, including EAAC1, has been largely documented to be controlled by protein kinase C (PKC) (4). In epithelial Madin-Darby canine kidney (MDCK) cells, EAAC1 localizes to apical surfaces and PKC activation induces a decrease in transport activity (5-7). However, it is not known whether the reduced activity is due to a decrease in ligand affinity or to a decrease in transporter surface density. Here we show that stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) induces a time-dependent decrease in glutamate transport activity that coincides with relocalization of the EAAC1 glutamate transporter from the apical surface of polarized MDCK cells to intracellular compartments. The PKC-induced relocalization of EAAC1 was negatively regulated by the calcineurin inhibitor cyclosporine A (CsA), and by the expression of a dominant negative mutant of the endocytic protein dynamin 1, a well known target of the phosphatase activity of calcineurin. Using ³²P-metabolic labelling experiments, we found unchanged levels of phosphorylated EAAC1, indicating that EAAC1 relocalization does not depend on its phosphorylation, while we found a decrease in phospho-serine⁷⁷⁸ dyamin1 that was abolished by a pre-treatment with CsA. Because calcineurin-mediated dephosphorylation of serine⁷⁷⁸ activates dynamin 1 endocytic functions, PMA-mediated relocalization of EAAC1 occurs through activation of a dynamin 1-dependent internalization pathway. By immunofluorescence experiments with endosomal markers, we demonstrated that internalized EAAC1 accumulates to recycling endosomes also containing the basolateral BGT1 transporter and activated PKC α . The sustained activation of PKC was required to maintain EAAC1 and BGT1 in the recycling compartment, as the transporters returned to their appropriate surfaces after post-treatment with a PKC specific inhibitor. Taken together these data indicate that PKC activity regulates EAAC1 surface density by inducing its internalization and retention to PKC α labeled recycling endosomes common to apical and basolateral proteins.

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MK801, a selective NMDA uncompetitive antagonist, attenuates excitotoxicity in an experimental model of spinal cord trauma**Paterniti Irene**

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Glutamate, the most abundant excitatory neurotransmitter at neuronal and glial cells in mammalian brain, is involved in fast excitatory transmission and plays important roles in neuronal function such as plasticity and cognitive processes, as well as in toxic events (1). It is stored in vesicles in the pre-synaptic cell and released by Ca^{2+} -dependent exocytosis (2) and exert its excitotoxic action through the binding with the N-methyl-D-aspartic acid (NMDA) receptors (3). An excessive release of glutamate into the extracellular space occurs in a range of pathological conditions, such as immediately after acute spinal cord injury (SCI), contributing substantially to exacerbate the inflammatory events, that play a central role in pathogenesis of acute and chronic SCI. In this model, spinal cord trauma was induced by the application of vascular clips to the *dura* via a four-level T5-T8 laminectomy (4), for replicating the persistence of cord compression that is commonly observed in human SCI. SCI in mice initiates a series of cellular and molecular cascade events, and a progressive neuronal injury results from a combination of secondary injury factors including: ischemia, biochemical alterations, excitotoxicity, neurotransmitter accumulation, and apoptosis (5). The aim of our study was to evaluate whether administration of uncompetitive NMDA receptor antagonist, such as dizocilpine melete (MK801), administered (2 mg/kg) i.p. at 30 min and 6 h after injury, may block the channel limiting the flow of Ca^{2+} into the CNS cells and consequent disruption of the myelin processes. In this study we clearly demonstrated that 24 h after injury, the administration of MK801 attenuated all the following endpoints of the inflammatory response: the degree of spinal cord inflammation and tissue injury (evaluated as histological score); infiltration of neutrophils [myeloperoxidase (MPO) activity], NF- κ B activation; cytokines levels (TNF- α and IL-1 β); phosphorylation of ERK1/2 and p38 MAPK; expression of adhesion molecules (ICAM-1, P-selectin, E-selectin) and expression of death signal. Moreover, in a separate set of experiments, in order to gain a better insight into the mechanisms of action of MK801, we have also demonstrated that this treatment significantly ameliorated the recovery of limb function (evaluated by motor recovery score). Taken together, our results clearly demonstrate that MK801 treatment ameliorates excitotoxicity induced by spinal cord injury.

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Drug-induced progressive multifocal leukoencephalopathy: data mining of international spontaneous ADR report databases**Piccinni Carlo**

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Progressive multifocal leukoencephalopathy (PML), a rare demyelinating infection of the central nervous system, is becoming a safety concern for monoclonal antibodies, as also recently warned by regulatory agencies. The interest in PML, a known HIV complication, reappeared when natalizumab, a new immunomodulating agent for multiple sclerosis, was withdrawn from US market because of PML cases. As with other monoclonal antibodies, e.g. rituximab and efalizumab, many PML cases were recently reported and efalizumab was consequently withdrawn from the market. The aim of this study was to review the cases of drug-induced PML reported in main international spontaneous ADR databases and in the literature, in order to identify active substances associated to this adverse reaction. Considering the rarity of PML, a post-marketing analysis based on different kind of sources seems essential to better define drug reaction relationship. From the main international spontaneous report databases, FDA-AERS and WHO-VigiBase, cases of drug-induced-PML recorded from 2004 to 2008 were searched. Moreover, from MEDLINE database, case-report/case-series published until 2008 and containing the MESH term "Leukoencephalopathy, Progressive Multifocal/chemically induced" were selected. In order to assess PML-drug relationship, drug-reaction-pairs in terms of patient characteristics, basal diseases, and co-suspected drugs were analysed. Overall, 214 PML cases in FDA-AERS, 118 in WHO-VigiBase and 140 in MEDLINE were retrieved. Drugs more frequently involved in PML cases belong to "antineoplastic/immunomodulating" or "antiviral" agents. Substances more frequently reported as the only suspected drug were rituximab (20 in WHO-VigiBase, 28 in FDA-AERS, and 5 in MEDLINE), natalizumab (4, 9, and 3) and tacrolimus (7, 8, and 2). The most frequent basal diseases were lymphoproliferative diseases (28%), autoimmune disorders (20%), and transplants (10%), without differences among sources. HIV/AIDS was more represented in FDA-AERS (27%) and WHO-VigiBase (19%), rather than in MEDLINE (10%). In HIV/AIDS, the role of drugs in PML occurrence is difficult to establish because of polypharmacy, whereas in autoimmune disorders, we found a strong relationship between PML and monoclonal antibodies (in particular, natalizumab in multiple sclerosis and rituximab in rheumatoid arthritis). Before the advent of biological agents, evidence of drug-induced PML among old immunosuppressants was very poor; thus, the risk of monoclonal antibodies-induced PML gains strength. In the past, PML has never been associated with autoimmune disorders (e.g. multiple sclerosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus), while recently it became a crucial issue of new immunosuppressive drugs used for the above mentioned diseases (1,2). Biological drugs are often wrongly considered safer than non-biological ones because of the lack of off-target effects. Actually, these new immunosuppressive drugs could cause severe ADRs through the imbalance of immune system (3,4). From the results of this study, it is reasonable to wonder whether PML can be considered a class effect of immunosuppressive monoclonal antibodies. Based on these results, careful monitoring of patients treated with any biological drugs should be recommended for early signs and symptoms of PML.

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Effects of resveratrol in an *in vitro* model of cellular aging**Pitozzi Vanessa**

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As the world population is increasingly aging, it is important to identify protective interventions aimed to reduce the deleterious effects of aging, such as the increased incidence of diseases which are often disabling. Resveratrol is a stilbene compound produced by different plants, and red wine is the main source in the human diet. Resveratrol is endowed with many interesting biological activities, including an anti-aging effect which has been demonstrated both *in vitro* in eukaryotic cells (1) and *in vivo* (2). The aim of this work was to study the anti-aging activity of resveratrol in human fibroblasts *in vitro*, a widely used model in aging studies. We evaluated age-associated changes such as the increase in cellular and nuclear dimensions, DNA content, DNA damage, and replicative arrest. Global transcriptional changes were also evaluated by genome-wide gene expression microarray analysis, both during *in vitro* aging and after resveratrol treatment. We observed a biphasic effect of resveratrol, which at high concentrations inhibited cell proliferation, while at lower concentrations delayed the onset of replicative senescence. We also observed protective effects of resveratrol on DNA oxidative damage and resistance to *in vitro* induced oxidative stress, on nuclear DNA content, and nuclear size in aging fibroblasts. Resveratrol treatment also had specific effects on the transcriptional profile of senescent fibroblasts, turning off complement and coagulation cascades and other innate immune response components typically activated during wound repair processes. In addition, resveratrol re-induced the basal expression of genes controlling cell cycle and DNA replication, usually down-regulated during aging. As a whole, these data are in agreement with an "anti-aging" activity of resveratrol.

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Anti-inflammatory effects of flavocoxid, a new dual inhibitor of cyclooxygenase and 5-lipoxygenase.**Polito Francesca**

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The flavonoids baicalin and catechin from *Scutellaria baicalensis* and *Acacia catechu*, respectively, have been used for various clinical applications. Flavocoxid is a mixed extract containing baicalin and catechin and acting as a dual inhibitor of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes (1). The anti-inflammatory activity, measured by protein and gene expression of inflammatory markers, of flavocoxid in rat peritoneal macrophages (MΦs) stimulated with *Salmonella enteritidis* lipopolysaccharide (LPS) was investigated. LPS-stimulated (1 µg/ml) peritoneal rat MΦs were co-incubated with different concentrations of flavocoxid (32-128 µg/ml) or RPMI medium for different incubation times. Inducible COX-2, 5-LOX, iNOS, and IκB-α levels were evaluated by Western blot analysis. NF-κB binding activity was investigated by EMSA. TNF-α gene and protein expression were measured by Real-Time PCR and ELISA, respectively. Finally, malondialdehyde (MDA) and nitrite levels in MΦ supernatants were evaluated. LPS stimulation induced a pro-inflammatory phenotype in rat peritoneal MΦs. Flavocoxid (128 µg/ml) significantly inhibited COX-2 (LPS=18.0±2.1; flavocoxid=3.8±0.9 integrated intensity), 5-LOX (LPS=20.0±3.8; flavocoxid=3.1±0.8 integrated intensity), and iNOS expression (LPS=15.0±1.1; flavocoxid=4.1±0.4 integrated intensity), but did not modify COX-1 expression. PGE-2 and LTB-4 secretion levels were consequently damped in the culture supernatants. Flavocoxid also prevented the loss of IκB-α protein (LPS=1.9±0.2; flavocoxid=7.2±1.6 integrated intensity), blunted the increased NF-κB binding activity (LPS=9.2±2.0; flavocoxid=2.4±0.7 integrated intensity) as well as the enhanced TNF-α mRNA levels (LPS=8.0±0.9; flavocoxid=1.9±0.8 n-folds/β-actin) induced by LPS. Finally, flavocoxid decreased MDA, TNF-α, and nitrite content in LPS-stimulated MΦs. Our data suggest that flavocoxid might be useful as a potential anti-inflammatory agent which works on the gene and protein expression level.

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Cytochrome P-450-dependent metabolism of newly synthesized monoamino oxidase inhibitor**Possidente Mariapia**

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The selective monoamine oxidase-B (MAO-B) inhibitor, *l*-deprenyl, is still used for treating Parkinson's patients. However, a disadvantage of its use lies in the formation of *l*-amphetamine and *l*-methamphetamine. Subsequently, this has promoted the design of new MAO-B inhibitors which presents propargylamino group. ASS94 was a new compound synthesized in the Laboratorio de Radicales Libres y Química Computacional, CSIC, Spain with the aim to inhibit MAO but also to possess protective properties against neurodegenerative diseases. The aim of this work was to study ASS94 phase I metabolism in human liver microsomes in order to study the influence of drug on major cytochrome P-450 (CYP) activities and to exclude potential drug-drug interaction. In fact, the presence of propargylamino moiety could represent a potential molecular site to the formation of suicide substrates. Effects of ASS94 on human CYP 3A4 were assessed with use of selective substrate, [3-[3(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)-phenyl]furan-2-(5H)-one] (DFB) (1). The interaction of DFB with human liver microsomes was assessed by a photofluorimetric method at excitation λ 360 nm and emission λ 460 nm. When human microsomes were incubated with DFB in presence of different concentration of ASS94, concentration-inhibition curves were obtained. ASS94 inhibition gave IC₅₀ value of 27 μ M. In a second set of experiments, human liver microsomes were pre-incubated for 15 min with ASS94, at IC₅₀ concentration, in presence or absence of NADPH. After this incubation time, DFB was added and activity measured. In all experiments, ASS94 inhibition did not result to be dependent on pre-incubation time and on the presence of NADPH in the medium. ASS94 reversible inhibition of DFB metabolism was assessed by dilution experiments. Reaction mixtures (containing microsomes, ASS94, NADPH, and various substrates) were pre-incubated for 15 min and then diluted 10 folds. The residual activity observed exhibited values close to those of control conditions, suggesting that inhibition was reversible. This data indicate that ASS94 inhibits the DFB metabolism promoted by human CYP 3A4 in a competitive manner suggesting that the MAO inhibitor is a substrate for this isoenzyme. Further experiments will be done in order to clarify the structure of the metabolite(s) and the interaction with other CYP-isoforms.

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Presence and inducibility of CYP2C isoforms in pig as a new animal model**Puccinelli Emanuela**

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Cytochrome P450s (CYPs) play an important role in the oxidative metabolism of numerous xenobiotics as well as endogenous compounds. In human, the CYP2C subfamily is one of the most important accounting for roughly 20% of total CYPs and being responsible for the metabolism of about 30% of drugs (1). In the latest years, pig has become of interest because it has been proposed as a new model for pharmacological and toxicological studies, and its liver may be used as bioreactor for patients waiting for liver transplantation. However, up to now, little information is available on its metabolic system, and in particular on its CYPs expression. In the present study we investigated by RT-PCR and marker activity experiments the presence and regulation of three recently cloned isoforms of the 2C subfamily (2C33, 2C42, and 2C49) in liver and some extrahepatic tissues of pig. It was evaluated the effects on the expression and activity of these CYP2Cs of three typical human CYP inducers: rifampin, phenobarbital, and β -naphthoflavone, inducers of CYP3A4 (through PXR activation), CYP2B6 (through CAR activation), and members of CYP1 family (through AhR activation), respectively. Seventeen male castrated pigs of about 20-30 kg were utilized: seven pigs were used as controls, three were treated with rifampin (40 mg/kg i.p. for 4 days), three were treated with β -naphthoflavone (30 mg/kg i.p. for 4 days), and four were treated with phenobarbital (20 mg/kg i.p. for 3 days). Total RNA was extracted from liver, kidney, and small intestine, retrotranscribed and amplified by PCR using specific primers. Hepatic and renal microsomes were also prepared in the standard way to investigate the activity of CYP2Cs through five reactions specific for human CYP2C isoforms (paclitaxel hydroxylase, tolbutamide hydroxylase, diclofenac 4'-hydroxylase, *S*-mephenytoin 4'-hydroxylase, and metoxytrifluoromethylcoumarin *O*-demethylase). Further inhibition studies were performed in liver microsomes with inhibitors specific for human CYP2Cs (quercetin, sulphaphenazole, ticlopidine). The three porcine isoforms considered were constitutively expressed in all the organs examined and their expressions were transcriptionally inducible by rifampin and phenobarbital, but not by β -naphthoflavone. In agreement with CYP2C9 human hepatocyte experiments (2), rifampin and phenobarbital treatments of pig increased the hepatic activity of tolbutamide hydroxylase and diclofenac 4'-hydroxylase but, unlike human CYP2C9, the porcine activities resulted not inhibitable by sulphaphenazole. Other differences between human and pig were also found in the metabolism of both paclitaxel and *S*-mephenytoin. Microsomes from rifampin- and phenobarbital-treated pigs oxidated paclitaxel forming an unknown metabolite, different from 6 α -hydroxypaclitaxel, the product of human CYP2C8 catalysis (3). Lastly, the typical oxidation of *S*-mephenytoin by human CYP2C19 forming the 4'-hydroxy derivative, was practically not observed with both control and rifampin- or phenobarbital-treated liver porcine microsomes. In conclusion, the results on the expression and activities of CYP2Cs presented in this study show many differences between human and pig CYP2Cs, suggesting that pig may not be a suitable model for the study of drugs metabolized in human by this subfamily.

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Antibacterial agents and risk of *torsade de pointes*: a critical evaluation by analyzing population exposure and the public version of the Adverse Event Reporting System (AERS)

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Because of regulatory and clinical implications, drug-induced *torsade de pointes* (TdP) represents a safety concern for several drug classes widely used in general practice such as antibacterials (1,2). The aim of my doctoral work was the critical evaluation of the risk of antibacterial-induced TdP using a two-fold approach: i) analyzing population exposure in 14 European countries; ii) investigating spontaneous reports submitted to the freely accessible version of the Adverse Event Reporting System (AERS). Each drug with published evidence on TdP-liability was classified according to an already proposed algorithm (3) based on the strength of evidence: from group A (any evidence) to group E (clinical reports and warnings of TdP). Consumption data, encompassing the period 1998-2005, were provided by ESAC (European Surveillance of Antibacterial Consumption) project and were expressed as defined daily doses per 1000 inhabitants per day (DID). In addition, spontaneous reports of TdP from January 2004 through December 2007 were retrieved from the public version of the AERS. All antibiotics reported as “suspected” or “interacting” in the DRUG files were selected and adverse reactions reported as TdP from REACTION files were identified. Duplicates were removed by an automated multi-step process. The adjusted reporting odds ratio (ROR), as a measure of disproportionality (4), was evaluated for each antibacterial drug using the statistical package Epi Info, version 3.4.3-2007. The presence of a Class I/III antiarrhythmic agents among concomitant drugs was used as confounding factor to calculate the adjusted ROR. Twenty-one antibacterials were identified through MEDLINE search: 9 of them [6 fluoroquinolones (FQs) and 3 macrolides (MACs)] belonged to group E. The use of group E drugs ranged from 1.3 (Sweden) to 4.1 DID (Italy) in 1998 and from 1.2 (Sweden) to 6.5 DID (Italy) in 2005. Italy displayed the highest increase: up to 1.6 fold (an increment of 2.4 DID). Significant exposure was observed also in Spain, Luxembourg (3.8 DID each), Hungary and Belgium (3.7 DID each). Only Denmark, Sweden and UK showed a slight decrease in use. Notably, exposure to clarithromycin increased in 10 out of 14 countries, with the highest increment in Hungary (up to 2 fold), where in 2005 it accounted for 2 DID (54% of total group E). Moreover, a peak of 3 DID was observed in Italy. Erythromycin use, instead, showed a decrease in all countries but was the most used drug in UK (1.7 DID in 2005). From the AERS database, 275 reports of TdP were retrieved: 20 antibacterials were involved in 166 cases. Agents more frequently reported were levofloxacin (49) and moxifloxacin (26) among FQs, clarithromycin (17) and azithromycin (14) among MACs. A high ROR was obtained for erythromycin (12.1) and moxifloxacin (9.8). A significant disproportionality was also observed for linezolid (3.5) and cotrimoxazole (2.8). Due to the amount of evidence, disproportion for MACs and FQs should be viewed as “expected”. By contrast, signals generated by linezolid and cotrimoxazole were considered “unexpected”. These drugs may belong to a hypothetical “black list”. Compounds with no published evidence, lack of signal, and extensive use (e.g. amoxicillin) may be included in a provisional “white list”. A “grey list” could include all drugs with questionable data on TdP liability. To sum up, several FQs and MACs are widely used in Southern European countries and associated with reports of TdP in the clinical setting. This pharmaco-epidemiological approach is a first step towards provisional risk stratification, in order to support physicians in appropriate use of antibiotics, especially in arrhythmia-susceptible patients.

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THE PPAR γ AGONIST PIOGLITAZONE STIMULATES NEURITE OUTGROWTH IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS**Rattazzi Lorenza**

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Neurite outgrowth, defined as the sprout of cell processes which will later become axons and dendrites, is a key event of neuronal differentiation and forms the basis of the proper connectivity within neuronal networks (1). Several evidences suggest that activation of peroxisome proliferator-activated receptor (PPAR) γ might influence neuronal differentiation. First, PPAR γ is expressed in the embryo mouse brain, in neuronal stem cells (NSC) from both embryo and adult mouse brains (2-4), and in distinct grey regions of the adult brain (5). Second, disorders of brain development have been reported in PPAR $\gamma^{-/-}$ and PPAR $\gamma^{+/-}$ embryo mice (4). Finally, *in vitro* experiments have shown that PPAR γ agonists, including the endogenous agonist 15-deoxy- Δ 12,14-PGJ2 (15d-PGJ2), modulate differentiation of murine NSC (4), rat embryonic midbrain cells, rat pheochromocytoma and human neuroblastoma cells (6-8). All these evidences indicate the opportunity to further explore the effects of PPAR γ agonists on events related to neuronal differentiation and neurite outgrowth. The aim of this study was to evaluate the effects of the synthetic PPAR γ agonist pioglitazone on neuronal differentiation and neurite outgrowth in SH-SY5Y human neuroblastoma cells. In addition, we have studied the pioglitazone effects on the activation of the mitogen-activated protein kinases (MAPK), a well-known event involved in the regulation of neuronal differentiation (1). SH-SY5Y cells were untreated or treated for 5 days with pioglitazone (10 pM-1 μ M) or 5 μ M all-*trans*-retinoic acid (atRA), a well-known pro-differentiating agent for these cells here used as a comparator. Neurite outgrowth was evaluated through morphometric analysis of cell processes. MAPK activation was evaluated by measuring the activation of p42/p44, p46/p54, and p38 by Western blot analysis. Pioglitazone promoted cell differentiation starting from day 2 (after 1 drug addition) and, more significantly, on day 5 (after 3 drug addition). During drug treatment, the average of processes length significantly increased: the length means were $8.4 \pm 0.6 \mu\text{m}$ and $30.9 \pm 1.9 \mu\text{m}$ ($P < 0.01$ vs cells treated with vehicle alone) on days 0 and 5, respectively. Pioglitazone increased the mean length of processes in a concentration-dependent manner with the maximal effect at 100 nM-1 μ M, both on days 2 and 5. By contrast, the EC₅₀s were 23.1 ± 0.9 nM and 2.1 ± 0.8 nM, on day 2 and 5, respectively, thus suggesting the increase in cell responsiveness throughout drug treatment. Finally, the effects of pioglitazone on neuronal differentiation were accompanied by significant activation of p42/p44 and p46 MAPK. In conclusion, our data suggest that PPAR γ stimulation might contribute to the development and maintenance of a proper neuronal connectivity within neuronal networks.

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Mechanisms of [³H]glycine release from mouse spinal cord and hippocampal glycinergic nerve terminals**Romei Cristina**

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The release of glycine has been the object of very few studies. Glycine is the major inhibitory transmitter in the spinal cord and brainstem where it activates strychnine-sensitive receptor channels which, in the adult, mediate Cl⁻ entry and membrane hyperpolarization (1,2). In addition to its inhibitory roles, glycine exerts important excitatory functions through the CNS; in fact, glycine is an obligatory co-agonist to activate glutamate receptors of the NMDA type (3). The multiplicity of glycine actions, particularly its primary roles in the activation of the NMDA receptors that are implicated in several physiological and pathological processes like schizophrenia and neurodegenerative disease, should have triggered much interest in the mechanisms of glycine release and its modulation. We recently studied glycine release from synaptosomes obtained from spinal cord (4), the region in which the density of glycinergic terminals is the highest and in which glycinergic transmission has been best established; on the other hand, studies on hippocampal glycine release are extremely rare. In the present work, we investigated release from mouse hippocampus glycinergic terminals selectively prelabelled with [³H]glycine through transporters of the GLYT2 type. Purified synaptosomes, prepared by homogenization of the hippocampal tissue and isolated by centrifugation, were incubated at 37°C for 15 min with [³H]glycine in the presence of the selective GLYT1 transporter blocker NFPS in order to permit [³H]glycine influx exclusively through GLYT2. Synaptosomes were superfused with standard medium and exposed in superfusion to three different depolarizing stimuli, KCl, 4-AP, and veratridine, at varying concentrations, and the modes of exit of the neurotransmitter were analyzed. Depolarization with high K⁺ (15-50 mM) provoked overflows totally dependent on external Ca²⁺, whereas in the spinal cord the 35 or 50 mM KCl-evoked overflow was only in part dependent on extraterminal Ca²⁺. The evoked overflow in hippocampus was lower than that in the spinal cord, suggesting that the exocytotic machinery in spinal cord is more efficient than that in hippocampus. Similar data were obtained with 4-AP: the Ca²⁺-dependent exocytosis of glycine provoked by 4-AP (0.3-1 mM) in hippocampus was much lower than that in the spinal cord. Differently, the component of the 10 μM veratridine-induced overflow that was dependent on external Ca²⁺ was higher in the hippocampus than in spinal cord, although the total overflow in the hippocampus was only half of that in spinal cord. We investigated the effects of GLYT2 selective blockers ORG25543 and ALX1393, and found that the glycine release evoked by 10 μM veratridine occurred in part by transporter reversal; in addition the overflow was significantly reduced by pre-treatment of synaptosomes with bafilomycin A₁ and by blocking the mitochondrial Na⁺/Ca²⁺ exchanger with CGP 37157, indicating that, in addition to the portion of release occurring by transporters reversal, a significant component of glycine release occurred by a vesicular process dependent on mobilization of internal Ca²⁺ originating from mitochondria. We unexpectedly found that glycinergic nerve terminals in the hippocampus are quite different from those in spinal cord in terms of efficiency of exocytosis as well as of mechanisms of release evoked by various depolarizing stimuli.

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Effects of obestatin on dopamine, norepinephrine, and serotonin release in the hypothalamus**Rugia Shohreh**

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Feeding and energy expenditures are modulated by the interplay of hormones, cytokines, and neurotransmitter in the hypothalamus, where peripheral hormones and neurotransmitter signalling convey timely updated information about energy needs and metabolic substrate availability (1,2). Obestatin is a 23 aminoacid peptide, the product of the preproghrelin gene, which is present in the circulation, mainly derived from the stomach. Despite the orexigenic activity of ghrelin, obestatin has been shown to have anorectic effects, possibly mediated by the activation of the orphan G-protein coupled receptor GPR-39, after both peripheral and central administration (3). In order to further elucidate the mechanisms of obestatin in the feeding regulatory pathways, in the present work we have evaluated the effects of obestatin on dopamine, norepinephrine, and serotonin release from rat hypothalamic neuronal endings (synaptosomes) *in vitro*. Hypothalamic synaptosomes were obtained from male Wistar rats (200-250 g) (4). They were loaded with either [3 H]dopamine, [3 H]norepinephrine, or [3 H]serotonin, perfused in water-jacketed superfusion chambers with Krebs-Ringer buffer (0.6 ml/min), and perfusate was collected (1 min fractions for serotonin, and 2 min fractions for dopamine and norepinephrine release) to detect released [3 H] by liquid scintillation scanning. The European Community guidelines for the use of experimental animals have been adhered to and the protocol was approved by the institutional ethics committee. In a first set of experiments, obestatin was added to the perfusion buffer, in graded concentrations (1-100 nM), for 15 min in the serotonin release experiments and for 10 min in the dopamine and norepinephrine release experiments, followed by 8 min with Krebs buffer alone. Amine release was calculated as the percentage of [3 H] recovered in the stimulus and return to basal fractions (a total of 11 fractions for serotonin, and 10 fractions for dopamine and norepinephrine), compared to total loaded [3 H]. A second set of experiments was run to evaluate the effects of obestatin on neurotransmitter release induced by a mild depolarizing stimulus. After a 30 min equilibration perfusion with buffer alone, a 23 min perfusion with the peptides (1-100 nM) was started, where in the final 3 min, K^+ concentration in the perfusion buffer was elevated to 15 mM (after removal of equimolar concentrations of Na^+). A time-response curve relative to the percentage of [3 H] recovered in each perfusate fraction compared to total loaded [3 H] was plotted, and amine release was calculated as the area under the time-response curve (AUC) corresponding to 3 min depolarization+return to basal period in Krebs-Ringer buffer (a total of 8 fractions). Preliminary experiments showed that monoamine intrasynaptosomal metabolism is negligible for dopamine and norepinephrine, while in the experiments evaluating serotonin release, a column chromatography of the perfusate proved necessary to separate serotonin from its metabolites (5). Treatment and control group means were compared by ANOVA followed by Student-Newman-Keuls multiple comparison test. Obestatin (1-100 nM) did not modify basal dopamine, norepinephrine, and serotonin release. After preincubating the synaptosomes with graded concentrations of obestatin and then perfusing with depolarizing buffer (15 mM K^+), we found that obestatin inhibited the stimulated release of dopamine, without affecting norepinephrine and serotonin. Considering the role played by amine neurotransmitters in the central modulation of feeding, the present findings support a central anorectic role for obestatin in modulating feeding behaviour that could be mediated, in part, by inhibition of hypothalamic dopamine release.

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A β ₁₋₄₀ peptide promotes brain endothelial cell proliferation through FGF-2 upregulation**Santini Roberta**

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Amyloid- β (A β) peptides are generated from APP (amyloid precursor protein), an ubiquitous transmembrane protein, after three subsequent cleavage processes operated by α , β and γ -secretases, respectively. A β peptides are naturally present in low nM quantities as circulating soluble monomers in the cerebrospinal fluid and blood of healthy individuals, but they form insoluble aggregates in Alzheimer's disease (AD) and other neurovascular pathologies. Fibroblast growth factor 2 (FGF-2), a well known pro-angiogenic factor, is a member of the FGFs family. FGFs exert their biological activities by binding to high affinity tyrosine kinase receptors (FGFRs) on the surface of target cells (1). Previous studies demonstrated that small amounts of A β ₁₋₄₀ and A β ₁₋₄₂ peptides, activate angiogenesis by promoting endothelial cell proliferation and migration as well as pseudocapillary formation (2,3). Furthermore it was demonstrated that A β peptides functionally synergize with FGF-2 (2,4). In this study, the activity of A β ₁₋₄₀ was studied *in vitro* on human brain endothelial cells (HBMECs). Proliferation and FGF-2 expression have been assessed to investigate whether the observed effect of amyloid peptide would be reproduced in brain endothelium. A β was administrated in the nM range (from 0.01 to 100 nM) for 24 and 48 h to evaluate the effect on proliferation. Furthermore, the FGF-2 expression was evaluated after 6 h administration of A β ₁₋₄₀ (same concentrations as described above) by Western blotting. Our results show that, when A β ₁₋₄₀ is administrated alone, there is a pro-angiogenic trend with regard to HBMECs proliferation, with a maximal stimulation at 10 nM. Correspondingly, we found an overexpression of FGF-2. However, threshold concentrations of A β ₁₋₄₀ and FGF-2 administered at the same time to HBMECs fail to reproduce the synergistic effect previously observed on other endothelial cell lines, suggesting the existence of inherent properties of the brain endothelium. In conclusion, in HBMECs nM concentrations of A β ₁₋₄₀ induced cell proliferation, which was combined with an upregulation of endogenous FGF-2. Future studies will be directed to characterize the innate replicative properties of HBMECs, the content/type of FGF and FGFR involved in the response to amyloid and the receptor through which amyloid exert its proliferative action on capillary endothelium.

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Lupin and peanut protein cross-reactivity: integrated proteomic approach to dissect the problem**Sirtori Elena**

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Lupin is an ancient legume typical of the Mediterranean area and produces seeds containing 40% proteins. Four species of lupin have been domesticated: *Lupinus albus* (white lupin), *L. angustifolius* (narrow-leaf lupin), *L. luteus* (yellow lupin), *L. mutabilis* (blue lupin). In recent years lupin has received much attention from the food industry, because of its nutritional properties, the low contents of anti-nutritional factors, and the presence of specific bioactive components. There is increasing evidence that lupin proteins may have hypocholesterolemic, hypotensive, and hypoglycemic effects (1-5). The developing use of lupin ingredients has, however, enhanced the concerns for possible allergic reactions (6). In numerous cases the cross-reactivity of lupin with peanut has been the suspected cause of these reactions. A controlled study in peanut allergic patients suggests a clinically relevant cross-reactivity rate from 30% to 68% (7). In 2007 lupin was officially added to the EU-list of known allergens (Commission Directive 2006/142/EC). Most of the studies on lupin bioactivity and allergenicity reported in the literature are focused on *L. albus*, the species traditionally used in Europe, whereas less attention has been dedicated to *L. angustifolius*, mainly produced in Australia, but now available also on the European food market. In this context, the final goal of the present investigation was a detailed comparison of these two lupin species, with respect to their structural and allergenic properties and potential cross-reactivity. As a preliminary approach, a protein profile study was carried out in order to compare the subunit composition of the main storage proteins (α , β , γ , and δ -conglutin) in the total protein extract of both species. Chromatographic and electrophoretic techniques in combination with mass spectrometry were used in order to attain a more general overview of the different lupin species and cultivars. 2D screening revealed noteworthy differences in all the globulins. In addition, anion-exchange chromatography pointed out several variations in the ratio of vicilin : legumin peak areas. Differences in glyco-protein profile between the two species were also detected. In order to determine the differences in the allergenicity of the two species, the lupin proteins were selectively purified and tested separately for IgE binding. ELISA assays (indirect and inhibition), Western blotting using individual and pooled serum, and mass spectrometry analysis were performed in order to identify the IgE-binding proteins. Sera from 34 peanut-allergic patients were provided by several hospitals in the Netherlands. The IgE response of the purified lupin protein fractions varied among the peanut allergic patients, with differences of intensity. The results indicated that there is a direct relationship between the peanut-specific IgE level and the cross-reactivity to lupin proteins: the lupin allergenic response of each patient was unique and individual, and strongly depends on the IgE level towards peanut proteins. The ELISA assays and the Western blotting analysis highlighted several reactive spots, indicating the presence of different important allergenic proteins, rather than one major allergen.

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Agonists of the cannabinoid receptor type 1 (CB1) promote rat cerebellar neural progenitor cell proliferation through activation of ERK and Akt pathways**Soffia Silvia**

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Endocannabinoids form a novel class of intercellular messengers, the functions of which include retrograde signalling in the brain and mediation or modulation of several types of synaptic plasticity. Endocannabinoid signalling not only regulates the proliferation, migration, specification, survival, and phenotypic differentiation of neural progenitors during CNS development (1), but has been shown to control proliferation and differentiation of neural stem/progenitor cells in the hippocampal subgranular zone and in the subventricular zone of the adult mammalian brain (2,3). To elucidate the cellular and molecular mechanisms underlying cannabinoid neurogenic action, we used neural progenitor cells isolated from primary cultures of rat postnatal cerebellum as an *in vitro* model of neural cell proliferation. These cells share some of the phenotypic and genotypic properties of stem cells, as characterized by immunocytochemistry and RT-PCR, respectively (4). The functional presence of the two cannabinoid receptors CB1 and CB2 in cerebellar neural progenitor cells at 10 days *in vitro* (DIV) was assessed by immunocytochemistry and Western blot analysis. Previous studies using [³H]-thymidine incorporation found a significant increase of cerebellar neural progenitor cell proliferation after 24 h incubation with the non-selective synthetic cannabinoid agonists WIN-55,212-2 (100 nM) or CP-55,940 (1 µM), which was completely abolished by treatment with the CB1 antagonist AM 251 (10-1000 nM). To evaluate the direct involvement of CB1 receptors, cerebellar neural progenitors were incubated for 24 h with ACEA (0.1-1000 nM), a potent CB1 selective agonist. ACEA (1 nM and 10 nM) significantly increased [³H]-thymidine incorporation (by 37.92±19.20% and 37.84±13.59%, respectively) and this effect was completely reverted by 100 nM AM 251. To investigate the involvement of the MEK/ERK1,2 and PI3K/Akt/GSK3β signalling pathways in CB1 receptor-induced cerebellar neural progenitor proliferation, we performed Western blot analysis. Short-term (5-60 min) incubation of cerebellar neural progenitor cells with 1 nM ACEA produced a significant activation of ERK1,2 and Akt while the pretreatment with the two kinase inhibitors U0126 and LY294002, respectively, reverted this effect. Experiments are currently underway to address the potential for TrkB receptor-dependent signaling pathway in mediating the observed cannabinoid induced proliferation in the absence of BDNF.

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Molecular mechanisms mediating the *in vitro* pro-angiogenic effect of urotensin-II**Sorato Elisa**

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Urotensin-II (U-II) is a cyclic peptide, originally isolated from the urophysis of the goby *Gillichthys mirabilis*. U-II was identified as the endogenous ligand of the orphan receptor GPR14 (1) which was renamed urotensin receptor (UR). UR is coupled to the Gαq/11 phospholipase C signal transduction pathway, the activation of which leads to an increase in inositol triphosphate and mobilization of intracellular Ca²⁺ (2), as it was noticed for example in human aortic endothelial cells (3). U-II, along with its receptor UR, is widely expressed in the cardiovascular system, where it exerts regulatory actions under both physiological and pathological conditions (4) and is involved in processes of morphological remodelling, such as cardiomyocyte hypertrophy (5). Recent data demonstrated that when the peptide is tested *in vitro* on endothelial cells of both animal (6) and human (7) origin it exerts a clearcut pro-angiogenic effect. Thus, in the present study *in vitro* models based on human umbilical vein endothelial cells (HUVEC), directly isolated from the vessel and cultured on Matrigel, were used to investigate possible molecular mechanisms mediating the pro-angiogenic effect of the peptide. The results indicated that the U-II-induced self organization of the cells in capillary-like structures is PKC dependent and involves the activation of the ERK1/2 transduction pathway. In fact, blocking PKC or ERK1/2 with the specific inhibitors calphostin C and PD98059, respectively, inhibited significantly the effect of the peptide. In contrast, U-II did not seem to activate the p38-MAPK, being the pro-angiogenic response not inhibited in the presence of the specific inhibitor of the p38-MAPK SB203580. Western blot analyses on the phosphorylated forms of these kinases provided further support to this finding. Interestingly, the pharmacological inhibition of PI3K (obtained by LY294002), hindered the capacity of U-II to induce a pro-angiogenic effect on HUVEC, indicating that the PI3K/Akt pathway is also involved in regulating the process.

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Cardiac lipid rafts and hyperpolarization-activated cyclic nucleotide gated channels**Spinelli Valentina**

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Caveolae are specialized lipid rafts present in the cell membrane and are characterized by the presence of caveolins (Cav1-3). These are scaffolding proteins that interact with cholesterol and provide the structural framework for macromolecular signals complexes (1). Caveolae are functional domains in the plasma membrane important for interaction, sub-localization, and function of proteins and ion channels. The hyperpolarization-activated cyclic nucleotide-gated (HCNs) channels encode for the alpha subunit of Na^+/K^+ channels present in primary and secondary cardiac pacemaker centers, in the adult and developing cardiac myocytes. Interestingly, f-channel expression and density are upregulated during cardiac disease, such as hypertrophy and failure. Recent studies indicated that HCNs channels localize into membrane lipid rafts of rabbit sino-atrial node cells and HEK293 cells expressing HCN4 isoform (2,3), where caveolin-channel interaction regulates current properties and autonomic regulation. No informations are available about developmental changes of HCN channels localization and function and its relationship HCN channel expression/function in adult cardiac myocytes. To this aim we performed immunocytochemistry of HCN4 isoform and caveolin-3, abundantly expressed in cardiac and skeletal muscles, in cardiomyocytes differentiated from human embryonic stem cells (hESC) and adult human atrial/ventricular cells. As positive controls we used HL-1 cells, a stable cardiac cell line, and HEK-293 cells, which transiently express HCN4 channels. The confocal analysis indicated that in undifferentiated cells HCN4 is expressed at cell membrane level, while caveolin-3 has a tiny signal in the cytoplasmatic space. Upon cardiac differentiation both HCN4 and caveolin-3 localize into the plasma membrane of hESC derived-cardiomyocytes. Additionally, caveolin-3 has a point appearance similar to that reported for sino-atrial node myocytes (4). A similar pattern of localization was detected in adult human ventricular myocytes, indicating that during cardiac maturation membrane lipid rafts recruit HCN4 channels and caveolin-3, suggesting that sub-cellular localization of f-channels in lipid rafts is a fundamental step in cardiac development. Future experiments are aimed at evaluating the occurrence of modifications of f-channel functional properties during cardiac development and maturation. Given that caveolae and lipid rafts are two distinct populations of free cholesterol (5), a different set of experiments will be aimed to evaluate the modification of distribution and function of different HCN isoforms due to cholesterol membrane depletion/enrichment of HEK293 cells.

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Interaction between PPAR ligands and albuterol in human bronchial smooth muscle cells**Stefanelli Fabio**

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The regular use of β_2 -adrenoceptor (β_2 -AR) agonists in the treatment of asthma has been associated with receptor desensitization which limits their efficacy and increases the risk of severe adverse reactions (1). Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-dependent transcription factors that play a role in the pathophysiology of lung-related diseases (2). The aim of the present study was to investigate the effect of the combination between the selective PPAR- γ agonist, prostaglandin J_2 (PGJ₂), and albuterol on airway smooth muscle responsiveness *in vitro*. An *in vitro* model of homologous β_2 -AR desensitization was performed in human bronchial smooth muscle cells chronically exposed to albuterol. Intracellular cAMP levels were measured by the cAMP-Glo™ assay, β_2 -AR gene expression was assessed by RT-PCR, and the presence of PPARs in cell nuclei was analyzed by electrophoretic mobility shift assay (EMSA). After albuterol treatment in the concentration range of 10^{-4} - 10^{-9} M, a dose-dependent increase in the intracellular cAMP levels was observed; the EC₅₀ mean value was 0.46 ± 0.13 μ M. Homologous β_2 -AR desensitization reduced albuterol responsiveness by 75% with respect to control samples. β_2 -AR down-regulation did not seem to be involved in homologous desensitization induced by albuterol since the β_2 -AR/GAPDH expression ratio was not altered significantly in desensitized vs control cells (0.78 ± 0.14 and 0.71 ± 0.12 , respectively; $P=0.37$). PGJ₂ at 10 μ M for 24 h was able to protect from β_2 -AR homologous desensitization, while PGJ₂ did not affect albuterol responsiveness in non-desensitized cells. Finally, the nuclear PPAR proteins were identified by EMSA in cell nuclei. In conclusion, these preliminary findings suggest the presence of a potential interaction between PGJ₂ and albuterol which may represent the basis for developing novel therapeutic strategies in the treatment of chronic pulmonary disease.

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Involvement of nitric oxide/ Ca^{2+} /calmodulin/MAP kinase extracellular signal regulated protein kinase signalling pathway in interleukin-1 β -induced astrocyte activation and neuron survival after brain injury

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Astroglial reactivity is a characteristic manifestation of brain pathology after many types of insults. It is characterized predominantly by cell proliferation (hyperplasia), morphological changes, such as hypertrophy, and an increased expression of glial fibrillary acidic protein (GFAP). A long term result of this astrocytic reaction is the formation of a densely interwoven glial scar at the lesion site, which is considered the major impediment to axonal regeneration after brain damage (1). Even though emerging evidences implicate a role for interleukin-1 β (IL-1 β) in the regulation of astrogliosis (2), the signalling pathway underlying this effect remains to be elucidated. In the present study, we investigated whether NO, intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), calmodulin and p42/44 extracellular signal-regulated protein kinase (ERK) were involved and the role played by them in neurodegenerative brain damage. *In vitro* experiments: serum starved human astrocytoma U-373 MG cells were treated with IL-1 β (0.1, 1, 10, and 50 ng/ml) for 1 h and then reincubated for different (6, 12, 24, or 48 h) periods in fresh medium. Phospho ERK and GFAP activities, cell proliferation and apoptotic cell death were assessed either by Western blotting assay or by immunocytochemistry followed by incubation with primary (anti-phospho-p42/44, anti-GFAP, or anti caspase-3) and secondary (immunoflorescent or HRP- conjugated) antibodies. Cell proliferation was determined by cell counting after DAPI staining of cell nuclei. $[\text{Ca}^{2+}]_i$ levels was determined by using Fura 2/AM indicator according to the method of Tsien et al. (3). Intracellular NO levels were measured electrochemically by using an NO electrode. *In vivo* experiments: following stereotaxic injection of IL-1 β (50 ng) into the brain striatum of adult male Sprague Dawley rats, the animals were perfused transcardially, decerebrated and the removed brain frozen at -80°C until use. Brain cryosections (25 μm) were obtained and processed for single or double immunohistochemistry. Results, in cells, showed that low IL-1 β concentrations induced a concentration-dependent ERK activation which paralleled upregulation of cell division and GFAP expression whereas high levels gradually reversed these responses. Pre-treatment with unselective or selective iNOS inhibitors, L-NAME and 1400W, respectively, antagonised IL-1 β -induced upregulation of cell proliferation, ERK and GFAP expression. Blocking endoplasmic reticulum Ca^{2+} release with ryanodine plus 2APB, or inhibiting calmodulin with W7, downregulated IL-1 β -induced cell proliferation as well as ERK and GFAP expression. The cytokine induced an increase of cellular NO levels which preceded by 15 min that of Ca^{2+} . *In vivo* results confirmed these data and showed that 48 h after brain insult, the IL-1 β treated animals presented a reduced number of apoptotic neurons compared to sham operated animals ($63.7 \pm 11.2\%$; $P < 0.01$) and this effect was reversed by ERK inhibition. These data identified the NO/ Ca^{2+} /calmodulin/ERK signalling pathway as a novel mechanism mediating IL-1 β -induced astrogliosis and neuronal survival. Therefore, this signalling may represent a new potential target for therapeutic intervention in neurodegenerative disorders.

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The nociceptin/orphanin FQ system modulates alcohol consumption and withdrawal associated to intoxication**Stopponi Serena**

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Alcoholism is a chronic relapsing disorder characterized by compulsive drug-seeking and use. Stress and environmental conditioning represent the major determinants of relapse in abstinent individuals. The significance of these factors for relapse risk is well documented also in the animal literature where using various reinstatement paradigms has consistently been shown that presentation of cues predictive of alcohol availability or footshock stress elicit reinstatement of ethanol-seeking behaviour in drug-free animals. Current literature demonstrates that cue reactivity is primarily under the control of the opioidergic, dopaminergic, and glutamatergic systems. On the other hand, stress-induced relapse is mainly controlled by the corticotropin-releasing factor (CRF) system. Pharmacological manipulation with the nonselective opioid antagonist naltrexone blunt cue reactivity in humans and reduces conditioned reinstatement of alcohol-seeking in laboratory animals. This compound is, however, ineffective in controlling reactivity to stress. Conversely, antagonism at CRF receptors results in prevention of stress but not cue-induced relapse. Nociceptin/orphanin FQ (N/oFQ), a recently isolated neuropeptide, is the endogenous ligand of the opioid receptor-like1 (NOP) receptor. This peptide exerts marked functional antagonist effects on endogenous opioid and corticotrophin-releasing factor (CRF) systems. Moreover, evidence exists that N/oFQ modulates dopaminergic, noradrenergic, and glutamatergic neurotransmission in different brain sites via a presynaptic inhibitory action. Studies conducted in our laboratories have demonstrated that activation of NOP receptors by i.c.v. N/oFQ (0-2 µg/rat) reduces ethanol self-administration, inhibits conditioned reinstatement of ethanol-seeking, and prevents ethanol-induced conditioned place preference in genetically selected alcohol preferring Sardinian Marchigian (msP) rats. Brain microinjection data also showed that the central but not the basolateral amygdala or the bed nucleus of the stria terminalis mediates the inhibitory action of nociceptin on ethanol drinking. Finally, we evaluated the effect of i.c.v. N/oFQ injection on alcohol withdrawal in Wistar rats. For this purpose, animals received an intoxication cycle and, following cessation of alcohol exposure, they were treated with N/oFQ (0, 1, and 3 µg/rat, i.c.v.). Results showed a highly significant reduction of withdrawal score in animals treated with both doses of N/oFQ. Overall these findings suggest that the N/oFQ-NOP system may have an important role in the control of alcohol related behaviours and identify this system as a promising target for “anti-relapse” medications.

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Modulatory role of the nociceptin/orphanin FQ-NOP receptor system in the allergen-induced airway hyperresponsiveness**Sullo Nikol**

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Asthma is a complex disease characterized by airway hyperresponsiveness (AHR) and chronic airway inflammation. Pharmacological studies have demonstrated that many processes involved in asthma, such as bronchoconstriction, mucus hypersecretion and plasma extravasation are mimicked by the release of sensory neuropeptides, such as calcitonin gene-related peptide (CGRP), substance P (SP) and neurokinin A (NKA). Nociceptin/orphanin FQ (N/OFQ), the endogenous ligand for the N/OFQ peptide receptor (NOP), inhibits tachykinin release in airway animal models. In fact, several studies have reported the role of N/OFQ-NOP receptor system in the airway, showing its ability to inhibit the contractions of the guinea pig isolated bronchus induced by electrical field stimulation. In the last two years we have documented a role for the N/OFQ-NOP receptor system in the modulation of capsaicin effects; in fact a reduction in endogenous N/OFQ or the lack of its receptor causes an increase in capsaicin induced bronchoconstriction. Moreover, for the first time, we also documented differential airway responsiveness to capsaicin between naive and ovalbumin-sensitized mice due, at least in part, to decreased endogenous N/OFQ levels in sensitized mice. At the light of these data, we investigated a possible involvement of N/OFQ-NOP receptor system in the mechanisms of airway sensitization, evaluating in particular a possible modulatory role of this system in ovalbumine sensitized mice. Balb/C mice were used through the study. The animals were systemically sensitized with i.p. injections of ovalbumine (OVA) and alum (0.4 µg/ml) on days 0 and 7. In a first group of animals, the NOP receptor agonist UFP-112 (0.1 µM) or antagonist UFP-101 (1 µM) were administered i.p., on day 0 and 7, 30 min before allergen injection. A second group of mice was treated with NOP receptor agonist or antagonist through osmotic pumps, implanted 24 h before allergen injection. Finally a third group of mice was treated, 2 days before allergen sensitization, with aerosol challenge of UFP-112 or UFP-101 for 5 days. On day 21, all the mice has been used for *in vivo* and *in vitro* evaluations: airway reactivity to acetylcholine (ACh) (10^{-9} - 10^{-4} M) and airway inflammation [cytokines and total cell count in bronchoalveolar lavage (BAL)] has been evaluated. In both *in vivo* and *in vitro* evaluations, treatment with agonist UFP-112 or antagonist UFP-101 significantly decreased or increased the bronchial responsiveness to ACh, respectively, in lung of sensitized mice but not in naive mice. Moreover, the NOP receptor agonist was able to reduce the airway inflammation, modulating the inflammatory cells and pro/anti inflammatory cytokines in the BAL, such as IL-2, IL-4, and IL-17. In conclusion, these results show a possible modulatory role for the N/OFQ-NOP receptor system in the allergen-induced hyperresponsiveness and airway inflammation, suggesting this system as possible target for novel therapies in pulmonary allergic inflammation and its related pathology.

Role of purinergic P1 and P2 receptors on synaptic transmission, neuronal survival, astrocytic response, and MAPK activation during oxygen-glucose deprivation in the rat CA1 hippocampus

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In the central nervous system (CNS), purines play a key role both in neurotransmission and in neuromodulation, acting through specific receptors divided into two subfamilies: adenosinergic P1 and ATPergic P2 (1). Currently, 4 metabotropic subtypes for P1 receptors (A_1 , A_{2A} , A_{2B} , A_3), 7 ionotropic (P2X), and 8 metabotropic subtypes (P2Y) for P2 receptors have been cloned. All purine receptors are expressed in many brain areas, including the hippocampus. The role of A_{2A} receptors and P2 receptors during ischemia, induced by oxygen and glucose deprivation (OGD), in the CA1 region of rat hippocampal slices was investigated. We conducted extracellular recordings of CA1 field excitatory post-synaptic potentials (fEPSPs) followed by histochemical (propidium iodide; PI) and immunohistochemical techniques coupled to Western blot. Seven-min OGD elicited an irreversible loss of fEPSP and the appearance of anoxic depolarization (AD), an unambiguous sign of neuronal damage (2). In a first series of experiments, we studied the effects of the selective adenosine A_{2A} receptor antagonist, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)phenol (ZM241385, 100-500 nM), during OGD. The antagonist prevented or delayed AD appearance induced by respectively 7- or 30-min OGD and protected from the irreversible fEPSP depression elicited by 7-min OGD. Two different selective adenosine A_{2A} receptor antagonists, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine (SCH58260, 50-500 nM) and 5-amino-2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (SCH442416, 5 nM) resulted less effective than ZM241385 during 7-min OGD. Substantial CA1 pyramidal neuronal damage occurred in control slices whereas injury was significantly prevented by 100 nM ZM241385. Glial fibrillary acidic protein (GFAP) immunostaining showed that 3 h after 7-min OGD definite astrogliosis was appreciable. Western blot analysis indicates an increase in GFAP 30 kDa fragment which was significantly reduced by treatment with 100 nM ZM241385. Thereafter, we tested the role of P2 purinergic receptors by using unselective and selective P2 receptor antagonists: pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS, unselective, 30 μ M), 2'-deoxy- N^6 -methyladenosine 3'-5'-bisphosphate (MRS2179, selective for P2Y₁ receptor, 10 μ M) and brilliant blue G (BBG, selective for P2X₇ receptor, 1 μ M). All antagonists applied during 7-min OGD significantly prevented AD appearance, allowed a significant fEPSP recovery and enhanced neuronal survival, as also confirmed by the reduction in the OGD-induced increase of PI fluorescence. In addition, MRS2179 and PPADS application, *per se*, decreased fEPSP amplitude, unmasking an excitatory tone of P2 receptors under normoxic condition. Western blot analysis indicates that 7-min OGD significantly increased ERK1/2 mitogen-activated protein kinase (MAPK) activation in comparison to control and all P2 antagonists tested significantly reduced ERK1/2 activation. Contrary to ERK1/2, 7-min OGD significantly reduced JNK MAPK levels in comparison to control. Among P2 receptor antagonists tested, PPADS and MRS2179 tended to reverse this effect. In agreement to biochemical data, hippocampal slices incubated with ERK1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene (U0126, 10 μ M) showed a significant fEPSP recovery after 7-min OGD. JNK inhibitor anthra[1,9-cd]pyrazol-6(2H)-one (SP600125, 20 μ M) did not affect synaptic recovery. In the CA1 hippocampus, the antagonism produced by ZM241385, PPADS, MRS2179 or BBG is protective during ischemia, indicating that A_{2A} adenosine and P2 purinergic receptors play a deleterious role during a severe OGD insult in hippocampal slices.

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Signalling pathways involved in the apoptotic action of ouabain in two different cancer cell lines**Trenti Annalisa**

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Cardiac glycosides, like ouabain, are specific inhibitors of plasma membrane Na^+/K^+ -ATPase, enzyme responsible for translocating Na^+ and K^+ ions across cell membrane using ATP as energy source. Recent findings suggest for Na^+/K^+ -ATPase a role as signal transducer, involved in the control of cell proliferation and growth or in the protection against apoptotic stimuli. In fact, the binding of ouabain to Na^+/K^+ -ATPase (at concentrations that do not inhibit the pump activity) triggers a complex signaling cascade that is initiated by interacting with neighboring membrane proteins and organized cytosolic cascades of signaling molecules. These signaling complexes send messages to intracellular organelles via the activation of the protein tyrosine kinase Src, transactivation of epidermal growth factor receptor (EGFR) by Src, activation of Ras and the extracellular signal-regulated kinase (ERK). Also a ROS dependent c-Jun N-terminal kinase (JNK) activation has been shown to be involved in the pathways activated by ouabain (1). In agreement with these findings, we have previously showed that ouabain has an antiapoptotic effect on HUVEC through the activation of phosphoinositide-3 kinase (PI3K) and ERK (2). On the other hand, several studies have suggested that cardiac glycosides may have an anticancer utilization. Evidences of the antineoplastic potential of cardiac glycosides have been obtained with *in vitro* studies, but the most relevant evidences of the beneficial effects of cardiac glycosides in cancer treatment were drawn from epidemiological data. The death rate and cancer recurrence turned out to be lower in women with breast cancer treated with digitalis than in non-treated patients. Moreover, it was observed a reduced incidence of leukemia/lymphoma and kidney/urinary tract tumours in subjects with elevated plasmatic concentrations of digitoxin (3). The aim of my doctoral work is to characterize the effect of nM concentrations of ouabain on two cancer cell lines: Jurkat (immortalized cell line of T lymphocytes) and A549 (carcinomic human alveolar basal epithelial cells) with particular attention to the signaling pathway involved. Cell treatment with ouabain (1-100 nM) for 24 h induced a concentration-dependent decrease in cell viability measured by the MTT reduction assay both in Jurkat and A549 cells. The decrease in cell viability at 100 nM was 67.0 ± 2.2 e 70.0 ± 2.1 in Jurkat and A549, respectively. Incubation of both cell lines with 100 nM ouabain for 24 h induced a significant raise in the number of apoptotic cells, as indicated by flow cytometric analysis of annexin V/propidium iodide binding: ouabain increased the proportion of annexin V positive (apoptotic) cells from $7.0 \pm 0.7\%$ to $33.0 \pm 6.0\%$ in Jurkat cells and from $7.0 \pm 0.6\%$ to $22.0 \pm 2.0\%$ in A549 cells. However, an increase in caspase-3 activation induced by ouabain treatment was observed only in Jurkat cells. In order to clarify the signaling pathways involved in the mechanism of action of ouabain, the role of ERK, Src kinase and JNK was investigated. A transient increase in ERK1/2 phosphorylation (determined by Western blotting analysis) was observed in Jurkat cells treated with 100 nM ouabain for 30 min. Nevertheless, incubation of the cells with a specific MEK inhibitor, PD98059 (25 μM) or U0126 (10 μM) did not abolish the apoptotic effect of ouabain, as shown by MTT test and flow cytometric analysis of annexin V/PI binding. Similar results were obtained with A549 cells. Furthermore, treatment of both tumour cell lines with the inhibitor of JNK, SP600125, did not affect the apoptotic action of ouabain. To ascertain whether the activation of Src kinase is required for the ouabain-effect on cancer cells we used the Src-kinase inhibitor PP2 (50 μM). This compound did not affect the ouabain-induced apoptosis in both Jurkat and A549 cells. Our results show that nM concentrations of ouabain are pro-apoptotic for Jurkat and A549 cells through a mechanism that does not involve the classical pathways of signal transduction activated by ouabain in non-tumoral cells.

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Hippocampal transcriptional profiles associated with vulnerability and resilience to stress in a behavioural model of depression**Valensisi Cristina**

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Depression is one of the top ten causes of morbidity and mortality worldwide and its prevalence is increasing. Despite this, unravelling the pathophysiology of depression is still a challenge. Major depression is a highly heritable disorder and genetic predispositions are thought to interact with environmental risk factors, among which stress is the most relevant, which can initiate a depressive episode in some patients. Notably, humans differ greatly in their way to cope with adversity and stressful life events. Although strong evidence exist that stressful events may be causally related with the outcome of depression, not everyone who experiences stressful life events become ill. Animal models have recently been used to provide some neurobiological insights into these clinical observations (1,2); but still little is known about the mechanisms that contribute to adaptive and resilient responses to stressors. To address this issue, we used a behavioural paradigm of depression, the chronic escape deficit (CED) model (3,4), in which exposure to intense and unavoidable stress (pre-test session) is able to modify the ability of the animals to avoid an adverse stimulus. This behavioural alteration is referred to as the escape deficit. The model begins as an acute escape deficit which can be indefinitely sustained by repeated administration of mild stressors. In this study we focused our attention on the transcriptional events in the hippocampus, as accumulating evidence suggests that maladaptive neuroplastic changes, occurring in this limbic area following stress, may be causally related to the development of depression. Clinical and preclinical studies support a crucial role of stress in altering neuroplastic process and adult neurogenesis in the hippocampus, supporting a correlation between these effects and depression as well as the outcomes of antidepressant treatments. In particular we addressed the early transcriptional events possibly involved in initiating long-lasting neuroplastic changes induced by a strong stressful procedure able to affect animal behaviour. Our behavioural results showed that exposure to an intense and unavoidable stressful procedure induced in animals the escape deficit when the rats were tested the day after the pre-test session for the capacity to avoid an adverse stimulus, but only in 60% of them. On the contrary, the remaining 40% of rats showed a behaviour not statistically different from control animals, which did not undergo the stressful procedure. To investigate transcriptional profiles in the hippocampus we used GeneChip Rat Exon Array (Affymetrix) technology, which provides both gene expression and alternative splicing analysis. Gene expression profiles were compared and functional analyses were performed between animals exposed to unavoidable stress and control animals, which never experienced stressful procedures. Multiple pathways were found that may be involved in the underlying mechanisms of a stress condition associated with escape deficit and that may mediate the effects of stress on hippocampal plasticity. Likewise gene expression profiling of stress-vulnerable and stress-resilient rats revealed distinct transcriptional profiles in the hippocampus, as has already been observed in other models of depression, such as the chronic mild stress models (1), as well as in the mesolimbic dopamine circuit in the social-defeat model (2). Taken together with these results, our data suggest that resilient behaviour represents a distinct, active neurobiological process, not simply the absence of vulnerability. The identification of anti-vulnerability processes may help to unravel the neurobiology of stress and the pathophysiology of depression. Moreover understanding the mechanisms underlying resilience to stress could provide insights in the discovery of new antidepressant drugs.

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Antinociceptive activity of soy isoflavone genistein in mouse models of painful diabetic and nerve injury-induced neuropathy: involved mechanisms**Valsecchi Anna Elisa**

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Nerve injuries or dysfunctions in the peripheral and central nervous system (CNS) are the leading causes for the development of neuropathies, which are frequently associated with allodynia (pain to previously innocuous stimuli) and hyperalgesia (a heightened pain response generated by a painful stimulus). Treatment of these disorders is often unsatisfactory due to side effects or insufficient analgesia of the currently available drugs. My doctoral work has examined the therapeutic effect of genistein, the major isoflavone in soy, against painful hypersensitivity induced in the chronic constriction injury (CCI) and streptozotocin (STZ) mouse models. The peripheral mononeuropathy was induced by right sciatic nerve CCI according to Bennett and Xie (1), diabetes was induced by a single injection of STZ (120 mg/kg, i.p.) in C57BL/6J adult male mice. Von Frey filament and Plantar test were used to examine changes in paw withdrawal threshold values. A treatment regimen with greater clinical applicability would involve compounds that are efficacious at reversing neuropathic pain symptoms once they are established, so three days after CCI and 14 days after STZ injection, genistein was injected subcutaneously daily for 11 days and over three weeks, respectively. The isoflavone repeated administration reversed thermal hyperalgesia and mechanical allodynia in nerve injured mice and mechanical allodynia in diabetic mice. Surely classical estrogen receptors (ERs), particularly ER β , are involved in phytochemical antinociceptive activity in CCI model, since a specific ER β antagonist reversed its antiallodynic and antihyperalgesic action. It is worth bearing in mind that genistein binds ERs with higher affinity for the ER β (2) that is particularly present in neurons, microglia, astrocytes (3), Schwann cells (4), and immune cells (5), but less expressed than ER α in hormone-dependent tissues (2). In pathological nociceptive disorders and particularly in the induction of painful peripheral neuropathy, reactive radical species are generated in excess of the regulatory capacity of the endogenous antioxidants to control them. Antioxidant effects are also implicated in the antinociceptive genistein activity, since the efficacious doses reversed the increase in ROS and malondialdehyde and increased or restored the activity of antioxidant enzymes and the content of reduced glutathione. Neuropathic pain is partially mediated by neuroinflammatory mechanisms and it also modulates local neurogenic inflammation. In fact, the release of inflammatory mediators from immune and glial cells in either peripheral and CNS may have an important role in the development and the maintenance of the neuropathic pain physiopathological processes. Genistein efficacy was due also to its neuroimmunomodulatory and anti-inflammatory properties, since it reduced peripheral and central NF- κ B, NO system, and proinflammatory cytokine overactivation in both peripheral neuropathy models. In conclusion, these results suggest that soy isoflavone genistein ameliorates the CCI- and diabetes-induced nociceptive hypersensitivity by its antioxidant, antiinflammatory, and neuroimmunomodulatory properties thus representing a possible therapeutic hope to treat neuropathic pain that is still now devoid of satisfactorily effective treatments.

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Purified phosphatidylglycerol from alveolar surfactant for studies with stable isotopes tracers**Vedovelli Luca F.**

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Phosphatidylglycerol (PG) is a key component of the alveolar surfactant, a mixture of lipids and proteins that permits normal breathing by reducing near to zero the alveolar surface tension. PG binds the hydrophilic etheropeptide KL-4 of surfactant specific protein B (SP-B) and the formed complex is fundamentally involved in lowering superficial tension (1), avoiding alveolar collapse during compression (expire). Interestingly, alveolar surfactant is where PG is most abundant in whole human body. PG is synthesized from early neonatal life and low PG percentage in alveolar surfactant is an index of pulmonary immaturity. Clinically, PG is involved in serious diseases like cystic fibrosis, acute respiratory distress syndrome in preterm infants (RDS) and adults (ARDS). In particular, ARDS patients shows a dramatically decrease of PG content in alveolar surfactant. This fact is exacerbated in patients who do not survive to the disease (2). Moreover, dioleoyl-PG was shown to inhibit secretion of secretory phospholipase A₂-II (sPLA₂-II) in alveolar macrophages by the inactivation of tumour necrosis factor α (TNF- α) and of nuclear factor κ -B (NF- κ B) (3). Stable isotopes tracers were showed in the last ten years as a powerful, safe, and non-invasive technique to understand kinetics of a significant number of pathways. In this research, we coupled PG purification with stable isotopes studies to understand its role in pulmonary diseases, specifically ARDS. The aim was to obtain highly purified PG from alveolar surfactant, readily to be applied in stable isotopes researches on pulmonary diseases like ARDS. Preliminary phosphorus (4) assay was made on the tracheal aspirate sample (TA) to avoid column saturation. Two aliquots of TA containing about 100 μ g of phospholipids were analyzed in duplicate: one aliquot was added with U-¹³C DPPC (0.5% of total phospholipids amount in the TA) to assess the PG purity by measuring the ¹³C contamination in the PG spot. Surfactant phospholipids were extracted (5) from both TA aliquots. Lipids were eluted in a 100 mg silica-NH₂ column (6) to separate PG from dipalmitoyl-phosphatidylcholine (DPPC), the most abundant phospholipid of alveolar surfactant. Five separate fractions were collected and the PG fraction eluted from the column with a mixture of chloroform/ethanol/water/triethylamine (30/35/7/35). The PG fraction was additionally separated from phosphatidyl-inositol (PI) and phosphatidyl-serine (PS) by thin layer chromatography (TLC) (7,8). PG identification was assessed by comparing retention factors (R_f) of lipids and lipids standards. Purity of the PG spots was assessed by measuring its ¹³C enrichments by gas-chromatography mass spectrometry (GC-MS) in duplicate. Mean sample PG R_f was 0.670 \pm 0.005 and a mean R_f of standard PG was 0.660 \pm 0.010. Mean sample DPPC R_f was 0.120 \pm 0.005 and a mean R_f of standard DPPC was 0.120 \pm 0.005. PG and DPPC mole percent excess (MPE) of the TA aliquot without the U-¹³C DPPC addition were 0.240 \pm 0.010% and DPPC 0.0250 \pm 0.003% MPE respectively; PG and DPPC MPE of the TA aliquot with U-¹³C DPPC were 0.210 \pm 0.003% and 13.9 \pm 1.0% MPE, respectively. In conclusion, the isolated surfactant PG did not show DPPC contamination. This method permit to plan future studies on surfactant PG metabolism in humans by stable isotope technique.

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Optimization of a co-culture system of hepatocytes and colorectal tumour cells to set up a bioreactor as an *in vitro* metastatic progression model**Vidimar Vania**

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Nowadays the main goal of solid tumour chemotherapy is the treatment of metastases, primary cause of death in most of the cancerous diseases. Growth of metastases is a highly selective, non-random process and takes place preferentially in organs that, in some way, provide a suitable environment (1). This study is part of a project developed in collaboration with the department of Engineering and Material Chemistry of the Università degli Studi di Trieste whose long-term aim is to set up a model system that allows simulating *in vitro* the metastatic dissemination of tumours on healthy tissues. This project involves the design of a prototype (bioreactor) consisting of two culture wells linked by an internal circuit. The first well is intended to contain cells to mimic a situation that occurs in a primary tumour, while the second well contains healthy cells to simulate a target tissue/organ; the internal fluidic system is used to mimic a blood vessel that allows the passage of a cell from the first to the second well. This system could be useful to identify innovative anticancer drugs and to evaluate their antimetastatic effect to find out a reliable alternative to *in vivo* models. Colorectal cancer, which is one of the most widespread types of solid tumour, is chosen as model for this study since it is known that it frequently evolves into liver metastasis and this represents the second cause of the cancer-related deaths after lung carcinoma (2). In order to recreate a metastatic colorectal tumour model, HT-29 cells (human colon adenocarcinoma cell line) are chosen as invasive and malignant cells; human non-malignant colon epithelial cell line (HCEC) is used to mimic a normal epithelial tissue derived from colon and (immortalized human hepatocytes cell line (IHH) is chosen to mimic the healthy hepatic tissue. The first issue of this study was to set up the optimal environment to simulate the physiopathological process of metastatization and liver invasion. Now we are trying to develop a co-culture system in which to grow together metastatic HT-29 cells and IHH healthy cells; the main obstacle we found while trying to establish a protocol for the co-culture experiments was to define optimal cell culture conditions which allow both cell lines to grow. Because IHH cells require a medium supplemented with insulin and dexamethasone (3), which are critical for maintaining the characteristics of differentiated hepatocytes of this cell line, we adapted the HT-29 cells to grow in the IHH medium. The effects of the new medium on HT-29 cells growth and proliferation were evaluated by different methods. We have seen that the number of cells does not change over time, but mitochondrial dehydrogenase activity measured by MTT assay is significantly decreased when HT-29 are grown in IHH medium in comparison to those kept in the conventional medium. Moreover, by performing SRB assay, it seems that there are no significant differences between the protein content of HT-29 cultivated in their own medium and in IHH medium. We have also decided to evaluate whether HT-29 cells adapted to IHH medium had retained the ability to migrate and invade using also IHH cells as a stimulus in a modified Boyden chamber. Simultaneously, we are analyzing the effect of three reference drugs for the treatment of colorectal cancer (irinotecan, 5- fluorouracil, and oxaliplatin) on HT-29 cells cultivated in IHH medium to evaluate whether this adaptation could vary their sensitivity to these drugs already known for their activity. Preliminary MTT assays show there is a significant decrease in the activity of the three drugs on HT-29 cultivate in IHH medium as compared to their own medium. Because the medium in which HT-29 are cultivated can influence many parameters, such as their proliferation rate and the effect of drugs, we are trying to understand if some components of IHH medium (such as insulin, dexamethasone or the higher content of glucose) could influence these parameters.

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Molecular and functional analysis of *Drosophila* EFHC1 gene**Zanarella Erica**

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Juvenile myoclonic epilepsy (JME), the most common cause of grand mal seizures, accounts for 3-12% of all epilepsies. This pathology is a collection of seizure patterns that are clinically distinct from those in other forms of idiopathic generalized epilepsies (IGEs), with the major characteristic being adolescence-onset myoclonic seizures. In 2004, Suzuki and collaborators (1) found 6 missense mutations in the EJM1A gene in chromosome 6p12 segregating in 25 epilepsy affected members of 6 unrelated families. This gene encodes for a protein called EFHC1. EFHC1 or myoclonin 1 is a protein of 640 aminoacids containing three DM10 domains, whose function is unknown, and an EF-hand motif, a typical domain of Ca^{2+} modulators (2). We have identified two *Drosophila* homologs (CG8959 and CG11048) of myoclonin/EFHC1. We are now using *Drosophila* as a model to study the function of CG8959 gene in normal development and in pathology to investigate the mechanisms whereby mutation of myoclonin causes human disease. Preliminary results suggest that ubiquitous CG8959 expression causes partial loss of veins in the adult wing. Because developmental vein patterning in the *Drosophila* wing is controlled by the coordinated action of several signalling pathways we are testing whether CG8959 is involved in the Notch and EGFR pathways. Moreover we are conducting a detailed analysis of the loss and gain of function phenotypes in the neuromuscular junction to verify whether this protein has a role in neuromuscular junction development or growth.

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Rapid detection and quantization by imaging mass spectrometry of curcumin and curcumin metabolites in rat tissue**Zivolic Fabio**

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My doctoral work will deal with the development of rapid analytical methods, based on MALDI mass spectrometry, for the study of the distribution of curcumin and curcumin metabolites in different rat tissues. Curcumin, a yellow pigment, is a polyphenol present in the rhizomes of the perennial herb *Curcuma longa*. It is the main ingredient of the spices turmeric, curry, and mustard and is used as a food colouring (E100). Curcumin has been used in traditional Asian medicine for over 2000 years and exhibits various biological activities as anti-inflammatory, anti-cancer, and potent antioxidant (1,2). The aim of this study is to investigate on the pharmacokinetic properties and pleiotropy of curcumin in rats, by i.p. administration and tissue examination after 2, 4, and 8 h. Frozen sections of the different rat tissues (brain, kidney, and liver) are placed on a stainless steel target plate or on a conductive slide, and then coated with a solution of MALDI matrix. The choice of matrix is critical, because the matrix ions can interfere with those of the analytes. For this reason different matrices and different operative conditions (in particular laser irradiance) will be tested. Once the most effective matrix is found out, each tissue will be coated with matrix crystal by a Sieve-based device (3). It will be dried and analysed by Matrix-assisted Laser Desorption/Ionization (MALDI) imaging (4), to get the distribution of the analyte of interest in the different portions of the tissue.

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Herpes Simplex Virus type 1 infection of myenteric ganglia: an *in vivo* animal model of small bowel neuromuscular dysfunction**Zoppellaro Chiara**

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Gastrointestinal motor disorders (GIMD) are a clinically heterogeneous group of diseases in which symptoms are presumed or proven to arise as a result of neuromuscular dysfunction (1). Infectious agents, such as neurotropic viruses, known to infect the central nervous system, have been suggested to infect and disrupt enteric nervous system (ENS) integrity (2). Among common pathogens Herpes Simplex Virus type 1 (HSV-1), orally inoculated to laboratory animals, targets neurons in the ENS and seems to be involved in the pathogenesis of GIMD (3). Moreover, HSV-1 induction of inflammatory cytokines is mediated by Toll-like receptor (TLR)-2, a member of TLR family that plays a critical role in the early innate immune response to invading pathogens (4). The aims of the present study were to evaluate the effect of HSV-1 infection on gut contractility and the influence of TLR-2 signalling in these changes. Male Wistar rats and C57Bl/6 mice were inoculated with HSV-1 intranasally and, after 4 weeks, intragastrically (IG). After 1-10 weeks, HSV-1 infection was determined in the brain and in freshly isolated myenteric ganglia by molecular analysis; neuromuscular contractility was evaluated by pharmacological/electrical stimulation of ileum segments, mounted vertically in organ baths. No signs of illness and histological abnormalities were observed in the gut of IG infected rodents. In the brain and ENS, HSV-1 established a latent infection demonstrated by the presence of viral tk-DNA and latency associated transcripts mRNAs. In rat ileum segments at 1 and 6 weeks post HSV-1 IG dose, a significant upward shift of the concentration-response curve to the muscarinic agonist carbachol (0.1-100 μ M) was observed, with a consequent increase in E_{max} values over control and no changes in pD_2 values. This suggests that the increased contractility is not due to altered sensitivity of muscarinic receptors. HSV-1 significantly increased contractions induced by 5 mM $CaCl_2$ in rat ileum segments maintained in a depolarizing Ca^{2+} -free salt solution at 1 and 6 weeks postinfection (PI); pretreatment with 0.1 μ M verapamil, an L-type Ca^{2+} channel blocker, significantly reduced tension increase in response to Ca^{2+} in sham as well as HSV-1 infected rats. Six weeks after the IG rechallenge, the blocker abolished the Ca^{2+} -induced responsiveness to HSV-1, indicating a role for voltage gated Ca^{2+} channels in the altered response to Ca^{2+} . Neurally-mediated contractions (electrical field stimulation, EFS; 2-40 Hz) and maximum response to KCl (3-80 mM) were significantly modified only at 1 week in HSV-1 infected rats. Tension increase evoked by 0.1-15 μ M R-PIA, a stable adenosine analogue, was augmented 1 week PI, whereas reduction of 0.1-1.25 mM adenosine-induced contractions occurred in 1 and 6 weeks inoculated rats, and was limited to the highest concentrations of the nucleoside. The contractile response to 0.1-1 mM ATP showed a clear concentration-dependency only in preparations from HSV-1 infected rats and was augmented at 1 week PI after IG challenge. Furthermore, relaxations of ileum segments induced by EFS (10 Hz) in the presence of 1 μ M atropine and 3 μ M guanethidine were significantly increased at 1 and 6 weeks PI. In TLR-2 deficient (KO), like in 1 week infected mice, ileum responses to 1 μ M carbachol and to 60 mM KCl were significantly increased, while EFS-evoked contractions (20 Hz) were decreased. These changes observed in TLR-2 KO animals were not affected by HSV-1 treatment (1 week), except for the contractions induced by carbachol that were further enhanced by HSV-1 infection. Following IG delivery, HSV-1 establishes a latent infection in the ENS affecting significantly gut contractility, enteric neurotransmission, and purinergic response. HSV-1 infection of the ENS appears to be an innovative animal model to uncover the basic mechanisms of the enteric neuropathies affecting gut motor function.

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Neurobehavioural and biochemical alterations in soluble β -amyloid-treated rats**Zotti Margherita**Dipartimento di Scienze Biomediche, Università degli Studi di Foggia, v.le Pinto 1, 71100
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The main features of Alzheimer's disease (AD) are represented, from a behavioural standpoint, by a progressive decline in cognitive functions (1). On the other hand, non-cognitive symptoms commonly accompany behavioural changes in AD, including neuropsychiatric symptoms, such as psychosis or depression that derive from neurobiological changes in specific brain areas. Several lines of recent evidences suggest that early memory deficits and neuropsychiatric symptoms may be explained by the presence of soluble forms of beta amyloid ($A\beta$) rather than by the form aggregated into insoluble plaques (2). Preclinical investigations have demonstrated deleterious effects of $A\beta$ on neurotransmitters related to major depression, such as serotonin (5-HT), raising the possibility that increased brain $A\beta$ levels may contribute to the development of depressive symptoms. Preservation of key neurotransmitters and neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) is considered to be crucial for the regulation of synaptic plasticity and neuronal survival, and a decline in their function is related to neurodegenerative disorders such as AD (3,4). Moreover, several studies have proposed these neurotrophins as a bridge between depression and AD (5). In this prospective, the aim of our study was to investigate whether an acute i.c.v. injection of soluble $A\beta$ was associated with impairment of short-term memory and whether the peptide was able to evoke a behavioural alteration linked to depressive state. Furthermore, we investigated whether soluble $A\beta$ could affect serotonergic neurotransmission in a key region involved in working memory and depression, namely prefrontal cortex (PFC). In addition, the effects of soluble $A\beta$ on BDNF and NGF levels were evaluated in the same area. Soluble $A\beta$, 7 days after a single dose, did not produce any deficit in cognitive and motor functions assessed, since no differences were found in parameters tested in the novel object recognition test and in the open field test in all experimental groups. Conversely, $A\beta$, at the same time point, induced a depressive-like state since, in the forced swimming test, immobility time was significantly increased in $A\beta$ -injected compared to either sham-operated or intact rats ($P<0.05$). Behavioural results were well corroborated by neurochemical analyses, since we found a significant reduction in 5-HT content in PFC of $A\beta$ -injected rats compared to intact animals ($P<0.05$). Likewise, PFC BDNF ($P<0.01$) and NGF ($P<0.05$) mRNA levels were significantly reduced only in $A\beta$ -injected rats. In conclusion, our study indicates for the first time that soluble $A\beta$ might be a critical player in producing functional and biochemical deficits linked to depression before any cognitive deficit become apparent. This approach will provide a fruitful basis for questioning not only the mechanisms underlying the acute effects of soluble $A\beta$, but also possible targets for therapeutic intervention in very early stages of the disease.

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