



IN – INSTITUTE OF NEUROSCIENCE
 Annual retreat – 2016
 Padova, Sept. 13-16, 2016
POSTER SCHEDULE & ABSTRACTS



N	Presenting Author	Title
Poster session 1. Physiology and biochemistry of behaviour and reward		
Attendance: Tuesday, 18:00-19:00		
P1.1	Anna Lisa Muntoni	PPAR- α modulation of mesolimbic dopamine transmission rescues depression-related behaviours
P1.2	Daniela Braida	MDMA, DOB and PMA induce rewarding and behavioural changes through serotonergic system in zebrafish
P1.3	Marta Busnelli	Novel targets and therapeutics for social impairments
P1.4	Luisa Ponzoni	Behavioural and neurochemical changes induced by Δ^9 -tetrahydrocannabinol in mice pre-exposed to nicotine
P1.5	Irene Lorrai	Suppressing effect of Saikosaponin A, an active ingredient of <i>Bupleurum falcatum</i> , on alcohol and chocolate self-administration in rats
P1.6	Paola Maccioni	Cor659: a novel positive allosteric modulator of the GABA _B receptor with unique <i>in vivo</i> pharmacological profile
P1.7	Liana Fattore	Methoxetamine, a novel psychoactive substance with serious adverse pharmacological effects
Poster session 2. Neurological diseases		
Attendance: Tuesday, 18:00-19:00		
P2.1	Silvia Ferrara	Mouse model of Polymicrogyria: proposal of therapeutic approaches
P2.2	Laura Baroncelli	Cortical plasticity in retinitis pigmentosa
P2.3	Silvia Bassani	Protocadherin-19 regulates GABA _A R surface expression and currents
P2.4	Emanuela Dazzo	Secretion-positive LGI1 Mutations Linked to Lateral Temporal Epilepsy Impair Binding to ADAM22 and ADAM23 Receptors
P2.5	Barbara Grillo	A possible pharmacological target to interfere with epilepsy in a Mecp ^{Y/-} mouse model
P2.6	Giovanna Crivellaro	Defective glutamate and K ⁺ clearance by cortical astrocytes in a mouse model of migraine with reduced expression of the glial alpha2
P2.7	Andrea Maset	OPHN1 regulates the migration of newly generated cells in the olfactory system
P2.8	Luisa Galla	Role of LRRK2 in neuronal connectivity in the olfactory system
P2.9	Nelly Redolfi	Mutation of OPHN1 impairs adult born granule cells development and network activity in the olfactory bulb
P2.10	Maura Francolini	The selective inhibition of the persistent sodium current INaP reverts precocious inter- and motoneurons hyperexcitability in the Sod1-G93R zebrafish ALS model
P2.11	Lara Pizzamiglio	New role of ATM in hippocampal neurons during development
P2.12	Alice Canzi	Hyperactivation of IL-1 pathway during brain development affects neuronal function and plasticity
P2.13	Chiara Elia	Response of healthy versus APP/PS1 microglia to A β 42 exposure
P2.14	Lorenza Culotta	Deciphering the role of SULT4A1 in brain development and Phelan-McDermid Syndrome pathogenesis
P2.15	Laura Gritti	The mutation of X-linked intellectual disability protein IL1RAPL1 alters dendritic arborization and excitatory synapse formation

P2.16	Giovanni Minervini	Heterozygous Reelin mutations cause autosomal-dominant lateral temporal epilepsy: a bioinformatics study
P2.17	Alessandro Papale	GBA expressing bone marrow-derived microglia for widespread and effecting degradation of alpha-synuclein pathological aggregates
P2.18	Marzia Baldareschi	The Florence stroke network: improvement in acute ischemic stroke care
P2.19	Antonio Di Carlo	Stroke awareness among high school students in Tuscany: effectiveness of an educational intervention
Poster session 3. Neurodegeneration and cell death		
Attendance: Tuesday, 18:00-19:00		
P3.1	Antonia Stefanov	Inner retinal remodeling in an inducible mouse model of Retinitis Pigmentosa
P3.2	Giorgia Pallafacchina	Loss-of-function mutations in the <i>SIGMAR1</i> gene cause distal hereditary motor neuropathy by impairing ER-mitochondria tethering and Ca ²⁺ signaling
P3.3	Rosa Pia Norante	Disturbed local Ca ²⁺ homeostasis in ALS astrocytes
P3.4	Maria Catia Sorgato	The Prion Protein Modulates the Activity of Glutamate Ionotropic Receptors and Mitochondrial Ca ²⁺ Uptake in Various Neuronal Types
P3.5	Giulia Zanetti	On the neurotoxicity of two Italian viper venoms
P3.6	Elisa Duregotti	Toxin-induced degeneration of dysfunctional nerve terminals: can the bad turn into the good?
Poster session 4. Aging		
Attendance: Tuesday, 18:00-19:00		
P4.1	Michele Azzolini	Pterostilbene and cognitive performance in the aged rat model: molecular and behavioral effects
P4.2	Ilaria Rocco	Structural equation modeling to analyze the determinants of healthy aging
P4.3	Federica Limongi	Prevalence and conversion to dementia of Mild Cognitive Impairment in an elderly Italian population
Poster session 5. Signalling		
Attendance: Wednesday, 14:00-15:00		
P5.1	Maria Lina Massimino	Generation and validation of novel tools for the analysis of Ca ²⁺ homeostasis in ALS motor neuron
P5.2	Simona Francia	Mechanism of activation and function of the odorant receptor expressed at the axon terminus-growth cone of olfactory sensory
P5.3	Elisabetta Menna	A new molecular pathway for PTX3 in the brain and its role in AMPA receptors regulation
P5.4	Fiorella Tonello	PLA2 snake myotoxins are internalized by cell surface nucleolin: a potential new pathway that leads directly to the cell nucleus
P5.5	Davide Pozzi	Role of the proinflammatory cytokine IL-6 in synaptogenesis
P5.6	Alessandra Gasparini	TANC2: a New Paradigm In Neuronal Signal Transduction
P5.7	Konstantinos Lefkimiatis	Phosphatases are key determinants of compartmentalized cAMP/PKA signaling at the Outer Mitochondrial Membrane
Poster session 6. Gliomas and other cancers		
Attendance: Wednesday, 14:00-15:00		
P6.1	Francesca Fasoli	Pharmacological characterisation of nicotinic acetylcholine receptors expressed in glioma and glioblastoma cells
P6.2	Maria Luisa Malosio	Validation and optimization of aptamers as novel diagnostic and therapeutic tools for pancreatic tumors
P6.3	Vanessa Mucchietto	Activation of α -bungarotoxin-sensitive nicotinic receptors in non small cell lung cancer A459 plays an important role in nicotine-induced proliferation

P6.4	Carlo Nobile	LGI1 tumor tissue expression and serum autoantibodies in patients with primary malignant glioma
P6.5	Luigi Leanza	Targeting mitochondrial potassium channel Kv1.3 to selectively kill glioblastoma cells
Poster session 7. Muscle and Nerve		
Attendance: Wednesday, 14:00-15:00		
P7.1	Samuele Negro	ATP released by injured neurons activates Schwann cells
P7.2	Alessandro Bertoli	Absolute quantification of myosin heavy chain isoforms using selected reaction monitoring highlights skeletal muscle alterations in a mouse model of amyotrophic lateral sclerosis
P7.3	Elisa Lidron	MCU knock-down impairs skeletal muscle and motor neuron development in zebrafish embryos
P7.4	Alessandra Zulian	Protective effect of Alisporivir – a Cyclophilin inhibitor without immunosuppressive activity – in Duchenne muscular dystrophy
P7.5	Marina Campione	SAN dysfunction underlie atrial fibrillation susceptibility in the PITX2 mouse model
Poster session 8. Glia – neuron interactions		
Attendance: Wednesday, 14:00-15:00		
P8.1	Michele Sessolo	Specific GABAergic interneuron signaling to cortical astrocytes
P8.2	Martina Gabrielli	Microglial MVs modulate synaptic transmission in a target-specific way
P8.3	Marta Gomez-Gonzalo	New insights into the mechanism of astrocytic glutamate release that elicits NMDAR-mediated neuronal slow inward currents
Poster session 9. Mitochondria		
Attendance: Wednesday, 14:00-15:00		
P9.1	Mirko Luoni	Exploring the role of Vps13 proteins family using CRISPR/Cas9 system in human cell lines
P9.2	Agnese De Mario	PrP-dependent perturbation of mitochondrial functions and Ca ²⁺ homeostasis by Aβ oligomers in cortical neurons
P9.3	Valentina Giorgio	The Ca ²⁺ regulatory site of the permeability transition pore is within the catalytic core of F-ATP synthase
P9.4	Michela Carraro	Shedding light on the Permeability transition through F-ATP synthase mutagenesis in <i>S. Cerevisiae</i>
P9.5	Marco Schiavone	The idebenone metabolite QS10 is an electron donor to complex III and rescues respiration in complex I-deficient cells and rotenone-treated zebrafish
P9.6	Giulia Rigotto	Study of mitochondria physiology in transgenic mouse models of Alzheimer's Disease
P9.7	Giulietta Di Benedetto	Mitochondrial cAMP signalling is involved in metabolic flexibility
P9.8	Elisa Greotti	mCerulean3-based cameleon sensors to explore mitochondrial Ca ²⁺ dynamics <i>in vivo</i>
Poster session 10. Posters a la carte		
Attendance: Wednesday, 14:00-15:00		
P10.1	Gabriele Sansevero	Intranasal BDNF administration promotes visual function recovery in adult amblyopic subjects
P10.2	Paola Binda	Adult plasticity of early visual areas after monocular deprivation measured with ultra-high field fMRI in humans
P10.3	Umberto Rodella	A novel model of Miller Fisher syndrome to study nerve terminal regeneration
P10.4	Laura Restani	Specificity and functionality of BoNT/A-intoxicated synapses following retrograde transport from the muscle to central neurons

P10.5	Moises Di Sante	The role of monoamine oxidases in formation and maturation of iPSC-derived cardiomyocytes
P10.6	Simona Casarosa	Impaired neuronal differentiation of En2 ^{-/-} neural stem cells
P10.7	Marta Pattaro	Effects of amino acids on insulin kinetics, pancreatic beta-cell function and insulin sensitivity
P10.8	Elena Vezzoli	High pressure freezing unveils the native ultrastructure of mouse hippocampus
P10.9	Marco Righi	Quantification of vascular angioarchitectures in the context of neurodegenerative diseases
P10.10	Lucia Biasutto	Absorption mechanisms of a pterostilbene aminoacidic prodrug
P10.11	Eliana Lauranzano	A Blood Brain Barrier model to investigate immune trafficking in neurological disorders
P10.12	Giovanni Bortolan	Automatic detection of EEG spikes: some experiments
P10.13	Benedetta Piccardi	Reperfusion injury after stroke study (RISKS study)

PPAR-ALPHA MODULATION OF MESOLIMBIC DOPAMINE TRANSMISSION RESCUES DEPRESSION-RELATED BEHAVIOURS.

Anna Lisa Muntoni¹, Marta De Felice², Sonia Aroni², Maria Graziella De Montis³, Simona Scheggi³,
Miriam Melis², Marco Pistis^{1,2}

¹CNR Neuroscience Institute, Cagliari, Italy; ²Department of Biomedical Sciences, Division of Neuroscience and Clinical Pharmacology, University of Cagliari, Italy; ³Department of Molecular and Developmental Medicine, University of Siena, Italy

Depression is a widespread illness with tremendous personal and socioeconomic consequences. The underlying causes of this heterogeneous spectrum of disorders remain poorly understood. Moreover, the available pharmacological therapies have important limitations, including relatively low efficacy, and time lag for treatment response.

Noteworthy, key depressive symptoms can be modeled in animals and enable the development of novel therapeutic interventions. Chronic unavoidable stress disrupts rats' competence to escape noxious stimuli and self-administer sucrose, configuring a depression model characterized by escape deficit and motivational anhedonia associated to impaired dopaminergic responses to sucrose in the nucleus accumbens shell (NAcSh). Repeated treatments that restore these responses also relieve behavioural symptoms. Ventral tegmental area (VTA) dopamine neurons encode reward and motivation and are implicated in the neuropathology of depressive-like behaviours. Peroxisome proliferator-activated receptors type- α (PPAR α) acutely regulate VTA dopamine neuron firing via β 2 subunit-containing nicotinic acetylcholine receptors (β 2*nAChRs) through phosphorylation and this effect is predictive of antidepressant-like effects.

Here, by combining behavioural, electrophysiological and biochemical techniques, we studied the effects of repeated PPAR α stimulation by fenofibrate on mesolimbic dopamine system. We found decreased β 2*nAChRs phosphorylation levels and a switch from tonic to phasic activity of dopamine cells in the VTA, and increased phosphorylation of dopamine and cAMP-regulated phosphoprotein Mr 32,000 (DARPP-32) in the NAcSh. We then investigated whether long-term fenofibrate administration to stressed rats reinstated the decreased DARPP-32 response to sucrose and whether this effect translated into antidepressant-like properties. Fenofibrate restored dopaminergic responses to appetitive stimuli, reactivity to aversive stimuli and motivation to self-administer sucrose. Overall, this study suggests PPAR α as new targets for antidepressant therapies endowed with motivational anti-anhedonic properties, further supporting the role of an unbalanced mesolimbic dopamine system in pathophysiology of depressive disorders. Translational studies in humans will be facilitated by the fact that fibrates are already well established therapeutic options for the treatment of hyperlipidemia.

MDMA, DOB AND PMA INDUCE REWARDING AND BEHAVIOURAL CHANGES THROUGH SEROTONERGIC SYSTEM IN ZEBRAFISH

Daniela Braida¹, Luisa Ponzoni¹, Mariaelvina Sala²

¹Università degli Studi di Milano, Italy; ²CNR Neuroscience Institute, Milan, Italy

In recent years, there has been a large increase in the number of synthetic drugs used recreationally. Among them, the synthetic phenethylamines, of which MDMA is the most known, produce psychostimulant and hallucinogenic effects. There's concern about the growing usage of synthetic drugs which can often be sold openly through internet websites (1), especially for the limited knowledge of their pharmacological effects. Even if hallucinogens have a relatively low addicting potential, they are frequently abused and there are not currently available data on their rewarding thus representing an important area of extensive investigation.

Recent evidence indicates that novel animal models are emerging as useful tool to screen addicting drugs. Among them, zebrafish show high physiological and genetic homology to humans (2) appearing a sensitive model to study hallucinogen-evoked states. Here, we investigated the rewarding effects of 2,5-dimethoxy-4-bromo-amphetamine hydrobromide (DOB) and *para*-methoxyamphetamine (PMA) in comparison with the classical 3,4-methylenedioxymethamphetamine (MDMA) using the conditioned place preference task (CPP) in zebrafish. The compounds were also tested for social preference, anxiety-like and hallucinatory behaviour. To investigate the mechanism, the role of serotonin 5-HT₂ like-receptors on the above mentioned effects was evaluated.

Zebrafish were i.m. treated DOB (0.1-20 mg/kg), PMA (0.0005-2 mg/kg) or MDMA (0.5-160 mg/kg) and then submitted to the different tasks.

MDMA and its derivatives induced a dose-dependent CPP following an inverted U-shape, being PMA the most potent. The rewarding effect of DOB (2 mg/kg) and PMA (0.1 mg/kg) was accompanied by a trance-like hallucinatory behaviour, in terms of complete immobility for 2 min. MDMA did not induce any hallucinatory behavior even at high doses. All the compounds induced a progressive increase of the time spent in proximity of a *nacre* fish picture in the social preference test. However, high doses were ineffective. Similarly, in the novel tank diving and light-dark tests these drugs elicited a progressive anxiolytic effect in terms of time spent in the upper half of the tank and in the light compartment, respectively.

The 5-HT_{2A/C} antagonist, ritanserin (0.025-2.5 mg/kg), in association with the maximal effective dose of MDMA, DOB and PMA blocked all the above mentioned effects.

Collectively, these findings demonstrate for the first time the rewarding, the prosocial and the anxiolytic properties of DOB and PMA and focus on the mechanisms of their action through the serotonergic-like system suggesting a potential clinical application.

References:

- 1) Lawn W, Barratt M, Williams M, Horne A, Winstock A.J *Psychopharmacol* 28:780-708, 2014.
- 2) Cheng KC, Xin X, Clark DP La Riviere P. *Curr Opin Genet Dev* 21: 620–629, 2011.

NOVEL TARGETS AND THERAPEUTICS FOR SOCIAL IMPAIRMENTS

Marta Busnelli^{1*}, Gunnar Kleinau², Mariaelvina Sala³, Daniela Braidà³, Maurice Manning⁴, Bice Chini¹

¹CNR Neuroscience Institute, Milan, Italy; ²Charité-Universitätsmedizin, Germany; ³Università degli Studi di Milano, Italy; ⁴University of Toledo, OH USA

Neurodevelopmental and neuropsychiatric disorders, i.e. autism spectrum disorders (ASD) and schizophrenia, are characterized by reduced social abilities often resistant to current treatments.

The neurohormone oxytocin (OT) facilitates the processing of social information, improves cognitive empathic abilities and increases interpersonal trust, and for these effects it has been recently proposed as treatment for these patients. However, OT efficacy is limited by its short half-life and low selectivity and thus we aim to identify novel therapeutic molecules and targets.

OT binds and activates a transmembrane receptor (OTR) that is coupled to G-proteins and that can form homo-dimers (OTR-OTR) and hetero-dimers with different receptor subtypes. First of all, **as novel therapeutic molecules, we synthesized and characterized bivalent ligands specific for OTR-OTR dimers.** These compounds consist of two OT-analogs tethered by a carboxylic linker, that simultaneously bind into the two protomers of the OTR-OTR dimer, generating an increase in affinity and selectivity towards the OTR. These bivalent ligands induce a three order magnitude boost in G-protein signaling of OTRs *in vitro* and a 100- and 40-fold gain in potency *in vivo* in the social behavior of mice and zebrafish compared to OT^a.

As novel therapeutic target, we started to investigate the OTR/Dopamine D2 receptor heterodimers. Indeed, it has been shown that OTR/D2R are expressed in the brain^b and preliminary clinical trials have reported that a combined treatment with OT and antipsychotics, acting on D2R, might improve negative symptoms in patients with schizophrenia^c. However the mechanisms of these therapeutical effects are still unidentified, and thus we evaluated the mechanism of dimerization and interaction between the OTR and D2R and we started to test the effects of a combined treatment with OT and dopamine.

In conclusion, the newly discovered OT bivalent ligands represent a powerful tool for targeting dimeric OTR and to rescue social defects in neurodevelopmental and psychiatric disorders; moreover the ongoing pharmacological evaluation of OTR/D2R dimers and their mechanism of action, modulation and interaction will provide important information for the development of novel molecules which may specifically target OTR/D2R complexes and that may improve several aspects of ASD and schizophrenia symptomatology.

BIBLIOGRAPHY:

^a Busnelli M et al. 2016 Design and characterization of superpotent bivalent ligands targeting oxytocin receptor dimers via a channel-like structure. *J. Med. Chem.*

^b Romero-Fernandez W et al. 2013 Evidence for the existence of dopamine D2-oxytocin receptor heteromers in the ventral and dorsal striatum with facilitatory receptor-receptor interactions. *Mol. Psychiatry*

^c Feifel D et al. 2016 A review of oxytocin's effects on the positive, negative, and cognitive domains of schizophrenia. *Biol. Psychiatry*

*M. Busnelli is the recipient of a Fondazione Umberto Veronesi Post-doctoral Fellowship

BEHAVIOURAL AND NEUROCHEMICAL CHANGES INDUCED BY Δ^9 -TETRAHYDROCANNABINOL IN MICE PRE-EXPOSED TO NICOTINE

Luisa Ponzoni¹, Daniela Braida¹, Milena Moretti^{1,2}, Francesco Clementi^{1,2}, Mariaelvina Sala^{1,2}, Cecilia Gotti^{1,2}

¹*Biometra, Università degli Studi di Milano, Italy,* ²*CNR Neuroscience Institute, Milan, Italy.*

Recently, many smokers have switched from standard (cig) to electronic cigarettes (e-cig) as an alternative to nicotine delivery because they look, feel and taste like traditional cigarettes but do not contain carcinogens. Furthermore, the use of e-cig is increasing used as a means to stop despite the contrary recommendation of the World Health Organization (World Health Organization, 2008) for the lack of scientific information.

Among adolescent tobacco smokers, who also smoke marijuana, the frequency of marijuana use is associated with greater levels of nicotine addiction (Rubinstein et al., 2014). As cigarette smoking (nicotine) appears to be a potent gateway drug for illegal drugs including marijuana and heroin it is possible that earlier nicotine use may indeed be of indirect relevance for cannabis use disorder risk. Therefore, it is important to verify whether exposure to tobacco or e-cig may be of indirect relevance for other drugs of abuse disorder risk.

The aim of this study was to verify whether cig smoke or e-cig vapour exposure facilitate the rewarding subsequent effects of Δ^9 -Tetrahydrocannabinol (Δ^9 -THC) during short and long term nicotine smoke/vapour cessation.

We used a rodent model of cig and e-cig exposure that mimics the intermittence and route of nicotine administration in humans and simulates the unique pharmacokinetic characteristics (rate of absorption and brain delivery) that are associated with smoking. Three month-old male BALB/cJ mice were exposed for 7 weeks, three times a day to the smoke of cig (7 commercial cigarettes containing 0.8 mg of nicotine/cigarette, 10 mg of tar and 10 mg of carbon monoxide) or e-cig vapour containing 19 mg of nicotine dissolved in 1 ml of aqueous solution, propylene glycol (55%), glycerin (35%), using a mechanical ventilator. Control mice were exposed to the same ventilator using the same schedule but receiving only air (Ponzoni et al., 2015). 2, 30 and 60 days after 7-weeks exposure, different groups of animals were injected i.p. with a subthreshold dose of THC (0.01 mg/kg) or vehicle and submitted to Conditioned Place Preference task.

The major effects of Δ^9 -THC in the central nervous system are mediated by cannabinoid CB1 receptors and in order to understand the neurobiological substrate of the altered sensitivity to rewarding effect of Δ^9 -THC we evaluated, in the nucleus accumbens of the different experimental groups, the level of CB1 cannabinoid receptors and the CB1 agonist-stimulated binding of the nonhydrolyzable GTP analog, ($[^{35}\text{S}]\text{GTP}\gamma\text{S}$).

Mice exposed to cig showed a higher sensitivity to Δ^9 -THC (0.01 mg/kg) compared to control group, in terms of increased time spent in the drug-associated compartment. E-cig exposed animals exhibited a greater sensitivity to a low dose of Δ^9 -THC compared to controls, although less pronounced than tobacco smoke treated group. In both exposed groups, Δ^9 -THC-induced increased response was most evident at 30 and particularly at 60 days after nicotine cessation compared to 2 days of nicotine cessation. CB1 cannabinoid receptor function, evaluated through $\text{GTP}\gamma\text{S}$ activity, was not affected in mice after seven weeks exposure or in mice that have performed CPP test after 2 days of nicotine cessation. However, in cig or e-cig pre-exposed mice, that have performed the CPP test 60 days after nicotine cessation, there was a decrease in CB1 receptors number and a lower CB1 agonist-stimulated $\text{GTP}\gamma\text{S}$ activity.

In conclusion, our results show that cig and e-cig exposure induce altered response to Δ^9 -THC-induced CPP probably through the involvement of multiple mechanisms.

References

- Rubinstein et al. (2014) *Drug Alcohol Depend.* 141:159-62.
Kandel and Kandel (2014) *N Engl J Med.* 371(21):2038-9.

SUPPRESSING EFFECT OF SAIKOSAPONIN A, AN ACTIVE INGREDIENT OF *BUPLEURUM FALCATUM*, ON ALCOHOL AND CHOCOLATE SELF-ADMINISTRATION IN RATS

Irene Lorrain, Paola Maccioni, Gian Luigi Gessa, Giancarlo Colombo

CNR Neuroscience Institute, Section of Cagliari, Monserrato (CA), Italy

Saikosaponin A (SSA) is an active ingredient of the roots of *Bupleurum falcatum*, a herb widely used in traditional Oriental medicine – alone or combined with other herbal compounds – to treat several psychiatric and neurological disorders. Recent, pioneering studies from a Korean lab demonstrated that acute treatment with SSA suppressed, likely via a GABA_B receptor-mediated mechanism, intravenous self-administration of morphine and cocaine in rats (Neurosci. Lett. 529:97-101, 2012; Neurosci. Lett. 555:198-202, 2013). The present study was designed to evaluate whether the capability of SSA to suppress morphine and cocaine self-administration extends to self-administration of alcohol (Experiment 1) and of a highly palatable chocolate solution (Experiment 2) in rats.

Experiment 1 was conducted using adult, male Sardinian alcohol-preferring (sP) rats, selectively bred for high alcohol preference and consumption. Administration of non-sedative doses of SSA (0, 0.25, 0.5 and 1 mg/kg, i.p.) produced a dose-related suppression of operant, oral alcohol (15%, v/v) self-administration in sP rats exposed to (a) fixed ratio (FR) 4 schedule of reinforcement (measure of the reinforcing properties of alcohol) and (b) progressive ratio (PR) schedule of reinforcement (measure of the motivational properties of alcohol). The possible involvement of the GABA_B receptor system, suggested by the “morphine” and “cocaine” studies (see above), was investigated testing the effect of (a) pretreatment with the GABA_B receptor antagonist, SCH50911, and (b) combined treatment with the positive allosteric modulator of the GABA_B receptor, GS39783, on alcohol self-administration under the FR4 schedule. Pretreatment with a *per se* ineffective dose of SCH50911 partially blocked the reducing effect of 0.5 mg/kg SSA on lever-responding for alcohol; combined treatment with doses of GS39783 and SSA (0.1 mg/kg) ineffective when given alone virtually halved lever-responding for alcohol.

Experiment 2 was conducted using adult, male Wistar rats trained to self-administer a chocolate solution [5% (w/v) Nesquik in water]. Several previous studies from this lab demonstrated the addictive-like behavior of rats exposed to this highly palatable food: rats lever-respond several thousands of times, in brief daily sessions, to access the chocolate solution, providing a valuable tool for investigations on pharmacological interventions on uncontrolled intake of highly palatable foods. Administration of doses of SSA ranging from 0.25 and 5 mg/kg (i.p.) (a) markedly reduced chocolate self-administration under both FR10 and PR schedules of reinforcement and (b) virtually completely suppressed reinstatement of chocolate-seeking behavior induced by a complex of stimuli previously associated to the availability of the chocolate solution (a validated animal model of relapse into exaggerated intake of highly palatable foods).

Together, the results of these experiments demonstrate, for the first time, that treatment with non-sedative doses of SSA potently and effectively reduced operant self-administration of alcohol and chocolate solution, as well as chocolate seeking, in rats. These results provide further pieces of experimental evidence to the preclinical, “anti-addictive” profile of SSA, as they extend to alcohol and a highly palatable food (known to generate addictive-like behaviors) the capacity of SSA to suppress morphine and cocaine self-administration in rats.

In terms of the mechanism of action underlying these effects of SSA, data from Experiment 1 suggest that the GABA_B receptor is likely part of the neural substrate mediating the reducing effect of SSA on alcohol self-administration. However, since SCH50911-induced blockade of SSA effect was only partial, and not complete, it is likely that SSA may recruit receptor systems other than the GABA_B one to produce its “anti-alcohol” effects; these receptor systems are presently unknown. Conversely, since activation of the GABA_B receptor is known not to produce any decrease in food intake, including that of highly palatable food, other receptor systems likely constitute the neural substrate mediating the reducing effect of SSA on self-administration of the chocolate solution. Studies are presently on-going in an attempt to unravel the receptor systems involved.

COR659: A NOVEL POSITIVE ALLOSTERIC MODULATOR OF THE GABA_B RECEPTOR WITH UNIQUE *IN VIVO* PHARMACOLOGICAL PROFILE

Paola Maccioni¹, Giancarlo Colombo¹, Irene Lorrai¹, Gian Luigi Gessa¹, Mauro A.M. Carai²,
Claudia Mugnaini³, Federico Corelli³

¹CNR Neuroscience Institute, Section of Cagliari, Monserrato (CA), Italy

²Cagliari Pharmacological Research, Cagliari (CA), Italy

³Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena (SI), Italy

Multiple lines of experimental evidence have demonstrated that pharmacological activation of the orthosteric GABA_B receptor suppresses several alcohol-related behaviors in rodents; as a result of these studies, the prototypic GABA_B receptor agonist, baclofen, is currently undergoing clinical evaluation as possible pharmacotherapy for alcohol use disorder (AUD). A recent, major step forward in the pharmacology of the GABA_B receptor is represented by the discovery – in the structure of the GABA_B receptor macroprotein – of a positive allosteric modulatory binding site. Its pharmacological activation appears to be a more favorable way manipulating the GABA_B receptor: treatment with the positive allosteric modulators (PAMs) of the GABA_B receptor, CGP7930, GS39783, BHF177, *rac*-BHFF, and ADX71441, reproduced several pharmacological effects of baclofen displaying a much greater separation between the “desired” (e.g.: anxiolysis, “anti-addiction”) and “unwanted” (motor-incoordination, sedation) effects. With regard to the “anti-alcohol” effects, acute and repeated treatment with non-sedative doses of all GABA_B PAMs tested to date has invariably resulted in robust and selective reductions in excessive alcohol drinking, binge- and relapse-like drinking, and lever-responding for alcohol in rodents exposed to experimental procedures that model, with remarkable predictive validity, different aspects of AUD. These data suggest that GABA_B PAMs may represent a novel class of agents with therapeutic potential for AUD. The recent transition of the first GABA_B PAM (ADX71441) to the initial steps of clinical trials finally makes testing this hypothesis a feasible option.

The present paper describes the behavioral characterization of the pharmacological profile of a recently synthesized GABA_B PAM, named COR659 [2-(4-chlorophenylcarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate] (J. Med. Chem. 56:3620-35, 2013). All “alcohol” studies have been conducted using adult, male rats of the Sardinian alcohol-preferring (sP) line, selectively bred for high alcohol preference and consumption. Notably, sP rats constitute a validated animal model of AUD and their alcohol drinking and alcohol reinforcing and motivational properties are highly sensitive to the suppressing effect of GABA_B-receptor activation. Administration of doses of COR659 (0, 2.5, 5 and 10 mg/kg, i.p.) – remarkably lower than those inducing motor-incoordination and sedation – resulted in the dose-related suppression of operant, oral alcohol (15%, v/v) self-administration in sP rats exposed to (a) fixed ratio (FR) 4 schedule of reinforcement (measure of the reinforcing properties of alcohol) and (b) progressive ratio (PR) schedule of reinforcement (measure of the motivational properties of alcohol). COR659 resulted to be 2-3 times more potent than the reference compound, GS39783.

Unexpectedly however, treatment with COR659 (0, 2.5, 5 and 10 mg/kg, i.p.) suppressed operant sucrose (1%, w/v) self-administration in sP rats exposed to FR4 and PR schedules of reinforcement. This test, initially conceived to assess the selectivity of COR659-induced reduction in alcohol self-administration, actually revealed the capacity of COR659 to produce a behavioral effect unlikely due to an action at the GABA_B receptor, as neither baclofen nor any GABA_B PAMs have ever been reported to reduce intake of a palatable food. To investigate more in-depth this unexpected activity of COR659, we conducted a series of experiments using adult, male Wistar rats trained to self-administer a chocolate solution [5% (w/v) Nesquik in water]. Several previous studies from this lab demonstrated the addictive-like behavior of rats exposed to this highly palatable food: rats lever-respond several thousands of time, in brief daily sessions, to access the chocolate solution, providing a valuable tool for investigations on pharmacological interventions on uncontrolled intake of highly palatable foods. Administration of COR659 (0, 2.5, 5 and 10 mg/kg, i.p.) (a) reduced chocolate self-administration under both FR10 and PR schedules of reinforcement and (b) suppressed cue-induced reinstatement of chocolate-seeking behavior (model of relapse-like consumption). Notably, treatment with the same dose range of COR659 failed to affect, even minimally, operant self-administration of regular food pellets in Wistar rats motivated to lever-respond for food – to levels comparable to those recorded in the “chocolate” study – by food deprivation. These data suggest that COR659 selectively suppressed behaviors motivated by palatable, rather than regular, foods.

Together, these results lead to hypothesize that COR659 might exert its unique, *in vivo* pharmacological effects *via* a dual mechanism: (i) positive allosteric modulation of the GABA(B) receptor, responsible for the reducing effects on alcohol-motivated behaviors, *plus* (ii) an action at presently unknown receptor system(s), through which COR659 likely affects seeking and consumption of highly palatable foods. Additional studies are presently on-going in an attempt to unravel the receptor system(s) involved.

METHOXETAMINE, A NOVEL PSYCHOACTIVE SUBSTANCE WITH SERIOUS ADVERSE PHARMACOLOGICAL EFFECTS

Liana Fattore¹, Mary Tresa Zanda², Annalisa Muntoni¹, Anna Mutti³, Laura Padovani³, Laura Mancini³, Roberto Collu², Sonia Aroni², Silvia Antinori², Marzia di Chio³, Walter Fratta², Paola Fadda², Cristiano Chiamulera³

¹CNR Neuroscience Institute, Cagliari, ²Dept Biomedical Sciences, Division of Neuroscience and Clinical Pharmacology, University of Cagliari and ³Dept Diagnostic and Public Health, Section of Pharmacology, University of Verona, Italy

An increasing number of novel psychoactive substances (NPS) are currently available and sold as 'legal highs' or 'research chemicals' accompanied by the indication that they are 'not for human consumption'. Among those that have emerged in the last few years, methoxetamine (MXE) owes its wide popularity to its easy access on the Internet and its reputation of being a 'safe' drug. MXE is an arylcyclohexylamine with a chemical structure analogous to ketamine and phencyclidine, and similar non-competitive glutamate N-methyl D-aspartate receptor antagonist properties. Although MXE-induced dissociative effects and acute toxicity have been reported, its toxicology and pharmacology is still poorly investigated.

In this study we evaluated (1) the behavioral effects induced by acute intraperitoneal (i.p.) administration of MXE (0.5-5 mg/kg) in rats, (2) MXE abuse liability and reinforcing properties, and (3) whether rapid changes in protein translation were involved in the observed behavioral responses. Data showed that (1) MXE (0.5-5 mg/kg) significantly affected spontaneous motor activity in a dose- and time-dependent manner, inducing hypermotility and hypomotility at low and high doses, respectively. At the highest dose tested (5 mg/kg), MXE induced transient analgesia (tail flick and hot plate test), caused sensory gating impairment (prepulse inhibition test) and significantly reduced immobility time while increasing swimming activity (forced swim test), suggesting an antidepressant effect. At lower doses (0.5 and 1 mg/kg), MXE induced anxious and/or obsessive-compulsive traits (marble burying test), increased sociability (social interaction test) but did not induce spatial anxiety (elevated plus maze test). Moreover, (2) MXE fully generalizes to ketamine discriminative stimulus in rats in a dose related manner starting from 0.125 to 1.0 mg/kg (i.p.), while intravenous (i.v.) MXE (0.125 and 0.25 mg/kg) substitutes for ketamine as a self-administered solution, increases dopamine extracellular levels in the nucleus accumbens (NAc) shell at 0.5 and 0.25 mg/kg (i.v.) and activates the mesolimbic dopamine transmission in a time-dependent manner when injected at cumulative doses (MXE 0.031–0.5 mg/kg, i.v.) by inducing a dose-dependent stimulation of firing and burst firing of NAc dopamine neurons projecting to the ventral tegmental area. Finally, (3) immunohistochemistry study showed that MXE (1 and 5 mg/kg) increases the expression of phosphorylated ribosomal protein S6 (rpS6P) in prelimbic and infralimbic cortices and hippocampus.

Altogether, our results indicate that MXE (1) depending on the dose tested, may alter spontaneous motor activity and sociability, cause transient analgesia and sensory disturbances, induce antidepressant effects and repetitive/perseverative behavior, (2) possesses discriminative and positive reinforcing properties, which likely underline its abuse potential and that (3) the increased expression of rpS6P protein provides a correlate of rapid neuroadaptive changes and potential antidepressant effect of MXE similarly to ketamine.

MOUSE MODEL OF POLYMICROGYRIA: PROPOSAL OF THERAPEUTIC APPROACHES

Silvia Ferrara¹, Giuliana Fossati², Cristina Zalfa³, Flavia Antonucci¹, Luisa Ponzoni¹, Pietro Avanzini^{4,5}, Mariaelvina Sala^{1,4}, Giacomo Rizzolatti^{4,5}, Angelo L. Vescovi³, Michela Matteoli^{2,4} and Elisabetta Menna^{2,4}

¹Dept. of Medical Biotechnology and Translational Medicine, University of Milan, Italy; ²Humanitas Clinical and Research Center, Milan, Italy. ³Biotechnology and Bioscience Department Bicocca University, Milan, Italy. ⁴CNR-Institute of Neuroscience, Milan, Italy. ⁵Dipartimento di Neuroscienze - sezione di Fisiologia- Università di Parma

Polymicrogyria (PMG) is a condition characterized by atypical prenatal brain development with an excessive number of abnormally small gyri in the cerebral cortex. Clinical signs include intractable epilepsy with an early onset, general developmental delay, language difficulties, movement and muscle weakness. No treatment is currently available for PMG and even the causes of the pathology are not completely defined. Researchers have identified several environmental and genetic factors that can be responsible for the disorder. Environmental causes of PMG include infections during pregnancy and lack of oxygen to the foetus. In addition, several causative gene mutations have been identified to be involved in the different forms of this pathology. Experimentally, the pathophysiological aspects of PMG can be reproduced by the focal freeze-lesion (FFL) model in neonatal rodents, resulting in the formation of microgyri in the mouse cortex. Previous studies in the FFL model showed enhanced excitatory and inhibitory synaptic transmission accompanied by increased connectivity in the paramicrogyral cortex and higher susceptibility to epilepsy.

Biochemical, morphological and behavioural analysis of the PMG model revealed, besides the alteration in the cortical laminar structure, a significant hypomyelination, astrogliosis and microglial activation with increased levels of inflammatory cytokines in the paramicrogyral cortex, indicating the occurrence of an inflammatory process. Furthermore, PMG mice displayed altered EEG profile and defective motor skills such as reduced brawn. Here we propose possible therapeutic approaches to treat this pathology and the more severe symptoms such as the refractory epilepsy. We have preliminary evidences that transplantation of human CNS neural stem cells, which has been demonstrated to exert positive effects on inherited or acquired myelinating disorders and to dampen brain inflammation, play a beneficial effect in the pathological condition of PMG ameliorating the myelination defect by promoting oligodendrocyte precursors proliferation and remodelling of myelin fibers. Our data also show that hNSC transplantation restores normal EEG brain activity and improves motor performances. Furthermore inhibition of IL-1R activation at P30 for one week by the IL-1R antagonist anakinra, leads to a significant improvement of EEG and motor skills in adult PMG mice thus suggesting a possible role of inflammation at the root of the pathology and identifying a therapeutic time window for the treatment.

Cortical plasticity in retinitis pigmentosa

Laura Baroncelli¹, Tatjana Begenisic¹, Maria Cristina Cenni¹, Alessandro Sale¹, Lucia Galli¹

¹CNR Neuroscience Institute, Via Moruzzi 1, I-56124 Pisa, Italy

Retinitis Pigmentosa (RP) is a family of inherited disorders caused by the progressive loss of retinal photoreceptors. There is no cure for RP, but research aimed at preventing further photoreceptor loss, or substituting new light-responsive elements of biological or artificial nature, is generating hope for these patients. These strategies require that the visual system downstream of the photoreceptors is capable of elaborating visual signals. Anatomical and functional studies have shown that retinal and thalamic structure are well preserved with RP, but the effect of photoreceptor degeneration on the visual cortex is still unknown. Here, we studied how visual cortical processing changed during the course of progression of RP, and whether the visual cortex retained the capability of plastic remodelling. We performed in vitro electrophysiological recordings of field excitatory post-synaptic potentials in V1. Basic synaptic transmission, as assessed by response vs stimulus amplitude, showed a significant shallower response in RP mice. Biochemical analysis suggests that this synaptic deficit could be related to the alteration of absolute levels of inhibition and excitation in the visual cortex, with an overexpression of inhibitory markers. These results suggest that cortical changes occur in the visual cortex that might further compromise vision by downregulating or suppressing visual processing, as the retinal input progressively deteriorates.

Protocadherin-19 regulates GABA_AR surface expression and currents

Silvia Bassani¹, Laura Gerosa¹, Giulia Maia Serratto¹, Maria Passafaro¹

¹CNR Neuroscience Institute, Milan, Italy

PCDH19 Girls Clustering Epilepsy (PCDH19 GCE), also known as Early Infantile Epileptic Encephalopathy-9 (EIEE9), is a debilitating neurological condition characterized by early onset seizures (6-36 months), intellectual disability and autistic features. PCDH19 GCE has a singular inheritance mode: heterozygous females are affected while hemizygous males are seizure-free.

Recently the X-linked gene PCDH19 (Xq22.3), which encodes for protocadherin-19 (Pcdh19), has been identified as the GCE causative gene and has rapidly become the second most relevant gene in epilepsy after SCN1A. Pcdh19 belongs to the cadherin superfamily of calcium-dependent cell adhesion molecules and is highly expressed in the brain, especially in cortex and hippocampus. A cellular interference model has been proposed to explain PCDH19 GCE pathogenic mechanism: PCDH19 mosaic expression in females due to random X inactivation would scramble neuronal recognition and communication. However, Pcdh19 function remains largely unknown.

We found that Pcdh19 C-terminal intracellular tail interacts with the alpha1 subunit of GABA_A receptor (GABA_AR). Pcdh19 shRNA-mediated downregulation in rat hippocampal neurons reduces GABA_AR α1 surface expression and affects the frequency and kinetics of mIPSCs, suggesting Pcdh19 involvement in GABA_AR trafficking and inhibitory transmission.

Secretion-positive LGI1 Mutations Linked to Lateral Temporal Epilepsy Impair Binding to ADAM22 and ADAM23 Receptors

Emanuela Dazzo¹, Emanuela Leonardi², Elisa Belluzzi^{3,§}, Sandro Malacrida⁴, Libero Vitiello³,
Elisa Greggio³, Silvio C.E. Tosatto^{1,4}, Carlo Nobile^{1,4*}

¹CNR-Neuroscience Institute, Padova, Italy; ²Department of Woman and Child's Health, University of Padua, Padova, Italy; ³Department of Biology, University of Padua, Padova, Italy; ⁴Department of Biomedical Sciences, University of Padua, Padova, Italy.

Autosomal dominant lateral temporal epilepsy (ADLTE) is a focal epilepsy syndrome caused by mutations in the *LGI1* gene, which encodes a secreted protein. Most ADLTE-causing mutations inhibit LGI1 protein secretion, and only a few secretion-positive missense mutations have been reported. Here we describe the effects of four disease-causing nonsynonymous *LGI1* mutations, T380A, R407C, S473L, and R474Q, on protein secretion and extracellular interactions. Expression of LGI1 mutant proteins in cultured cells shows that these mutations do not inhibit protein secretion. This finding likely results from the lack of effects of these mutations on LGI1 protein folding, as suggested by 3D protein modelling. In addition, immunofluorescence and co-immunoprecipitation experiments reveal that all four mutations significantly impair interaction of LGI1 with the ADAM22 and ADAM23 receptors on the cell surface. These results support the existence of a second mechanism, alternative to inhibition of protein secretion, by which ADLTE-causing *LGI1* mutations exert their loss-of-function effect extracellularly, and suggest that interactions of LGI1 with both ADAM22 and ADAM23 play an important role in the molecular mechanisms leading to ADLTE.

A possible pharmacological target to interfere with epilepsy in a *Mecp2*^{Y/-} mouse model

Barbara Grillo¹, Luisa Ponzoni¹, Francesco Rusconi¹, Emanuela Toffolo¹, Mariaelvina Sala^{1,2}, Elena Battaglioli^{1,2}

¹Dept Medical Biotech & Translational Medicine, University of Milan, Italy; ²CNR Institute of Neuroscience (IN) Milan, Italy.

Rett syndrome is a neurodevelopmental disorder characterized by early arrest of normal developmental milestones and seizures are among the major debilitating symptoms. RTT is caused by mutations in the X-linked *Mecp2* gene, which has been characterized as transcriptional repressor although very few of its targets have been directly implicated in RTT pathogenesis. We have identified a new MeCP2 direct target namely the splicing regulator NOVA1, whose expression is upregulated in symptomatic *Mecp2*^{Y/-} mice and that has been recently proposed as a homeostatic regulator of excitability in the mouse hippocampus (Rusconi et al., 2014). Upregulation of NOVA1 protein and mRNA levels in *Mecp2*^{Y/-} mice causes upregulation of the histone demethylase LSD1/KDM1A splicing isoform neuroLSD1 that was unraveled as positive modulator of neuron excitability (Rusconi et al., 2014). NeuroLSD1 is characterized by the inclusion of microexon E8a and it acts as a dominant-negative splicing isoform of LSD1 (Zibetti et al., 2010).

Recent studies demonstrate disease reversibility in RTT mouse models, suggesting that the neurological defects in *Mecp2*-related disorders are not permanent and can be therapeutically modulated (Garg et al., 2013). Relevantly to *Mecp2*^{Y/-} mice hyper-excitable phenotype and severe epilepsy in humans, loss of neuroLSD1 protects against seizures, and its upregulation could concur to set severity of convulsive behaviour and health deterioration. Therefore, we hypothesize that modulation of LSD1 neurospecific splicing in *Mecp2*^{Y/-} mice would be instrumental to ameliorate aspects of hyper-excitability of neuronal circuitry proper of RTT symptoms. Our goal is to test the possibility to prevent or reduce altered excitability profile of *Mecp2*^{Y/-} mice by two complementary approaches a genetic one (a) and a pharmacological one (b) aimed at restoring the physiological LSD1/neuroLSD1 ratio. (a) We intend to interfere with the altered excitability profile of *Mecp2*^{Y/-} mouse model of RTT with a genetic strategy based on the generation of *neuroLSD1*^{Het}/*Mecp2*^{Y/-} mice. We obtained the first double mutant F1 offsprings, crossing female *Mecp2*^{+/-} mice with male *neuroLSD1*^{het} showing that this crossing is productive. The characterization just started and is in progress. (b) Moreover, we propose to explore the possibility to modify altered excitability profile of *Mecp2*^{Y/-} mice by interfering with the generation of the neurospecific isoform of LSD1 using modified antisense oligonucleotides (2'OMOE-PS AON) as neuropharmacological drug. This methodology should allow us to restore a physiological LSD1/neuroLSD1 splicing ratio without affecting overall LSD1 expression levels representing therefore the most suitable approach to our goal. Considering the 100% conservation between mouse and human of the LSD1 exon E8a and its flanking intronic regions positive results could have immediate translational potential.

Defective glutamate and K⁺ clearance by cortical astrocytes in a mouse model of migraine with reduced expression of the glial alpha2 Na⁺.K⁺ ATPase.

Giovanna Crivellaro¹, Angelita Tottene¹, Clizia Capuani¹, Marcello Melone^{2*}, Luca Bragina², Mirko Santello³, Giorgio Casari⁴, Fiorenzo Conti², Daniela Pietrobon^{1,5}

¹ *Department of Biomedical Sciences, University of Padova, Italy;* ² *Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, Ancona, Italy;* ³ *Institute of Pharmacology and Toxicology, University of Zurich, 8057 Zürich, Switzerland;* ⁴ *Vita-Salute San Raffaele University and San Raffaele Scientific Institute, Milano, Italy;* ⁵ *CNR Institute of Neuroscience, Padova, Italy*

Migraine is a common disabling brain disorder. A subtype of migraine with aura (familial hemiplegic migraine type 2: FHM2) is caused by loss-of-function mutations in the alpha2 Na⁺,K⁺ ATPase, an isoform almost exclusively expressed in astrocytes in adult brain. Cortical spreading depression (CSD), the phenomenon that underlies migraine aura and activates migraine headache mechanisms, is facilitated in W887R/+ FHM2 knockin mice showing a 50% reduced expression of alpha2 Na⁺,K⁺ ATPase. To investigate the unknown mechanisms underlying facilitation of CSD in FHM2 and to study the functional consequences of the W887R FHM2 mutation on glutamate and K⁺ clearance by cortical astrocytes during neuronal activity, we measured the synaptically activated glutamate transporter current and the [K⁺]_e-dependent slow inward current elicited in astrocytes by extracellular stimulation in acute cortical slices from heterozygous FHM2 knockin mice. We show reduced rates of K⁺ and glutamate clearance by cortical astrocytes during neuronal activity and reduced density of GLT-1a glutamate transporters in perisynaptic astrocytic processes in FHM2 knockin mice, demonstrating key physiological roles of the alpha2 Na⁺,K⁺ ATPase and supporting its tight coupling with GLT-1a. Using ceftriaxone to enhance expression of GLT-1a in FHM2 mice and TBOA to partially inhibit the glutamate transporters in wild-type mice, we obtain evidence that defective glutamate clearance contributes to facilitation of CSD initiation in FHM2, thus supporting the idea that excessive glutamatergic transmission is a common mechanism underlying vulnerability to CSD ignition in migraine.

OPHN1 REGULATES THE MIGRATION OF NEWLY GENERATED CELLS IN THE OLFACTORY SYSTEM

Andrea Maset¹, Luisa Galla^{1,2}, Claudia Lodovichi^{1,2,3}

¹*Venetian Institute of Molecular Medicine, Padua, Italy;* ²*CNR Neuroscience Institute, Padua, Italy;*
³*Armenise Harvard CDA.*

OPHN1 is a X-linked gene associated to intellectual disability that encodes a Rho GTPase activating protein that plays a crucial role in the conformational rearrangements of actin filaments involved in several developmental processes including axon outgrowth, dendritic maturation and cell migration. How these cellular events affect circuit formation and functions remains unknown. To address this point we analyzed the neurogenesis in the olfactory system, in OPHN1^{-/-} mice and controls.

The olfactory bulb constantly receives newly generated interneurons from the subventricular zone. The neuronal precursors exit the subventricular zone and converge in chains supported by tubular structure made by specialized astrocytes, and migrate along the rostral migratory stream to reach the olfactory bulb. Once the neuronal precursors reach the core of the olfactory bulb, they detach from these chains and begin to migrate radially towards more superficial layers of the olfactory bulb. Along this radial migration they undergo a precisely orchestrated series of morphological and electrophysiological changes to become fully mature interneurons.

We found that loss-of-function mutation of OPHN1 did not affect the generation of new neuronal precursor cells in the subventricular zone. However the number of adult-born cells (i.e. granule cells) that reached the olfactory bulb was dramatically reduced in OPHN1^{-/-} mice, suggesting that the migration from the subventricular zone to the olfactory bulb was perturbed. To explore whether and how the migration was altered in OPHN1^{-/-} mice, we combined injections of lentiviral vectors expressing GFP in the subventricular zone, to label neuronal progenitor cells and immunostaining against Doublecortin, a neuronal precursor marker, BrdU, a cell division marker, and Glial Acid Fibrillary Protein, an astrocytes marker. We found that distribution, morphology, directionality of neuronal precursor cells were deeply perturbed in OPHN1^{-/-} mice.

Neuroblast migration is regulated by several factors, including GABA and Ca²⁺ signalling. To dissect the mechanism underlying altered migration, we are currently performing in vivo time-lapse imaging of migrating neuroblasts, in basal conditions and upon stimulations with drugs able to modulate GABA and Ca²⁺ signalling in controls and OPHN1^{-/-} mice.

Role of LRRK2 in neuronal connectivity in the olfactory system

Luisa Galla^{1,2}, Andrea Maset¹, Claudia Lodovichi^{1,2}

¹ Venetian Institute of Molecular Medicine, Padua, Italy; ² CNR Neuroscience Institute, Padua, Italy

Parkinson disease (PD) is a common adult-onset neurodegenerative disorder, that affects an increasing number of people in aging population. The precise causes of PD remain still uncertain. PD is characterized by a wide spectrum of symptoms including motor symptoms such as resting tremor, bradykinesia, rigidity as well as non-motor symptoms including sleep disorders, constipation, cardiac abnormalities, cognitive and sensory dysfunctions. Olfactory dysfunction is an early symptom of PD. About 90% of PD patients present olfactory deficits years before the appearance of motor symptoms. The mechanism underlying olfactory deficit are still unknown. The genetic contribution to PD is now well established and numerous genes and genetic loci having been found to cause familial PD or affect the risk for PD. Mutations in leucine-rich repeat kinase, *LRRK2*, are associated to an autosomal-dominant inherited form of Parkinson's disease that mimics the sporadic form of PD. *LRRK2* encodes a large multidomain protein that includes a central catalytic tridomain with GTPase and kinase activities surrounded by a series of potential protein–protein interaction domains.

While molecular and cellular evidence places LRRK2 at the cytoskeleton, it is still unclear how these cellular events affect neuronal morphology and wiring *in vivo*, leading to a progressive neurodegeneration. Our aim is to disclose the role of LRRK2 in neuronal morphology and connectivity in the olfactory system. To address this question we are studying adult neurogenesis in the olfactory system in two different transgenic mice lines, LRRK2 KO and LRRK2 G2019S BAC mice. Analyzing the olfactory behavior, we found that both transgenic mice lines, LRRK2 KO and LRRK2 G2019S BAC mice, exhibited significant olfactory deficits. Combining BrdU injections, a marker of newly generated cells and injections of lentiviral vector expressing GFP in the subventricular zone to label the newly generated cells, we studied the generation of new cell in the subventricular zone, the migration and the morphological maturation of new granule cells in the olfactory bulb. We found that the generation, migration and morphological maturation of adult-born granule cells were deeply affected in LRRK2 KO and in LRRK2 G2019S BAC mice.

Mutation of OPHN1 impairs adult born granule cells development and network activity in the olfactory bulb

Nelly Redolfi^{1,2}, Luisa Galla^{1,2}, Andrea Maset¹, Luca Murru³, Maria Passafaro³, Claudia Lodovichi^{1,2}

¹*Venetian Institute of Molecular Medicine, Padua, Italy;* ²*CNR Neuroscience Institute, Padua, Italy;* ³*CNR Neuroscience Institute, Milan, Italy*

Inhibition plays a prominent role in shaping electrical activity and neural synchrony and is thought to contribute to the pathophysiology of neurodevelopmental disorders.

OPHN1, a X-linked gene associated to intellectual disability, encodes for a Rho GTPases-activating protein, that is thought to mediate several key neurodevelopmental processes, such as neurite outgrowth, dendrite formation and cell migration. Whether OPHN1 mutation leads to alterations of inhibitory interneurons remains unknown. To address this question we studied development and function of granule cells, the major population of inhibitory interneurons of the olfactory bulb. Noteworthy granule cells constantly regenerate throughout life, offering a unique useful model to investigate development and function of inhibitory interneurons and their effects on neuronal circuitry in the olfactory bulb.

By using BrdU, a cell division marker, and lentivirus expressing GFP to label the new cells in the subventricular zone, we found that the number and the morphology of new granule cells was deeply perturbed in OPHN1 ko mice. mIPSC and sIPSC recorded from mitral cells, the postsynaptic target of granule cells, were larger in OPHN1 ko mice than in controls. Electrophysiological recording *in vivo*, in the olfactory bulb, indicated that the amplitude of the gamma oscillations was significantly reduced in OPHN1 ko mice respect to controls. Behavioural test showed that odor discrimination and learning were altered in OPHN1 ko mice. Morphological and electrophysiological alterations of adult born granule cells were successfully rescued by pharmacological treatment.

Our data demonstrate that OPHN1 plays a critical role in regulating development and function of adult-generated inhibitory interneurons in the olfactory bulb. Furthermore we found that olfactory discrimination and gamma oscillations in the olfactory bulb were impaired in OPHN1 ko mice. Alterations in the inhibitory circuits could contribute to the altered behaviour and neuronal oscillatory activity.

THE SELECTIVE INHIBITION OF THE PERSISTENT SODIUM CURRENT INaP REVERTS PRECOCIOUS INTER- AND MOTORNEURONS HYPEREXCITABILITY IN THE Sod1-G93R ZEBRAFISH ALS MODEL

Maura Francolini¹, Lorena Benedetti¹, Anna Ghilardi², Elsa Rottoli¹, Laura Prospero², Luca Del Giacco²

¹*CNR Neuroscience Institute and Dept. Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Italy;* ²*Dept. of BioSciences Università degli Studi di Milano, Italy*

To comprehend SOD1 mutations pathogenetic role in Amyotrophic Lateral Sclerosis (ALS), we employed a zebrafish disease model stably expressing the ALS-linked G93R mutation. Besides the main pathological ALS-features shown by adult fish, we report remarkable precocious alterations on motor nerve circuitry development and embryos behavior. We suggest that such alterations are prompted by the inter-and motorneurons hyperexcitability, which in turn is triggered by anomalies in the persistent pace-maker sodium current INaP. Modulating INaP through riluzole we reduced spinal neurons excitability, reverting the behavioral phenotypes and ameliorating motor nerve circuitry developmental deficits, shedding new light on the employment of riluzole for ALS management. Finally, we provide a valid phenotype based tool for in-vivo unbiased drug screening in search for potential new therapies

NEW ROLE OF ATM IN HIPPOCAMPAL NEURONS DURING DEVELOPMENT

Lara Pizzamiglio¹, Elisa Focchi^{1,3}, Luca Murru², Matteo Tamborini³, Maria Passafaro², Elisabetta Menna^{2,3}, Michela Matteoli^{2,3} and Flavia Antonucci^{1,2}

¹Department of Medical Biotechnology and Translational Medicine (BIOMETRA), University of Milan, Milan, 20100, Italy; ²Institute of Neuroscience, C.N.R., Milan, 20129, Italy; ³Humanitas Clinical and Research Center, IRCCS Rozzano, Italy.

ATM (Ataxia Telangiectasia mutated) is a large protein kinase of approximately 350 kDa, whose best-known function is associated to the DNA damage response (DDR). Despite its activity in dividing cells has been largely investigated, a high number of evidences have demonstrated new roles of ATM kinase also in adult neurons, such as its involvement in adult neurogenesis and its crucial function in oxidative stress response. Moreover, it has been shown that, at the synapses of cerebellar granule cells, ATM interacts with β -adaplin in synaptic vesicles and also, in cortical neurons, it is able to form a complex with two synaptic vesicle proteins, VAMP2 and synapsin-I, participating to the synaptic vesicles release. These evidences suggest that the absence of the protein can be responsible for the generation of neuronal dysfunctions. Coherently, the neurodegenerative condition associated to genetic mutations in *Atm* gene, the Ataxia Telangiectasia (A-T), exhibits, besides cerebellar ataxia, a variable phenotype with impairment in cognition. Furthermore, it has been found by positron emission tomography (PET), a decreased glucose metabolism not only in cerebellum but also at hippocampal level in heterozygous A-T patients, indicating that halved amount of ATM generates functional alterations in neurons. Thus, ATM may impact brain functions through distinct mechanisms, in different brain regions and in diverse cell populations. To directly address these new and unclear aspects of ATM kinase in adult neurons, we prepared hippocampal neuronal cultures from ATM heterozygous mouse embryos. Here we found a significant excitatory/inhibitory unbalance toward inhibition as indicated by a higher frequency of miniature inhibitory postsynaptic current events, an increased number of GABAergic synapses and a more precocious development of the inhibitory system (i.e. excitatory to inhibitory GABA switch). *In vivo*, the enhanced inhibition still persists and, even if a higher excitation is also present, a reduced neuronal excitability is shown, as indicated by the lower action potential frequency generated in response to high-current intensity stimuli. One of the mechanisms underlying these rearrangements consists in the unbalanced phosphorylation/de-phosphorylation cycle. In particular, we found an elevated extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in heterozygous hippocampi, associated with lower expression levels of PP1 phosphatase. These data unveil an unexpected role of ATM in the maintenance of an appropriate GABAergic development and transmission in hippocampal formation, laying the basis for a more clear comprehension of cognitive defects occurring in A-T and opening to novel therapeutic strategies.

HYPERACTIVATION OF IL-1 PATHWAY DURING BRAIN DEVELOPMENT AFFECTS NEURONAL FUNCTION AND PLASTICITY

Alice Canzi^{1,2}, Romana Tomasoni², Raffaella Morini², Irene Corradini^{2,3}, Marco Rasile^{2,4}, Cecilia Garlanda², Alberto Mantovani^{1,2}, Elisabetta Menna^{2,3} and Michela Matteoli^{2,3}

¹Hunimed University, Rozzano, Italy; ²IRCCS Humanitas, Rozzano, Italy; ³IN-CNR, Milano, Italy; ⁴University of Milano, Milano, Italy

In recent years, it has been demonstrated that inflammatory mediators can contribute to the regulation of specific neuronal processes, affecting synapse structure and function and possibly impacting the manifestation of brain diseases. In this study we took advantage of a genetic mouse model of immune deregulation, the IL1R8^{-/-} mice to examine the role of inflammation on synapse structure and function.

IL-1R8 belongs to the toll-like receptors (TLRs) and interleukin-1R-like receptors (ILRs). The IL-1R subfamily includes components of signaling receptor complexes and regulatory molecules. IL-1R8 dampens the activation of the TLR and IL-1R signaling pathways by interfering with the associations between the receptor complexes and adaptor molecules. As a consequence, IL-1R8^{-/-} mice display exaggerated symptoms of inflammatory conditions. IL-1R8 is also present in the brain. Here we used both hippocampal neurons and brain slices of WT and IL1R8^{-/-} mice to investigate the morphology and function of excitatory and inhibitory synapses by using confocal analysis and patch clamp electrophysiology.

IL1R8^{-/-} cultured neurons display a reduction in the density of mature dendritic spines, and lower levels of PSD95. Furthermore IL1R8^{-/-} neurons show a decreased mEPSC frequency and are unable to undergo LTP (long-term-potential). Analysis of ex vivo preparations revealed IL1R8^{-/-} mice display a reduction of dendritic spines formed onto apical dendrites of CA1 pyramidal neurons and of vGLUT1 positive puncta. Accordingly, IL1R8^{-/-} mice display clear behavioral defects, when tested for hippocampus-dependent spatial reference memory and novel place recognition ability. We are currently performing analysis of inhibitory interneuron subtypes and inhibitory synapses in the hippocampus and cerebral cortex. Preliminary data showed a reduction in the number of parvalbumin positive -GABAergic interneurons and vGAT positive puncta in the hippocampus.

Hyperactivation of IL1 pathway disrupts dendritic spine morphology and plasticity. These changes likely underlie the learning and memory defects observed in IL1R8^{-/-} mice and point to a strong correlation between excessive inflammatory stimuli during brain development and synaptic defects similar to ones described in intellectual disabilities. Furthermore the preliminary results on the parvalbumin subpopulation of GABAergic interneurons, associated with the reduction of inhibitory synapses in the hippocampus, suggest that the lack of IL1R8 and the consequent increased susceptibility to inflammatory challenge can interfere also with the correct formation of the inhibitory network in the brain.

Response of healthy versus APP/PS1 microglia to A β 42 exposure

Chiara Adriana Elia¹, Marco Rasile¹, Melissa Sorosina², Ferdinando Clarelli², Miriam Colombo³, Filippo Martinelli Boneschi² and Michela Matteoli^{1,4}

¹Humanitas Research Hospital Institute, Rozzano (MI), Italy; ²San Raffaele Scientific Institute, Milan, Italy; ³Bicocca University, Milan, Italy; ⁴CNR, National Research Council, Institute of Neuroscience, Milan, Italy.

Microglia play a paradoxical role in Alzheimer's Disease (AD). On the one hand, activated microglia show an enhanced ability to phagocytose and degrade A β , thus having beneficial effects. However, in a longer time window, activated microglia have detrimental consequences, by promoting the release of inflammatory cytokines, which in turn drive the chronic progression of AD by exacerbating A β deposition and neuronal death. Consistently, microglial activation increases linearly throughout the disease and correlates with AD neurodegeneration. In particular, in the last years it has been proposed that activated microglia, in the presence of excess A β 42, produce extracellular vesicles (EVs) containing neurotoxic oligomers that, through white matter tract damage, spread the disease to neighbouring and connected areas (Agosta et al., 2014).

Aim of our study is to compare the response of microglia from wt or from transgenic AD chimeric murine model (APP/PS1) upon exposure to human-A β 1-42 (h- A β 1-42) for 24h.

Data derived from the whole genome expression profiling showed that many genes are differently modulated in APP/PS1 microglia exposed to human h- A β 1-42, in comparison with WT microglia. In particular, pathways analysis with KEGG or GO databases revealed a specific modulation of genes related to the formation of endosomes or extracellular vesicles in APP/PS1, but not wt, microglia treated with h- A β 1-42. Consistently, significant differences in the formation of EVs (microvesicles and exosomes) were detected between wt and APP/PS1 microglia exposed to human A β 1-42 , as assessed by Nanosight quantitation.

We now plan i) to characterize the two populations of EVs by western blot analysis, in order to evaluate their protein composition, and ii) to define their functional effects in order to understand whether these organelles can contribute to the pathological progress in AD.

Deciphering the role of SULT4A1 in brain development and Phelan-McDermid Syndrome pathogenesis

Lorenza Culotta, Carlo Sala and Chiara Verpelli

*CNR Neuroscience Institute and Department of Medical Biotechnology and Translational Medicine,
University of Milan, Milan 20129, Italy*

The 22q13 deletion/Phelan-McDermid Syndrome (PMS) is a genetic disease orphan of cure which causes a severe form of intellectual disability and autism, mainly characterized by neonatal hypotonia, global developmental delay, absent to severely delayed speech, and minor dysmorphic features.

Although it is widely recognized that SHANK3 is the major gene contributing to the neurological phenotype of PMS, the wide clinical heterogeneity among PMS patients suggests that the haploinsufficiency of genes in the 22q13 region, beside SHANK3, might contribute to altered neurological symptoms associated with PMS. One of these genes is SULT4A1 that encodes a brain specific cytosolic sulfotransferase. SULT4A1 is highly expressed in specific regions of the brain but its function in the central nervous system was not fully addressed. Moreover, some SNPs and microsatellites of SULT4A1 have been recently associated with the susceptibility to schizophrenia and altered levels of SULT4A1 protein have been observed in bipolar and Alzheimer's disease patients. Therefore, we decided to investigate the still-unknown role of SULT4A1 in brain development and functioning.

Using biochemical and morphological analysis of SULT4A1 expression in rat primary neuronal cultures and wild type mice brain areas, we found that the expression of SULT4A1 in rat cortical neurons increases fourfold during neuron maturation and that it is widely and differentially expressed throughout mice brain. The same rise of SULT4A1 expression was appreciable during the differentiation of iPSC-derived NSC to mature neurons.

Furthermore, the silencing of SULT4A1 in rat cortical neurons led to the simplification of neuronal branching and the reduction of spine density, suggesting a role for SULT4A1 in neuronal maturation. Although the exact biological function of SULT4A1 is yet to be revealed, our data suggest that this gene has a relevant role for neuron development in brain.

THE MUTATION OF X-LINKED INTELLECTUAL DISABILITY PROTEIN IL1RAPL1 ALTERS DENDRITIC ARBORIZATION AND EXCITATORY SYNAPSE FORMATION

Laura Gritti¹, Caterina Montani¹, Mariana Ramos-Brossier², Pierre Billuart², Chiara Verpelli¹ and Carlo Sala¹

¹*CNR Neuroscience Institute and Department of Medical Biotechnology and Translational Medicine, University of Milan, Italy;* ²*Institut Cochin, INSERM U1016, CNRS UMR8104, Université Paris Descartes, Paris 75014, France.*

Mutations and deletions of Interleukin-1 receptor accessory protein like 1 (IL1RAPL1) gene are associated to X-linked intellectual disability and autism spectrum disorder (1). The IL1RAPL1 transmembrane protein belongs to the family of IL1/Toll receptors and it is localized to the post-synaptic density where it plays a role in synapse formation and stabilization. Mice carrying a null mutation of the *Il1rapl1* gene show a reduction of both dendritic spine density and excitatory synapses, in the CA1 region of the hippocampus (2). These structural abnormalities are associated with specific deficits in hippocampal long-term synaptic plasticity (3).

The aim of presented work was to evaluate how mutations and deletions of IL1RAPL1 found in patients (4) affect the neuronal morphology.

We first analyzed neurons differentiated from induced pluripotent stem cells (iPSC) derived from fibroblast of a patient lacking the exon 6 of IL1RAPL1 gene. These neurons show an increased complexity of dendritic arborisation compared to neurons differentiated from a healthy donor. Interestingly a similar result was observed in pyramidal neurons from hippocampus of IL1RAPL1-KO mice.

We then investigated the role of different domain of the protein on neuronal morphology by *in vitro* experiments. Overexpression of IL1RAPL1 full length and mutants lacking the C-terminal domain in hippocampal neurons lead to a simplified dendritic arborization, instead this effect was abolished when we overexpressed mutants in the N-terminal domain.

These results suggest the key role of IL1RAPL1, and in particular of the extracellular domain of the protein, in dendrite development as well as in synapse formation.

HETEROZYGOUS REELIN MUTATIONS CAUSE AUTOSOMAL-DOMINANT LATERAL TEMPORAL EPILEPSY: A BIOINFORMATICS STUDY

Giovanni Minervini¹, Emanuela Dazzo², Carlo Nobile², Silvio Tosatto^{1,2}

¹Department of Biomedical Sciences, University of Padova, Italy; ²CNR Institute of Neuroscience, Padova, Italy

Autosomal-dominant lateral temporal epilepsy (ADLTE) is a genetic epilepsy syndrome characterized by focal seizures with prominent auditory symptoms. Traditionally, ADLTE is linked with mutations in LGI1. However, this explain fewer than 50% of affected families. Experimental investigation showed ADLTE-related mutations significantly decrease serum levels of Reelin, suggesting an inhibitory effect of mutations on protein secretion. Here, we report an in silico characterization of causal mutations in reelin (RELN) in seven ADLTE-affected families without LGI1 mutations. 3D modeling and in silico functional analysis were used to assess the mutations effects on protein-domain folding (1). We found that mutations disrupt RELN fold suggesting a functional impairment. Our findings can be used to extend the spectrum of neurological disorders associated with RELN mutations and to establish its regulatory roles in neuronal cell function.

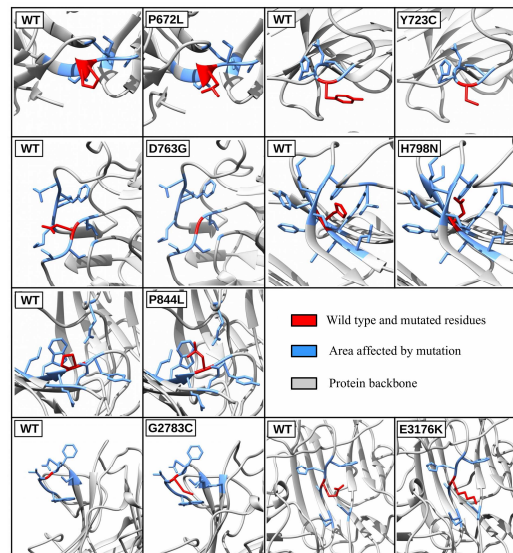


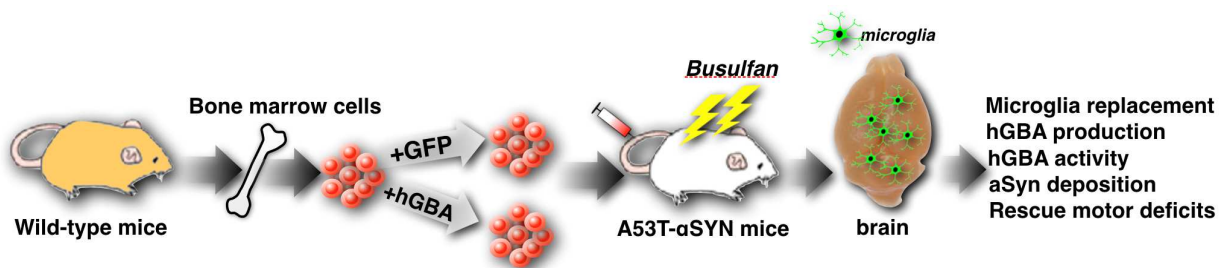
Fig. 1 Zoom panels comparing wild type and mutated residues

GBA expressing bone marrow-derived microglia for widespread and effecting degradation of alpha-synuclein pathological aggregates

Alessandro Papale^{1,2}, Luca Massimino^{1,2}, Chiara Elia^{1,3}, Rita Milazzo², Pietro Giuseppe Mazzara^{1,2}, Mirko Luoni^{1,2}, Gabriele Ordazzo^{1,2}, Serena Giannelli^{1,2}, Giuseppe Morabito^{1,2}, Alessandra Biffi^{2,4}, Michela Matteoli^{1,3}, Vania Broccoli^{1,2}

¹CNR Neuroscience Institute, Milano, Italy; ²IRCCS Ospedale San Raffaele, Milano, Italy; ³Humanitas Clinical and Research Center, Rozzano, Italy; ⁴Children's Hospital, Boston

Current therapies in Parkinson's disease (PD) are exclusively symptomatic, treating the motor dysfunctions without altering the course and progression of the disease processes. Therefore, new therapeutic strategies are needed that can directly counteract the pathological mechanisms. Recent studies have shown that PD progression is strictly associated with the accumulation and propagation of alpha-synuclein (α -Syn) toxic aggregates throughout the brain. In fact, over time α -Syn accumulates in neurons leading to their severe intoxication resulting in neuronal dysfunctions and eventual degeneration. This process is slow and can take years in humans leaving enough time for applying new treatments aiming to block this deadly α -Syn accumulation and spreading. With this in mind we have designed a strategy based on empowering brain microglia to overexpress the lysosomal enzyme Glucocerebrosidase (GCase) in order to enhance degradation of α -Syn oligomers and other pathological aggregates. In fact, although GCase viral transduction in brain has proven to strongly enhance α -Syn degradation, this approach is highly invasive and targeted to small sites. Herein, we plan a strategy where GCase lentiviral transduced bone marrow-derived microglia can colonize and diffuse throughout the brain and release GCase for a global degradation of α -Syn in the brain parenchyma. This approach will be tested in A53T- α -Syn transgenic mice to validate its efficacy in promoting bone marrow-derived microglia reconstitution in brain, diffuse GBA expression, α -Syn toxic species degradation and rescue of motor defects. This approach will provide the first proof-of-concept in vivo for the release of a therapeutic enzyme degrading α -Syn aggregates through microglia colonization in A53T- α -Syn mouse brains.



THE FLORENCE STROKE NETWORK: IMPROVEMENT IN ACUTE ISCHEMIC STROKE CARE.

Marzia Baldereschi¹, Antonio Di Carlo¹, Benedetta Piccardi^{1,2}, Francesco Bellomo³, Domenico Inzitari^{1,2}

¹CNR Neuroscience Institute, Florence, Italy; ²Department of Neurofarba, Neuroscience Section, University of Florence, Italy; ³Clinical Networks Management, Tuscany Health System, Florence, Italy.

AIMS. An organized, evidence-based approach to managing stroke can reduce mortality and morbidity and improve functional outcome for stroke patients. Variations in stroke care have been highlighted in Tuscany, with many patients not receiving evidence-based care. To address these concerns, a strategic planning process has been launched by the Tuscany Health System in early 2015 to develop and implement a new governance for the regional stroke network. The network was piloted throughout the Florence area: Florence Stroke Network (FSN).

MATERIALS AND METHODS. A coordinated system of care where Emergency Medical Services and hospitals function as a unified whole across Florence area was set up in 2015 to improve acute stroke care. The FSN relies on the development and implementation of standardized protocols related to each step of the acute stroke care: prehospital stroke code activation, in-hospital acute stroke coordinated procedures, inclusion and exclusion criteria for t-PA and thrombectomy treatments, transfer and drip&ship procedures. We included all patients with acute ischemic stroke consecutively admitted to each FSN hospitals from January 1, 2014 to December 31, 2015, using hospital discharge diagnoses (SDO). The 434.*1 and 433.*1 ICD9 codes were aggregated to determine a primary diagnosis of ischemic stroke; procedure code 99.10 identified t-PA treatments, code 39.74 thrombectomy. We measured short-term FSN efficacy by estimating and comparing numbers and rates of acute ischemic stroke treatments before (2014) and after (2015) FSN implementation.

RESULTS. The network spans across 3500 Km² and 1million inhabitants, with 1 hospital with no stroke services, 3 urban spoke hospitals, 1 suburban spoke hospital, and 1hub hospital. Through 2014, 956 patients with acute ischemic stroke were admitted and 57 (6%) were treated with t-PA. Number and proportions of t-PA treatments increased up to 88 and 9.2% (p=.008) in 2015. Number of treatments significantly increased in every spoke hospital as well in the hub hospital. A total of 16 drip&ship transfers were activated with 4 thrombectomies performed eventually by the hub hospital in 2015, compared to none in 2014.

CONCLUSIONS AND IMPLICATIONS. The logistic interventions provided by the FSN proved associated with a significant increase in acute ischemic stroke treatments. More stroke patients have received the benefits of t-PA and thrombectomy. This study could evaluate only short-term hospital-based care, information on longer-term outcomes are being collected. Next steps include the geographical expanding of the stroke network to the whole Tuscany region.

1. Alonso de Leciñana M, et al. A collaborative system for endovascular treatment of acute ischaemic stroke: the Madrid Stroke Network experience. *Eur J Neurol.* 2016 Feb;23(2):297-303.
2. Fargen KM, et al. Needed dialog: regionalization of stroke systems of care along the trauma model. *Stroke.* 2015 Jun;46(6):1719-26.
3. Spolaore P, et al. Measuring accuracy of discharge diagnoses for a region-wide surveillance of hospitalized strokes. *Stroke.* 2005 May;36(5):1031-4.

STROKE AWARENESS AMONG HIGH SCHOOL STUDENTS IN TUSCANY: EFFECTIVENESS OF AN EDUCATIONAL INTERVENTION

Antonio Di Carlo¹, Marzia Baldereschi¹, Benedetta Piccardi^{1,2}, Francesca Bovis², Domenico Inzitari^{1,2}

¹CNR Neuroscience Institute, Florence, Italy;

²Department of Neurofarba, Neuroscience Section, University of Florence, Italy

Objective. Differences in stroke incidence, care and outcomes may reflect different degrees of implementation of evidence-based interventions.^{1,2} Unawareness of stroke, its risk factors and treatments limits application of effective care. We aimed to evaluate knowledge of stroke and the effect of an educational intervention among high school students.

Methods. As part of the Program 'Ictus: Comunicazione & Innovazione', funded by Ente Cassa di Risparmio di Firenze and in collaboration with stroke patients' Association ALICe Tuscany, we performed a prospective evaluation of stroke knowledge in students of the fourth and fifth years in 10 high schools in Tuscany. A close-ended questionnaire on stroke knowledge, symptoms, risk factors, reaction to stroke, awareness of thrombolytic therapy and stroke units was administered at baseline. After questionnaire administration, a standardized presentation of about 1 hour was given, focusing mainly on risk factors, stroke symptoms, reaction and therapy. Questions from audience were allowed. After 3 months, the same questionnaire was re-administered to evaluate the long-term impact of the educational intervention.

Results. Overall, 585 students (50.8% males) were enrolled. At both examinations, more than 90% of participants indicated stroke as a brain disease, involving old as well as young people. Improved identification of stroke risk factors between baseline and follow-up examinations was found for diabetes, 41.1% vs. 58.5% ($P<0.001$); high cholesterol, 59.4% vs. 69.6% ($P=0.001$); obesity, 55.9% vs. 76.1% ($P<0.001$); alcohol abuse, 49.4% vs. 66.8% ($P<0.001$); smoking, 62.1% vs. 83.2% ($P<0.001$); and illegal drugs use, 73.8% vs. 87.2% ($P<0.001$). Among possible stroke symptoms, more than 90% of students identified hemiparesis and aphasia at both examinations. Recognition of severe headache and sudden visual loss improved from 70.4% to 78.9% ($P=0.002$) and from 69.5% to 86.4% ($P<0.001$), respectively. Knowledge of thrombolysis as a therapy for acute stroke was reported by 36.5% of students at baseline and 83.7% at follow-up ($P<0.001$). The existence of stroke-units, specialized in stroke care and providing a better outcome, was known by only 25.3% of students at baseline, which increased to 59.6% at follow-up ($P<0.001$).

Discussion and Conclusions. Baseline knowledge of stroke among high school students in Tuscany indicated a relatively good awareness of symptoms, and a fair awareness of some risk factors particularly relevant in young people, such as alcohol and smoking. A limited knowledge of thrombolysis and stroke units was also evidenced. The comparison between the two examinations indicated a global improvement of stroke awareness, which was dramatic for stroke units and thrombolysis, emphasizing the role of educational interventions in changing knowledge and behavior towards stroke in young populations.

1. Di Carlo A, et al. Methods of Implementation of Evidence-Based Stroke Care in Europe: European Implementation Score Collaboration. *Stroke* 2015;46:2252-2259.
2. Heuschmann PU, et al. Incidence of stroke in Europe at the beginning of the 21st century. *Stroke* 2009;40:1557-1563.

Inner retinal remodeling in an inducible mouse model of Retinitis Pigmentosa

Antonia Stefanov¹, Elena Novelli¹, Enrica Stretto¹

¹CNR Neuroscience Institute, Pisa, Italy;

In Retinitis Pigmentosa (RP), primary degeneration of rods occurs because of a genetic defect in a retinal-specific gene. The process of photoreceptor death triggers a complex chain of events among other retinal cells, which remodel to various extents, typically in a regressive manner. Studies on rodent models of RP have been shown that the first neurons to remodel are those connected to photoreceptors, namely bipolar and horizontal cells. These undergo gradual dendritic atrophy, loss of synaptic receptors, formation of ectopic synapses and secondary cell death. This cascade of events affects profoundly the possibility of repairing RP retinas by intervention on inner retinal neurons, which might eventually be lost in the long run. However, present knowledge of remodeling has been achieved by studying developmental rodent models, in which retinal degeneration and late stages of synaptogenesis are overlapped. This casts a shadow on the possibility to extend the results to human RP, in which photoreceptor death takes place well after completion of retinal development. Here we show the first results of remodeling studies on the retina of *Tvrm4* mice, which carry a dominant mutation of the rhodopsin gene, activated upon brief exposures to strong, white light. In 48 hours, light induction triggers a typical rod-cone degeneration with all the features of RP. Our hypothesis is that remodeling of second order neurons in *Tvrm4* adult mice conforms to stereotyped features previously described in other RP models, i.e. that cells connected to rods are among the first neurons to regress.

Tvrm4 mice (with a I307N mutation of RHO) aged 2-4 months and wt littermates were given eye drops of atropine, placed in an illuminating box built ad hoc and exposed to 2' of 12,000 Lux neon light. Groups of mice were harvested at 21 and 42 days, their eyes enucleated, fixed and processed for retinal immunocytochemistry to stain rod and cone bipolar cells, horizontal cells, glutamate metabotropic receptors and glial cells. These were counted in retinal whole mounts after confocal microscopy imaging and acquisition.

No variation in the number of rod bipolar cells was observed in *Tvrm4* mutant mice with respect to wt controls. Survival of these neurons was close to 100% even 6 weeks after phenotype induction. However, we observed a clear process of dendritic arborization retraction and loss of spatial order in these cells, with loss and misplacement of synaptic glutamate receptors. Horizontal cells also lost a relevant number of dendrites and their density dropped sensibly in the central retina, where they died out.

As these events have been described before in other RP mutants in which photoreceptor death overlaps with retinal development, we conclude that major regression of second order neurons is a hallmark of RP triggered by the primary death of rods and that is not influenced by the age of onset of the disease phenotype and by the underlying genetic mutation.

Funded by Macula Vision Research Foundation (USA), Roma Foundation (Italy), European Project H2020-MSCA-ITN-2014.

Loss-of-function mutations in the *SIGMAR1* gene cause distal hereditary motor neuropathy by impairing ER-mitochondria tethering and Ca²⁺ signaling

Giorgia Pallafacchina¹, Sofia Zanin¹, Elisa Gregianin², Valeria Crippa³, Paola Rusmini⁴, Angelo Poletti⁴, Antonio Petrucci⁵, Gian Maria Fabrizi⁶, Rosario Rizzuto¹, Giovanni Vazza²

¹CNR Neuroscience Institute and Department of Biomedical Sciences, University of Padova, Italy;

²Department of Biology, University of Padova, Italy; ³Experimental Neurobiology Lab, IRCCS "C. Mondino" National Neurological Institute, Pavia, Italy; ⁴Department of Pharmacological and Biomolecular Sciences, University of Milan, Italy; ⁵Neuromuscular and Rare Neurological Diseases Centre, Neurology & Neurophysiopathology Unit, ASO San Camillo-Forlanini Hospital of Rome, Italy; ⁶Section of Neuropathology, Neurological and Movement Sciences, University of Verona, Italy

Distal Hereditary Motor Neuropathies (dHMNs) are clinically and genetically heterogeneous neurological conditions characterized by degeneration of the lower motor neurons. So far, 18 dHMN genes have been identified, however about 80% of dHMN cases remain without a molecular diagnosis.

By a combination of autozygosity mapping, identity-by-descent segment detection and whole-exome sequencing approaches we identified two novel homozygous mutations in the *SIGMAR1* gene (p.E138Q and p.E150K) in two distinct Italian families affected by an autosomal recessive form of HMN.

SIGMAR1 encodes for the sigma non-opioid intracellular receptor 1 (sigma-1R), an integral membrane protein of the endoplasmic reticulum (ER) with chaperone activity, localized at the mitochondria-associated ER membrane (MAM) and implicated in many aspects of cellular homeostasis in the nervous system, including regulation of ion channels, Ca²⁺ signaling, neurite outgrowth and autophagy.

Functional analyses in several neuronal cell lines strongly support the pathogenicity of the sigma-1R mutations and provide insights into the underlying pathomechanisms involving the regulation of ER-mitochondria tethering, Ca²⁺ homeostasis and autophagy. Indeed, *in vitro*, both mutations reduce cell viability, induce the formation of abnormal protein aggregates thus preventing the correct targeting of sigma-1R protein to the mitochondria-associated ER membrane (MAM) and impinging on the global Ca²⁺ signaling.

Our data definitively demonstrate the involvement of *SIGMAR1* in motor neuron maintenance and survival by correlating, for the first time in the Caucasian population, mutations in this gene to distal motor dysfunction and highlight the chaperone activity of sigma-1R at the MAM as a critical aspect in motor neuron survival and dHMN pathology.

Disturbed local Ca²⁺ homeostasis in ALS astrocytes

Rosa Pia Norante¹, Maria Lina Massimino², Paolo Lorenzon¹, Agnese De Mario¹, Maria Catia Sorgato^{1,2}, Alessandro Bertoli¹

¹Department of Biomedical Science, University of Padova, and ²CNR Neuroscience Institute, Padova, Italy

Amyotrophic lateral sclerosis (ALS) is an invariably fatal neurodegenerative disorder characterized by the selective injury and death of motor neurons (MNs) in the spinal cord, brainstem and cerebral cortex. In spite of extensive studies, the mechanism underlying ALS pathogenesis is poorly understood, and no effective treatment is yet available.

To fill this gap, we have undertaken a thorough comparative investigation of local Ca²⁺ homeostasis in spinal astrocytes from a mouse model of ALS and healthy controls. This choice stems from two notions. Firstly, Ca²⁺ homeostasis – which controls a wide range of physiologic processes and triggers cell death events once its fine tuning is compromised – has been intimately related to ALS pathogenesis. Secondly, it has been recently recognized that non-neuronal cells, in particular astrocytes, are a primary target of ALS pathology and may play a role (by in-trans effects) in ALS-related MN demise, possibly through Ca²⁺-dependent mechanisms.

To accomplish the proposed aim, we use primary spinal cord astrocytes from transgenic mice expressing the G93A missense mutant of human superoxide dismutase 1 (SOD1), which is a well recognized animal model for ALS, and control (healthy) Tg mice expressing wild-type (WT) human SOD1. For Ca²⁺ measurements, we employ a Ca²⁺-sensitive photo-protein (aequorin, AEQ), genetically targeted to different cell compartments including the cytosol, cytosolic domains adjacent to the plasma membrane and the mitochondrial matrix. Such AEQ probes are transduced into primary astrocytes by means of lentiviral vectors.

Our study indicates altered Ca²⁺ fluxes in the cytosol and mitochondria of SOD1(G93A)-expressing astrocytes, following both store-operated Ca²⁺ entry and stimulation of purinergic receptors by ATP, with respect to the SOD1(WT) counterpart. In both cases, astrocytes expressing the mutant SOD1 display higher Ca²⁺ mobilization with respect to controls, suggesting the occurrence of (local) Ca²⁺ overloads in ALS astrocytes.

The Prion Protein Modulates the Activity of Glutamate Ionotropic Receptors and Mitochondrial Ca²⁺ Uptake in Various Neuronal Types

Maria Catia Sorgato^{1,2}, Agnese De Mario¹, Maria Lina Massimino², Caterina Peggion¹, Alessandro Bertoli¹

¹Department of Biomedical Science, University of Padova, and ²CNR Neuroscience Institute, Padova, Italy

It is well established that a conformation remodeling of the cellular prion protein (PrP^C), located to the external side of the plasma membrane (PM), originates the infectious prion agent causing fatal prion diseases (Prusiner, 1998). The physiologic function of PrP^C remains, however, elusive in spite of the many suggestions intimately connecting PrP^C to processes against cell damage and death (Linden et al., 2008). Following the finding that PrP^C limits Ca²⁺ entry via store-operated Ca²⁺ channels (SOCC) by downregulating Fyn tyrosine kinase, which is implicated in SOCC activation (De Mario et al., 2015), we explored whether PrP^C could also regulate ionotropic glutamate receptor-channels (iGluR) and metabotropic GluR-induced ER Ca²⁺ release. To this end, we used aequorin probes to assessing local Ca²⁺ movements and primary cerebellar granule (CGN), or cortical, neurons expressing, or not, PrP^C.

Combination of these approaches demonstrated that the presence of PrP^C prevented NMDAR-mediated Ca²⁺ overflow in both neuronal types, in accord with previous data in hippocampal paradigms (You et al., 2012). However, for the first time we also demonstrated that PrP^C downregulates both AMPA- and kainate-R in CGN, as well as total iGluR Ca²⁺ entry after glutamate addition. The finding that Ser845 of AMPAR GluR1 subunit was around 30% less phosphorylated in PrP^C-expressing neurons could explain the restricted Ca²⁺ entry in these neurons, given that Ser845 phosphorylation is directly linked to the channel open probability, and its stabilization at the synaptic surface.

Intriguingly, when assessing mitochondrial Ca²⁺ uptake, we detected only a minimal contribution of IP₃-sensitive channels to endoplasmic reticulum-mitochondrial Ca²⁺ transfer in both CGN types, the majority of Ca²⁺ accumulation deriving from Ca²⁺-induced Ca²⁺-release (CICR) via ryanodine receptor channels. Being PrP-KO CICR stimulated to a higher extent by the more abundant glutamate-induced Ca²⁺ entry, this explains mitochondrial Ca²⁺ overload in PrP-KO neurons compared to PrP^C-expressing counterparts.

Our data thus emphasize that PrP^C is part of the cell apparatus controlling Ca²⁺ homeostasis, and, concurrently, that the action of PrP^C protects neurons against Ca²⁺ excitotoxicity. In addition, data also demonstrate that the action of PrP^C extends beyond the PM by defending mitochondria from dangerously high Ca²⁺ accumulations.

Prusiner, S.B. (1998) Proc. Natl. Acad. Sci. U S A 95, 13363-13383.

Linden R., et al. (2008) Physiol. Rev. 88, 673-728.

De Mario A., et al. (2015) Front. Cell. Neurosci. 9, 416.

You H., et al. (2012) Proc. Natl. Acad. Sci. U S A 109, 1737-1742.

ON THE NEUROTOXICITY OF TWO ITALIAN VIPER VENOMS

Giulia Zanetti¹, Marco Pirazzini¹, Cesare Montecucco^{1,2}, Davide Lonati³ and Ornella Rossetto¹

¹University of Padova, Department of Biomedical Sciences, ²National Research Council Institute of Neuroscience, Padova, and ³Pavia Poison Center and National Toxicology Information Centre, Toxicology Unit, IRCCS Maugeri Foundation and University of Pavia, Italy

Venomous snakes in Europe are essentially restricted to the vipers of the genus *Vipera*, which is characterized by many subfamilies (*V. berus*, *V. ammodytes*, *V. ursinii* and *V. aspis*). From the clinical point of view, viper snakebites are generally characterized by local symptoms (pain, edema, swelling and in some case local necrosis) accompanied by systemic effects such as gastrointestinal manifestations, chest pain, hypotension, and coagulopathy. Neurological implications are rarely reported in Italy and France. Within the limitations linked to the retrospective determination of the biting viper species, it has been reported that neurologic complications were associated to *V. aspis*, but not to *V. berus* bites. We report here a molecular and toxicological investigation of *V. aspis* and *V. berus* venoms aimed at defining their neurotoxic profile. We determined the electrophoretical pattern of the two venoms, finding that both display bands around 14 kDa, typical of snake phospholipases A2 (PLA2). Accordingly, we investigated PLA2 activity *in vitro*, finding both venoms capable to hydrolyze phospholipids. However, only *V. aspis* venom generates "neuron bulges", the hallmark of PLA2-neurotoxins activity in primary cultures of neurons. For the *in vivo* analysis we injected the venoms in the mouse hind limb, and then evaluated the NMJ functionality by mean of electrophysiological measurements on soleus muscle. Consistently with the experiments on neuron cultures, we found that the venom from *V. aspis* causes paralysis, as assessed by the lack of evoked junction potentials and muscle twitch. At variance, the venom of *V. berus* induced a huge hemorrhagic effect, but did not hinder either evoked potentials or the capability of muscle to twitch. The immunohistochemical analysis of the same muscles with pre- and post-synaptic markers, showed that *V. berus* venom does not cause evident morphological alterations, while *V. aspis* venom produces major damage both to muscle fibers and to motor axon terminals. We also tested the neutralizing activity of an antiserum, routinely used as antidote. Using immunoblotting, we found that the antiserum similarly recognizes the majority of components found in both venoms, but, surprisingly, it poorly provides protection *in vivo*. Collectively, our results show that the venom of *V. aspis* displays both myotoxic and neurotoxic activity, reconcilable with its PLA2 activity. At variance, *V. berus* venom, even though equipped with an active PLA2 cause neither evident myotoxic nor neurotoxic activity.

TOXIN-INDUCED DEGENERATION OF DYSFUNCTIONAL NERVE TERMINALS: CAN THE BAD TURN INTO THE GOOD?

Elisa Duregotti^{1*}, Giulia Zanetti¹, Marco Pirazzini¹, Michele Scorzeto¹, Michela Rigoni¹, Ornella Rossetto¹, and Cesare Montecucco^{1,2}

¹ *Department of Biomedical Sciences, University of Padua, Via U. Bassi 58/B, 35131 Padova, Italy;*

² *CNR Institute of Neuroscience, Padua*

Botulinum neurotoxins (BoNTs) are metalloproteases acting inside peripheral nerve terminals, where they cleave different SNAREs causing a persistent, but reversible, inhibition of neuroexocytosis. Most patients survive botulism, but complete recovery is slow: among BoNTs, BoNT/A has the longest-lasting effect, causing a paralysis which persists for years in humans.

SPANs (snake neurotoxins with PLA2 activity) and alpha-Latrotoxin (from black widow spiders) are animal presynaptic neurotoxins which cause a runaway Ca^{2+} influx that triggers an acute degeneration of motor axon terminals, with consequent paralysis of denervated skeletal muscles. Such paralysis is completely reversible: in humans functional re-innervation is fully restored in few weeks.

Despite the fact that BoNTs and animal neurotoxins share similar outcomes on patients, the mechanism and duration of intoxication are very different: BoNTs induce a long-lasting blockade of neurotransmission without damaging the structure of nerve endings, whereas animal neurotoxins induce a rapid and complete degeneration of axon terminals, followed by a rapid recovery.

In this study, the injection of animal neurotoxins in muscles previously intoxicated with BoNT/A accelerates the rescue from paralysis in mice. Electrophysiological recordings on *ex vivo* nerve-muscle preparations as well as phenotypical observations on intoxicated mice limbs indicate that the effect of animal neurotoxins overcomes the long-lasting paralysis induced by BoNT/A, switching the kinetics of recovery from several weeks to few days. This is a proof of principle that, by destroying a dysfunctional nerve terminal via an acute, localized but reversible insult, one could favour nerve recovery. These observations could be extended to many dying-back neuropathies, where pathological changes first occur distally at the NMJ and then progress proximally toward the cell body.

Pterostilbene and cognitive performance in the aged rat model: molecular and behavioral effects

Michele Azzolini^{1,2}, Martina La Spina², Gabriele Sansevero³, Giulietta Di Benedetto^{1,2}, Silvia Morea³,
Laura Baroncelli³, Alessandro Sale³, Nicoletta Berardi³, Mario Zoratti^{1,2}, Lucia Biasutto^{1,2}

¹CNR Institute of Neuroscience, Padova; ²Department of Biomedical Sciences, University of Padova;
³CNR Institute of Neuroscience, Pisa

Pterostilbene is a major polyphenolic component of blueberries. Recent papers report that Pterostilbene is potentially very beneficial for health care, improving, for example, cognitive performance and short-term memory impaired by old age or neurodegeneration. While the literature reports effects of Pterostilbene in prevention/treatment of cognitive impairment in old individuals, no studies have investigated the detailed mechanisms underlying these observations.

The intent of this work is to identify novel downstream effectors of Pterostilbene treatment in aged rats. In an ongoing study we are evaluating whether the effects of Pterostilbene may be ascribed to cell renewal, through modulation of autophagy and mitochondrial homeostasis.

Aged (18 months) rats were initially evaluated through behavioral tests. Half cohort was then subjected to Pterostilbene treatment for 20 consecutive days at the end of which the cognitive assessment was repeated. Ventromedial prefrontal cortex, perirhinal cortex, dentate gyrus and the remaining hippocampus were then collected and analyzed by RT-qPCR or western blot.

Behavioral data indicate that Pterostilbene-treated animals show an improvement in memory and learning tests. Biochemical studies, although in progress, indicate that Pterostilbene induce important rearrangements at the cellular level, specific for each area of the brain considered. Major effects were seen in mitochondria- and synapse-specific proteins, suggesting remodeling, probably through the modulation of autophagy (see abstract by La Spina et al.) and activation of the transcription factor CREB. Previous analyses conducted in our laboratory suggest that cAMP and ROS may be the main effectors of Pterostilbene's effects (cf. La Spina et al.)

Concluding, protein analysis suggests that behavioral improvement may be related to: 1. a rearrangement in the synaptic architecture 2. modulation of mitochondrial homeostasis and 3. an increase in autophagy.

STRUCTURAL EQUATION MODELING TO ANALYSE THE DETERMINANTS OF HEALTHY AGING

Ilaria Rocco, Barbara Corso, Nadia Minicuci

CNR Neuroscience Institute, Padova, Italy

Structural Equation Modeling (SEM) is a statistical methodology that provides a very general and convenient framework for statistical analysis that includes several traditional multivariate procedures, for example factor analysis, regression analysis, discriminant analysis and canonical correlation.

The purpose of SEM is to investigate the real world complexity by taking into account a number of causal relationships among variables, both measurable and not directly observable (latent factors).

The first component of these models, called measurement model, allows us to build the latent factors by specifying which observed variables define each latent factor. The second component, the structural model, describes the relationships between the latent factors defined by the measurement model.

In the multiple equations that describe the model, the response variable in one regression equation can appear as an explanatory variable in another equation. For this reason, the terms dependent and independent variables of the canonical regressions are replaced by the terms endogenous and exogenous variables. If a variable assumes the role of independent variable in all the equations then it is called exogenous variable, otherwise if it assumes the role of dependent variable in at least one equation then it is called endogenous variable.

Moreover, one of the strong points of SEM is that it can estimate both the direct and indirect effects of a factor on the outcome. The direct effects are those influences unmediated by any other variable in the model, while the indirect effects are mediated by at least one intervening variable. The total effect of a factor on an outcome is equal to the sum of all the direct and indirect effects of the factor on that outcome.

An example of SEM application is presented. It concerns a study aimed to analyse the main factors that influence the health status of old Europeans and the effect of health status on wellbeing. The sample comes from two waves of the Survey of Health, Ageing and Retirement in Europe (SHARE) and comprises about 7000 respondents aged 65 or older.

Wellbeing and health are two complex concepts without a universal definition or measure. In the measurement model, the first factor was measured using the 12 items of the Control, Autonomy, Self-realisation and Pleasure quality of life scale (CASP-12), while the health status considering several health variables: difficulties in performing ADL and IADL, drugs consumption, morbidity, mobility and hospitalisation.

The possible determinants of healthy ageing considered are: the circumstances in which a respondent lived during childhood, the economic situation of the interviewee, the traumatic event of death of a child.

One of the results obtained showed that, even if the direct effect of the experience of traumatic events during childhood on the wellbeing in older ages is not significant, the total effect is significant, due to the mediation of the good health (indirect effect). This is just one example of the potential utility and power of SEM. Decomposing the effect makes it possible to perform more detailed analysis needed to address complex topics and problems that can be applied to a wide variety of fields of research.

PREVALENCE AND CONVERSION TO DEMENTIA OF MILD COGNITIVE IMPAIRMENT IN AN ELDERLY ITALIAN POPULATION

Federica Limongi¹, Paola Siviero¹, Marianna Noale¹, Antonella Gesmundo², Gaetano Crepaldi¹, Stefania Maggi¹, for the Dementia Registry Study Group

¹CNR Neuroscience Institute - Aging Branch, Padova, Italy; ²University of Padova, Italy

Background: Mild Cognitive impairment (MCI) is defined as a transitional phase between normal cognitive aging and dementia and represent a significant risk factor for dementia. There are a few Italian population studies on the prevalence of MCI and its rate of conversion to dementia.

Our study assessed the prevalence of MCI and its 4 subtypes and their rates of conversion to dementia after 1 year in an elderly Italian population.

Methods: The data are based on an Italian multicenter population-based cohort study with both cross-sectional and longitudinal components. Two thousand three hundred thirty-seven community-dwelling individuals over 65 underwent screening, clinical confirmation for MCI and 1-year follow-up.

Results: The prevalence of MCI was 21.6% and the amnesic multiple domain was the most frequent subtype (63.2%). The conversion rate to dementia was 4.1% and was found only in the amnesic multiple domain and the unclassifiable subjects, persons with cognitive deficit but neither demented nor diagnosed with MCI.

Conclusion: Besides providing data on the prevalence and rate of conversion of MCI to dementia in an elderly Italian population, this study examined MCI's subtypes. Special attention should be reserved for unclassifiable subjects who risk going undiagnosed and untreated.

Generation and validation of novel tools for the analysis of Ca²⁺ homeostasis in ALS motor neuron

Maria Lina Massimino¹, Rosa Pia Norante², Paolo Lorenzon², Raffaele Lopreiato², Agnese De Mario², Mattia Albiero³, Maria Catia Sorgato^{1,2}, Alessandro Bertoli²

¹CNR Neuroscience Institute, and ²Department of Biomedical Science, University of Padova, Italy, ³Department of Medicine, and Venetian Institute of Molecular Medicine, Padova, Italy.

A finely tuned Ca²⁺ homeostasis is of fundamental importance for the life of excitable cells and of neurons in particular, in which transient local Ca²⁺ oscillations direct the proper spatio-temporal coordination of electro-chemical signals, overall neuronal metabolism, and cell survival.

The mechanisms underlying amyotrophic lateral sclerosis (ALS) are poorly understood, and no effective treatment for the disease is available. Recent research, however, has established that ALS-related mutant SOD1 (mSOD1), which is responsible for about 20% of familial (f) ALS forms, alters Ca²⁺ homeostasis in motor neurons (MNs). This may render MNs particularly vulnerable to the activation of a subset of harmful pathways, such as those triggered by the elevated Ca²⁺ levels found in the cytosol and other cell compartments of fALS MNs.

To the purpose of studying local Ca²⁺ homeostasis in MNs from primary spinal cord cell cultures, we have generated and functionally validated adeno-associated viral (AAV) vectors for the expression of fluorescent (FRET-based)ameleon Ca²⁺ probes targeted to different cell domains (i.e., cytosol, mitochondrial matrix or endoplasmic reticulum lumen) under the transcriptional control of a MN-specific promoter.

We have demonstrated that:

- (i) Such probes are expressed in immortalized NSC-34 cells only after differentiation to a MN phenotype.
- (ii) The probes are not expressed in primary cultures of spinal or cortical astrocytes, or of cortical, hippocampal or cerebellar granule neurons.
- (iii) In primary spinal cord cultures, the cameleons are expressed only in MNs, enabling us to specifically record MN Ca²⁺ fluctuations in mixed spinal cord cultures (thus in a more physiologic setting than pure MN cultures), with no need for complex MN purification steps.
- (iv) All generatedameleon probes have the expected sub-cellular localization.
- (v) The probes allow Ca²⁺ measurements in the three cell compartments of primary MNs. Preliminary analyses have shown higher basal Ca²⁺ levels, and a larger Ca²⁺ response following stimulation of AMPA-sensitive ionotropic glutamate receptors, in the cytosol of mSOD1-expressing MNs with respect to the healthy counterpart.
- (vi) Theameleon probes can be effectively delivered to spinal cord MNs *in vivo*, following intravenous injection in newborn mice. This achievement will potentially allow local Ca²⁺ measurements in MNs of spinal cord slices.

MECHANISM OF ACTIVATION AND FUNCTION OF THE ODORANT RECEPTOR EXPRESSED AT THE AXON TERMINUS-GROWTH CONE OF OLFACTORY SENSORY

Simona Francia^{1,2}, Ilaria Zamparo³, Sira Angela Franchi⁴, Claudia Lodovichi^{1, 2,5}

¹Venetian Institute of Molecular Medicine, Padua; ²CNR, Neuroscience Institute, Padua; ³Department of Biomedical Sciences, University of Padua; ⁴San Raffaele Hospital, Milan; ⁵Armenise Harvard CDA

A unique feature in the topographic organization of the olfactory bulb is the dual role of the odorant receptor. It does detect odors in the olfactory epithelium but it is also plays an instructive role in the convergence of axons of olfactory sensory neurons expressing the same odorant receptor to form glomeruli in specific loci of each olfactory bulb. This spatial segregation of sensory afferents gives rise to the sensory map of the olfactory bulb, that has a critical role in odor coding. The odorant receptor, a G protein-coupled receptor, is expressed at the cilia, where it binds odors, but also at the axon terminus, a suitable location to act as an axon guidance molecule.

In previous works, we found that the odorant receptor at the axon terminus is functional and coupled to local increase of cAMP and Ca²⁺. The question that remained to be addressed was the mechanism of activation of the odorant receptor at the axon terminus. We hypothesized that few molecules expressed in the olfactory bulb, could bind and activate the odorant receptor at the axon terminus of olfactory sensory neurons.

Here, by studying the spatio-temporal dynamics of Ca²⁺ in olfactory sensory neurons in response to a pool of molecules extracted from the olfactory bulb, we found that this pool of molecules, locally applied, was able to induce Ca²⁺ rise at the olfactory sensory neuron axon terminus. To ascertain that this Ca²⁺ rise was due to the activation of the odorant receptor, we expressed specific odorant receptors in HEK cells. We found that the active pool of molecules from the olfactory bulb was able to elicit Ca²⁺ rise in HEK cells transfected with specific odorant receptors, but not in HEK cells transfected only with the vector, used as controls.

By mass spectrometry of the active pool of molecules from the olfactory bulb, we identified a putative ligand of the odorant receptor at the axon terminus. This ligand, locally applied, was able to elicit Ca²⁺ rise in olfactory sensory neuron axon terminus and in HEK cells transfected with specific odorant receptors.

All together our data demonstrated that the odorant receptor at the axon terminus appears to act as an axon guidance molecule, activated by molecules expressed in the olfactory bulb, that contribute in providing the olfactory sensory neurons with instructions to reach the proper target.

A new molecular pathway for PTX3 in the brain and its role in AMPA receptors regulation

Elisabetta Menna^{1,2}, Giuliana Fossati², Davide Pozzi², Sonia Valentino², Alice Canzi³, Annalisa Savardi², Filippo Mirabella¹, Milena Moretti⁴, Cecilia Gotti¹, Barbara Bottazzi², Cecilia Garlanda², Michela Matteoli^{1,2}

¹CNR-Institute of Neuroscience, Milan, Italy; ²Humanitas Clinical and Research Center, Rozzano (Milan), Italy; ³Humanitas University, Rozzano (Milan), Italy; ⁴BIOMETRA, University of Milan.

Pentraxin3 (PTX3) is a molecule of the innate immune system with well-known functions such as pathogens opsonization, interaction with C1q protein of the complement and tissue remodeling. Nowadays, inflammation is increasingly recognized as a key factor influencing the brain physiology and pathology. Indeed, a number of inflammatory molecules has been found to regulate specific neuronal processes, but almost nothing is known about the role of PTX3 in brain development and function, except for a few studies showing that PTX3 deficiency is accompanied by increased neuronal death during epileptic events and stroke. Here, we demonstrated that astrocytes, but not neurons, produce and release PTX3. Exogenous administration of PTX3 to hippocampal neurons leads to MAPK phosphorylation and enhanced excitatory neurotransmission, which is blocked by the specific MAPK inhibitor, PD98059. Confocal analysis excluded any alterations in synapse number and structure, and showed a significant increase of surface AMPA receptors at the synapse in PTX3-treated neurons. Of note, PTX3 ^{-/-} neurons display a reduction of AMPA receptors at the postsynaptic membrane. Moreover, N-terminal region of PTX3, but not the PTX3-pentraxin C-terminal domain, showed the same ability as the full-length protein to exert the effects we observed. The N-terminal region of the protein has been reported to interact with hyaluronan, a component of the extracellular matrix (ECM) playing a role in ECM organization. Indeed, perturbation of the ECM completely prevented the PTX3-induced increase of AMPA receptors at the synapse. We then hypothesized that integrins could have a strategic role in mediating PTX3 effects. Integrins bind to both ECM and AMPA receptors, and they are also involved in the activation of MAPK signaling. We therefore used an RGD sequence-containing peptide to block the interaction between integrins and different ECM components. Indeed, we found that co-incubation of RGD with PTX3 prevents AMPA receptors insertion. In conclusion, our results demonstrate that astrocyte-derived PTX3 regulates glutamatergic neurotransmission via MAPK activation, an intact neuronal ECM and integrin involvement.

PLA2 snake myotoxins are internalized by cell surface nucleolin: a potential new pathway that leads directly to the cell nucleus

Fiorella Tonello¹, Giorgio Arrigoni², Julian Fernandez³, Bruno Lomonte³,
Maria Lina Massimino¹, Morena Simonato¹, Barbara Spolaore⁴

¹CNR Institute of Neuroscience, Padova; ²Department of Biomedical Sciences, University of Padova;
³Instituto Clodomiro Picado, San José, Costa Rica, ⁴CRIBI Biotechnology Centre, University of Padova.

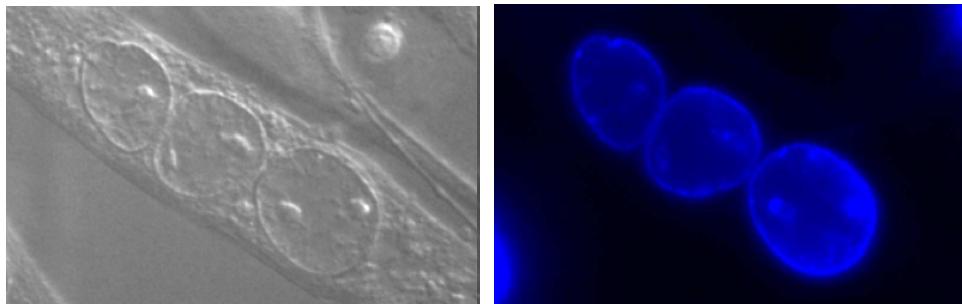
Myotoxins from *Bothrops* snake venom are group II basic phospholipases A2, but some of them are enzymatically inactive natural mutants. Beside the myotoxic activity some snake PLA2 possess a generic cytotoxic activity and many were reported to have specific toxic action against tumor cells.

The subcellular site of action of these toxins is still unknown, their toxicity is uncorrelated to the catalytic activity and many experimental observations indicate that they stimulate intracellular signalling events, suggesting that they have a cell surface receptor (1, 2, 3).

Here, live fluorescence experiments with PLA2 toxins, obtained from *Bothrops asper* venom and derivatized with a fluorophore, demonstrated that these toxins are internalized in cells in perinuclear and nuclear zones.

In pull-down assays, with a *Bothros asper* myotoxin derivatized with biotin used as bait, different proteins were isolated from cellular lysate and identified by mass spectrometry. Nucleolin and nucleophosmin, proteins overexpressed in the surface of different tumoral cells, resulted to be among the most abundant pulled-down proteins. The interaction between the phospholipase A2 and nucleolin was confirmed by crosslinking experiments on isolated proteins and on cells. A specific nucleolin aptamer was found to inhibit the internalization of *Bothrops asper* PLA2s and their cytotoxicity.

In conclusion, surface nucleolin appears to interact with snake venom phospholipases and guide them to the cell nucleus.



C2C12 myotubes treated with *B. asper* myotoxin I – DNS (blue fluorophore)

1. Tonello et al. A Lys49-PLA2 myotoxin of *Bothrops asper* triggers a rapid death of macrophages that involves autocrine purinergic receptor signaling, *Cell death & disease*, 3,7,e343, 2012
2. Simonato et al. Production in *Escherichia coli*, folding, purification and characterization of notexin with wild type sequence and with N-terminal and catalytic site mutations, *Toxicon*, 88, 11-20, 2014
3. Tonello & Rigoni, Cellular Mechanisms of Action of Snake Phospholipase A2 Toxins, *Snake Venoms*, Toxinology series 2015

ROLE OF THE PROINFLAMMATORY CYTOKINE IL-6 IN SYNAPTOGENESIS

Davide Pozzi¹, Filippo Mirabella¹, Giuliana Fossati¹, Genni Desiato¹, Marco Rasile¹, Raffaella Morini¹, Elisabetta Menna^{2,3} and Michela Matteoli^{1,2,3}

¹Laboratory of Pharmacology and Brain Pathology Humanitas Clinical and Research Center, Rozzano, Milan, Italy; ²Dept of Medical Biotechnology and Translational Medicine, University of Milan, Italy; ³CNR Institute of Neuroscience, Milan, Italy.

Inflammatory conditions occurring at central nervous system have been associated with several brain diseases. In particular epilepsy and autism show a tight association with a neuroinflammatory state characterized by high levels of circulating cytokines, such as the pro-inflammatory cytokine Interleukin-6 (IL-6). Hence, we started investigating the role of IL-6 in synaptogenesis and synaptic transmission in primary cultured neurons, a simplified model which recapitulate the in vivo stages of synaptic formation. Our preliminary results show that elevated levels of IL-6 during the development of neuronal cultures are able to significantly modify the normal formation of excitatory synapses, leading to an increase in the number of glutamatergic synaptic contacts. Interestingly, the same treatment failed to alter GABAergic synapses, thus suggesting that altered levels of IL-6 during neuronal development might affect the proper balancing between excitatory and inhibitory (E/I) inputs. In line with a possible alteration of E/I balance, IL-6 treated neurons exhibited an increase in spontaneous firing rates. Furthermore, we also found that IL-6 treatment alters the expression pattern of JAK2 and STAT3, two central protein in IL-6 signaling, thus suggesting their possible involvement in such process. These evidences show that increased level of IL-6 affects the proper neuronal development by altering the E/I balance. As many brain disorders, including autism and epilepsy, are characterized by an unbalance between excitatory and inhibitory inputs, we propose that altered IL-6 levels during brain development might play a role in the pathogenesis of these diseases.

TANC2: a New Paradigm In Neuronal Signal Transduction

Alessandra Gasparini^{1,2}, Fiorella Tonello³, Geppo Sartori², Emanuela Leonardi¹, Silvio C.E. Tosatto^{2,3}

¹*Molecular Genetics of Neurodevelopmental disorders, Department of Woman and Child's health, University of Padova;* ²*Department of Biomedical Sciences and CRIBI Biotechnology Center, University of Padova;* ³*CNR Institute of Neuroscience, Padova.*

CDKL5 (cyclin-dependent kinase-like 5) functions in synapse development and plasticity. Upon NMDAR stimulation, CDKL5 is dephosphorylated by protein phosphatase 1 (PP1), causing its proteasome-dependent degradation. This step seems to be important for the activity-dependent signaling cascade regulating the composition, shape, and strength of the synapse. However, the mechanism targeting PP1 activity towards CDKL5 is still unknown¹. Here we performed a detailed *in silico* analysis of TANC2, a novel PSD-95-interacting scaffold protein located at postsynaptic densities and involved in regulation of synaptic strength². TANC2 contains multiple domains for protein-protein interaction, such as the ankyrin-repeat containing domain and the tetratricopeptide domain, and several conserved linear motifs for protein binding, suggesting a role in integrating multiple incoming signals (paper under revision). Given the presence of two highly conserved PP1 docking motifs (RVxF) in N-terminal tail, we speculate that TANC2 could bind the phosphatase and direct its substrate specificity to CDKL5, as other PP1 regulative subunits³. To confirm this hypothesis, binary interaction combinations among TANC2, PP1 and CDKL5 were validated using the yeast two hybrid system. As expected, TANC2 directly binds PP1 through its N-terminus, which we proved to be mandatory for the protein complex formation. Conversely, CDKL5 binds the TPR and nearby regions of TANC2. Supporting our hypothesis, the results were replicated by co-immunoprecipitation assay. This findings suggest that TANC2 could function as a scaffold linking the enzyme (PP1) to its substrate (CDKL5) allowing its dephosphorylation and subsequent degradation. Thus, TANC2 seems to be the connection among different signaling pathways converging to modulate neuronal activity.

This work is supported by italian ministry of health young researchers grant GR-2011-02347754.

REFERENCES:

1. La Montanara, P. et al. Synaptic Synthesis, Dephosphorylation, and Degradation: a novel paradigm for an activity-dependent neuronal control of CDKL5. *J. Biol. Chem.* 290, 4512–4527 (2015).
2. Han, S. et al. Regulation of Dendritic Spines, Spatial Memory, and Embryonic Development by the TANC Family of PSD-95-Interacting Proteins. *J. Neurosci.* 30, 15102–15112 (2010).
3. Peti, W., Nairn, A. C. & Page, R. Structural basis for protein phosphatase 1 regulation and specificity: Protein phosphatase 1 regulation and specificity. *FEBS J.* 280, 596–611 (2013).

Paper under revision: Gasparini, A. et al. Dynamic scaffolds for neuronal signaling: *in silico* analysis of the TANC protein family. *Sci Rep.*

Phosphatases are key determinants of compartmentalised cAMP/PKA signalling at the Outer Mitochondrial Membrane

Konstantinos Lefkimmiatis^{1,2,3,4}, Alex Burdyga¹, Nicoletta C. Surdo¹, Stefania Monterisi¹, Mario Bortolozzi³, Pawel Swietach¹, Manuela Zaccolo^{1,2}

¹Burdon Sanderson Cardiac Science Centre Department of Physiology, Anatomy and Genetics, Oxford UK;

²BHF Centre of Research Excellence, Oxford; ³Foundation for Advanced Biomedical Research, Venetian Institute of Molecular Medicine, Padua, Italy. University of Padua, Department of Physics and Astronomy, Padua, Italy; ⁴CNR Neuroscience Institute and Dept. of Biomedical Sciences, Padua, Italy.

The second messenger cyclic AMP (cAMP) achieves its functional pleiotropy thanks to the compartmentalization of its signaling cascade. This is accomplished *via* the coordinated actions of two protein families, phosphodiesterases (PDEs) and A-kinase anchoring proteins (AKAPs). According to this paradigm, PDEs selectively hydrolyze cAMP allowing it to reach cellular domains of enriched PKA (protein kinase A) generated by AKAPs. However, another group of proteins can regulate the cAMP/PKA axis, the phosphatases. These enzymes dephosphorylate the proteins modified by PKA to effectively terminate the cAMP cascade. While the role of phosphatases in regulating PKA is intuitively important, their contribution into shaping the cAMP/PKA pathway is underappreciated.

Here we combined biochemistry, molecular biology and FRET-based sensors to monitor cAMP, PKA and phosphatase activity at the cytosol and outer mitochondrial membrane (OMM) of primary cardiomyocytes. Low levels of cAMP, that did not activate PKA in the cytosol, induced strikingly high PKA activity at the OMM. Interestingly, these differences did not depend on PDEs but rather on differential phosphatase activity between the two compartments. We found that phosphatase activity at the cytosol blocked activation of CREB (cAMP response element-binding protein) while low phosphatases at the OMM allowed mitochondrial elongation in response to cAMP elevating agents. We conclude that the OMM of cardiomyocytes constitutes a privileged cAMP microdomain and pinpoint phosphatases as important contributors in shaping the OMM/cAMP pathway in the heart.

Pharmacological characterisation of nicotinic acetylcholine receptors expressed in glioma and glioblastoma cells

Francesca Fasoli^{1*}, Vanessa Mucchietto^{1,2**}, Roberta Benfante^{1,2}, Annalisa Maroli^{1,2}, Matteo Tamborini^{1°}, Michela Matteoli^{1,3}, Francesco Clementi^{1,2} and Cecilia Gotti^{1,2}

¹CNR Neuroscience Institute, Milan, Italy; ²Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy; ³Humanitas Clinical and Research Center, Rozzano, Italy

Recipients of fellowship from the Fondazione Giancarla Vollaro[°] and Fondazione Confalonieri^{°°}, Milan, Italy

Gliomas and glioblastomas (GBMs) are a family of highly proliferative, migratory and invasive malignant brain tumours arising from different glial elements and more than 70% of the affected patients die within two years of diagnosis.

Cigarette smoke is an environmental risk factor for the induction and development of human malignancies, and many glioma patients are cigarette smokers.

The aim of this study was to characterise the nAChRs in gliomas and glioblastoma cells, analyse whether they regulate cell proliferation and intracellular signalling and investigate whether this signalling is modulated by nicotine.

Quantitative real-time PCR confirmed the expression of a number of nAChR subunits in the U87MG glioma cell line and in primary glioblastoma cultures derived from patients at different tumoral stages. MTS assay and cell counting showed that nicotine, in a dose-dependent manner, significantly increased cell proliferation.

These effects were blocked by co-treatment of the cells with specific nAChR antagonists.

We also found that treatment of glioma cell line and glioblastoma cells with stylobene-derived antagonists reduced the viability of the cells in a dose-dependent manner, but had no effect on neuroblastoma and hepatocyte cells. Moreover, we found that nicotine exposure induced a time-dependent changes in the phosphorylation of Erk and Akt, and these changes are prevented by co-incubation of the cells with nicotinic antagonists.

Collectively, our data suggest that nicotine activates signalling pathways involved in the proliferation and invasiveness of glioma and glioblastoma cells and, α -bungarotoxin-sensitive receptors mediate these effects, thus representing a possible target for new therapeutic strategies.

Validation and optimization of aptamers as novel diagnostic and therapeutic tools for pancreatic tumors

Maria Luisa Malosio^{1,2}, Francesca Davi², Cristina Brigatti³, Davide Cittaro⁴, Dejan Lazarevic⁴, Elia Stupka⁴, Laura Cerchia⁴, Vittorio de Franciscis⁴

¹*Istitute of Neuroscience (IN) Consiglio Nazionale delle Ricerche (MI), Italy, email: m.malosio@in.cnr.it;* ²*Humanitas Clinical and Research Center, Laboratory of Pharmacology and Brain Pathology, Rozzano (MI), Italy;* ³*Diabetes Research Institute, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Hospital & San Raffaele University School of Medicine, Milan, Italy.*

Pancreatic tumors are difficult to diagnose and especially the exocrine-derived are among the most lethal human cancers. RNA aptamers are an emerging class of drugs and diagnostic tools able to interact, due to their specific three-dimensional structure, with a great number of different targets, including proteins, small molecules, viruses and whole cells with high selectivity and specificity. Similar to antibodies, RNA aptamers can be used for targeted diagnosis and therapy.

The cell-SELEX approach has been employed to perform two screenings for identifying aptamers binding to pancreatic tumor cells and to a molecule up-regulated on endothelial cells following inflammation, a feature accompanying neoangiogenesis associated to tumor metastasis.

We have characterized the aptamers obtained following the two screening approaches by Next Generation Sequencing and have performed with selected aptamers binding studies on several cell lines of mouse, rat and human origin representing two types of pancreatic tumors the endocrine insulinoma and the ductal adenocarcinoma.

Around 10 aptamers have been characterized more in detail, also using short variants and show some binding specificities for the different cell lines. Studies are ongoing in order to identify their binding targets on the tumor cell lines.

RNA aptamers are promising versatile diagnostic and therapeutic tools, which offer several advantages compared to antibodies and are suitable to being modified by introducing fluorochromes, radionuclides or nanoparticles suiting several diagnostic imaging modalities. Moreover aptamers can be used to achieve targeting specificity, such as for miRNA and drug delivery.

References

Catuogno S, Esposito CL, de Franciscis V Developing aptamers by Cell-based SELEX Methods Mol Biol 2016 1380:33-46; doi: 10.1007/978-1-4939-3197-2_3.

Esposito CL, Catuogno S, de Franciscis V Aptamer miRNA conjugates for cancer cell-targeted delivery Methods Mol Biol 2016 1364:197-208; doi: 10.1007/978-1-4939-3112-5_16.

Activation of α bungarotoxin-sensitive nicotinic receptors in non small cell lung cancer A549 plays an important role in nicotine-induced proliferation

Vanessa Mucchietto^{1,2*}, Francesca Fasoli^{1,2**}, Roberta Benfante^{1,2}, Annalisa Maroli^{1,2}, Marco Pallavicini², Cristiano Bolchi², Susanna Pucci^{1,2}, Francesco Clementi^{1,2}, Michael McIntosh³ and Cecilia Gotti^{1,2}

¹CNR Neuroscience Institute and ²Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy; ³Departments of Psychiatry and Biology, University of Utah, Salt Lake City, Utah, USA.

Recipients of fellowship from Fondazione Confalonieri° and the Fondazione Giancarla Vollaro°, Milan,

Nicotinic acetylcholine receptors (nAChRs) are expressed in normal bronchial epithelial and in non-small cell lung cancer (NSCLC) cells, and are involved in cell growth regulation. Nicotine is an nAChR exogenous ligand and through activation of nAChRs induces cell proliferation and stimulates lung cancer growth.

To verify whether nicotine-induced A549 NSCLC cell proliferation was the result of nAChR activation, cells exposed to nicotine were co-incubated with the nicotinic $\alpha 7$ or $\alpha 9$ subtype-specific antagonists α -bungarotoxin (α -Bgtx) or methyllycaconitine (MLA).

As nicotine-induced cell proliferation was observed using the selective $\alpha 7$ agonist ICH3, we also treated the cells with Rg1 toxin, an $\alpha 9$ -selective antagonist, in order to detect the possible involvement of $\alpha 9$ -containing receptors. Co-incubation with Rg1 toxin completely blocked nicotine-induced proliferation and signalling.

We have previously characterised 4-oxystilbene derivatives as selective $\alpha 7$ antagonists, and have now found that two of these compounds, F1 (previously called MG624) and F3, bind and block not only $\alpha 7$ - but also $\alpha 9$ - and $\alpha 10$ -containing receptors. F1 and F3 treatment of A549 cells blocked their nicotine-induced proliferation and viability in a dose-dependent manner, but were much less active on SH-SY5Y and HepG2 cells.

Finally, as A549 cells express high $\alpha 5$, $\alpha 7$ and $\alpha 9$ mRNA levels, and nAChRs containing these subunits are involved in nicotine-induced proliferation, we also screened for at least two short interfering RNAs (siRNAs) against the *CHRNA5*, *CHRNA7* and *CHRNA9* genes in order to knock down the expression of the subunits.

RT-PCR and Western blotting showed that the siRNAs against *CHRNA5* and *CHRNA7* blocked nicotine-induced proliferation and intracellular signalling.

In conclusions, our data show that the inhibition of Bgtx-sensitive receptors ($\alpha 7$ and/ or $\alpha 9$ - containing receptors) by subtype-specific antagonists or RNA interference can inhibit A549 NSCLC cell proliferation *in vitro*.

LG11 TUMOR TISSUE EXPRESSION AND SERUM AUTOANTIBODIES IN PATIENTS WITH PRIMARY MALIGNANT GLIOMA

Carlo Nobile^{1,2}, Emanuela Dazzo¹, Elena Pasini³, Sandra Furlan¹, Dario de Biase⁴, Matteo Martinoni³, Roberto Michelucci³

¹CNR-Neuroscience Institute, Section of Padua, and ²Department of Biomedical Sciences, University of Padua, Padua, Italy; ³IRCCS-Institute of Neurological Sciences, Bellaria Hospital, Bologna, Italy; ⁴Department of Pharmacology and Biotechnology (FaBIT), University of Bologna, Bologna, Italy

The LG11 protein is thought to be implicated in malignant progression of glioma tumors, and mutations in the encoding gene, *LG11*, cause autosomal dominant lateral temporal epilepsy, a genetic focal epilepsy syndrome. We investigated the possible involvement of LG11 in high-grade glioma-associated epilepsy by analyzing its expression in tumor specimens of patients with and without epilepsy and by searching for LG11 autoantibodies in the sera these patients. We examined tumor tissue samples from 24 patients with high-grade gliomas (12 with and 12 without epilepsy) by immunoblot and detected variable amounts of LG11 in tumor tissues from 9/24 (37%) patients. LG11 was detected in 7/12 (58%) patients with epilepsy and in 2/12 (16%) patients without epilepsy ($p = 0.0894$; Fisher's exact test). Moreover, testing blood sera of five patients for antibodies against LG11 revealed LG11 autoantibodies in two patients, both suffering from epilepsy and expressing LG11 in tumor tissue. Our findings suggest that there may be a preferential expression of LG11 in high-grade glioma tumors of patients with epilepsy. We also unveil the presence of serum LG11 autoantibodies in some patients with high-grade gliomas, where they might play an epileptogenic role.

Targeting mitochondrial potassium channel Kv1.3 to selectively kill glioblastoma cells

Luigi Leanza¹, Elisa Venturini², Michele Azzolini³, Stephanie Kadow², Michael Weller⁴, Ghazaleh Tabatabai⁵, Mario Zoratti³, Ildikò Szabó^{1,3}, Erich Gulbins², Katrin Anne Becker^{2*}

¹Department of Biology, University of Padova, Italy; ²Department of Molecular Biology, University of Duisburg-Essen, Germany. ³CNR Institute of Neurosciences and Department of Biomedical Sciences, University of Padova, Italy. ⁴Laboratory of Molecular Neuro-Oncology, Department of Neurology and Neuroscience Center, University Hospital and University of Zurich, Switzerland; ⁵Interdisciplinary Division of Neuro-Oncology, Departments of Vascular Neurology and Neurosurgery, University Hospital Tübingen, Germany.

Glioblastoma (GBM) is one of the most aggressive cancers, accounting for half of the newly diagnosed patients with central nervous system (CNS) cancers in the United States and Europe. The major problem with the current standard of care, which includes temozolomide in combination with radiation therapy, is the high resistance, the highly invasive behavior of GBM cells and the heterogeneity of the blood brain barrier (BBB). Kv1.3, a potassium channel of the *shaker* family, is expressed in the inner mitochondrial membrane (IMM) of many cancer cells, where its inhibition by different membrane permeant blockers PAP-1, Psora-4 and clofazimine was shown to induce apoptosis [Leanza et al. EMBO Mol Med. 2012]. Here, we report for the first time that Kv1.3 is expressed in mitochondria of GL261, A172 and LN308 glioma cell lines, and that their treatment with clofazimine, a drug already used in the clinic against leprosis, induces significant apoptosis in these cells. Cytochrome c release followed an increased mitochondrial reactive oxygen species (ROS) production and mitochondrial membrane depolarization, indicating the induction of the intrinsic pathway of apoptosis. We further demonstrate that the effects were specific for Kv1.3, since transient transfection with siRNA against Kv1.3 abolished channel inhibitor-induced cell death. These *in vitro* data suggest that inhibition of Kv1.3 in glioma cells might be a novel strategy to target these cells. However, *in vivo* pharmacokinetics reveal that clofazimine was not able to pass the BBB and, in accordance, reduction of tumor volume did not take place using the *in vivo* syngeneic mouse GBM model.

ATP RELEASED BY INJURED NEURONS ACTIVATES SCHWANN CELLS

Samuele Negro¹, Elisanna Bergamin¹, Umberto Rodella¹, Elisa Duregotti¹, Michele Scorzeto¹, Kees Jalink³, Cesare Montecucco^{1,2} and Michela Rigoni¹

¹*Department of Biomedical Sciences, University of Padua, Via U. Bassi 58/B, 35131 Padova, Italy;*

²*CNR Institute of Neuroscience, Padua;* ³*Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands*

Injured nerve terminals of neuromuscular junctions (NMJs) can regenerate. This remarkable and complex response is governed by molecular signals that are exchanged among the cellular components of this synapse: motor axon nerve terminal (MAT), perisynaptic Schwann cells (PSCs), and muscle fibre. The nature of signals that govern MAT regeneration is ill-known. In the present study the spider toxin α -Latrotoxin has been used as tool to investigate the mechanisms underlying peripheral neurodegeneration. Indeed this neurotoxin induces an acute, specific, localized and fully reversible damage of the presynaptic nerve terminal, and its action mimics the cascade of events that leads to nerve terminal degeneration in injured patients and in many neurodegenerative conditions.

Here we provide evidence of an early release by degenerating neurons of ATP as *alarm* messenger, that contributes to the activation of a series of intracellular pathways within SCs that are crucial for nerve regeneration: Ca^{2+} , cAMP, ERK1/2, and CREB. These results contribute to define the cross-talk taking place among degenerating nerve terminals and PSCs, involved in the functional recovery of the NMJ.

Absolute quantification of myosin heavy chain isoforms using selected reaction monitoring highlights skeletal muscle alterations in a mouse model of amyotrophic lateral sclerosis

Alessandro Bertoli¹, Caterina Peggion¹, Maria Lina Massimino², Giancarlo Biancotto³, Roberto Angeletti³, Carlo Reggiani¹, Maria Catia Sorgato^{1,2}, Roberto Stella³

¹*Department of Biomedical Science, University of Padova and* ²*CNR Neuroscience Institute, Padova, Italy;* ³*Department of Chemistry, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy*

Skeletal muscle fibers contain different myosin heavy chain (MyHC) isoforms that define distinct muscle contractile properties. Variations in MyHC expression pattern are specific of muscle fiber adaptation to physiological and pathological conditions and may represent valuable diagnostic tools. For this reason, novel techniques and protocols for the rapid assessment of muscle MyHC composition, requiring little amounts of tissue biopsies and allowing the simultaneous analysis of numerous samples, are of fundamental importance to screening and diagnostics.

Until now the most widely used protocols for the detection and relative quantification of MyHC isoforms relied on gel electrophoresis-based methods that suffers from several drawbacks, including time-consuming procedures and low reproducibility. For the first time to our knowledge, here we applied a targeted proteomic approach based on selected reaction monitoring in order to obtain – with high reproducibility – the absolute quantification of slow (type-I) and fast (type-IIa, -IIb, -IIx) MyHC isoforms in different mouse skeletal muscles. Such approach can be easily applied to the analysis of human skeletal muscles, for a precise, rapid and simultaneous comparison of samples, e.g., in the screening of muscular or neuromuscular diseases.

In order to validate the robustness and applicability of this technique, we quantitatively analysed the MyHC expression profile in different skeletal muscles from a genetic mouse model of amyotrophic lateral sclerosis (i.e., mice expressing the SOD1(G93A) mutant) compared to control mice. By this approach we confirmed that in terminally ill mice a fast-to-slow shift in fiber type composition occurs in fast muscles, such as Tibialis anterior and gastrocnemius.

MCU knock-down impairs skeletal muscle and motor neuron development in zebrafish embryos

Elisa Lidron¹, Sofia Zanin¹, Enrico Moro², Francesco Argenton³, Rosario Rizzuto¹, Giorgia Pallafacchina¹

¹CNR Neuroscience Institute and Department of Biomedical Sciences, University of Padova, Italy;

²Department of Molecular Medicine, University of Padova, Italy; ³Department of Biology, University of Padova, Italy

Ca²⁺ is a fundamental signalling molecule which decodes a variety of extra-and intra-cellular inputs and regulates diverse biological processes, from egg fertilization to organogenesis and to tissue specific function, such as contraction in skeletal muscle and neuronal firing in brain.

Mitochondria are one of the most important targets and regulators of cellular Ca²⁺ signalling. Their strategic subcellular distribution ensures the efficient coupling between the release of Ca²⁺ from the stores and its uptake inside the organelle, thus guaranteeing a tight control of the cell death/cell survival pathways and of the energy homeostasis through regulation of oxidative metabolism.

In 2011, the molecular complex responsible for the entry of Ca²⁺ in mitochondria, the mitochondrial Ca²⁺ uniporter (MCU) channel and its regulators, was identified by our and Mootha's groups, opening the path for the biochemical and molecular characterization of the mechanisms underlying mitochondria contribution to Ca²⁺ signalling. The MCU^{-/-} mouse model was produced two years later, and its unexpected mild phenotype raised a quite lively discussion within the scientific community. However, the viable phenotype of the knockout mouse has been reported only when mice were kept in a mixed genetic background, while the same genotype is lethal in the pure BL6 background, thus underlying a role of MCU during embryogenesis. Since the MCU^{-/-} mouse description, tissue-specific and inducible knockout animals have been developed, in order to discern the contribution of MCU in single organs and to overcome possible compensatory mechanisms that may occur during development. In this line, our work aims to explore the contribution of MCU and mitochondrial Ca²⁺ dynamics in the regulation of vertebrate development and organogenesis implementing the zebrafish (*danio rerio*) as a model organism.

Our experimental strategy consists in knocking down *drMCU* expression during zebrafish embryonic development by injecting morpholino antisense oligonucleotides in fertilized eggs and monitoring the phenotype of developing embryos. Western blot analysis reveals an efficient MCU knocking down after 48-72 hpf, accompanied by reduced Ca²⁺ uptake in morphant embryo cells. The down regulation of MCU is extraordinarily maintained up to 8 dpf, suggesting a strong maternal contribution to MCU expression during early stages. Despite the MCU morphant fishes develop without gross morphological abnormalities, to a deeper analysis, they present noteworthy defects in several tissues. In particular we observe: 1) an impaired locomotor activity, as resulting from touch test response performed at 48 hpf, 2) an altered skeletal muscle structure, as assayed by birefringence and phalloidin staining, and, finally, 3) a compromised motor neuron differentiation, as revealed by imaging of motor neuron branching in *Tg(HB9-mGFP)* embryos. Concluding, our data indicate a role of MCU in early zebrafish development and in particular we found that MCU is required for the differentiation and maturation of skeletal musculature and of motor neuron network.

PROTECTIVE EFFECT OF ALISPORIVIR - A CYCLOPHILIN INHIBITOR WITHOUT IMMUNOSUPPRESSIVE ACTIVITY - IN DUCHENNE MUSCULAR DYSTROPHY

Alessandra Zulian¹, Marco Schiavone¹, Sara Menazza¹, Erika Rizzo¹, Francesca Sardone², Luciano Merlini², Patrizia Sabatelli^{2,3}, Francesco Argenton⁴ and Paolo Bernardi^{1,5}

¹Department of Biomedical Sciences, University of Padova, I-35131 Padova, Italy; ²Laboratory of Musculoskeletal Cell Biology, Istituto Ortopedico Rizzoli, I-40136 Bologna, Italy; ³Consiglio Nazionale delle Ricerche, Institute of Molecular Genetics, I-40136, Bologna Italy; ⁴Department of Biology, University of Padova, I-35131 Padova, Italy; ⁵Consiglio Nazionale delle Ricerche Neuroscience Institute, I-35131 Padova, Italy

Duchenne muscular dystrophy (DMD) is due to mutations in the gene encoding dystrophin, an important structural component of muscle tissue that provides structural stability linking the cytoskeleton to the dystroglycan complex of sarcolemma. A potential mechanism for skeletal muscle death in DMD, as well as in other forms of muscular dystrophy, is increased propensity to open of the permeability transition pore (PTP), an inner mitochondrial membrane channel that requires matrix Ca^{2+} . It was already reported that D-MeAla³-EtVal⁴-cyclosporin (Alisporivir), a non-immunosuppressive cyclophilin inhibitor that desensitizes the PTP to Ca^{2+} , was able to reduce disease outcome in *mdx* mice, a murine model of DMD. Since *mdx* mice typically show a very mild phenotype compared to DMD patients, here we tested the effect of Alisporivir in the zebrafish *sapje* model, which is affected by a severe form of muscular dystrophy generated by a T→A mutation at codon 222 of the dystrophin gene. The *sapje* zebrafish is characterized by the production of a truncated non-functional dystrophin, which leads to ultrastructural abnormalities that can be demonstrated by birefringence *in vivo* as well as by electron microscopy already at 5 days post fertilization (dpf). Structural abnormalities were largely prevented when Alisporivir was administered at 48 hours post fertilization, and long term treatment was able to increase the number of surviving larvae up to 30 dpf – a time point at which *sapje* mutants would otherwise be dead. Beneficial effects of Alisporivir were also detected in primary muscle-derived cell cultures from three DMD patients, where (i) the observed mitochondrial alterations and depolarization in response to oligomycin treatment were significantly reduced and (ii) an improvement of respiration measured with the Seahorse technology was detected. Our results suggest that early treatment with Alisporivir could be a potential therapy for DMD.

SAN DYSFUNCTION UNDERLIE ATRIAL FIBRILLATION SUSCEPTIBILITY IN THE PITX2 MOUSE MODEL

Marina Campione^{1,2}, Frantisek Vostarek³, Nicola Pianca⁴, Tania Zaglia⁴, Marco Mongillo^{2,4}

¹CNR Neuroscience Institute, Padova, Italy; ²Department of Biomedical Sciences, Padova, Italy;
³Czech Academy of Sciences, Institute of Physiology, Prague, Czech Republic; ⁴Venetian Institute of
Molecular Medicine (VIMM), Padova, Italy

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with significant morbidity, mostly due to resulting heart failure and ischemic stroke. Many important unanswered questions remain about the underlying mechanisms, resolution of which is needed to improve AF management. AF is considered a “left” disease, due to localization of its triggering foci in the pulmonary vein (PV) myocardium and its propagation through the left atrium (LA). Noticeably, AF is frequently associated with sick sinus syndrome (SSS), a condition of impaired sino-atrial node (SAN) functionality which leads to tachy-brady syndrome. It is still unknown whether AF develops as a result of the underlying SAN dysfunction, or viceversa, or whether a common pathophysiological process can cause both AF and SSS.

We have approached this problem by *in-vivo* optogenetic analysis of the Pitx2 heterozygous mice, which are a recognized model for AF.

We show that adult Pitx2 het mice present reduced SAN activity, which defines the basis for SSS. Additionally, optogenetic-driven regional stimulation of the atrial region has resulted in strong tachy-brady response, which is possibly due to SAN dysfunction coupled to abnormal electrical propagation. Our data suggest that *that* SAN dysfunction, coupled with altered atrial electrophysiological properties, will contribute to AF onset and perpetuation in the Pitx2 model. These findings define a novel paradigm to better dissect the abnormal substrate triggering AF.

Specific GABAergic interneuron signaling to cortical astrocytes

Michele Sessolo^{1,2}, Gabriele Losi^{1,2}, Letizia Mariotti^{1,2}, Michele Speggin² and Giorgio Carmignoto^{1,2}

¹CNR Neuroscience Institute and ²Department of Biomedical Sciences, University of Padova, Italy

Astrocytes are glial cells that are active partners of neurons in regulating brain functions. Indeed astrocytes respond to different neurotransmitters and, through Ca^{2+} oscillations and gliotransmitter release, signal back to neurons modulating network excitability. Although the astrocytic response to several glutamatergic neurons is well described, astrocyte response to specific GABAergic interneuron populations is still poorly defined because of the high heterogeneity of these cells. Among different types of interneurons, one of the major subpopulation in the neocortex is represented by somatostatin (SST) interneurons that modulate signal integration and synaptic plasticity. Here we asked whether GABA selectively released by SST interneurons could activate astrocytes Ca^{2+} responses. To selectively stimulate SST interneurons and evaluate Ca^{2+} responses of astrocytes, we expressed the light-gated cation channel channelrhodopsin-2 in SST-CRE mice and the genetically encoded Ca^{2+} indicator GCaMP6f in astrocytes. In the mouse somatosensory cortex, both in *in vivo* and in slice preparations, we found that optogenetic activation of SST interneurons evoked Ca^{2+} elevations in astrocytes both at soma, proximal and distal processes, mediated by GABA_B receptors. We next investigated whether SST interneuron activated astrocytes signal back to neurons modulating network excitability. Spontaneous excitatory post-synaptic currents (sEPSCs) recorded from pyramidal neurons (PyN) in acute somatosensory cortical slices revealed that after optogenetic activation of SST interneurons excitatory inputs onto cortical PyN are potentiated for few minutes in a large subset of cells tested. Pharmacological tools revealed that potentiation of sEPSCs was mostly dependent on metabotropic glutamate receptor 1 (mGluR1) activation. These preliminary results suggest that astrocytes activated by SST interneurons via GABA_B receptors potentiate excitatory synapses in the neocortex by releasing glutamate that acts on mGluR1 receptors. Future experiments on IP3R2 KO mice, in which gliotransmission is impaired, will be used to confirm the role of astrocyte in the modulation of sEPSCs on PyN. Altogether our data reveal that SST interneurons, beside their direct fast inhibitory effect, may induce a delayed increase of excitatory synaptic transmission by activating astrocytes. This intercellular communication may be relevant in the modulation of synaptic plasticity and signal integration mediated by SST interneurons in neocortical circuits.

MICROGLIAL MVs MODULATE SYNAPTIC TRANSMISSION IN A TARGET-SPECIFIC WAY

Martina Gabrielli¹, Loredana Riganti¹⁻⁴, Natalia Battista²⁻³, Ilaria Prada¹, Flavia Antonucci⁴, Pooja Joshi⁶, Michela Matteoli¹⁻⁶, Mauro Maccarrone^{3,5}, Claudia Verderio¹⁻⁶

¹CNR Institute of Neuroscience, Milano, Italy; ²Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy; ³European Center for Brain Research/IRCCS Santa Lucia Foundation, Rome, Italy; ⁴Department of Medical Biotechnology and Translational Medicine, University of Milano, Milano, Italy; ⁵Center of Integrated Research, Campus Bio-Medico, University of Rome, Italy; ⁶IRCCS Humanitas, Rozzano (MI), Italy.

Extracellular vesicles (EVs) are membrane structures of endosomal (exosomes) or plasma membrane (microvesicles - MVs) origin, which act as shuttles for bioactive molecules from donor to specific target cells. In the last few years, we collected evidence indicating a role for MVs released from microglia in the modulation of neurotransmission. The first evidence came from *in vivo* recordings after injection of microglial MVs into the rat visual cortex. These experiments showed that microglial MVs induce a significant increase in VEP amplitude and an enlargement of neuron receptive fields measured by single cell recordings, which is typically explained by an increase in the excitation-inhibition balance (Antonucci et al, 2012).

Further *in vitro* whole cell patch clamp electrophysiological experiments were performed on cultured rat hippocampal neurons in order to gain insight into the molecular mechanisms underlying synaptic modulation of microglial MVs. Analysis of MV effects on synaptic transmission showed that MVs induce an increase in presynaptic release probability at excitatory synapses through activation of sphingolipid metabolism in neurons (Antonucci et al, 2012), while cause a decrease in spontaneous GABA release (Gabrielli et al, 2015). Further experiments revealed that this effect was due to the activation of the endocannabinoid type I receptor (CB1) by vesicular endocannabinoids (eCB). In fact: i) mass spectrometry measurements detected an enrichment of the eCB anandamide (AEA) in MVs compared to donor cells; ii) electrophysiological experiments showed that the CB1 antagonist SR141716A was able to completely abolished MV effects on spontaneous GABA release; iii) western blot analysis showed that MVs induces an increase in ERK phosphorylation, which was completely inhibited by SR141716A, indicating that CB1 activation by eCB-storing MVs translates into downstream signaling in neurons. Experiments performed in the presence of pharmacological block of sphingolipid metabolism, clarified that the potentiation of excitatory transmission evoked by MVs did not cross-affect inhibitory transmission. Similarly, pharmacological block of eCB signaling showed that microglial MVs directly potentiate excitatory transmission, independently of modulation on the inhibitory tone (Gabrielli et al, 2015). This indicates that MVs activate distinct signaling pathways and deliver distinct messages depending on the GABAergic or Glutamatergic nature of recipient neurons.

In order to define whether microglia-derived MVs play a physiological or pathological role in neurotransmission, we are now evaluating whether neuronal activity impacts MV production from microglia. In particular we are setting a methodology to selectively quantify microglial MVs in neuron-microglia co-cultures, taking advantage of the innovative TRPS (Tunable Resistive Pulse Sensing) technique using an IZON qNano instrument.

New insights into the mechanism of astrocytic glutamate release that elicits NMDAR-mediated neuronal slow inward currents

Marta Gómez-Gonzalo¹, Paola Bezzi², Giorgio Carmignoto¹

¹*CNR Neuroscience Institute and Department of Biomedical Sciences, University of Padova, Padova, Italy;* ² *Department of Fundamental Neurosciences, University of Lausanne, Lausanne, Switzerland*

The release of glutamate from astrocytes of different brain areas induces slow inward currents (SICs) in neighboring neurons by activating extrasynaptic NMDARs. It is well established that Ca^{2+} elevations evoked in astrocytes by different stimuli triggers neuronal SICs, suggesting an exocytotic glutamate release mechanism of SIC generation. To gain new insights into SIC-mediated molecular mechanism, in hippocampal slices of young mice we investigated the relationship between spontaneous Ca^{2+} elevations in astrocytes and spontaneous SICs in neurons. In the present study, we demonstrate that both spontaneous and hypotonicity-evoked SICs remain unchanged in the absence of Ca^{2+} elevations at different astrocytic compartments, including the soma and spatially restricted microdomains. Similarly, spontaneous and hypotonicity-evoked SICs are not impaired following a hypotonic pre-stimulus and a long incubation with Bafilomycin A1 that, by inhibiting the electrochemical H^+ gradient generated by the vesicular proton pump, impairs glutamate loading into the vesicles. Furthermore, we found that hemichannels and TREK family channels, that were proposed to be involved in glutamate release in astrocytes, are not at the basis of SIC generation. Finally, we found that SICs are reduced by DIDS, quinine and fluoxetine, suggesting a possible contribution of volume-sensitive anion channels in SIC generation. In conclusion, our data suggest that the release of glutamate generating SICs is not involved in synaptic transmission modulation by astrocytes, that appears mediated by a Ca^{2+} -dependent glutamate release mechanism.

Exploring the role of Vps13 proteins family using CRISPR/Cas9 system in human cell lines

Mirko Luoni¹, Gabriele Ordazzo², Serena Gea Giannelli¹, Elena Ziviani³, Vania Broccoli^{1,4}

¹*Stem Cells and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Italy;*
²*Vita-Salute San Raffaele University, Italy;* ³*Department of Biology, University of Padova, Italy;* ⁴*National Research Council (CNR), Institute of Neuroscience, Italy*

The VPS13 gene family is highly conserved in all eukaryotic cells. In yeast, there the single VPS13 ortholog is involved in several cellular processes including protein sorting to the vacuole, sporulation and maintenance of mitochondrial integrity. Humans have four VPS13 orthologs (A,B,C,D) and loss-of-function mutations in three out of the four genes are responsible for brain disorders. In particular, inactivation of VPS13A and VPS13C, which are highly conserved in sequence, are associated with the neurodegenerative disorders chorea acanthocytosis (ChAc) and Parkinson's disease, respectively. Conversely, mutations in VPS13B causes the developmental disorder Cohen syndrome, associated with autism-spectrum disorder and severe cognitive disability. Despite the relevance of the Vps13 protein family, the functions of the different human orthologs remains yet to be explored. In order to dissect the role of human Vps13 proteins, we employed the CRISPR/Cas9 system to specifically inactivate the VPS13A,B,C genes in human cell lines. Given the high similarity between Vps13A and Vps13C, we decided to inactivate both at the same time to avoid any compensatory mechanisms. Preliminary data showed that double mutant VPS13A/C cells have a fragmented mitochondria network both in basal and stress conditions. These data suggest a fundamental role of these two proteins in mitochondrial homeostasis and function. On this line, candidate protein interactors of these family members have a known role in mitochondria biology. On the contrary, Vps13b appears to have a mitochondria-independent function while might be important to regulate specific functions in the cilium. The establishment of VPS13 targeted mutant human cells will be a fundamental tool to unveil the functions of these proteins in the regulation of the activity of mitochondria and other organelles.

PrP-dependent perturbation of mitochondrial functions and Ca²⁺ homeostasis by A β oligomers in cortical neurons

Agnese De Mario¹, Maria Lina Massimino², Alessandro Bertoli¹, Chiara Bianchimani¹, Rosa Pia Norante¹, M.C. Sorgato^{1,2}

¹Department of Biomedical Science and ²CNR Neuroscience Institute, University of Padova, Italy

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by the accumulation of amyloid β (A β) peptides, which self-assemble to form oligomers of increasing mass. Between different types of A β aggregates, soluble assemblies of A β (1-42) oligomers (A β 42) are considered the neurotoxic species able to stimulate the production of reactive oxygen species (ROS), and cause mitochondrial dysfunctions and Ca²⁺ dysomeostasis in neurons. It has been recently proposed that the cellular prion protein (PrP^C) acts as a high affinity receptor for A β 42, and that PrP^C-A β 42 binding conveys toxic signal into neurons.

In light of the above premises, the aim of this work is analysing whether acute treatment with A β 42 affects mitochondrial physiology in a PrP^C-dependent way.

To this purpose, we have used primary cultures of cortical neurons isolated from wild-type (WT), or PrP-knockout (PrP-KO) mice, subjected to acute treatment (1 h) with A β 42, and then analysed cells for the following parameters.

- 1- Cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay.
- 2- Mitochondrial ROS (mROS) and mitochondrial membrane potential ($\Delta\psi_m$) by means of fluorescent dyes.
- 3- Cytosolic and mitochondrial Ca²⁺ homeostasis by means of gene-encoded fluorescent or luminescent probes, transduced into primary neurons by means of viral vectors.
- 4- Activation of signalling pathways by Western blot analysis.

Following this experimental setting, we have found that:

- 1- The acute treatment of primary cortical neurons with A β 42 reduces cell viability, increases the production of mROS and decreases $\Delta\psi_m$ in WT but not in PrP-KO neurons. Treatment with A β 42 also affects mitochondrial Ca²⁺ accumulation following NMDA stimulation in a PrP^C-dependent way.
- 2- The acute treatment with A β 42 increases the activation of Fyn kinase in WT but not in PrP-KO neurons, while no significant difference was observed in the basal cytosolic Ca²⁺ levels between the two PrP genotypes.

In conclusion, A β 42 affect mitochondrial physiology in a PrP^C-dependent way, opening the possibility of a new therapeutic approach in the treatment of AD.

THE Ca^{2+} REGULATORY SITE OF THE PERMEABILITY TRANSITION PORE IS WITHIN THE CATALYTIC CORE OF F-ATP SYNTHASE

Valentina Giorgio^{1,2}, Victoria Burchell¹, Marco Schiavone¹, Claudio Bassot³, Giovanni Minervini³, Silvio Tosatto³, Francesco Argenton³, Valeria Petronilli^{1,2}, Mike Forte⁴, Giovanna Lippe⁵, Paolo Bernardi^{1,2}

¹Department of Biomedical Sciences, University of Padova, I-35131 Padova (Italy); ²Consiglio Nazionale delle Ricerche Neuroscience Institute, I-35131 Padova (Italy); ³Department of Biology, University of Padova, I-35131 Padova (Italy); ⁴Vollum Institute, Oregon Health and Sciences University, Portland, OR, 97239; ⁵Department of Agricultural, Food, Environmental and Animal Sciences, I-33100 Udine (Italy)

The mitochondrial permeability transition pore (PTP) is a key regulator of apoptotic cell death. It is a high-conductance channel that forms from F-ATP synthase in a process that requires matrix Ca^{2+} and is favored by oxidative stress [1]. An absolute requirement for PTP opening is matrix Ca^{2+} . Since Mg^{2+} and ADP—physiological inhibitors of the channel—compete with Ca^{2+} , we have explored the potential role of the catalytic cation/nucleotide binding pocket of F-ATP synthase as the Ca^{2+} binding site of the PTP. Mutation of a conserved threonine in the β subunit to serine (T163S) in HeLa cells did not affect ATP synthesis in the presence of Mg^{2+} , while it significantly reduced both ATPase activity in the presence of Ca^{2+} (as in *Rhodospirillum rubrum* [2]) and sensitivity of the PTP to this cation. This same mutation also protected cells from death induced by arachidonic acid and ionomycin, both of which induce PTP opening. Expression of the mutant β subunit reduced apoptotic cell death in zebrafish embryos, resulting in morphological defects and highlighting a new role for the PTP in regulating apoptosis during development. Molecular dynamics studies of the enzyme revealed that Ca^{2+} binding to the catalytic site of F-ATP synthase generates conformational changes that are transmitted to the lateral stalk, suggesting a mechanism that couples Ca^{2+} binding to the catalytic site to PTP opening in the inner membrane.

References:

1. V. Giorgio, S. von Stockum, M. Antoniel, A. Fabbro, F. Fogolari, M. Forte, G. D. Glick, V. Petronilli, M. Zoratti, I. Szabó, G. Lippe, P. Bernardi, Dimers of mitochondrial ATP synthase form the permeability transition pore, PNAS 110 (2013) 5887-92
2. L. Nathanson and Z. Gromet Elhanan, Mutations in the beta-subunit Thr¹⁵⁹ and Glu¹⁸⁴ of the *Rhodospirillum rubrum* F₀F₁ ATP synthase reveal differences in ligands for the coupled Mg^{2+} - and decoupled Ca^{2+} -dependent F₀F₁ activities, J. Biol. Chem. 275 (2000) 901-5

SHEDDING LIGHT ON THE PERMEABILITY TRANSITION THROUGH F-ATP SYNTHASE MUTAGENESIS IN *S. Cerevisiae*

Michela Carraro¹, Lishu Guo¹, Valentina Giorgio¹, Vanessa Checchetto², Roza Kucharczyk³, Justina Šileikytė¹, Geppo Sartori¹, Michael Forte⁴, Giovanna Lippe⁵, Mario Zoratti¹, Ildikò Szabò² and Paolo Bernardi¹

¹Department of Biomedical Sciences, University of Padova and CNR Neuroscience Institute, Padova, Italy; ²Department of Biology, University of Padova, Padova, Italy; ³Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; ⁴Vollum Institute, Oregon Health and Sciences University, Portland, OR, USA; ⁵Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Udine, Italy

The Permeability Transition Pore (PTP) might play a role in the yeast programmed cell death signaling cascade in response to high cytosolic $[Ca^{2+}]$ and ROS [1]. Purified F-ATP synthase dimers of *S. cerevisiae* have been recently shown to form a 300-pS channel with features matching those of the mammalian PTP, *i.e.* activation by Ca^{2+} and oxidants (phenylarsine oxide and copper-*o*-phenanthroline) and inhibition by ADP/Mg²⁺ [2]. Null mutant strains for subunits e and/or g, which are involved in dimerization of the enzyme, displayed PTP opening at higher Ca^{2+} loads and formed dimers detectable by BN-PAGE only upon oxidation with copper. Our findings indicate that ATP6 (subunit a) Cys23 mediates the copper-induced stabilization of dimers but is not involved in the sensitivity of the PTP to oxidants. Moreover, preliminary data show that dimers devoid of e and g subunits generate a channel with a substantially smaller conductance, suggesting that these subunits are required for the activity in the full-conductance state. We carried out mutagenesis of F-ATP synthase cysteines to identify those responsible for the PTP sensitivity to oxidants. Preliminary findings indicate that the unique Cys100 of OSCP (a lateral stalk subunit) mediates PTP induction by diamide, a thiol oxidant. Taken together, our findings indicate that (i) F-ATP synthase dimers of yeast form PTP-like channels activated by Ca^{2+} and oxidants and (ii) sensitivity to oxidants may be conferred by specific cysteine residues of the enzyme.

1. M. Carraro and P. Bernardi, Calcium and reactive oxygen species in regulation of the mitochondrial permeability transition and of programmed cell death in yeast, *Cell Calcium*. (2016), doi.org/10.1016/j.ceca.2016.03.005.
2. M. Carraro, V. Giorgio, J. Šileikytė, G. Sartori, M. Forte, G. Lippe, M. Zoratti, I. Szabò, P. Bernardi, Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition, *J Biol Chem*. 289 (2014) 15980–5.

The idebenone metabolite QS10 is an electron donor to complex III and rescues respiration in complex I-deficient cells and rotenone-treated zebrafish

Marco Schiavone¹, Valentina Giorgio¹, Valeria Petronilli¹, Francesco Argenton², Tatiana Da Ros³, Maurizio Prato³ and Paolo Bernardi¹

¹*Department of Biomedical Sciences, University of Padova and CNR Neuroscience Institute, Padova (Italy);* ²*Department of Biology, University of Padova, Padova (Italy);* ³*Department of Chemical and Pharmaceutical Sciences, University of Trieste, I-34127 Trieste (Italy)*

Mitochondrial diseases involving mutations in genes encoding for respiratory chain complex I subunits range from mild to severe phenotypes such as Leigh syndrome and Leber's Hereditary Optic Neuropathy (LHON). Idebenone is a short chain CoQ analogue used in clinical trials for a number of mitochondrial diseases, although its efficacy is still debated [1]. Improved frequency of visual recovery has been observed in LHON patients in therapy with idebenone yet (i) idebenone may cause sensitization of the mitochondrial permeability transition pore (PTP) to opening, which would be an untoward effect [2], and (ii) it is rapidly metabolized *in vivo*, generating metabolites of decreasing side chain length (QS10, QS8, QS6 and QS4) that may be responsible for the beneficial effect. Here we show that QS10 has no PTP-inducing effects, and that its reduced form allows maintenance of the mitochondrial membrane potential in the presence of rotenone by providing electrons to respiratory chain complex III. In complex I-deficient cells (XTC.UC1 cells and RJ206 cybrids derived from a LHON patient bearing the G3460A/MT-ND1 mutation) QS10 increased respiration, which was insensitive to rotenone and inhibited by antimycin A. Pretreatment with QS10 improved spontaneous coiling events and survival of *Danio rerio* exposed to rotenone, while no protective effect was seen with idebenone. These results suggest that QS10 rather than idebenone should be tested for its potential therapeutic efficacy in complex I diseases.

1. S. Jaber and B.M. Polster, Idebenone and neuroprotection: antioxidant, pro-oxidant or electron carrier?, *J Bioerg Biomembr*, 47 (2015) 111-8
2. V. Giorgio, V. Petronilli, A. Ghelli, V. Carelli, M. Rugolo, G. Lenaz, P. Bernardi, The effect of idebenone on mitochondrial bioenergetics, *Biochim Biophys Acta*, 1817 (2012) 363-9

Study of mitochondria physiology in transgenic mouse models of Alzheimer's Disease

Giulia Rigotto¹, Tullio Pozzan^{1,2,3}, Emy Basso^{1,2}

¹Department of Biomedical Sciences, University of Padua, Padua, Italy; ²CNR Neuroscience Institute, Padua, Italy; ³Venetian Institute of Molecular Medicine, Padua, Italy

Alzheimer's Disease (AD) is the most common cause of dementia in the elderly, marked by progressive loss of memory and impairment of cognitive ability. The most obvious histological lesions in AD brains are extracellular plaques formed by amyloid beta (A β) aggregation and intracellular neurofibrillary tangles of hyperphosphorylated Tau protein (NFT). The majority of AD cases is sporadic with unknown etiology and aging is the main risk factor. Only about the 1% is characterized by autosomal dominant mutations on three genes coding for presenilin 1 and 2 (PS1, PS2) or the amyloid precursor protein (APP). PSs are essential components of the γ -secretase complex which, in turn, by cleaving APP in concert with β -secretase, produces the neurotoxic β -amyloid peptide. APP is a trans-membrane protein expressed in many tissues whose proteolysis generates β -amyloid peptides, and it is involved in axonal transport and cell signalling. The identification of genetic factors contributing to AD, and the intense investigation into the cell biology of amyloid precursor protein (APP), has led to the development of several transgenic mouse models of the disease.

It is now widely accepted that mitochondrial dysfunctions are implicated in aging-related neurodegenerative diseases, including AD. We perform our experiments using transgenic mice carrying the human FAD-linked PS2-N141I mutation, either alone or together with the APP Swedish mutation (K670N, M671L). Measurements of oxygen consumption, mitochondrial membrane potential and calcium retention capacity in isolated brain-cortex mitochondria from WT and transgenic animals of different ages didn't show any significant difference. In order to study mitochondria functionality in more physiological conditions, we used hippocampal cultures from newborn mice and we measured the mitochondrial membrane potential variations in the cells upon addition of respiratory chain inhibitors (rotenone or antimycin). Interestingly, mitochondria from double transgenic neurons seem to be less efficient compared to the single transgenic and wt neurons in maintaining the membrane potential after inhibition of the respiratory chain.

We are presently investigating whether this difference is caused by an impairment of the ATP synthase activity, unable to maintain the mitochondrial membrane potential through its reverse activity, or by the incapability of glycolysis to provide a sufficient amount of ATP to keep the mitochondrial membrane potential.

*Kindly provided by L. Ozmen and F. Hoffmann, La ROCHE Ltd (Basel, Switzerland)

Mitochondrial cAMP signalling is involved in metabolic flexibility

Giulietta Di Benedetto^{1,2} and Tullio Pozzan^{1,2,3}

¹CNR Neuroscience Institute, Padova, Italy; ²VIMM (Venetian Institute of Molecular Medicine, Padova, Italy); ³Department of Biomedical Sciences, University of Padova, Italy

Cytosolic cAMP cannot reach the mitochondrial matrix but is synthesized *in situ* by a mitochondrial isoform of the soluble adenylyl cyclase (sAC). While the presence of an autonomous cAMP signalling cascade is accepted in the matrix, the function and local targets of this pathway remain largely unknown. We developed a FRET-based biosensor to measure mt-cAMP dynamics within mitochondria of living cells, showing that raises in matrix $[Ca^{2+}]$ stimulate *de novo* mt-cAMP synthesis through the activity sAC. In addition we found that increases in mt-cAMP result in increased efficiency of mitochondrial ATP synthesis. We concluded that OXPHOS regulation, which modulates ATP generation in response to nutrient availability and cellular demand, is at least partially achieved by mitochondrial cAMP (Di Benedetto et al., Cell Metab, 2013). Our findings opened a number of questions regarding the identity of the cAMP effectors, targets and regulator proteins within the mitochondrial matrix, as well as the existence of additional cellular functions regulated by mt-cAMP. The first cell-specific mt-cAMP role has been discovered in adrenocortical glomerulosa cells, which secrete aldosterone in response to agonists coupled to Ca^{2+} increases, such as angiotensin II. We showed that mt-cAMP synergizes with intramitochondrial Ca^{2+} in regulating aldosterone biosynthesis (Katona et al., Mol Cell Endocrinol, 2015).

A working hypothesis on mt-cAMP-regulating proteins concerns the PDE8A phosphodiesterase, which has been found enriched at mitochondria. PDE8 family (PDE8A and PDE8) are cAMP-specific and IBMX-insensitive PDEs, broadly expressed, particularly in steroidogenic cells, but also in cardiomyocytes, T cells, pancreatic β cells and in brain. PDE8A regulates testosterone and corticosterone release from, respectively, Leydig cells and adrenal cells. Our preliminary data indicate that PDE8A has a role in the regulation of mt-cAMP levels.

A second working hypothesis is the involvement of mt-cAMP in the ability of living cells to adapt fuel preference to changes in nutrient availability termed as *metabolic flexibility* (MF). MF is a hallmark of healthy metabolism, and mitochondria are emerging as determinant in its regulation; indeed, MF involves the control of mitochondrial biogenesis and activity, which results in the fine-tuning of oxidative metabolism. The transcriptional regulatory networks governing mitochondrial biogenesis, as well as proteins involved in MF and the anabolism/catabolism shift, are highly conserved. Interestingly, cAMP is involved in both bacteria and eukaryotic cells in the regulation of diverse processes, including the response to fasting (e.g.: bacterial catabolite repression and biofilm formation; mammalian glycogen degradation; mitochondrial network re-organization). We are currently testing the hypothesis of the existence of a feedback loop where nutrient and oxygen availability could regulate mt-cAMP level, which, in turn, would participate in the processes underpinning metabolic flexibility through its action on mitochondrial metabolism. Our preliminary data indicate that sugars, α -ketoacids, aminoacids and oxygen availability modulate cAMP in the mitochondrial matrix.

mCERULEAN3-BASED CAMELEON SENSORS TO EXPLORE MITOCHONDRIAL Ca²⁺ DYNAMICS *IN VIVO*

Elisa Greotti^{1,2}, Diana Pendlin^{1,2}, Ilaria Fortunati⁴, Camilla Ferrante⁴, Renato Bozio⁴, Lorena Zentilin⁵, Nina Kaludercic¹, Letizia Mariotti^{1,2}, Annamaria Lia², Giorgio Carmignoto^{1,2}, Tullio Pozzan^{1,2,3}

¹Neuroscience Institute, Padova Section, National Research Council, Italy; ²Dept. Biomedical Sciences, University of Padova, Padova, Italy; ³Venetian Institute of Molecular Medicine (VIMM), Padova, Italy; ⁴Chemical Science Dept., University of Padova and INSTM, Padova; ⁵International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste

Calcium (Ca²⁺) signalling modulates a variety of functions in cells, regulating many physiological and pathological processes. Cellular organelles contribute in shaping Ca²⁺ signals, allowing its fine spatial and temporal regulation. In particular, the mitochondrial ability to take up Ca²⁺ plays a key role in several cellular pathways, e.g., regulation of Ca²⁺ homeostasis, oxidative stress response, ATP production, apoptosis and innate immune response, putting these organelle in a central position in the control of cellular function and fate.

Genetically encoded calcium indicators (GECIs) allow quantitative Ca²⁺ measurements in different experimental models. Organelle-specific targeting signals fused with the GECI's sequence allow selective targeting to a specific organelle or cytoplasmic domains. Moreover, GECI's coding sequences can be placed under the control of tissue specific or inducible promoters, allowing the control of their cellular expression in complex tissues. Ca²⁺ dynamics in organelles have been extensively investigated in cultured living cells using GECIs targeted to specific cellular compartments; in contrast, few examples of *in vivo* studies are available.

The aim of this study was to develop novel molecular sensors and new methodologies to express these probes *in vivo* allowing interrogation of Ca²⁺ signalling in the intact animal models. To this end we have employed FRET-based Ca²⁺ sensors, named Cameleons, that have been extensively used in cultured cells. Cameleon structure consists of a Ca²⁺-responsive element that alter the efficiency of FRET (Förster resonance energy transfer) between two fluorescent proteins (FPs): a cyan fluorescent protein (ECFP), the donor, and a variant of the yellow fluorescent protein (cpV), the acceptor. The Ca²⁺-responsive element is represented by Calmodulin (CaM) and the CaM-binding domain of myosin light chain kinase. One of the limitations of these probes is the low fluorescence of ECFP and the multi-exponential lifetime of this fluorescent protein. Recently, a brighter, more photostable FP has been developed, mCerulean3. Compared to ECFP, mCerulean3 is brighter and possesses a single exponential lifetime. For this reason, ECFP has been replaced with mCerulean3 in two different Cameleons: the cytosolic D3cpv and the mitochondria-targeted probe named 4mtD3cpv. The new probes have been first tested in different cell types in culture: HeLa, neonatal rat cardiomyocytes and neonatal mouse neurons, demonstrating improved brightness, photostability and low pH-sensitivity *in situ* compared to the original Cameleons, in both the cytosolic and mitochondrial probes. The only drawback of the new probes is a slightly reduced dynamic range (about 20%). To increase the dynamic range different approaches have been used and, eventually, the addition of 16 glycines between the two Ca²⁺ responsive elements allowed us to recover the dynamic range of the original probes. The mitochondrial targeting sequence has been also modified with a much better mitochondrial localization compared to the original probe. The improved brightness, photostability and the low pH-sensitivity *in situ* were maintained also after the glycines linker addition. Finally, we evaluated the Ca²⁺ affinity *in situ*, obtaining a K_d of 3 μM for 4mtD3cpV and a K_d of 5 μM for mCerulean3-based mitochondrial Cameleon (named 4mtD3mCerulean3+16). In parallel, we are also assessing another method to biophysically characterize FRET-GECIs, employing the FLIM technique, that measures the lifetime of FPs (the time the molecule spend in the excited state), which is totally independent from phenomena such as probe photobleaching, expression level, image shading. FRET efficiency and resting Ca²⁺ level have been evaluated by FLIM in HeLa cells expressing the new generated 4mtD3mCerulean3+16. Finally, to express the new probes *in vivo* we exploited viral vectors: adeno-associated virus (serotype 9), containing either the cytosolic or the mitochondrial targeted new probe, with a CMV promoter has been generated and used to express the probes in adult mouse cardiomyocytes; whereas a synapsin promoter has been used to investigate Ca²⁺ changes in neurons of mice brain slices. With both strategies a good and cell specific expression of the Ca²⁺ probes has been obtained. Experiments to monitor Ca²⁺ dynamics in the whole brain *in vivo* are currently ongoing and will be briefly discussed.

Intranasal BDNF administration promotes visual function recovery in adult amblyopic subjects

Gabriele Sansevero^{1,2}, Laura Baroncelli¹, Alessandro Sale¹

¹CNR Neuroscience Institute, Pisa, Italy; ²University of Florence, NEUROFARBA department, Italy

Amblyopia is the most diffused form of visual impairment affecting one eye, with a prevalence of 1-5% in the world population. While amblyopia can be efficiently treated in children, it becomes hard to be reversed in adults, due to the dramatic decline in neural plasticity past the end of the critical period in the primary visual cortex. We report that intranasal BDNF administration restored visual acuity, ocular dominance and stereopsis in adult amblyopic rats, both in animals subjected to reverse occlusion and in those with unrestricted binocular sight. Visual function recovery was long-lasting, and was prevented by pharmacological blockade of BDNF-ERK signaling in the visual cortex.

Adult plasticity of early visual areas after monocular deprivation measured with ultra-high field fMRI in humans

Paola Binda^{1,2}, Jan Kurzawski³, Claudia Lunghi^{1,2}, Laura Biagi³, Maria Concetta Morrone^{2,3}

¹CNR Neuroscience Institute, Pisa, Italy; ²University of Pisa, Italy; ³IRCCS Stella Maris, Calambrone (Pisa), Italy

While plasticity of the developing brain is subject of much research, there is relatively little information about plasticity in the visual cortex of adult humans. Recent psychophysical studies have shown that short-term monocular deprivation alters visual perception in adult humans. Specifically, after about 2h of monocular deprivation, the deprived eye strongly dominates the dynamics of binocular rivalry, reflecting homeostatic plasticity. In this study we investigate the neural mechanisms underlying this form of short-term plasticity by measuring BOLD (Blood Oxygenation Level Dependent) signal changes during monocular visual stimulation, before and after 120 min of monocular deprivation. Deprivation was achieved by having observers wear a translucent patch, which leaves only light perception eliminating all form perception. BOLD signal was measured in 10 young human adults, at ultra-high field (7 Tesla magnet), which allows us to study with high sensitivity and spatial precision visual responses in early visual areas (V1-V3) and subcortical visual structures (Lateral Geniculate Nuclei and Superior Colliculi). Visual stimuli consisted of random noise matrices, filtered to preferentially stimulate group of neurons with matched spatial frequency selectivity. We find that monocular deprivation has opposite effects on the amplitude of the BOLD responses for the deprived and non-deprived eye stimulation. After deprivation, responses to stimuli in the deprived eye are boosted compared to responses to stimuli in the non-deprived eye. This is observed in early visual cortex, as well as in LGN and SC. Deprivation affects not only the strength of the response, but also its spatial frequency dependency. We use mathematical modeling (akin to the “population Receptive Field” approach presented in Dumoulin & Wandell, *Neuroimage*, 2008) to extract the preferred spatial frequency of each “voxel” (patch of cortex imaged within one spatial unit of fMRI acquisitions). We estimate preferred spatial frequencies in V1 separately for each eye, before and after monocular deprivation. We find that, after deprivation, there is a shift towards higher preferred spatial frequencies for the deprived eye compared to the non-deprived eye outside the foveal region. Because voxels preferring higher spatial frequencies must include a majority of neurons with small receptive field sizes, this shift of preferred spatial frequency is indicative of a re-size (or re-weight) of visual receptive fields population.

These results indicate that a brief period of monocular deprivation affects two basic properties of activity in early visual areas of adult humans. Not only the strength of visual responses is boosted for the deprived eye, compared to the non-deprived eye, but also the spatial selectivity (receptive field size, hence spatial frequency preference) is altered, so that the deprived eye dominates most strongly is signaling the fine details of the image. These observations indicate that the adult human visual cortex retains a high level of homeostatic plasticity in adulthood (in line with VEP results by Lunghi et al. *JPhysiol* 2015), and this might even extend to earlier subcortical sites like the Lateral Geniculate Nucleus and the Superior Colliculus. Importantly, the plasticity effect is shown not only as an excitability change but also as a reshape of the receptive fields of early visual neurons, which may both result from changes in inhibitory signaling - as suggested by the GABA concentration change with monocular deprivation (Lunghi et al., *CurrBio* 2015).

A NOVEL MODEL OF MILLER FISHER SYNDROME TO STUDY NERVE TERMINAL REGENERATION

Umberto Rodella¹, Samuele Negro¹, Elisa Duregotti¹, Michele Scorzeto¹, Nobuhiro Yuki³, Michela Rigoni¹ and Cesare Montecucco^{1,2}

¹Department of Biomedical Sciences, University of Padova, Italy; ²CNR Institute of Neuroscience, Padova, Italy;

³Departments of Medicine and Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

The neuromuscular junction (NMJ) is a 'tripartite' synapse, composed of the presynaptic nerve terminal (NT), the muscle fiber and perisynaptic Schwann cells (PSCs). NMJ functionality is essential for the execution of body movements and is compromised in a number of disorders.

In the Miller Fisher Syndrome (MFS) autoantibodies against specific gangliosides (>90% GQ1b) bind to NT and in turn activate the complement cascade at its surface, leading to nerve degeneration [1]. Such process is reversible, as the motor neuron is able to fully regenerate and restore neurotransmission. It is well-known that PSCs are main supporters of NMJ regeneration, and it was recently reported that *alarm* signals released by degenerating NT play an important role in PSCs response [2].

To study the complex cross-talk between NT and PSCs during neurodegeneration/regeneration, we recently developed a novel *in vivo* MFS model. The combination of FS3, an anti-GQ1b antibody [3], and normal human serum (NHS) as a source of complement, administered subcutaneously in mice, causes the complete degeneration of NT, followed by its engulfment by PSCs. Within 3-4 days post-injection NT regrowth is complete, in line with the known reversibility of MFS.

To identify the neuronal *alarmins* responsible for PSCs activation and the signaling pathways engaged, we have parallelly set-up an *in vitro* MFS model consisting of FS3+NHS addition to primary neurons which causes a complement-dependent massive calcium overload in neurons together with the formation of neurite enlargements, termed here *bulges*. *Bulges* are sites of swollen mitochondria accumulation and localized H₂O₂ production of mitochondrial origin. In neurons-SCs co-cultures, H₂O₂ produced by neurons induces ERK phosphorylation within SCs, a crucial event for their pro-regenerative phenotype switch [4].

Furthermore, primary neurons exposed to FS3+NHS release ATP, which in turn evokes calcium-spikes and activation of the transcription factor CREB within SCs.

We are currently testing the *in vivo* involvement of neuronal H₂O₂ and ATP in NT regeneration, in order to provide a deeper understanding of the molecular mechanisms underlying neuroregeneration.

References:

- [1] Plomp et al., J Physiol. 2009
- [2] Duregotti et al., Proc Natl Acad Sci USA 2015
- [3] Koga et al., Neurology 2005
- [4] Napoli et al., Neuron 2012

Specificity and functionality of BoNT/A-intoxicated synapses following retrograde transport from the muscle to central neurons

Laura Restani¹, Francesca Colosimo¹, Matteo Spinelli¹, Francesca Biondi¹,
Ornella Rossetto², Matteo Caleo¹

¹CNR Institute of Neuroscience, Pisa; ²Department of Biomedical Sciences, University of Padua

Botulinum neurotoxins (BoNTs) block neurotransmitter release at the neuromuscular junction, inducing a flaccid paralysis. Since few years ago, the established view that BoNT trafficking is restricted to the neuromuscular junction has been challenged. Evidence coming from animal studies, has in fact shown that BoNT/A could act at central level, as a fraction of BoNT/A can undergo retrograde transport following endocytosis. New literature suggests that these central effects by BoNT/A could contribute to its therapeutic efficacy.

However, many aspects of this mechanism remain unclear. For our experiments we used two central target systems: motoneurons within the *nucleus facialis* and motoneurons within lumbar spinal cord, intoxicated by BoNT/A injection into, respectively, the whisker-pad and the hindleg of rats. First, we detected BoNT/A action in these central targets, by immunohistochemistry and Western blotting for BoNT/A-truncated SNAP25. Then, we systematically investigated the neurochemical phenotype of cleaved-SNAP25-positive neurons, performing double immunohistochemistry for specific synaptic markers (cholinergic, GABAergic, glutamatergic) together with cleaved-SNAP25. Data show a significant colocalization between cleaved-SNAP25-positive terminals and the vesicular acetylcholine transporter VACHT. VACHT appears to stain synaptic terminals impinging onto motoneurons, likely suggesting BoNT/A transcytosis in central synapses following peripheral injection and retrograde transport. Other synaptic markers, like vGlut2, did not display colocalization with cleaved-SNAP25. To further study the specificity of BoNT/A for cholinergic terminals, we injected minute amount of BoNT/A into the striatum or the hippocampus of adult mice. Then we performed colocalization experiments to investigate if BoNT/A, at this very low concentration, preferentially enters cholinergic terminals also locally.

To examine in depth the functionality of BoNT/A-intoxicated synapses following retrograde transport, we measure the size of terminals double-positive for VACHT and cleaved-SNAP25 into the facial nucleus. We found that terminals of BoNT/A-intoxicated neurons are larger than normal, suggesting an accumulation of synaptic vesicle and thus an impaired neurotransmitter release. Moreover, we are setting a functional assay to test synaptic vesicle recycling rate of synapses into the facial nucleus following muscle injection of BoNT/A.

Our data will clarify cellular aspect of BoNT/A action and trafficking and may hold relevant clinical implications.

THE ROLE OF MONOAMINE OXIDASES IN FORMATION AND MATURATION OF iPSC-DERIVED CARDIOMYOCYTES

Moises Di Sante¹, Soni Deshwal², Elisa Greotti^{1,2}, Diana Pendin^{1,2}, Fabio Di Lisa^{1,2}, Nina Kaludercic¹

¹CNR Neuroscience Institute, Padova, Italy; ²Department of Biomedical Sciences, University of Padova, Italy.

Monoamine oxidases (MAOs) are mitochondrial flavoenzymes that exist in two isoforms, MAO-A and -B, and are responsible for neurotransmitter and biogenic amines catabolism. During this process they generate hydrogen peroxide and aldehydes, products that have been shown to contribute to the development of cardiovascular diseases. To date, the potential role of either MAO isoform in cardiac development and cardiomyocyte differentiation remains elusive. Therefore, we established a human model based on cardiomyocytes derived from human induced pluripotent stem cells (iPSCs), in which both MAO-A and -B activity and protein expression have been manipulated by means of pharmacological inhibition or small RNA interference (siRNA). During cardiomyocyte formation and maturation, mRNA and protein levels for both isoforms increased progressively, although MAO-A appeared to be the most abundant isoform. Interestingly, MAO-A predominance was attenuated in mature cardiomyocytes. In fact, after 40 days of differentiation levels of both isoforms were equivalent, with a delayed but remarkable increase of MAO-B in the latter stages. Next, to assess whether MAOs might affect cardiomyocyte differentiation and function, iPSCs were treated either with pan MAO inhibitor pargyline, or with siRNA against MAO-A or -B isoform. After 15 days of differentiation, both vehicle- or scramble RNA-treated iPSC-derived cardiomyocytes were beating in a regular manner and displayed a fully organized contractile element structure, with a regular sarcomere striation pattern. On the contrary, cells treated with pargyline or siRNA against MAO-A showed dysynchrony and beat in an arrhythmic manner. As assessed by calcium handling properties, beat rate, a measure of the interval between beats, was irregular and increased in pargyline- and MAO-A siRNA-treated cells. No differences in the beating rate have been detected in cells treated with siRNA against MAO-B, in line with the data showing low levels of MAO-B expression at this stage. Interestingly, loss of sarcomeric integrity was apparent only in MAO-A siRNA, but not pargyline-treated iPSC-derived cardiomyocytes. The regularly striped staining pattern of α -actinin staining was lost in favour of a diffused one, thus denoting lack of sarcomere organization. Although isolated areas of well-organized myofilament structure were present in siRNA-treated cardiomyocytes, the majority of cells exhibited myofilament disarray.

In conclusion, abolition of MAO-A activity/expression during cardiac differentiation negatively affects contractile properties of the iPSC-derived cardiomyocytes resulting in fewer calcium transients and poorly coordinated and irregular cardiomyocyte beats. Moreover, it appears that lack of MAO-A protein during iPSCs differentiation into cardiomyocytes leads to disruption in sarcomere integrity. Further studies are necessary in order to address whether the arrhythmia and sarcomere disorganization observed in iPSC-derived cardiomyocytes following MAO-A ablation are due to the absence of MAO-A activity and/or previously unrecognized structural effects of this protein.

IMPAIRED NEURONAL DIFFERENTIATION OF En2^{-/-} NEURAL STEM CELLS

Simona Casarosa^{1,2}, Angela Bozza¹, Andrea Messina¹, Yuri Bozzi^{1,2}, Camilla Boschian¹

¹University of Trento, Italy; ²CNR Neuroscience Institute, Pisa, Italy

Autism spectrum disorders (ASD) are characterized by impaired relationships, impaired verbal and non-verbal communication, restricted and repetitive behaviours. Their identification and classification are difficult as patients show different typical behaviors and phenotypes. Post-mortem studies of both patients and animal models reveal neuroanatomical abnormalities in different brain regions. Defects have also been shown at a cellular level, such as an important reduction of cortical GABAergic interneurons and of cerebellar Purkinje cells, proposed as causes of the pathology. Two single-nucleotide polymorphisms (SNPs) in the human Engrailed-2 (EN2) gene are associated with ASD, and one of these was shown to markedly affect EN2 promoter activity. Accordingly, mice lacking the homeobox domain of En2 (En2^{hd/hd} mice; referred to as En2^{-/-}) have been proposed as models for ASD, due to their complex neurodevelopmental, neuroanatomical and behavioral phenotype. From behaviour analyses, these mice display reduced social interactions, locomotor impairment, defects in spatial learning and memory. En2^{-/-} mice display cerebellar hypoplasia, including a reduced number of Purkinje cells, and a reduced number of GABAergic neurons in the hippocampus and cerebral cortex. Cortical GABAergic interneurons originate from the basal ganglia region between E14 and E18, subsequently migrating to the cortex by precise tangential migration routes. The molecular mechanisms linking the loss of En2 with the reduction in GABAergic interneurons is still not known. For this reason we have set up an *in vitro* model in which we investigated the role of En2 in neuronal differentiation, and more specifically in GABAergic differentiation. We derived neural stem cells (NSCs) from the basal ganglia of E14 wild-type and En2^{-/-} embryos and assessed their proliferative and differentiation potential. Preliminary results show an increased proliferation and a reduced capability of neuronal differentiation of the En2^{-/-} derived lines with respect to the wild type ones. This is the first molecular evidence linking the transcription factor En2 to the differentiation of GABAergic interneurons.

EFFECTS OF AMINO ACIDS ON INSULIN KINETICS, PANCREATIC BETA-CELL FUNCTION AND INSULIN SENSITIVITY

Marta Pattaro¹, Andrea Tura¹, Yanislava Karusheva², Daniel Markgraf², Julia Szendrödi^{2,3}, Giovanni Pacini¹, Michael Roden^{2,3}

¹CNR Institute of Neuroscience, Padova, Italy; ²Institute for Clinical Diabetology, German Diabetes Center (DDZ), Leibniz Center for Diabetes Research, Düsseldorf, Germany; ³Department of Endocrinology and Diabetology, Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany

Background: Existing evidences suggest that amino acids may influence glucose metabolism. Specifically, during an oral glucose tolerance test, or a meal test, amino acids appear having some effects on insulin secretion and, possibly, on insulin action and on insulin degradation. With the aim of quantitatively analysing this, we developed a mathematical model of insulin kinetics in plasma, where the effect of amino acids is considered. **Methods:** In the model (Figure 1), we assumed that insulin kinetics, i.e., the plasma insulin temporal variations during a metabolic test depend on the following main elements: (i) Insulin disappearance, due to factors such as insulin utilization, insulin clearance from plasma, hepatic insulin extraction; (ii) Insulin appearance, due to pancreatic secretion from the beta-cells, triggered by plasma glucose (the main contributor to the secretory phenomenon); (iii) Insulin appearance (secretion), or, possibly, disappearance, due to amino acids (smaller, but possibly not negligible contributor). We studied 11 patients with type 1 diabetes, that underwent a 3-hr meal test, with measure of glucose, insulin, and all the amino acids. These data were analysed by the model.

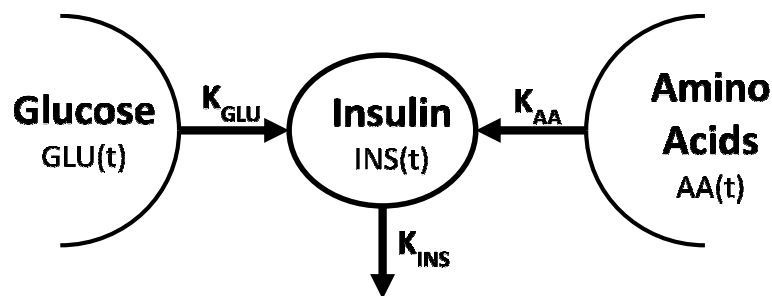


Figure 1: Conceptual scheme of the amino acids-based model of insulin kinetics

Results: Our findings suggest that the AA effect seems to differ among subjects. Specifically, AA effect appears negligible in 2 patients, negative (that is, contributing to insulin disappearance) in 4 patients, positive (that is, contributing to insulin appearance, i.e., insulin secretion) in 5 patients. Results also suggests that the behaviour of the different AAs is similar in a given patient (apart for a couple of AAs, which were not measured in all patients). In regression analyses between the model parameter K_{AA} (representing the sensitivity to AA of insulin appearance) and some indices of pancreatic beta-cell function and insulin sensitivity, we found strong correlation for the beta-cell function indices (up to $R=0.93$, $p<0.0001$), whereas less clear correlation was found for the insulin sensitivity indices.

Conclusions: To our knowledge, the developed model is the first mathematical model of insulin kinetics that includes possible effects of amino acids. We conclude that all (or almost all) AAs seem to have similar effects in a given patient, but AAs effect may be different in the different patients: predominant effect on insulin appearance (thus presumably on insulin secretion) or, at contrast, on insulin disappearance. Furthermore, regression analyses suggested that AAs seem to have in fact significant effects on insulin secretion (beta-cell function), but marginal effects on insulin sensitivity. This may depend on the specific population studied (patients with type 1 diabetes). Future studies on different populations should allow clarifying these issues.

HIGH PRESSURE FREEZING UNVEILS THE NATIVE ULTRASTRUCTURE OF MOUSE HIPPOCAMPUS

Elena Vezzoli^{1,2}, Eleonora Curzi¹, Maura Francolini^{1,3}

¹Università degli Studi di Milano – Dept. of Medical Biotechnology and Translational Medicine, Milano, Italy; ²Università degli Studi di Milano – Dept. of Pharmacological and Biomolecular Sciences, Milano, Italy; ³Institute of Neuroscience – National Research Council (CNR), Milano, Italy

By using transmission electron microscopy (TEM) imaging we compared the neuropil ultrastructure focusing on excitatory synapses of adult mouse hippocampus, processed according to three different protocols: i) cryo-immobilization of acute brain slices by High-Pressure-Freezing (HPF) followed by Freeze Substitution (FS) (Korogod, 2015); ii) aldehyde perfusion followed by HPF and FS (Sosinsky, 2008), and iii) transcordial aldehyde perfusion followed by room temperature processing and embedding in epoxy resin. We observed that the appearance of several neuropil structures differ depending on the protocol used (Figure 1). In HPF/FS processed samples, membrane contours are smoother, intracellular organelles are more regular in shape and the cytoplasm and mitochondrial matrices are denser if compared to the chemically fixed specimens. Quantitative measurements revealed that the surface area of the presynaptic bouton was significantly higher in cryo-immobilized sections if compared to aldehyde-perfused brain, even if the synaptic vesicle density was unchanged. The length and the thickness of the post-synaptic density (PSD) were comparable in all analysed samples. We then confirmed previously reported observations (Korogod, 2015), indicating that the extracellular space in cryo-immobilized acute slices is broader than in perfused brains.

Sosinsky G.E. et al., *Journal of Structural Biology* 2008,161, 359–371.
Korogod N. et al., *eLife* 2015, 4 doi:10.7554/eLife.05793.

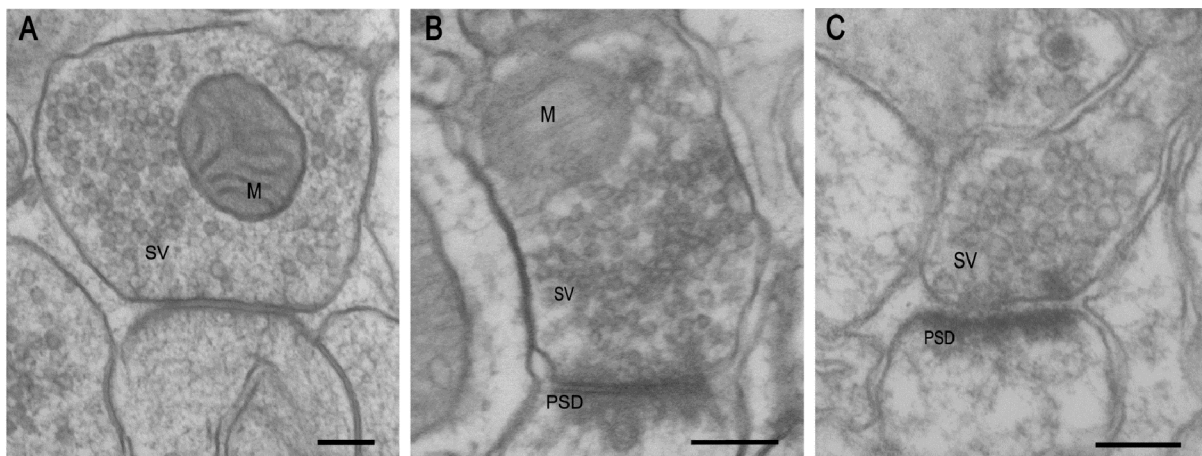


Figure 1. (A) TEM images of cryo-immobilized and chemically fixed (B-C) excitatory hippocampal synapses show reduction in the synaptic surface after chemical fixation. (Scale bars 100 nm). Synaptic surface has been determined in samples processed both by HPF-FS and by chemical fixation at room temperature (C).

QUANTIFICATION OF VASCULAR ANGIOARCHITECTURES IN THE CONTEXT OF NEURODEGENERATIVE DISEASES

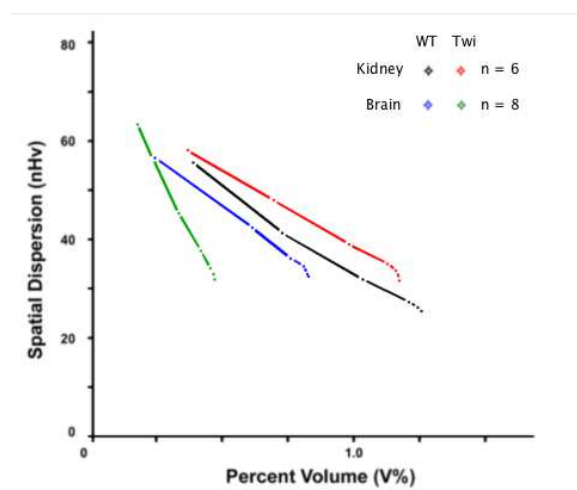
Marco Righi¹, Arianna Giacomini², Mirella Belleri², Marco Presta², Carmelo Carlo-Stella³

¹CNR Neuroscience Institute, Milano, Italy; ²University of Brescia, Italy; ³Humanitas Clinical & Research Center, Rozzano (Milano), Italy;

Vascular architectures play a pivotal role in the pathophysiology of CNS and PNS. Indeed, even minor chronic alterations that reduce oxygen and nutrient availability may affect neurological functions in the long term. Therefore, it is important to monitor, discover and prevent alterations in vascular trees that might affect the correct resupply of nervous cells, especially at the microvascular level.

However, attempts to perform easy and significant comparisons among different angioarchitectures using only a minimal number of parameters represent a challenging task. In this respect, we had reported an unexpected relationship between vascular amounts of caliber-classified microvessels and their spatial dispersion in cancer cells, calculated by normalized Euclidean Distances (nHv 90%) (Righi, M. et al., 2013). This relationship allowed plotting curves that characterized different angioarchitectures. Nevertheless, this approach did not permit a comparison of the observed microvascular networks nor the identification of simple, descriptive mathematical indicators.

Here we present an improved approach based on not only the amount and spatial dispersion of the different classes of microvessels, but also on the implicit relationships between vessels of close calibers (contiguous classes). To this aim, we recalculated the data of percent volume and normalized spatial dispersion (nHv 90%) starting from the class of vessels with largest calibers. Then, we summed the voxels derived from the contiguous class of lower caliber vessels and recalculated volume and spatial dispersion. To these cumulated vessels we summed the voxels derived from the next class of vessels with lower calibers and recalculated parameters. The process was reiterated over the 6 or 7 classes of caliber-classified microvessels we could usually obtain after confocal analysis of sulphobiotin- or antiCD31-stained tissue samples, and data were plotted. When considering 6 or more different samples we could obtain a near linear organisation of points which were describing the angioarchitecture of a representative microvascular tree as observed in the tissue under analysis. Our plotted "lines" could be quantified in term of line length, position and inclination and provided a way to compare angioarchitectures. Re-appraisal of our published data on Twitcher mice (Giacomini et al., 2015) provided an immediate visual description of the cerebral angioarchitecture in this model (see aside). In addition we observed how Twitcher kidneys - which marginally accumulate psychosine - did not suffer marked vascular alterations, although their vessels might be a little more clustered than WT.



We are now approaching the neuropathological issues due to possible microvascular alterations observed in pro-diabetic mice on high fat diet. Follow-up of increasingly diabetic mice should allow to investigate changes in cerebral microvascular angioarchitectures responsible, or relatable, to the cognitive impairment that occurs under these pathologic conditions.

Righi, M. et al., PLoS ONE, 8, e59691 (2013).

Giacomini, A. et al., Angiogenesis, 18, 499-510 (2015).

ABSORPTION MECHANISMS OF A PTEROSTILBENE AMINOACIDIC PRODRUG

Lucia Biasutto^{1,2}, Michele Azzolini^{1,2}, Martina La Spina², Andrea Mattarei³, Cristina Paradisi³, Mario Zoratti^{1,2}

¹CNR Neuroscience Institute, Padova, Italy; ² University of Padova, Department of Biomedical Sciences, Padova, Italy; ³University of Padova, Department of Chemical Sciences, Padova, Italy

The biomedical effects of the natural phenol pterostilbene (Pt) are of great interest (see e.g. poster P4.1 by Michele Azzolini et al.) but its bioavailability is negatively affected by the phenolic group in position 4', which is an ideal target for the conjugative enzymes of phase II metabolism.

A "library" of pterostilbene prodrugs was thus synthesized, in which the hydroxyl moiety is protected via a carbamoyl linkage to a natural amino acid. Prodrugs comprising amino acids with hydrophobic side chains (Ile, Leu, β -Ala, Val, Phe) were readily absorbed after intragastric administration of a bolus to rats. The Area Under the Curve for Pt in blood was optimal when prodrugs with isoleucine or β -alanine were used. The prodrug incorporating isoleucine was used for further studies to map distribution into major organs. When compared to Pt itself, administration of the isoleucine prodrug afforded increased absorption, reduced metabolism and higher concentrations of pterostilbene, sustained for several hours, in most of the organs examined.

The high circulating and tissue levels obtained after administration of the isoleucine prodrug prompted an investigation into the mechanisms involved in its absorption. The presence of a carboxylic group (which can be ionized at physiological pH) and the carbamate ester bond, in fact, render the prodrug more polar and hydrophilic than pterostilbene, which is expected to permeate biological membranes by passive diffusion; the involvement of active transport systems was thus plausible. Experiments using Caco-2 cells as an in vitro model for human intestinal absorption suggest that the uptake of the isoleucine prodrug is partly due to passive diffusion, and partly mediated by H⁺-dependent transporters expressed on the apical membrane of enterocytes such as PepT1 and OATP.

A BLOOD BRAIN BARRIER MODEL TO INVESTIGATE IMMUNE TRAFFICKING IN NEUROLOGICAL DISORDERS

Eliana Lauranzano¹, Raffaella Molteni², Fabia Filipello¹, Ruggero Pardi^{2,3}, Michela Matteoli^{1,4}

¹ *Humanitas Clinical and Research Center, IRCCS Rozzano, Milan, Italy;* ² *San Raffaele Scientific Institute, Milan, Italy;* ³ *Vita-Salute San Raffaele University School of Medicine, Milan, Italy;* ⁴ *CNR Institute of Neuroscience, Milan, Italy*

The blood-brain barrier (BBB) is a highly specialized barrier separating the brain parenchyma from the vascular compartment. It plays a crucial role in regulating the entry of blood-borne molecules and preserving homeostasis within brain microenvironment. Progressive BBB breakdown and loss of solute barrier are associated with a diverse range of neuroinflammatory and neurodegenerative conditions. We are developing an *in vitro* BBB model consisting of a contact co-culture of brain endothelial cells (EC) growing as a monolayer on top of a matrix-coated permeable membrane (vascular BBB side), and primary astrocytes cultured on the opposite side (CNS BBB side) with astrocyte endfeet taking contact with EC. We monitored transendothelial electrical resistance (TEER) as the main indicator of the functional formation of the barrier. Barrier integrity was confirmed by the formation of tight junctions between adjacent EC, by immunofluorescence for claudin-5 and ZO-1, connexin-43, with restricted paracellular diffusion of water-soluble substances. Permeability assays using the impermeable dye LY and the hydrophilic dye NaF confirmed low ($MW \leq 550\text{Da}$) solute passive transport. The ability of the barrier to prevent protein extravasation was confirmed by measurements of permeability to 10kDa-Dextran. Inserts with fully differentiated BBB were placed in a microfluidic platform to assess transmigration of immune cells. Real-time 4D tracking of cells under flow conditions revealed the initial rolling and adhesion of leukocytes to the endothelium, followed by subsequent diapedesis across the BBB and interstitial migration. This microfluidic platform provides a new and versatile tool to investigate *in vitro* the stepwise process of circulating immune cell extravasation across the BBB and to test novel therapeutics targeting various steps of the process.

AUTOMATIC DETECTION OF EEG SPIKES: SOME EXPERIMENTS

Giovanni Bortolan¹, Matteo Caleo², Chiara Cerri², Manuela Allegra²

¹CNR Neuroscience Institute, Padova, Italy; ²CNR Neuroscience Institute, Pisa, Italy;

Background. In biomedical signal processing, spike detection in nonstationary environments is an important task for characterize particular events. In Electroencephalogram (EEG), spikes characterize epileptic seizures. The quantitative description of temporal and frequency parameters of spikes which varies from signal to signal and even from time to time in the same subject, reveal a great research interest.

Methods and Material. This paper presents some experiments on methods for automatic detection of interictal epileptiform discharges in electroencephalogram (EEG) signals. A particular set of EEG signals has been considered (sampling rate 200 Hz, total duration 600 sec), and all the considered algorithms consider the raw EEG data. Time domain and frequency domain have been considered.

In the time domain, three non-linear operators which are sensitive to any discontinuity of the signal were considered in the identification problem, based on spatial differences ("spatial velocity", CL_SV) and product ("energy", CL_EN) of EEG intervals and a specific energy operator (CL_NEO) .

$$CL_SV(i) = \text{abs} (\text{eeg}(i-k) - \text{eeg}(i+k))$$

$$CL_EN(i) = \text{abs} ((\text{eeg}(i-k) - \text{eeg}(i)) * (\text{eeg}(i) - \text{eeg}(i+k)))$$

$$CL_NEO(i) = \text{eeg}(i)^2 - (\text{eeg}(i-k) * \text{eeg}(i+k))$$

where the window parameter k takes appropriate values in the set [1, 3, 5].

An additional smoothing procedure has been performed, and the second part of the spike detection algorithm is computed with the use of variable threshold functions.

The ictal events in EEG signals have been detected with the use of these algorithms, and the following parameters have been computed: mean duration of the spikes, mean inter-spike durations, and spike ratio (ratio between mean value and standard deviation of inter-spike duration in an ictal event) .

In frequency domain, the energy in certain ranges was often stronger or weaker during seizures, and the analysis of these components can identify or characterize seizure activity. The FFT was computed for the various frequency ranges (delta, theta, alpha, beta, gamma) and their behavior inside and outside ictal events were considered and compared.

Results . The initial database the EEG signals are extracted from experiments with mice with temporal lobe epilepsy (sampling rate 200 Hz, total duration 600 sec). All the considered algorithm consider the raw EEG data. Algorithm based on temporal domain and frequency domain have been tested and the main characteristics will be outlined and illustrated.

REPERFUSION INJURY AFTER STROKE STUDY (RISKS STUDY)

Benedetta Piccardi^{1,2}, Antonio Di Carlo², Marzia Baldereschi², Domenico Inzitari^{1,2}

¹*Department of NEUROFARBA, Neuroscience Section, University of Florence, Florence, Italy;*

²*CNR Neuroscience Institute, Florence, Italy.*

Background. Stroke is a major cause of death and disability. Revascularization techniques are able to re-open occluded vessels, salvaging the ischemic tissue from death. However, recanalization may cause blood brain barrier (BBB) disruption due to activation of molecular pathways eventually determining reperfusion injury and hemorrhagic transformation. Preliminary data show that BBB disruption can be traced in vivo by perfusion CT (CTP). Our aim is to evaluate relative effects of biomarkers (circulating factors versus imaging) in relation to clinical outcomes after revascularization in patients with acute ischemic stroke.

Method. Consecutive acute stroke patients candidates to intravenous thrombolysis or to endovascular treatment are currently being enrolled in our hospital. Circulating levels of pro-, anti-inflammatory, immunomodulatory factors, metalloproteinases and their inductors/inhibitors, factors of endothelial dysfunction and fibrin resistance to lysis (measured in blood samples of each patients pre-thrombolysis and 24 hours after thrombolysis), will be determined in relation to in vivo measurement of BBB permeability assessed by CTP.

Results. Enrollment started on October 2015. As of June 2016, 30 patients have been included. Results are expected by the end of 2017 with an estimated sample size of 120 patients. Using a definite protocol, a prospective collection of data, and an adequate number of patients assuring statistically powered data, this study will integrate clinical information about biological factors involved in reperfusion injury after cerebral ischemia. Data obtained may help design of randomized control trials to test putative therapeutic strategies in contrasting reperfusion injury.