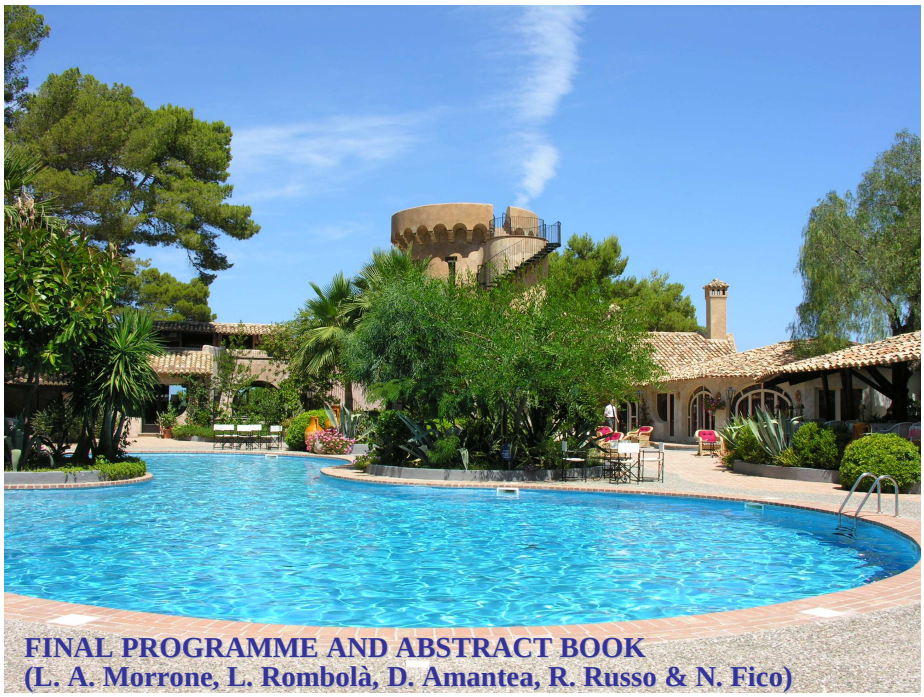
IX WORKSHOP ON APOPTOSIS IN BIOLOGY AND MEDICINE:
Neuroinflammation in neuronal death and repair

Porto Pirgos Hotel (www.portopirgos.com)
Parghelia (VV), Calabria, Italy
13-16 September, 2006

UNDER THE AUSPICES

University of Calabria, Cosenza (www.unical.it)
University "Magna Græcia", Catanzaro (www.unicz.it)
Department of Pharmacobiology, UNICAL (www.unical.it)
Calabria Region (www.regione.calabria.it)
National Institute of Health (www.iss.it)
Italian Society of Pharmacology(http://farmacologiasif.unito.it)
Italian Society of Neuroscience (www.sins.it)



FINAL PROGRAMME AND ABSTRACT BOOK
(L. A. Morrone, L. Rombolà, D. Amantea, R. Russo & N. Fico)

PRESIDENTS

Bagetta Giacinto	(Cosenza)
Lipton A. Stuart	(La Jolla)

INTERNATIONAL ORGANIZING COMMITTEE

Bagetta Giacinto	(Cosenza)
Corasaniti M. Tiziana	(Catanzaro)
Lipton A. Stuart	(La Jolla)
Wyss-Coray Tony	(Stanford)

INTERNATIONAL ADVISORY BOARD

Annunziato Lucio	(Naples)
Berliocchi Laura	(London)
Bernardi Giorgio	(Rome)
Caltagirone Carlo	(Rome)
Di Renzo Gianfranco	(Naples)
Garcia Augustina	(Barcelona)
Genchi Giuseppe	(Cosenza)
Melino Gerry	(Rome)
Mercuri B. Nicola	(Rome)
Mollace Vincenzo	(Catanzaro)
Moroni Flavio	(Florence)
Nicotera Pierluigi	(Leicester)
Pellegrini-Giampietro Domenico	(Florence)
Pittaluga Anna	(Genoa)
Rotilio Giuseppe	(Rome)
Rotiroti Domenicantonio	(Catanzaro)

LOCAL ORGANIZING COMMITTEE

Amantea Diana	(Cosenza)
Iannone Michelangelo	(Catanzaro)
Morrone A. Luigi	(Cosenza)
Nisticò Robert	(Cosenza)
Palma Ernesto	(Catanzaro)
Paoletti Annamaria	(Catanzaro)
Rombolà Laura	(Cosenza)
Russo Rossella	(Cosenza)

LOCAL EXECUTIVE COMMITTEE

Fico Guido	(Cosenza)
Marzullo Michele	(Cosenza)
Politi Giovanni	(Catanzaro)
Russo Francesco	(Cosenza)

SPONSORS
Calabria Region
University Magna Graecia, Catanzaro
ISN-CNR, Catanzaro
National Institute of Health (ISS, Rome)
Department of Pharmacobiology, Cosenza
International Society of Neurochemistry
Italian Society of Pharmacology
Astra-Zeneca SpA
Pharmacists Association of Catanzaro, Cosenza, Kroton and Vibo Valentia
Federation of Private Pharmacy Holders of Catanzaro, Cosenza and Kroton

SCIENTIFIC INFORMATION
Prof. Luigi A. Morrone, Department of Pharmacobiology, University of Calabria, Via P. Bucci, Edificio Polifunzionale, 87036 Arcavacata di Rende (CS), Italy; Tel 39 0984 493054, fax 39 0984 493462, Email: luigimorron@libero.it

SECRETARIAT
Hilary Rowe, Publishing Editor, Neuroscience, at Elsevier, San Diego, CA
Nicola Fico, Department of Pharmacobiology, University of Calabria, Via Pietro Bucci, I – 87036 Rende (CS), Italy – Tel 39 0984 493248 – Fax 39 0984 493462 – E-mail: fico@unical.it

LOCAL SECRETARIAT
J&B srl www.jbprof.com

MEETING VENUE
Porto Pirgos Hotel (tel. 39 0963 600 351) is located at Parghelia (Vibo Valentia), in one of the loveliest spots of the Tirrenian Coast facing the still active Stromboli volcano. The international airport of Lamezia Terme (Catanzaro) is 30 Km from the Hotel. Further travelling information are available on the web at www.portopirgos.com. A reduced price has been agreed for the full board accommodation of participants to the workshop.

LANGUAGE
The official language of the conference is English.

ABSTRACTS SUBMISSION
Participants are welcome to submit abstracts for presentation at the meeting. All abstracts will be for poster presentation and should be written according to the instructions below. Deadline for submission: 15th July 2006. Language:

English (abstract and poster). Abstracts must be submitted by e-mail to the secretariat (fico@unical.it), indicating "IX Workshop" as subject of the correspondence. Authors will be notified the acceptance of the abstract by August, 10th , 2006. Each abstract, written in times new roman (12 character), should contain a sentence stating the study objective; a brief statement of methods, if pertinent; a summary of the results and a statement of the conclusions. It is not satisfactory to say, "the results will be discussed". Use a short, specific title. Capitalize initial letters of trade names. Use standard abbreviations for units of measure. Other abbreviations should be spelled out in full at first mention, followed by the abbreviation in parentheses. Include the source of research support on the bottom line of the abstract. The presenting author assures the merit of the presentation and that all authors listed have had a significant role in the research being reported. The size of the poster will be 70 cm width and 100 cm height. Posters should be mounted on the numbered boards at the corresponding number as listed in the book of abstract (e.g. P1 to P23) and should be attended by one of the Authors during the whole duration of the two poster sessions as indicated in the scientific programme. Posters should be dismounted at the end of the poster session on September 14th.
Also, Elsevier will be publishing the proceedings in the highly respected serial International Review of Neurobiology. This serial, as with all Elsevier serials, is indexed by ISI and linked to Pubmed, ensuring that articles can be cited on Medline.

REGISTRATION FEES

A registration fee of 150 € must be payed at the time of abstract submission. The fee includes access to the meeting room, congress kit, cocktail at the opening ceremony, coffee breaks. Payment should be made to "IX Workshop on Apoptosis in Biology and Medicine", Department of Pharmacobiology, University of Calabria, Via P. Bucci 87030 Rende (CS) Italy. **Bank details:** MONTE DEI PASCHI DI SIENA FILIALE 8473, Via Ponte Pietro Bucci, 87036 Arcavacata di Rende (Cosenza). Cin W, ABI 01030, CAB 80880, Bank Account 000000010947, IBAN IT 52 01030 80880 000000010947; BIC PASCITMMXXX

FELLOWSHIPS AND FINANCIAL ASSISTANCE

A limited number of grants from the Italian Society of Pharmacology will be available to support the participation of PhD students and young researchers presenting their original scientific data as poster communication. Applications for these grants should be submitted directly to the Italian Society of Pharmacology. Also, financial support for PhD students and young Post-Docs which will participate in the meeting are available from the International

Society of Neurochemistry. Applicants should send their request via email to Prof. Giacinto Bagetta (g_bagetta@virgilio.it).

SCIENTIFIC PROGRAMME

Wednesday, 13th September

18.00 Welcome address

18.15 Invited Opening Lecture

Chairperson: ***Oliver J. Dolly*** (Ireland)

Stuart A. Lipton (USA)

Inflammatory mediators, protein misfolding, and uncompetitive/fast off-rate (UFO) drug therapy for neurodegenerative disorders

19.00-20.0 Poster Discussion (P1-P23)

Chairpersons: ***Barbara Viviani*** (Italy) *&* ***Nicola B. Mercuri*** (Italy)

Thursday, 14th September

Morning session

8.30-13.00 BASIC MECHANISMS OF NEUROINFLAMMATION
Chairpersons: ***Diana Boraschi*** (Italy) & ***Tony Wyss-Coray*** (USA)

8.30 *Gasque P. (Cardiff)*
Innate immunity and inflammation in the brain

9.00 *Volterra A., Bezzi P., Brambilla L., Vesce S., Giaccone.G., Kingston A. E. and Rossi D. (Lausanne)*
Altered astrocyte glutamate signalling in *in vitro* inflammation and in an animal model of Alzheimer's disease

9.30 *Bonanno G. (Genoa)*
Release of glutamate by high mobility group box 1 protein from glia re-sealed particles (gliosomes) freshly prepared from adult rat brain

10.00 *Pekny M. (Goteborg)*
The role of astrocytes and the complement system in neural plasticity

10.30-11.00 ***Coffee Break***

Chairpersons: ***Natalia N. Nalivaeva*** (UK) & ***Stuart A. Lipton*** (USA)

11.00 *Turner A. J. and Nalivaeva N. N. (Leeds)*
New insights into the roles of metalloproteinases in neurodegeneration and neuroprotection

11.30 *Fossati S., Faraco G., Santini R., Bianchi M., Patrone M., Pedrazzi M, Sparatore B., Moroni F. and Chiarugi A. (Florence)*
HMGB1: a proinflammatory mediator released from neural cells. Pathophysiological implications for stroke

12.00 *Amantea D., Gliozzi M., Russo R., Bagetta G. and Corasaniti M.T. (Cosenza, Catanzaro)*
Early up-regulation of matrix metalloproteinases (MMPs) triggers neuroinflammatory mediators in brain ischemia in rat

12.30 *Hunt P. S. (London)*
Chronic inflammatory and neuropathic pain: the role of non-neuronal cells

13.00 General Discussion

Afternoon session

18.30-20.00 Poster Discussion (P1-P23)
Chairpersons: ***Marina Marinovich*** (Italy) *&* ***Mauro Maccarrone*** (Italy)

Friday, 15th September

Morning session

8.30-13.00 NEW TARGETS TO TREAT NEUROINFLAMMATORY DISEASES

Chairpersons: ***Linda J. Van Eldik*** (USA) & ***Flavio Moroni*** (Italy)

8.30 *Centonze D., Furlan R., Mercuri N. B. and Martino G. (Rome, Milan)*
Early synaptic dysfunction in experimental multiple sclerosis

9.00 *Ransohoff R. M. (Cleveland)*
Chemokines, chemokine receptors and neuroinflammation

9.30 *Wyss-Coray T. (Stanford)*
Neuroinflammatory mediators in Alzheimer's disease

10.00 *Rogers J. (Sun City)*
Commonalities and differences in Alzheimer's and Parkinson's neuroinflammatory mechanisms

10.30-11.00 ***Coffee break***

Chairpersons: ***Anna Pittaluga*** (Italy) & ***Howard E. Gendelman*** (USA)

11.00 *Viviani B., Gardoni F., Bartesaghi S., Galli C. L., Di Luca M. and Marinovich M. (Milan)*
Cytokines and neuronal channels in disease

11.30 *Minghetti L. (Rome)*
COX-2, PGE_2 and microglial activation in prion diseases

12.00 *Van Eldik J. L. and Watterson D. M. (Chicago)*
Minozac: A new class of disease-modifying therapeutic that targets glia proinflammatory cytokine up-regulation

12.30 *Gendelman E. H. (Omaha)*
Synuclein-linked neuroimmunity and the pathogenesis of Parkinson's disease

13.00 General Discussion

Saturday, 16th September
Closing remarks and Departure

Poster Communications

P01 *Longordo F., Musante V., Severi P., Pittaluga A. and Raiteri M.*
RANTES regulates the spontaneous and the evoked release of glutamate from human cortical synaptosomes.

P02 *Spagnuolo P., Oddi S., Bari M., D'Agostino A., Finazzi-Agrò A. and Maccarrone M.*
Differential modulation of CB1 and CB2 receptors along the neuroimmune axis

P03 *Musante V., Neri E., Pittaluga A. and Raiteri M.*
Presynaptic NMDA receptors located on noradrenergic terminals are differently affected by gp120 and SDF1α

P04 *Luccini E., Neri E., Severi P., M. Raiteri M. and Pittaluga A.*
mGluR1 receptors in Central Nervous System as a selective cellular targets of the HIV-1 protein Tat

P05 *Chiappetta O., Siviglia E., Gliozzi M., Amantea D., Corasaniti M.T. and Bagetta G.*
Early modulation of IL-1β expression underlies neuroprotection by estradiol (E_2) in rats undergone transient middle cerebral artery occlusion (MCAo)

P06 *Mazzei C., Amantea D., Spagnuolo P., Bari, M., Bagetta G., Corasaniti M.T. and Maccarrone M.*
Modulation of the endocannabinoid system is implicated in the neuroprotection afforded by 17β-estradiol (E_2) against brain damage caused by transient middle cerebral artery occlusion (MCAo) in rat

P07 *De Palma C., Panzeri C., Martinuzzi A., Daga A., De Polo G., Bresolin N., Miller C. C., Tudor E. L., Clementi E. and Bassi M. T.*
The first *ALS2* missense mutation associated to jpls reveals new aspects of alsin biological function

P08 *Maiuolo J., Maida S., Navarra M., Bagetta G. and Corasaniti M. T.*
Mechanisms underlying neuroprotection afforded by bergamot essential oil (BEO) against NMDA-induced cell death in vitro

P09 *Bagetta V., Picconi B., Barone I., Nicolai R., Benatti P., Bernardi G., Calvani M. and Calabresi P.*
On the mechanism of Acetyl-L-Carnitine (ALC) neuroprotection against *in vitro* ischemia

P10 *Pelle C., Amantea D., Morrone L.A., Rombolà L., Corasaniti M.T. and Bagetta G.*
Neuroprotection by Bergamot essential oil (BEO) involves inhibition of excitatory aminoacid release in the ischemic rat brain

P11 *Nisticò R., Piccirilli S., Giampà C., Fusco F. and Mercuri N. B.*
Protective role of ATP-sensitive potassium channels blockers in OGD deprived hippocampal slices

P12 *Fratto V., Amantea D., Morrone L. A., Bagetta G. and Corasaniti M. T.*
Akt contributes to neuroprotection afforded by bergamot essential oil (BEO) against focal cerebral ischemia in rats

P13 *Greco R., Amantea D., Sandrini G., Nappi G., Bagetta G., Corasaniti M. T. and Tassorelli C.*
Neuroprotective effect of nitroglycerin in an animal model of ischemic stroke: interference with Bcl2 expression

P14 *Nucci C., Tartaglione R., Cerulli A., Cavaliere F., Rombolà L., Bagetta G. and Morrone L. A.*
Neurochemical and neuropathological evidence that coenzyme Q10 prevents retinal damage caused by high intraocular pressure (IOP)-induced transient ischemia in rat

P15 *Tassorelli C., Greco R., Armentero M. T., Cappelletti D., Mandrini G., Blandini F. and Nappi G.*
A role for brain COX2 and prostaglandins in migraine: insights from an animal model

P16 *Sakurada S., Nakayama D., Mizoguchi H., A. Yonezawa A. and Sakurada T.*
The histamine-induced allodynia in neuropathic pain model mice

P17 *Levato A., Rombolà L., Morrone L.A., Corasaniti M.T., Bagetta G. and Berliocchi L.*
(-)-Linalool attenuates allodynia in the spinal nerve ligation model of neuropathic pain in C57BL6 mice

P18 *Cerullo A., Bulotta S., Ierardi V., Clementi E., Rotiroti D. and Borgese N.*
Studies on the role of endothelial NO synthase in cell migration

P19 *De Palma C., Perrotta C., Sciorati C. and Clementi E.*
Acid sphingomyelinase: a new biological target of the nitric oxide/cyclic GMP pathway

P20 *Facciolo R. M., Giusi G., Alò R., Carelli A. and Canonaco M.*
Role of heat shock proteins 90 and 70 on apoptotic processes in teleost brain following exposure to environmental stressors

P21 *Rufini A., Candi E., Terrinoni A., Ranalli M. and Melino G.*
Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice

Formattato: Tipo di carattere: 11 pt, Colore carattere: Automatico, Inglese (Regno Unito)

P22 *Tucci P., Cione E., Senatore V. and Genchi G.*
All-*trans*-retinoic acid (RA) induces apoptosis in leydig (TM-3) cells

P23 *Granata T., Madeo M., Carelli A., Facciolo R. M. and Canonaco M.*
Lesions of some amygdalar sites induce neurodegenerative events in hibernating rodents

SCIENTIFIC PROGRAMME

ABSTRACTS

MAIN LECTURES

(L1-L17)

L1

INFLAMMATORY MEDIATORS, PROTEIN MISFOLDING, AND UNCOMPETITIVE/FAST OFF-RATE (UFO) DRUG THERAPY FOR NEURODEGENERATIVE DISORDERS

Lipton A. S.
Professor, Burnham/Salk/Scripps Research Institutes and University of California, San Diego; Scientific Director, Burnham Institute for Medical Research, La Jolla, California 92037

Inflammatory mediators, including free radicals such as nitric oxide (NO) and reactive oxygen species (ROS), can contribute to neurodegenerative diseases in part by triggering protein misfolding. In this talk, we will discuss a newly discovered pathway for this phenomenon and possible novel treatments.

Excitotoxicity, defined as overstimulation of glutamate receptors, has been implicated in a final common pathway contributing to neuronal injury and death in a wide range of acute and chronic neurologic disorders, ranging from Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Alzheimer's disease (AD) to stroke and trauma. Excitotoxic cell death is due, at least in part, to excessive activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, leading to excessive Ca^{2+} influx through the receptor's associated ion channel and subsequent free radical production, including NO and ROS.

These free radicals can trigger a variety of injurious pathways, but newly discovered evidence suggests that some proteins are S-nitrosylated (transfer of NO to a critical thiol group), and this reaction can mimic the effect of rare genetic mutations. This posttranslational modification can contribute to protein misfolding, triggering neurodegenerative diseases. One such molecule affected is protein-disulfide isomerase (PDI), an enzyme responsible for normal protein folding in the ER. We found that when PDI is S-nitrosylated (forming SNO-PDI), the function of the enzyme is compromised, leading to misfolded proteins and contributing to neuronal cell injury and loss. Moreover, SNO-PDI occurs at pathological levels in several human diseases, including AD and PD. This discovery thus links protein misfolding to excitotoxicity and free radical formation in a number of neurodegenerative disorders.

Blockade of excessive NMDA receptor activity can in large measure protect neurons from this type of injury and death. However, inhibition of the NMDA receptor by high-affinity antagonists also blocks the receptor's normal function in synaptic transmission and leads to unacceptable side effects. For this reason, many NMDA receptor antagonists have disappointingly failed in advanced clinical trials. Our group was the first to demonstrate that gentle blockade of NMDA receptors by Memantine, via a mechanism of uncompetitive open-channel block with a rapid "off-rate," can prevent this type of damage in a clinically efficacious manner without substantial side effects. We showed that Memantine blocks excessive NMDA receptor activity without disrupting normal activity. Memantine does this by preferentially entering the receptor-associated ion channel when it is excessively open, and, most importantly, its off-rate from the channel is relatively fast so that it does not accumulate to interfere with normal synaptic transmission. Hence, Memantine is well tolerated, has been used

in Europe for PD for many years, and recently passed multiple phase 3 trials for dementia, leading to its approval by the FDA for AD. Clinical studies of Memantine for additional neurologic disorders, including other dementias, neuropathic pain, and glaucoma, are currently underway. We have also developed a series of second-generation drugs that display greater neuroprotective properties than Memantine. These second-generation drugs take advantage of the fact that the NMDA receptor has other modulatory sites, including critical thiol groups that are S-nitrosylated. In this case, in contrast to PDI, S-nitrosylation proves to be neuroprotective by decreasing excessive NMDA receptor activity. Targeted S-nitrosylation of the NMDA receptor can be achieved by coupling NO to Memantine, yielding second-generation "smart" drugs known as NitroMemantines.

References

1. Choi Y-B, Tenneti L, Le DA, Ortiz J, Bai G, Chen H-SV, Lipton SA. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. Nature Neurosci. 2000;3:15-21.
2. Lipton SA, Choi Y-B, Takahashi T, Zhang D, Li W, Godzik A, Bankston LA. Cysteine regulation of protein function — as exemplified by NMDA-receptor modulation. Trends Neurosci 2002;25:474-480
3. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-Nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science 2002;297:1186-1190.
4. Lipton SA. Concepts: Turning down but not off—Neuroprotection requires a paradigm shift in drug development. Nature 2004;428:473.
5. Yao D, Gu Z, Nakamura T, Shi Z-Q, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z, Masliah E, Uehara T, Lipton SA. Nitrosative stress linked to sporadic Parkinson's disease: S-Nitrosylation of parkin regulates it E3 ligase activity. Proc Natl Acad Sci USA 2004;101:10810-10814.
6. Lipton SA, Nakamura T, Uehara T, Shi Z-Q, Gu Z. Comment on S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science 2005;308:1870.
7. Uehara T, Nakamura T, Yao D, Shi Z-Q,Gu Z, Masliah E, Nomura Y, Lipton SA. S-Nitrosylation of protein-disulphide isomerase links protein misfolding to neurodegeneration. Nature 2006;441:513-517.
8. Lipton SA. NMDA receptors, glial cells, and clinical medicine. Neuron 2006;6:9-11.
9. Lipton SA. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. Nature Rev Drug Disc 2006;5:160-170.
10. Chen H-SV, Lipton SA. The chemical biology of clinically-tolerated NMDA receptor antagonists. J Neurochem 2006;97:1611-1626.

L2

INNATE IMMUNITY AND INFLAMMATION IN THE BRAIN

Gasque P.
Cardiff University, UK and University of la Reunion, France

In invertebrates and primitive vertebrates, the brain contains large numbers of "professional" macrophages associated with neurones, ependymal tanycytes and radial glia to promote robust regenerative capacity. In mammals, peripheral immune cells are largely excluded from the brain, and innate immune molecules and receptors produced by the resident "amateur" macrophages (microglia, astrocytes and ependymal cells) control pathogen infiltration and clearance of toxic cell debris. However, there is minimal capacity for regeneration. This evolutionary pattern may have been necessary to reduce the risk of autoimmune attack while preserving the neuronal web but the ability to repair central nervous system damage may have been sacrificed in the process. Remarkably, it may be possible to re-educate and stimulate the resident phagocytes to promote clearance of pathogens (e.g. Prion), toxic cell debris (e.g. amyloid fibrils and myelin) and apoptotic/necrotic cells. Hence, it has now been shown that glial cells and neurons can be induced to express a plethora of innate immune receptors/molecules involved in sensing, decoding and removing of "danger" signals while promoting tissue repair. I will introduce the concept of *self* and *non-self* and the role of complement, lectins, PSR and other associated receptors in the local innate immune response. I will further illustrate the emerging role of a new family of receptors (**CD93**, CD141 and CD248) in controlling the local innate immune response. Understanding the mechanisms involved in the nurturing of damaged neurons by protective glial stem cells with the safe clearance of cell debris could lead to remedial strategies for chronic brain diseases.

L3

ALTERED ASTROCYTE GLUTAMATE SIGNALLING IN *IN VITRO* INFLAMMATION AND IN AN ANIMAL MODEL OF ALZHEIMER'S DISEASE

Volterra A.[1,2], Bezzi P.[1], Brambilla L.[2], Vesce S.[1], Giaccone.G.[3], Kingston A. E.[4] and Rossi D.[2]

[1]Department of Cell Biology and Morphology, University of Lausanne, Switzerland and [2]Department of Pharmacological Sciences, Centre of Excellence on Neurodegenerative Diseases, University of Milan, Italy; [3]National Neurological Institute "Carlo Besta", Milan, Italy; [4]Lilly Research Center, Eli Lilly and Co. Ltd., Windlesham, Surrey, UK and Indianapolis, USA

Several lines of evidence suggest that astrocytes are not only passive supportive elements responsible for maintaining the optimal environment for neurons, but actively participate to the function of the central nervous system (CNS). They were found to express a broad range of neurotransmitter receptors and possess synaptic-like vesicles that release glutamate via a calcium-dependent mechanism. The latter event can be triggered by various stimuli, including the pro-inflammatory cytokine tumour necrosis factor α (TNFα), acting via prostaglandins (PGs) (Bezzi et al. Nat. Neurosci., 2001). In our 2001 study we reported that glial TNFα-dependent glutamate release is normally part of a physiological interplay between neurons and astrocytes. However, acute inflammation *in vitro*, induced by adding reactive microglia to astrocyte cultures, leads to enhanced TNFα production and amplified glutamate release, switching the pathway into a neurodamaging cascade.

In order to investigate whether a similar event occurred in chronic neurodegenerative conditions characterised by brain inflammatory processes, we studied how the properties of the glial glutamate system changed in the hippocampus of a transgenic animal model of Alzheimer's disease (AD), the PDAPP mice. We found that TNFα-mediated glutamate release from hippocampal acute slices was dramatically reduced in 12-month-old, but not in 4-month-old, PDAPP mice. Interestingly, the defect correlated with the presence of numerous β-amyloid deposits. Further, astrocytes displayed a reactive phenotype and expressed 10-times more GFAP than the astrocytes of the animals analysed at the early age. As the release was normal in the cerebellum, a region devoid of β-amyloid deposition and astrocytosis, it is tempting to conjecture that the identified alteration is associated with the phenotypic switch to reactive astrocytes. We then studied the signal-transduction events potentially responsible for the deficient glutamate release, identifying an alteration in the coupling between TNFR1 signalling and PG formation via the arachidonic acid cascade (Rossi et al., JBC, 2005). In view of the emerging functional roles of glial glutamate release at the synapses, deficient TNFα-dependent glutamate release from astrocytes may have an impact on the strength of synaptic connectivity and participate in the cellular mechanisms leading to cognitive dysfunction in AD.

L4

RELEASE OF GLUTAMATE BY HIGH MOBILITY GROUP BOX 1 PROTEIN FROM GLIA RE-SEALED PARTICLES (GLIOSOMES) FRESHLY PREPARED FROM ADULT RAT BRAIN

Bonanno G.
Department of Experimental Medicine - Pharmacology and Toxicology Section and Center of Excellence for Biomedical Research, University of Genoa, Italy
bonanno@pharmatox.unige.it

Glial subcellular re-sealed particles (here referred as to gliosomes) have been purified from rat cerebral cortex. Confocal microscopy showed that the glial specific proteins GFAP and S-100B β but not the neuronal proteins PSD-95, MAP-2, and β-tubulin III were enriched in purified gliosomes. Furthermore, gliosomes exhibit labelling nor for integrin αM neither for myelin basic protein, specific for microglia and oligodendrocyte, respectively.
Firstly, gliosomes were investigated for their ability to release glutamate. The Ca^{2+} ionophore ionomycin efficiently stimulated release of tritium from gliosomes pre-labelled with [^{3}H]D-aspartate and of endogenous glutamate. Gliosomal glutamate release was dependent on external Ca^{2+} and sensitive to bafilomycin-A1. Accordingly, ionomycin induced Ca^{2+}-dependent increase of the vesicular fusion rate, when monitoring exocytosis with acridine orange. Confocal microscopy revealed that the gliosomal fraction contains proteins of the exocytotic machinery (syntaxin-1, VAMP-2, SNAP-23, SNAP-25) coexisting with GFAP immunoreactivity. Moreover, GFAP or VAMP-2 co-expresses with the vesicular glutamate transporter type 1 in purified glial particles. Consistent with ultrastructural analysis, several ~30 nm non-clustered vesicles are present in the gliosome cytoplasm. Also high KCl and veratrine can release [^{3}H]D-aspartate and endogenous glutamate by a mechanism partly dependent on external Ca^{2+} and partly blocked by glutamate uptake inhibitors. It is concluded that gliosomes purified from adult brain can release excitatory amino acid neurotransmitters by a process resembling neuronal exocytosis as well as by reversal of the glutamate membrane transport.
The multifunctional protein high mobility group box 1 (HMGB1) is expressed in hippocampus and cerebellum of adult mouse brain. Our aim was to determine whether HMGB1 affects glutamatergic transmission by monitoring neurotransmitter release from gliosomes and synaptosomes isolated from cerebellum and hippocampus. HMGB1 concentration-dependently induced release of [^{3}H]D-aspartate form gliosomes, whereas nerve terminals were insensitive to the protein. The release of [^{3}H]D-aspartate relied on the presence of external Ca^{2+} but it was independent on modifications of cytosolic Ca^{2+} and was blocked by DL-TBOA, an inhibitor of glutamate transporters. HMGB1 also stimulated the release of endogenous glutamate in a Ca^{2+}-independent and DL-TBOA-sensitive manner. These findings suggest the involvement of carrier-mediated release. Of note, dihydrokainic acid, a selective inhibitor of the glutamate transporter GLT1, does not block the effect of HMGB1, indicating a role for the glial glutamate-aspartate transporter GLAST in this response. HMGB1/glial particles association is promoted by Ca^{2+}. Furthermore, although

L4

HMGB1 can physically interact with GLAST and the Receptor for Advanced Glycation End Products (RAGE), only its binding with RAGE is promoted by Ca^{2+}. Altogether, these results suggest that the HMGB1 cytokine could act as a modulator of glutamate homeostasis in adult mammal brain.

L5

Mylos Pekny

ABSTRACT NOT ARRIVED

L6

NEW INSIGHTS INTO THE ROLES OF METALLOPROTEINASES IN NEURODEGENERATION AND NEUROPROTECTION

Turner A. J. and Nalivaeva N. N.
Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT

Proteolytic enzymes constitute around 2% of the human genome and provide important therapeutic targets in many diseases. Proteases are involved in many stages of cell development from fertilisation to death (apoptosis). The identification of many novel proteases from genome sequencing programmes has identified potential new therapeutic targets. In addition, several well characterised metallopeptidases were recently shown to possess new biological roles making them important players in the arena of neurodegeneration and neuroprotection. As a result of these studies, metabolism of the neurotoxic amyloid peptide (Aβ) is considered now as a physiologically relevant dynamic process with several metallopeptidases being suggested for the role of amyloid-degrading enzymes. These include the neprilysin (NEP) family of metalloproteinases (including its analogue endothelin-converting enzyme), insulin-degrading enzyme (IDE), angiotensin-converting enzyme, plasmin and, possibly, some other enzymes. The existence of natural enzymatic mechanisms for removal of amyloid peptides has extended the therapeutic avenues in Alzheimer's disease (AD) and neurodegeneration. The proteolytic events underlying AD are highly compartmentalised in the cell. Formation of amyloid peptide from its precursor molecule APP (amyloid-precursor protein) was demonstrated to take place both within intracellular compartments of the cell and in the plasma membrane, including segregation of key components into lipid raft domains. On the other hand, degradation of amyloid peptide by metallopeptidases can be both intra- and extra-cellular events depending on the activity of membrane-bound enzymes and their soluble partners as well as on the oligomeric state of the amyloid. Soluble forms of proteases can be secreted by the cells or released from the cell surface through the activity of "sheddases" – another group of proteolytic enzymes involved in key cellular regulatory functions. The activity of proteases involved in amyloid metabolism depends on numerous factors (genetic, environmental, age) and some conditions (e.g. hypoxia and ischemia) shift the balance of amyloid metabolism towards accumulation of higher concentrations of Aβ in the brain. This could explain the higher risk of development of AD in patients with brain ischemia and stroke. Also it was demonstrated that in the cortex and hippocampus there is endogenous age-dependent deficit of such metallopeptidases as NEP and IDE. In this relation regulation of the activity of amyloid-degrading enzymes should now be considered as a viable strategy in neuroprotection.

L7

HMGB1: A PROINFLAMMATORY MEDIATOR RELEASED FROM NEURAL CELLS. PATHOPHYSIOLOGICAL IMPLICATIONS FOR STROKE

Fossati S., Faraco G., Santini R., Bianchi M.[1], Patrone M.[2], Pedrazzi M.[3], Sparatore B.[3], Moroni F. and Chiarugi A.

Dept. Pharmacology, University of Florence, [1]San Raffaele Research Institute, [2]Dept. Environmental and Life Sciences, University of Eastern Piedmont, [3]Dept. Experimental Medicine, University of Genoa, Italy

High Mobility Group Protein Box-1 (HMGB1) is a nuclear protein which bends DNA and regulates transcription. Recent studies demonstrate that necrotic cells release HMGB1 in the extracellular space, whereas those undergoing apoptosis retain the protein into the nucleus. Upon release, HMGB1 acts on RAGE, TLR-2 and -4 receptors to activate immune cells. The latter, in turn, release HMGB1 thereby establishing an auto-amplifying inflammatory signaling cascade. To investigate the neuropathological role of HMGB1, we sought to determine its expression and dynamics in in vitro and in vivo models of neuronal death. By means of immunocytochemistry and Western blotting, we found that HMGB1 was highly expressed in the nuclei of cultured mouse cortical neurons and glial cells. The protein was promptly released from neurons and glia into the cytoplasm and culture media upon necrotic stimuli such as 10 mM sodium azide + 6 mM deoxyglucose, 1 mM hydrogen peroxide, 3 mM glutamate or 1 mM methyl-nitroso-guanidine (MNNG). Conversely, HMGB1 was retained into the nucleus of apoptotic neurons and glia exposed to 1 µM staurosporine. Inhibition of histone deacetylases by trichostatin A or of poly(ADP-ribosyl)ation by PJ34 and phenanthridinone did not alter nuclear release of HMGB1 from both cell types. Importantly, addition of pure HMGB1 to mixed mouse cortical cultures induced expression of inflammatory mediators such as TNF, iNOS and COX-2, and increased glutamate-dependent neuronal cell death. In a mouse model of middle cerebral artery occlusion release of nuclear HMGB1 occurred in ischemic neurons and astrocytes 3 h after the insult, and increased in the subsequent 6 and 12 h. Partial release of HMGB1 was detected in the penumbral region. Of note, cortical microinjection of HMGB1 dose-dependently worsened the ischemic brain injury. These results taken together suggest an active role of cellular HMGB1 extrusion in post-ischemic brain damage.

L8

EARLY UP-REGULATION OF MATRIX METALLOPROTEINASES (MMPs) TRIGGERS NEUROINFLAMMATORY MEDIATORS IN BRAIN ISCHEMIA IN RAT

Amantea D.[1], Gliozzi M.[2], Russo R.[1], Bagetta G.[1] and Corasaniti M. T.[2]
[1]*Department of Pharmacobiology, University of Calabria, 87036 Rende, Italy;* [2]*Department of Pharmacobiological Sciences, Faculty of Pharmacy, University "Magna Graecia" of Catanzaro, Catanzaro, Italy*

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, classically recognised as matrix-degrading enzymes involved in tissue remodelling during development and homeostasis. In addition to their physiological roles, MMPs are markedly upregulated in the central nervous system (CNS) in response to injury and have been implicated in the propagation and regulation of neuroinflammatory processes that accompany most CNS pathologies (Cunningham et al. 2005, Glia 50:329-339; Rosenberg 2002, Glia 39:279-291). In particular, the gelatinases MMP-2 and MMP-9 become activated following permanent or transient focal brain ischemia and participate to the disruption of the blood brain barrier and hemorrhagic transformation following injury both in experimental animals (Rosenberg et al., 1998, Stroke 29:2189-95; Romanic et al., 1998, Stroke 29:1020-30; Ashai et al., 2000, J Cereb Blood Flow Metab 20:1681-9) and in humans (Horstmann et al., 2003, Stoke 34:2165-72). Moreover, recent work has emphasized the role of MMPs in the regulation of neuronal cell death and apoptosis through MMP modulation of excitotoxicity (Jourquin et al., 2003, Eur J Neurosci 18:1507-17), anoikis (Gu et al., 2002, Science 297:1186-90), death receptor activation (Wetzel et al., 2003, Eur J Neurosci 18:1050-60) and neurotrophic factors bioavailability (Lee et al., 2001, Science 294:1945-8). Original *in situ* zymography experiments demonstrate that a significant increase in gelatinolytic MMPs activity occurs in the ischemic brain hemisphere (cortex and striatum) after 2 h middle cerebral artery occlusion (MCAo) followed by 2 h reperfusion in rat. Accordingly, gelatin zymography reveals that expression and activity of MMPs are enhanced. In fact, both MMP-2 and MMP-9 are significantly up-regulated following ischemia/reperfusion and the latter effect appears to be instrumental for development of delayed (seen 22 hours after reperfusion had been initiated) brain damage. The latter deduction is supported by the observed inhibition of MMP-2 and MMP-9 up-regulation and neuroprotection afforded by systemic, intra-arterial, administration of a broad spectrum, highly specific MMPs inhibitor, e.g. GM6001, but not by its negative control. Increased gelatinase activity in the ischemic cortex is coincident with elevation (166% vs sham) of mature IL-1beta after 2 h reperfusion and this does not seem to implicate a caspase-1-dependent processing of pro(31 KDa)-IL-1beta to yield mature (17 KDa) IL-1beta. Quite surprisingly, a pre-treatment with a neuroprotective dose of GM6001 abolished the early IL-1beta increase in the ischemic cortex and reduced the cleavage of the cytokine pro-form supporting the deduction that MMPs may initiate IL-1beta processing.

L8

In conclusion, our results emphasize the crucial interplay between MMPs and mediators of neuroinflammation (e.g. IL-1beta) during the early development of tissue damage observed following transient ischemia, further underscoring the potential of MMPs inhibitors in stroke therapy.

Financial support from University of Calabria (Ex quota 60% 2006) is gratefully acknowledged.

L9

CHRONIC INFLAMMATORY AND NEUROPATHIC PAIN: THE ROLE OF NON-NEURONAL CELLS

Stephen P. Hunt
Professor of Molecular Neuroscience Department of Anatomy and Developmental Biology, University College London, Medawar Building, Gower Street, WC1E 6BT, UK

Chronic pain particularly neuropathic pain has remained difficult to treat effectively with only around one in three patients experiencing adequate pain relief. In part, this is because we have yet to understand the underlying pathology that gives rise to pain particularly when the original injury is thought to have resolved. Research has concentrated on both peripheral and central areas of the nervous system. Considerable attention has been focused on the peripheral nervous system as causal in the generation of chronic pain. Given the recent explosion of knowledge of peripheral pharmacology and the cloning of large numbers of receptor genes specifically expressed by nociceptors it is at first sight surprising that new treatments have not been forthcoming. Targeted knockout or disruption of identified genes has suggested that particular nociceptor specific transcripts may well be involved in maintaining abnormally high levels of nociceptive sensitivity following either inflammation or nerve injury but specific drugs that mimic these molecular interventions have yet to be successfully developed. This may be because the contribution of the central nervous system as well, as non-neuronal cells such as glial cells and macrophages, to maintaining chronic pain states has not been fully appreciated. For many years it was implied that this plasticity of the dorsal horn was an intrinsic function of the dorsal horn neurons and was unrelated to brain processing or neuro-immune interactions, but this seems not to be the case at least in experimental models of neuropathic and inflammatory pain. It has been repeatedly shown that ablation or inactivation of descending pathways from the brainstem alleviate pain states. This suggests that the maintenance of chronic pain states requires the cooperation of descending pathways and that inappropriate activation of brain systems that modulate nociception is in some way contributing to the pathology. A second aspect of this story is that currently high profile of non-neuronal cells to the maintenance of chronic pain states. In both the periphery and the central nervous system cells of the macrophage-monocyte lineage have been shown to play important roles in progression of the pain state. In my presentation I will highlight the role of tumour necrosis factor in macrophage recruitment during arthritis, the importance of nerve growth factor in maintaining pain in bone cancer and the role of dorsal horn microglial cells in neuropathic pain states.

L10

EARLY SYNAPTIC DYSFUNCTION IN EXPERIMENTAL MULTIPLE SCLEROSIS

Centonze D.[1], Furlan R.[2], Mercuri N. B.[1] and Martino G.[2]
[1]Department of Neuroscience, Tor Vergata University, & European Brain Research Institute (CERC)/ Santa Lucia Foundation, Rome, Italy; [2]Neuroimmunology Unit, DIBIT, & Department of Neurology and Neurophysiology, San Raffaele Scientific Institute, via Olgettina 58, 20132, Milan, Italy martino.gianvito@hsr.it

Although classically regarded as a disorder of the white matter, alterations of the neuronal compartment of the central nervous system occur precociously and largely independent of demyelinization in multiple sclerosis (MS) (Bjartmar et al., 2001; Peterson et al., 2001; De Stefano et al., 2002; Kuhlmann et al., 2002; Filippi et al., 2003; Filippi and Rocca, 2005). Glutamate excitotoxicity has been proposed as a major determinant of neurodegeneration in MS (Stover et al., 1997; Werner et al., 2001; Sarchielli et al., 2003; Srinivasan et al., 2005; Vallejo-Illarramendi et al., 2006;) and in experimental MS (Hardin-Pouzet et al., 1997; Pitt et al., 2000; Smith et al., 2000; Ohgoh et al., 2002). However, a physiological study addressing synaptic transmission during immuno-mediated aggression of the central myelin is still lacking. Here, we addressed neuronal and synaptic functioning in major oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), a preclinical model of MS (Pluchino et al., 2003). Striatal neurons, which are particularly prone to degenerate in MS (Bermel et al., 2003), exhibited complex and dynamic alterations of glutamate- and GABA-mediated neurotransmission, starting in the presymptomatic phase of the disease and evolving independently of inflammatory infiltrates, demyelinization or axonal injury. Changes in the activity of NMDA and non-NMDA glutamate receptors, of sodium-calcium exchanger and of voltage-dependent sodium channels, all contributed to up-regulate excitatory inputs to striatal neurons, while GABA-mediated transmission was persistently down-regulated due to pre- and postsynaptic alterations. These data reveal an important role of synaptic dysfunction in the pathological process of EAE, and provide a rationale for the use of neuroprotective strategies since the very early stages of MS.

L11

CHEMOKINES, CHEMOKINE RECEPTORS AND NEUROINFLAMMATION

Ransohoff R. M.
MD, Director, Neuroinflammation Research Center, Cleveland Clinic

Abstract
Inflammation of the central nervous system (CNS) entails the activation of resident microglia and recruitment of hematogenous leukocytes. Thus defined, inflammation accompanies most neurological disorders, including multiple sclerosis (MS), stroke, neoplasia, trauma and HIV-1-associated dementia, as well as Alzheimer's disease and other primary neurodegenerations. Chemokines comprise a family of peptides that act through G protein-couple receptors (GPCRs) to regulate leukocyte migration throughout all tissues, in an exquisitely specific and flexible fashion. Initial studies asked how chemokines and chemokine receptors governed inflammatory cell recruitment to the CNS during immune-mediated or virus-induced inflammation. More recently, it has become clear that the CNS complement of constitutive chemokines supports developmental and neurophysiological functions as well as regulating the activation of microglia. Because GPCRs can serve as drug targets, these results have implications for the understanding and treatment of disease by neurologists and neuroscientists.

References

1. Liu,L., Graham,G.J., Damodaran,A., Hu,T., Lira,S.A., Sasse,M., Canasto-Chibuque,C., Cook,D.N., and Ransohoff,R.M. 2006. Cutting edge: the silent chemokine receptor d6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis. *J.Immunol.* 177:17-21.
2. Liu,L., Huang,D., Matsui,M., He,T.T., Hu,T., DeMartino,J., Lu,B., Gerard,C., and Ransohoff,R.M. 2006. Severe disease, unaltered leukocyte migration, and reduced IFN-{gamma} production in CXCR3-/- mice with experimental autoimmune encephalomyelitis. *J.Immunol.* 176:4399-4409.
3. Huang,D.R., Wang,J., Kivisakk,P., Rollins,B.J., and Ransohoff,R.M. 2001. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J.Exp.Med.* 193:713-726.
4. Cardona,A.E., Pioro,E.P., Sasse,M.E., Kostenko,V., Cardona,S.M., Dijkstra,I.M., Huang,D., Kidd,G., Dombrowski,S., Dutta,R., Lee,J.C., Cook,D.N., Jung,S., Lira,S.A., Littman,D.R., and Ransohoff,R.M. 2006. Control of microglial neurotoxicity by the fractalkine receptor. *Nat.Neurosci.* 9:917-924.
5. Charo,I.F. and Ransohoff,R.M. 2006. The many roles of chemokines and chemokine receptors in inflammation. *N.Engl.J.Med* 354:610-621.
6. Ransohoff,R.M., Kivisakk,P., and Kidd,G. 2003. Three or more routes for leukocyte migration into the central nervous system. *Nat.Rev.Immunol.* 3:569-581.
7. Tsai,H.H., Frost,E., To,V., Robinson,S., ffrench-Constant,C., Geertman,R., Ransohoff,R.M., and Miller,R.H. 2002. The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 110:373-383.

L12

NEUROINFLAMMATORY MEDIATORS IN ALZHEIMER'S DISEASE

Wyss-Coray T.
Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305, USA

Alzheimer's disease (AD) is a progressive dementia with unknown etiology that affects a growing number of the aging population. Based initially on increased expression of inflammatory mediators in postmortem AD brains and epidemiological studies that link the use of anti-inflammatory drugs with reduced risk for AD, inflammation has been proposed as a possible cause or driving force of the disease. If true, this could have important implications for the development of new treatments. Alternatively, inflammation is simply a bystander product of the disease process and does not significantly alter its course, or, as suggested by some, components of the inflammatory response are beneficial and slow the disease. To address these possibilities we need to determine if inflammation in AD is an early event, if it is genetically linked with AD, and if manipulation of inflammatory pathways changes the course of disease. While there is still little support that inflammation triggers or significantly promotes AD. I will review some of the increasing evidence from mouse models to suggest that several inflammatory mediators are potent drivers of the disease. Related factors, on the other hand, elicit beneficial responses and can reduce disease.

In addition to the studies linking AD with altered immune and injury responses in the brain immune responses may be activated or perturbed in the periphery as well. We thus hypothesized that the cellular communication network consisting of secreted signaling molecules, would be dysregulated in AD and produce characteristic protein expression patterns in plasma. We measured more than 100 cytokines, chemokines, growth factors, and related proteins using filter membrane arrays and found a large number were present at significantly different levels in plasma from AD compared with non-demented controls. Protein measurements were confirmed independently by ELISA or Luminex bead assays and expression patterns produced by a small set of protein markers could predict AD or mild cognitive impairment (MCI) with high accuracy in an independent set of samples. Our findings point to a dysregulation of the cellular communication network in AD in the periphery and offer potential opportunities for diagnosis.

L13

COMMONALITIES AND DIFFERENCES IN ALZHEIMER'S AND PARKINSON'S NEUROINFLAMMATORY MECHANISMS

Rogers J., Ph.D.
Sun Health Research Institute, 10515 West Santa Fe Drive, Sun City, USA

Many of the mechanisms of intrinsic brain inflammatory responses, particularly those related to activated microglia, originally attracted widespread research interest in the context of Alzheimer's disease (AD). Major histocompatibility complex type II cell surface expression by microglia in pathologically-vulnerable regions of AD brain, first reported in 1986 and 1987, was a seminal event. Basic research studies on a virtual textbook of inflammatory markers--cytokines, complement, chemokines, mediators and products of the respiratory burst such as iNOS and reactive oxygen and nitrogen species, growth factors, receptors, transcription factors, and many others--ensued over the next two decades, and culminated in several dozen epidemiologic surveys and direct clinical trials.

More recently, lessons learned about AD neuroinflammation have begun to be applied to Parkinson's disease (PD). Activated microglia appear to cluster around dystrophic, pigmented dopamine (DA) neurons in the PD substantia nigra. Activation of microglia--for example, by injection of lipopolysaccharide into or near the nigra--causes a selective, permanent depletion of nigral DA, sparing other neurotransmitters. Nigral and/or striatal microglia are activated in MPTP and 6-OHDA animal models of PD, and prior treatment with anti-inflammatory drugs may be protective against DA loss in these paradigms. As occurred in AD, upregulation of a wide variety of inflammatory mediators is being demonstrated in PD, and several epidemiologic studies have reported decreased risk for PD in chronic users of anti-inflammatory drugs. Though obviously speculative, it might also be argued that the majority of proposed etiologies for PD, such as repeated head trauma, CNS infections, and environmental toxins, have brain inflammation as a common denominator.

A final, crucial commonality of AD and PD neuroinflammation is lack of any definitive evidence that inflammatory mechanisms play a pathogenic rather than detritus-clearing role in either disorder. Treatment trials in AD have generally not been promising, and one group of investigators has even suggested that AD brain inflammation may be more beneficial than harmful by helping clear cortical and limbic amyloid β peptide (Aβ) deposits. However, in both AD and PD, substantial pathology and damage may exist before overt clinical symptoms are manifest, rendering treatment trials moot, and efforts to enhance Aβ removal by stimulating inflammation with anti-Aβ antibodies apparently results in both Aβ clearance and lethal inflammatory responses.

Despite parallels in AD and PD inflammation research, the two disorders are likely to differ with respect to the stimuli and targets of inflammation. Studies in my laboratory strongly suggest, for example, that Aβ deposition and (though less well-studied) neurofibrillary tangle formation stimulate AD inflammatory responses, with neurons as a non-selective, bystander target. By contrast, our recent studies of PD suggest that DA itself may stimulate chemotaxis to and attack on DA neurons by activated microglia, with DA neurons as a direct, selective target.

CYTOKINES AND NEURONAL CHANNELS IN DISEASE

Viviani B., Gardoni F., Bartesaghi S., Galli C. L., Di Luca M. and Marinovich M.
Laboratory of Toxicology and Center of Excellence on Neurodegenerative Diseases, Department of Pharmacological Sciences, University of Milan, Italy

The biophysical properties and the spatial distribution of receptor and ion channels define the signalling of individual neurons and endow their specific character. Function, number and localization of receptor and ion channels are dinamically modulated in response to diverse stimuli and undergo plastic changes in both physiological and pathological conditions. There is increasing evidence that cytokines may specifically interact with receptor and ion channels regulating neuronal excitability, synaptic plasticity and responses to injury. Cytokines are thus regarded as novel neuromodulators, opening up new perspective in the current view of brain behaviour.

Interleukin (IL)-1β is one of the proinflammatory cytokines implicated in various pathophysiological conditions of the CNS. We recently demonstrated that recombinant IL-1β increases NMDA receptor function through activation of tyrosine kinases and subsequent NR2B subunit phosphorylation at tyr1472. Activation of tyrosine kinases appears to be a crucial pathway for the stabilization of the NMDA receptor NR2B subunit at the synaptic sites, the occurrence of a sustained elevation of intracellular Ca^{2+} in neurons and neuronal death in the presence of IL-1β. This effect occurs also in neurons exposed to HIV-envelope glycoprotein gp120 in the presence of glia due to IL-1β release, thus suggesting the recruitment of this pathway in a pathological condition. IL1β, by affecting NMDAR localization and function, could contribute to glutamate-mediated action.

L15

COX-2, PGE_2 AND MICROGLIAL ACTIVATION IN PRION DISEASES

Minghetti L.
Department of Cell Biology and Neurosciences, National Institute of Health, Rome, Italy

Cyclooxygenase (COX), also known as prostaglandin H synthase, catalyses the first committed step in the synthesis of prostanoids, a large family of arachidonic acid metabolites comprising prostaglandins (PGs), prostacyclin and thromboxanes. COX has become a very popular enzyme since, in 1971, it was demonstrated that non steroidal anti-inflammatory drugs (NSAIDs) exert their anti-inflammatory properties through the inhibition of COX enzymatic activity. COX exists as constitutive (COX-1) and inducible (COX-2) isoforms, which are coded by two distinct genes. While COX-1 gene represents a classical "house-keeping" gene, COX-2 gene is characterized by the presence of a TATA box and a multitude of binding sites for transcription factors in its promoter region, which account for the complex regulation of COX-2 expression. Indeed, COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines and pro-inflammatory molecules. Since its discovery in early 1990s, COX-2 is emerged as a major player in inflammatory reactions in peripheral tissues. Similarly, COX-2 expression in brain has been associated with pro-inflammatory activities, thought to be instrumental in neurodegenerative processes of several human neurological diseases, including chronic diseases such as Alzheimer's disease and prion diseases.

Prion diseases are a heterogeneous group of infectious, sporadic and genetic disorders characterized by amyloid deposition of the proteinase-resistant prion protein, astrocytosis and spongiform degeneration. A further typical sign is the extensive microglial activation, which supports the occurrence of a local chronic inflammatory response. To investigated the involvement of COXs in prion diseases, we have used animal models in which mice are infected with scrapie, the prion form affecting sheep, or with brain homogenates obtained from cases of Creutzfeldt-Jakob disease (CJD), the most known human form of prion disease. In these models, we observed a substantial raise in the hippocampal levels of PGE_2 and a strong induction of COX-2 expression, which increased with the progression of disease and was specifically localized to microglial cells. Few scattered COX-1 positive microglia-like cells were found in control and infected brains. The increased COX activity in experimental prion diseases was consistent with the elevated levels PGE_2 found in the cerebro-spinal fluid of a group subjects affected by genetic or sporadic CJD. In sporadic CJD patients, higher CSF levels of PGE_2 were associated with shorter survival. Experimental evidence suggests that PGE_2 synthesis and COX-2 expression in microglial cells may be associated with the clearance of apoptotic neurons and in CJD, abundance of apoptotic neurons correlated well with microglial activation. However, whether COX-2 over-expression is a cause or a consequence of neuronal death in prion diseases or whether CSF levels of PGE_2 represent an index of disease severity remain to be established.

L16

MINOZAC: A NEW CLASS OF DISEASE-MODIFYING THERAPEUTIC THAT TARGETS GLIA PROINFLAMMATORY CYTOKINE UP-REGULATION

Van Eldik L. J. and Watterson D. M.
Center for Drug Discovery and Chemical Biology, Northwestern University, Chicago IL USA

Background: Excessive or uncontrolled neuroinflammation arising from chronically activated glia is a key contributor to the progression of neurologic injury and disease, such as Alzheimer's disease (AD), Parkinson's disease, traumatic brain injury (TBI), stroke, and neuropathic pain. Development of therapeutics that suppress neuroinflammatory responses of activated glia, such as elevations in proinflammatory cytokines, would have broad impact across diverse neurological disease indications [1]. Recently, we used a novel integrative drug discovery approach that exploited the established success of modulating proinflammatory cytokine pathways in anti-inflammatory drug discovery for peripheral tissues and combined it with a CNS focus by using glia as the primary target for discovery [2-4]. A *de novo* lead compound discovery approach was used. An inactive pyridazine fragment was incrementally diversified following chemoinformatics guidelines and the resultant compounds subjected to hierarchal biological screens. An emergent lead compound, MW01-5-188WH, is a selective suppressor of excessive glia proinflammatory cytokine production in an animal model of AD, with resultant attenuation of synaptic dysfunction and hippocampal-dependent behavioral deficits [2]. Analysis of marketed drugs has led to an emerging consensus that multi-property refinement is critical to improvement of the success rate in moving compounds through development. Solubility is a key molecular property that should be addressed early in the development process. Because the lead compound had the desired *in vivo* functions for a clinical candidate, we decided to test the hypothesis that a molecular property-driven medicinal chemistry refinement could improve aqueous solubility without increasing molecular weight, yet retain the highly attractive *in vivo* functions.
Objective: The goal was to develop a clinical candidate compound by molecular properties-focused medicinal chemistry refinement while retaining *in vivo* efficacy and safety in animal models.
Methods: Medicinal chemistry refinement of the lead compound was done using computed molecular properties and established synthetic chemistry schemes to probe how diversification at two critical points in the active scaffold could be varied with retention of cell-based activity. An emergent refined, aqueous-soluble, safe, orally bioavailable, brain-penetrant compound was tested for *in vivo* function in an AD model and shown to be efficacious. A synthetic production protocol amenable to GMP production was developed for this clinical candidate.
Results: Medicinal chemistry refinement [4] yielded Minozac, a water-soluble, efficacious, safe, novel drug candidate that retains the *in vivo* functions of MW01-5-188WH [2]. Minozac has a promising safety and bioavailability profile, and is efficacious in animal models of AD and TBI. A chemical production process scheme

[4] amenable to large scale GMP production of Minozac for clinical studies was developed. The clinical development campaign is in progress by NeuroMedix, Inc. (NMX).

Conclusions: Our results provide an integrative chemical biology causative link supporting the neuroinflammation hypothesis of disease progression, and indicate the feasibility of developing innovative, potentially disease-modifying therapeutics for neurodegenerative disorders by targeting key glia activation pathways.

(Supported in part by grants from the NIH (NS047586, AG013939, AG000260), Institute for the Study of Aging, and NeuroMedix, Inc.)

References

1 Craft JM et al. (2005) Neuroinflammation: a therapeutic target. Exp Opin Ther Targets 9: 887-900.

2 Ralay Ranaivo H et al. (2006) Glia as a therapeutic target: selective suppressionn of human Aβ-induced up-regulation of brain proinflammatory cytokine production attenuates neurodegeneration. J Neurosci 26: 662-670.

3 Wing LK et al. (2006) De novo and molecular target-independent discovery of orally bioavailable lead compounds for neurological disorders. Curr Alz Res 3: 205-214.

4 Hu W et al. (2006) Molecular property-driven refinement yields a novel drug candidate that inhibits brain cytokine production and attenuates synaptic dysfunction and behavioral deficits, submitted.

L17

SYNUCLEIN-LINKED NEUROIMMUNITY AND THE PATHOGENESIS OF PARKINSON´S DISEASE

Gendelman E. H.
Center for Neurovirology and Neurodegenerative Disorders and the Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska 68198

The pathology of Parkinson´s disease (PD) includes loss of dopaminergic neurons in the substantia nigra, nitrated alpha-synuclein (a-syn) enriched inclusions or Lewy bodies and neuroinflammation. We reasoned that PD-associated oxidative protein modifications create novel antigenic epitopes capable of both microglial and peripheral adaptive T cell responses exacerbating nigrostriatal degeneration. Nitrated a-syn was readily detected in cervical lymphoid tissue in 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) intoxicated mice. Using a combination of genomic (gene arrays) and proteomic (SELDI-TOF, liquid chromatography-tandem mass spectrometry, Ettan DIGE, and protein array) methods, a fingerprint of a-syn activated microglia was also generated. Nuclear factor-kB transcriptional activation and its related signaling cascades that affect cell metabolism and immune response signalled this a-syn microglial response. Microglial reactive oxygen species production was also induced, in a dose-dependent manner, by activation with aggregated nitrated a-syn. This was inhibited by voltage-gated potassium current blockade, and to a more limited degree, by chloride current blockade. Transfer of T cells from mice immunized with oxidized a-syn to MPTP-intoxicated animals led to prolonged neuroinflammation and significant increases in dopaminergic cell loss. These data support the notion that nitrotyrosine modifications in a-syn induce both an innate neuroinflammatory response as well as serving to break immunological tolerance to self. Both processes serve to exacerbate the pathobiology of PD.

ABSTRACTS

POSTER COMMUNICATIONS

(P1 – P23)

P1

RANTES REGULATES THE SPONTANEOUS AND THE EVOKED RELEASE OF GLUTAMATE FROM HUMAN CORTICAL SYNAPTOSOMES.

Longordo F.[1], Musante V.[1], Severi P.[3], Pittaluga A.[1,2] and Raiteri M.[1,2]
[1]Pharmacology and Toxicology Section, Department of Experimental Medicine, [2]Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy and [3]Division of Neurosurgery, Galliera Hospital, Genoa, Italy (G.C.)

Background Chemokine are believed to play a role in the neuropathogenesis of AIDS. Levels of chemokines, in particular stromal derived factor 1α (SDF1α) and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) are elevated in brain of HIV-1 infected patients where they are thought to mediate and control neuronal death. In particular, while SDF1α is thought to favour neurotoxicity, RANTES, by acting at CCR5 receptors, has been proposed to protect mixed cultures of human neurones and astrocytes from viral protein or NMDA-induced apoptosis. CCR5 receptors are widely express in Central Nervous System and their localization at the presynaptic level has been inferred by electrophysiological recordings. The aim of our study was to investigate the existence and the functional role of presynaptic CCR5 on human cortical glutamatergic nerve terminals.
Methods Human cortical specimens were obtained from informed and consenting HIV-1 negative patients undergoing neurosurgery to reach deeply seated tumours. Once isolated by subsequent centrifugations, synaptosomes were prelabelled with [^{3}H]D-aspartate ([^{3}H]D-ASP), stratified at the bottom of superfusion chambers and then superfused following an experimental technique developed in our laboratory. After 38 minutes of superfusion to equilibrate the system, synaptosomes were exposed to human RANTES (hRANTES). In a set of experiments hRANTES was applied contemporary to a mild depolarazing stimulus (12 mM KCl). Superfusate fractions were collected and the tritium content quantified by radioactive counting.
Results When human cortical synaptosomes were exposed in superfusion to 1 nM hRANTES a significant, long lasting evoked release of tritium occurred. hRANTES exerted its action in a concentration dependent manner. The effect of the protein was totally prevented by Pertussis toxin, to suggest the involvement of a Pertussis toxin-sensitive G protein linked receptor. The releasing effect depended on intraterminal Ca^{2+} ions released from IP3-sensitive stores, since it was more than halved by Xestospongin-C. Transient exposure of synaptosomes to 12mM K^+ causes the Ca^{2+}-dependent exocytotic–like release of [^{3}H]D-ASP: 1 nM hRANTES co-applied with the depolarizing stimulus almost halved the K^+-evoked release of [^{3}H]D-ASP. The inhibitory effect depended on G protein-dependent inhibition of adenilyl cyclase activity.
Conclusions Our data seem to suggest that hRANTES can mediate both potentiation and inhibition of the release of [^{3}H]D-ASP of human cortical synaptosomes. hRANTES-mediated potentiation of spontaneous glutamate release depends on activation of G-protein receptors coupled to phosphatidyl inositol specific phospholipase C, while hRANTES-induced inhibition of K^+-evoked release of

glutamate relies on activation of inhibitory G proteins negatively coupled to adenylil cyclase activity.

Supported by *grants from Istituto Superiore di Sanità (Programma nazionale di Ricerca sull'AIDS - Progetto "Patologia, Clinica e Terapia dell'AIDS") and from Italian Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica [Projects n. 2004052809_004]*

P2

DIFFERENTIAL MODULATION OF CB1 AND CB2 RECEPTORS ALONG THE NEUROIMMUNE AXIS

Spagnuolo P.[1], Oddi S.[2], Bari M.[3], D'Agostino A.[2], Finazzi-Agrò A.[3] and Maccarrone M.[2,4]

[1]Department of Pharmacobiology, University of Calabria, 87036 Rende, Italy; [2]Department of Biomedical Sciences, University of Teramo, 64100 Teramo, Italy; [3]Department of Experimental Medicine and Biochemical Sciences, University of Rome Tor Vergata, 00133 Rome, Italy; [4]IRCCS C. Mondino, Mondino-Tor Vergata Center for Experimental Neuropharmacology, 00133 Rome, Italy

Recently we have shown that treatment of rat C6 glioma cells with the raft disruptor methyl-beta-cyclodextrin (MCD) doubles the binding of anandamide (AEA) to type-1 cannabinoid receptors (CB1R), followed by CB1R-dependent signaling via adenylate cyclase (AC) and p42/p44 mitogen-activated protein kinase (MAPK) activity. Here, we investigated whether also type-2 cannabinoid receptors (CB2R), widely expressed in immune cells, are modulated by MCD. We show that treatment of human DAUDI leukemia cells with MCD does not affect AEA binding to CB2R, and that receptor activation triggers similar [^{35}S]GTPγS binding in MCD-treated and control cells, similar AC and MAPK activity, and similar MAPK-dependent protection against apoptosis. The other AEA-binding receptor TRPV1, the AEA synthetase NAPE-PLD and the AEA hydrolase FAAH were not affected by MCD, whereas the AEA membrane transporter AMT was inhibited (~55%) compared with controls. Furthermore, neither diacylglycerol lipase nor monoacylglycerol lipase, which respectively synthesize and degrade 2-arachidonoylglycerol, were affected by MCD in DAUDI or C6 cells, whereas the transport of 2-arachidonoylglycerol was reduced to ~50%. Instead, membrane cholesterol enrichment almost doubled the uptake of AEA and 2-arachidonoylglycerol in both cell types. Finally, transfection experiments with human U937 immune cells, and the use of primary cells expressing CB1R or CB2R, ruled out that the cellular environment could account *per se* for the different modulation of CB receptor subtypes by MCD. In conclusion, the present data demonstrate that lipid rafts control CB1R, but not CB2R, and endocannabinoid transport in immune and neuronal cells.

References

1 Bari M. et al. (2005) *J. Biol. Chem*. 280: 12212-12220.

P3

PRESYNAPTIC NMDA RECEPTORS LOCATED ON NORADRENERGIC TERMINALS ARE DIFFERENTLY AFFECTED BY GP120 AND SDF1α

Musante V.[1], Neri E.[1], Pittaluga A.[1,2] and Raiteri M.[1,2]
[1]*Pharmacology and Toxicology Section, Department of Experimental Medicine,* [2]*Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy*

Background The envelope viral protein gp120 has been considered one of the main effector of neuronal impaired functions and neuronal death observed during CNS HIV-1 infection. A great body of evidence support its ability to affect neuronal functions. In particular, data from our laboratory supported the role of gp120 as potent high selective glycine like agonist at presynaptic NMDA receptors (NMDARs), since the protein was shown to reverse and surmount the blockade of NMDARs caused by 7-chloro kynurenate, the selective glycine antagonist. One possible criticism to our thesis considers that gp120 may exert its positive effect because of the activation of chemokine receptors localized nearby the NMDARs, whose functions can potentiate the effect of the glutamate receptors. In an attempt to answer this question, we investigate the role of chemokine receptors in the positive effect of gp120 on NMDA-mediated functions. In particular, we focussed on the presence and the functional involvement of CXCR4 receptors, as the previous results were obtained by using gp120 strain IIIB.

Methods Rat hippocampal synaptosomes were isolated by homogenizing and then prelabelled with [^{3}H]NA (release of [^{3}H]NA). When indicated, Pertussis toxin (PTx) was entrapped into synaptosomes to prevent G protein-mediated events. Superfusion was carried out following an experimental technique developed in our laboratory several. After 38 minutes of superfusion to equilibrate the system, synaptosomes were exposed 100 µM NMDA plus 1 µM glycine in presence or in absence of 100pM gp120 or 1 nM SDF 1α, the natural ligand of CXCR4 receptors; antagonists or enzyme inhibitors were added 8 minutes before agonists. Superfusate fractions were collected and the tritium content quantified by radioactive counting.

Results SDF1α, inactive on its own, potentiated in a concentration dependent fashion the NMDA-evoked release of [^{3}H]NA; the effect of 1 nM SDF1α was comparable to that caused by 100pM gp120. SDF1α, but not gp120-mediated effect was reversed by entrapped PTx. Notably, the effect of 1µM glycine on the NMDA-evoked release of tritium was totally insensitive to PTx. Consistently with the activation of a G-protein linked event the potentiation by SDF1α, but not that by gp120 or glycine, was prevented by the phospholipase inhibitor U73122, while both effects were impeded by PKC inhibitors.

Preliminar conclusions CXCR4 receptors are possibly co-localized with NMDA receptors on noradrenergic nerve terminals. Their activation by the natural ligand SDF1α potentiates the NMDA-evoked release of [^{3}H]NA through a PTx-sensitive PLC-dependent mechanisms. When added up to 100 pM, gp120 potentiates the NMDA-evoked release in an almost CXCR4-indipendent fashion. At the

concentration applied, the effect of the protein scarcely depends on G-protein coupled PLC-mediated intracellular events. Experiments are in progress to further investigate the role of CXCR4 in mediating the NMDA-mediated functions.

Supported *by grants from Istituto Superiore di Sanità (Programma nazionale di Ricerca sull'AIDS - Progetto "Patologia, Clinica e Terapia dell'AIDS").*

P4

mGLUR1 RECEPTORS IN CENTRAL NERVOUS SYSTEM AS A SELECTIVE CELLULAR TARGETS OF THE HIV-1 PROTEIN TAT

Luccini E.[1], Neri E.[1], Severi P.[3], M. Raiteri M.[1,2] and Pittaluga A.[1,2]
[1]Pharmacology and Toxicology Section, Department of Experimental Medicine,[2] Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy and [3]Division of Neurosurgery, Galliera Hospital, Genoa, Italy

Background Acquired immunodeficiency syndrome (AIDS) is often accompanied by neuropsychiatric symptoms known as Human Immunodeficiency Virus-1 (HIV-1)-Associated Dementia (HAD), the origins of which are poorly understood. We hypothesized that impairments of central neurotransmission induced by viral component may cause subtle changes to neurotransmission in part responsive of the central symptoms observed. Accordingly to this thesis, the HIV-1 proteins gp120 and Tat were shown to affect neuronal functions through glutamate receptor activation, although their exact targets remain undetermined.
We here summarize some recent findings concerning the effects of the human immunodeficiency virus-1 (HIV-1) transactivator of transcription (Tat) on the release of neurotransmitters from isolated nerve endings (synaptosomes) prepared from samples of human cerebral cortex.
Methods Human cortical specimens were obtained from informed and consenting HIV-1 negative patients undergoing neurosurgery to reach deeply seated tumours. Once isolated by subsequent centrifugations, synaptosomes were prelabelled with [^{3}H]Ch (release of [^{3}H]Acetylcholine, [^{3}H]ACh) or [^{3}H]NA (release of [^{3}H]NA), then stratified at the bottom of superfusion chambers and superfused following an experimental technique developed in our laboratory. After 38 minutes of superfusion to equilibrate the system, synaptosomes were exposed Tat or agonist; antagonists or enzyme inhibitors were added 8 minutes before agonists. Superfusate fractions were collected and the tritium content quantified by radioactive counting.
Results Tat (1nM) elicits the release of [^{3}H]ACh from human neocortical nerve endings. Tat-mediated effect was found to be mediated by presynaptic Pertussi toxin (PTx)-sensitive metabotropic glutamate receptors belonging to I group (I group mGluRs), whose activation causes inflow of Ca^{2+} and subsequent mobilization of Ca^{2+} ions from IP_3-sensitive intraterminal stores. The pharmacological profile of the mGluR targeted by Tat revealed that it belongs to the mGlu1 subtype. Furthermore, the release of preloaded [^{3}H]noradrenaline ([^{3}H]NA) induced by activation of presynaptic NMDA receptors from human neocortical and rat hippocampal brain synaptosomes could be further potentiated by Tat. In noradrenergic terminals, Tat could not evoke directly release of [^{3}H]NA, but NMDA concentrations unable to release (human synaptosomes) or slightly releasing (rat synaptosomes) [^{3}H]NA became very effective in presence of Tat (1nM). Tat-binding at NMDA receptors (NMDARs) however was excluded. Tat-effect involves activation of presynaptic PTx-sensitive metabotropic receptors. Also in this case, the pharmacological profile of the metabotropic receptor unveiled the involvement of I group mGluRs, in particular of the subtype mGlu1.

Conclusions Tat can represent a potent pathological agonist at mGlu1 receptors able to i) release of ACh from human cholinergic terminals; ii) upregulate NMDARs mediating NA release from human and rat noradrenergic terminals. This finding may be relevant to the proposal of new therapeutic approaches to HAD.

Supported *by grants from Istituto Superiore di Sanità (Programma nazionale di Ricerca sull'AIDS - Progetto "Patologia, Clinica e Terapia dell'AIDS").*

P5

EARLY MODULATION OF IL-1β EXPRESSION UNDERLIES NEUROPROTECTION BY ESTRADIOL (E_2) IN RATS UNDERGONE TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (MCAo)

Chiappetta O.[1*], Siviglia E.[2*], Gliozzi M.[2*], Amantea D.[1], Corasaniti M. T.[2] and Bagetta G.[1]

[1]Department of Pharmacobiology, University of Calabria, Cosenza, Italy; [2]Department of Pharmacobiological Sciences, Faculty of Pharmacy, University "Magna Graecia" of Catanzaro, Catanzaro, Italy

Both physiological and pharmacological levels of E_2 have been shown to exert neuroprotection against brain damage caused by MCAo in male and female rats, though the underlying mechanisms remain to be discovered (Garcia-Segura et al., 2001, Prog Neurobiol 63, 29-60). Previous studies have demonstrated that E_2 modulates IL-1β levels in rat brain following NMDA-induced excitotoxic insult (Nordell et al., 2003, Aging 24, 733-743). Moreover, inhibition of cytokine and chemokine expression by estrogen has been described in an animal model of multiple sclerosis (Matejuk et al., 2001, J Neurosci Res 65, 529-42), strengthening the hypothesis that blockade of inflammatory factors may represent an important mechanism involved in estrogenic neuroprotection (Maggi et al., 2004, Annu Rev Physiol 66, 291-313). Here we report the original observation that neuroprotection exerted by E_2 in an animal model of transient focal brain ischemia is accompanied by reduced citochrome c translocation from mitochondria and involves early modulation of IL-1β production.

MCAo was induced in male Wistar rats (280-300 g) by intraluminal silicon-coated nylon filament (0.28 mm diameter); two hours after occlusion, the filament was withdrawn to allow reperfusion. Cerebral infarct volume was evaluated 22 hours following reperfusion by staining 2 mm-thick consecutive coronal brain slices with 2,3,5-triphenyltetrazolium chloride (2% in saline) and measuring the infract area (unstained) using a computer assisted image analysis (ImageJ 1.30v software). Rats received E_2 (0.2 mg/kg, i.p.) 1 h before MCAo or the estrogen receptor (ER) antagonist ICI182,780 (0.25 mg/kg, i.p.) 1 h before E_2. Cytosolic cytochrome c-like immunoreactivity was determined in the cortices (penumbra) and striata (core) 3 h after reperfusion by western blotting, using a mouse anti- cytochrome c monoclonal antibody (1:1000 dilution; PharMingen, CA, USA). Immunoreactive IL-1β levels were assayed in individual brain cortical tissue homogenates by an established, rat specific, sandwich ELISA as previously described (Corasaniti et al., 2001, J Neurochem 78:611-8).

E_2 pre-treatment resulted in a significant reduction of brain infarct volume and this was reverted by the ER antagonist ICI182,780 (vehicle=555 ± 41; E_2=314 ± 44*, E_2+ICI=437 ± 21 mm^3, *P<0.001 vs vehicle, ANOVA followed by Dunnett's, n = 5). Neuroprotection by E_2 was accompanied by reduced cytochrome c translocation both in the striatum and in the cortex as revealed by western blotting after 3 h reperfusion.

P5

Two hours MCAo followed by 2 h reperfusion resulted in a significant, 3-fold increase of IL-1β levels in the cortical tissue ipsilateral to the ischemic damage. Interestingly, a pre-treatment with a neuroprotective dose of E_2 reverted the cytokine increase and this appears to occur through ER activation (IL-1β cortical levels: sham, 4.06 $\pm$ 0.31; vehicle, 11.84 $\pm$ 2.02**; E_2, 8.53 $\pm$ 2.01, E_2+ICI, 10.28 $\pm$ 0.79* pg/mg protein; *P<0.05 and **P<0.01 vs sham, ANOVA followed by Dunnett's, n = 3-4).

Financial support from MIUR (COFIN2004 prot. N. 2004053099_004 to GB) is acknowledged

*O. Chiappetta, E. Siviglia and M. Gliozzi have contributed equally.

P6

MODULATION OF THE ENDOCANNABINOID SYSTEM IS IMPLICATED IN THE NEUROPROTECTION AFFORDED BY 17BETA-ESTRADIOL (E_2) AGAINST BRAIN DAMAGE CAUSED BY TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION (MCAo) IN RAT

Mazzei C.[1], Amantea D.[1], Spagnuolo P.[1,4], Bari M.[4], Bagetta G.[1], Corasaniti M. T.[2] and Maccarrone M.[3,4]

[1]Department of Pharmacobiology, University of Calabria, 87036 Rende, Italy; [2]Department of Pharmacobiological Sciences, Faculty of Pharmacy, University Magna Graecia, 88100 Catanzaro, Italy; [3]Department of Biomedical Sciences, University of Teramo, 64100 Teramo, Italy and [4]Mondino-Tor Vergata Neuropharmacology Center, Laboratory of Neurochemistry, 00133 Rome, Italy

Both physiological and pharmacological levels of 17β-estradiol (E_2) afford neuroprotection against brain damage caused by middle cerebral artery occlusion (MCAo) in rats, though the underlying mechanisms remain to be discovered (Maggi *et al.*, 2004, Annu Rev Physiol 66, 291-313). E_2 exerts its activity through the interaction with intracellular estrogen receptors (ERα and ERβ), resulting in the modulation of transcription of target genes. Female sex hormones have been shown to provide pivotal modulation of the endocannabinoid system (see Maccarrone 2005, Life Sci 77, 1559-68). Here we investigate whether modulation of the endocannabinoid system by estrogen may contribute to the mechanism underlying its neuroprotection against ischemia-induced brain damage.

MCAo was induced in male Wistar rats (280-300g) by intraluminal silicon-coated filament (0.28mm diameter); 2h after occlusion, the filament was withdrawn to allow reperfusion. Cerebral infarct volume was evaluated 22h after reperfusion by the triphenyltetrazolium chloride staining technique. Endogenous AEA, *N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) activities were determined in cortical (penumbra) and striatal (core) specimens as previously described (see Maccarrone et al. 2003, J Neurochem 85, 1018-25).

2h MCAo significantly elevated endogenous AEA (~3-fold over controls; $P < 0.01$) in the ischemic core, but not in the penumbra and this was accompanied by increased activity of the AEA synthesizing enzyme NAPE-PLD (~1.7-fold; $P < 0.01$), whereas the activity of the metabolic enzyme FAAH was reduced (~0.6-fold; $P < 0.01$). Elevation of AEA in the ischemic core may contribute to brain damage, since the CB1 receptor antagonist, SR141716 (3 mg/kg, i.p., 15min before MCAo), but not the agonist, R-(+)-WIN55,212-2 (1 mg/kg, i.p., 15min before MCAo), significantly reduced infarct size detected 22h after reperfusion (vehicle=509 ± 29, n=5 vs SR141716=341 ± 63 mm^3, n=4; $P<0.05$, t-test). Interestingly, a neuroprotective dose of E_2 (0.2 mg/kg, i.p., 1 h before MCAo) (vehicle=555 ± 41; E_2=314 ± 44*, E_2+ICI=437 ± 21 mm^3, n=5, *$P<0.01$ vs vehicle, ANOVA) minimized the effect of brain ischemia on endogenous AEA levels, NAPE-PLD and FAAH activity and this was reverted by the ER antagonist ICI 182,780 (0.25 mg/kg i.p., 1h before E_2).

P7

THE FIRST *ALS2* MISSENSE MUTATION ASSOCIATED TO JPLS REVEALS NEW ASPECTS OF ALSIN BIOLOGICAL FUNCTION

De Palma C.[1,5], Panzeri C.[2], Martinuzzi A.[2], Daga A.[3], De Polo G.[2], Bresolin N.[2,4], Miller C. C.[5], Tudor E. L.[5], Clementi E.[2,6] and Bassi M. T.[6]

[1]Dept. of Pharmacobiology, University of Calabria, 87036, Rende, Italy; [2]IRCCS E. Medea, 23842 Bosisio Parini, Italy; [3]Dulbecco Telethon Institute at the Dept. of Pharmacology, University of Padova, Italy; [4]Dept. of Neurological Sciences University of Milan, 20129 Milan, Italy; [5]Dept of Neuroscience and Neurology, The Institute of Psychiatry, Kings College, London SE58AF, UK; [8]Dept. of Preclinical Sciences University of Milan, 20157 Milan, Italy

Primary lateral sclerosis is a rare progressive paralytic disorder that results from dysfunction of the upper motor neurones (UMN). Although PLS is a sporadic disorder of adult middle age, it has also been described in children as Juvenile PLS or JPLS. The causative gene for JPLS was found to be ALS2, which is also responsible for a recessive form of amyotrophic lateral sclerosis (ALS), for infantile onset ascending hereditary spastic paralysis (IAHSP) and for a form of complicated spastic paraplegia (cHSP). ALS2 gene encodes a protein termed alsin, containing multiple guanine nucleotide exchange factor domains, specifically binding to small GTPase Rab5 and acting as a GEF for Rab5. In vitro studies performed with full length and truncating forms of alsin protein support its role in endosomal dynamics and trafficking of mitochondria. All ALS2 mutations so far reported generate alsin protein truncation. Here we describe the first homozygous missense mutation in ALS2, p.G540E. The mutation, that falls within the RCC1 domain, was identified in a 34 year old patient with typical signs of JPLS such as ascending generalized and severe spasticity involving the limbs and the bulbar district, dysphagia, limb hypothrophy, preserved cognition and sensation. The father and two proband's sisters were found to be heterozygous carriers of the mutation with no signs of the disease.

Studies in the neuronal cell line SK-N-BE indicated that the known subcellular localization of wild type alsin with the early endosome antigen 1, in enlarged endosomal structures, and transferrin receptor is completely lost by the mutant protein, thus indicating that this mutation leads to protein delocalisation. Mutant alsin induced both neuronal death itself, and significantly enhanced the apoptogenic effect of NMDA and staurosporine. This effect was associated with decreased Bcl-xL/Bax ratio. By contrast, wild-type alsin was neuroprotective and increased Bcl-xL/Bax ratio. Our results provide the first demonstration that a missense mutation in alsin is cytotoxic. In addition, the identification of Bcl-xL/Bax as target of protection by alsin and of cytotoxicity by the mutant form provides a new signalling event regulated by alsin protein that may be important to define its role in neuronal physiology and neurodegeneration. Finally, the phenotype-genotype correlation in our patient, in view of all other ALS2 mutant cases previously reported, suggests a functional interplay of long and short forms of alsin in relation to disease onset and progression.

P8

MECHANISMS UNDERLYING NEUROPROTECTION AFFORDED BY BERGAMOT ESSENTIAL OIL (BEO) AGAINST NMDA-INDUCED CELL DEATH IN VITRO

Maiuolo J.[1*], Maida S.[1*], Navarra M.[1], Bagetta G.[2] and Corasaniti M.T.[1]
[1]Department of Pharmacobiological Sciences, University "Magna Graecia" of Catanzaro, Catanzaro, Italy; [2]Department of Pharmacobiology, University of Calabria, Cosenza, Italy

The essential oil of bergamot (BEO), is obtained by cold pressing of the epicarp and, partly, of the mesocarp of the fresh fruit of bergamot, a citrus growing almost exclusively in a restricted area of the Calabrian coast, in Italy. BEO comprises a volatile fraction (93-96% of total) and a non volatile fraction (4-7% of total) containing waxes, polymethoxylated flavones, coumarins and psoralens i.e. bergapten (5-methoxypsoralen) and bergamottine (5-geranyloxypsoralen) (Dugo et al., 2000). The most abundant compounds found in the volatile fraction are the monoterpene hydrocarbons limonene, γ-terpinene, and β-pinene, the monoterpene alcohol, linalool, and the monoterpene ester, linalyl acetate (Verzera et al., 2003). Previous experiments have shown that BEO concentration-dependently reduces death of SH-SY5Y human neuroblastoma cells caused by exposure to the excitotoxin N-methyl-D-aspartic acid (NMDA) (Navarra et al., 2005). Protection from NMDA-induced cell death is also afforded by a fraction of the essential oil deprived of bergapten (BEO-BF) supporting the deduction that psoralens are not implicated. Quite importantly, a fraction of the oil deprived of bergapten and of monoterpene hydrocarbons (BEO-BF-MHF) failed to rescue SH-SY5Y neuroblastoma cultures from cell death induced by NMDA thus suggesting that monoterpene hydrocarbons found in the volatile fraction of BEO may be responsible for neuroprotection. Pretreatment of SH-SY5Y cultures with BEO prevents accumulation of intracellular ROS induced by NMDA, suggesting that the ability of abrogating NMDA-induced ROS accumulation may be implicated in the mechanisms of neuroprotection.
In the present study, we further investigated the mechanisms underlying neuroprotection afforded by BEO against NMDA-triggered cell death and this by dissecting the molecular pathways implicated in death caused by NMDA.
Under our experimental conditions, cell death triggered by NMDA is caspase-independent. In fact, fluorimetric caspase-3 activity assay revealed no activation of caspase-3, the main executioner caspase in neurones, following exposure (2 minutes-20 hours) of SH-SY5Y cells to the excitotoxin (1 mM; n= 3 experiments per exposure time). In contrast to caspase-3, activation of the Ca^{2+}-activated neutral protease calpain I was detectable early after exposure to NMDA. Activation of calpain was studied by western blot analysis of generation of α-spectrin cleavage fragments (150-145 kDa) characteristic of calpain-mediated proteolysis. The accumulation of the calpain-cleaved 145-150 kDa α-spectrin breakdown product was evident within 5-10 minute exposure to NMDA (n= 3) and it reached statistically significant levels at 5 minutes after NMDA addition. In contrast, no accumulation of the α-spectrin fragment derived

from caspase-mediated proteolysis (120-kDa) was detectable and this in accordance with the lack of caspase-3 activation evaluated by fluorimetric assay. Interestingly, calpain I has been reported to inhibit the processing of procaspase-3 and -9 into their active subunits (Lankiewicz et al., 2000) and to down-regulate caspase activity (Neumar et al., 2003). Despite reports of caspase-dependent excitotoxic neuronal death, lack of caspase-3 activation has been previously observed in primary rat hippocampal neurones treated with NMDA (Lankiewicz et al., 2000; Luo et al., 2003), suggesting that excitotoxicity does not require activation of caspases. Interestingly, a pretreatment with a neuroprotective concentration of BEO (0.01%; n= 3) abrogates accumulation of calpain-specific 145-150 kDa α-spectrin breakdown product caused by 5 minutes exposure to 1 mM NMDA.

Several experimental evidence suggest a role for decreased Akt activity in the mechanisms of neuronal cell demise following brain ischemia whereas transient upregulation of Akt activity is regarded as a neuroprotective response to ischemic insult (see Fukunaga & Kawano, 2003). Deactivation of Akt kinase has also been reported in vitro, following exposure of primary hippocampal neurones to NMDA (Luo et al., 2003). Akt is activated by phosphorylation of Ser473 and Thr308 by a signalling cascade involving phosphatidylinositol 3-kinase (PI3K) and 3-phosphoinositide-dependent kinase-1 (PDK-1). Activated Akt promotes cell survival by phosphorylating and, thus, inactivating proteins implicated in promoting cell death such as Bad, caspase-9 and glycogen synthase kinase-3β (GSK3β) (see Brazil & Hemmings, 2001).

Interestingly, exposure of SH-SY5Y cells to NMDA induces a fast and transient deactivation of Akt kinase. Phosphorylation status of Akt was monitored by western blot analysis of proteins obtained from cellular homogenates by using a polyclonal antibody specific for Akt phosphorylated at Ser473. Nitrocellulose membranes were subsequently immunoblotted with an antibody specific for total Akt. Reduced phosphorylation of Akt is evident ($P<0.01$ versus control) at 2 and 5 minutes after NMDA exposure (n= 4), then it is followed by a trend towards an increase of phosphorylation reaching a peak at 15-30 min and returning to control levels at 1-3 hours after treatment (n= 4 experiment per exposure time). At variance with modification of phospho-Akt (P-Akt) levels, NMDA does not affect the expression of total Akt. Under these experimental conditions, preincubation with a neuroprotective concentration of BEO (0.01%; n= 3) prevents deactivation of Akt induced by 2 and 5 minutes exposure to NMDA. In NMDA-stimulated cells, deactivation of Akt precedes activation of GSK-3β monitored by western blot analysis of phospho-GSK-3β levels at Ser9. In fact, reduction of Ser9 phosphorylation of GSK-3β is evident at 5 but not 2 minutes after NMDA exposure. It has been shown that reduced levels of GSK-3β phosphorylation at Ser9 correlate with its activation (see Grimes & Jope, 2001). In comparison to control cultures, the reduction of P-GSK-3β induced by 5 minute exposure to NMDA did not reached statistically significance; however, NMDA-triggered decrease of phosphorylation at Ser9 is accompanied by an elevation of GSK3β activity that underlies cell death because incubation of SH-SY5Y cells with GSK3β inhibitor IX (0.001-1 μM; n= 3 experiments per group) concentration-dependently reduced ($P<0.01$) cell death triggered by 1 mM NMDA. Interestingly,

preincubation with a neuroprotective concentration of BEO (0.01%; n= 3) enhanced phosphorylation of GSK-3β reduced by 5 minutes exposure by NMDA, thus suggesting that Akt activation and GSK-3β inactivation by BEO contribute to neuroprotection.

To investigate the intracellular pathways through which BEO reduces Akt deactivation, cells were preincubated with LY294002, an inhibitor of PI3K, for 30 min prior to addition of BEO (0.01%) and NMDA (1 mM). Pre-treatment with LY294002 (0.002-20 μM; n= 4 experiments per concentration) concentration-dependently reverted the neuroprotection by BEO, whereas NMDA-induced cell death was not affected by any concentration of the enzyme inhibitor. These findings suggest that PI3K is involved in neuroprotection afforded by BEO.

Acknowledgements. *Financial support from MIUR (PRIN 2004055288_1) is gratefully acknowledged.*

References

1 Brazil D.P. & Hemmings B.A. (2001). *Trends Biochem. Sci.,* **26,** 657-664.
2 Dugo P. et al. (2000). *J. Pharm. Biomed. Anal.,* **24,** 147-150.
3 Fukunaga K. & Kawano T. (2003). *J. Pharmacol. Sci.,* **92,** 317-327.
4 Grimes C.A. & Jope R.S. (2001). *Prog. Neurobiol.,* **65,** 391-426.
5 Lankiewicz S. et al. (2000). *J. Biol. Chem.,* **275,** 17064-17071.
6 Luo H.R. et al. (2003). *Proc. Natl. Acad. Sci. U.S.A.,* **100,** 11712-11717.
7 Navarra M. et al. (2005). XXXII National Congress of the Italian Pharmacological Society, Naples, Italy, June 1-4, 2005, p.221.
8 Neumar R.W. et al. (2003). *J. Biol. Chem.,* **278,** 14162-114167.
9 Verzera A. et al. (2003). *J. Agric. Food Chem.,* **51,** 206-210.

J. Maiuolo and S. Maida have contributed equally.

P9

ON THE MECHANISM OF ACETYL-L-CARNITINE (ALC) NEUROPROTECTION AGAINST *IN VITRO* ISCHEMIA

Bagetta V.[1], Picconi B.[1], Barone I.[1], Nicolai R.[3], Benatti P.[3], Bernardi G.[1,2], Calvani M.[3] and Calabresi P.[1,4]

[1]Laboratory of Neurophysiology, Santa Lucia Foundation IRCCS-CERC, Rome; [2]Clinica Neurologica, Department of Neuroscience, "Tor Vergata" University , Rome; [3]Sigma-tau Industrie Farmaceutiche Riunite S.p.A., Pomezia, Rome; [4]Clinica Neurologica, Dip. Specialità Medico Chirurgiche e Sanità Pubblica, University of Perugia, Perugia Italy

Acetyl-L-Carnitine (ALC) is widely distributed in the mammalian brain and it is involved in the regulation of carbohydrate, lipid, and protein metabolism and also in the maintenance of key mitochondrial proteins for maximum energy production.
Several studies have suggested that ALC may play a neuroprotective role in some pathological conditions.
In this study we have analyzed ALC-mediated neuroprotection on an *in vitro* model of brain ischemia, a pathological condition closely linked to inhibition of mitochondrial respiratory chain leading to a reduced ATP generation, dysregulation of ion metabolism and finally neuronal death.
For this purpose we used extracellular and intracellular recordings from rat corticostriatal brain-slice preparations.
In the extracellular recordings, ten minutes of *in vitro* ischemia caused an irreversible loss of the field potential amplitude whilst pretreatment with ALC produced a progressive and dose-dependent recovery of the field potential amplitude. Conversely, the application of ALC after the ischemic insult failed to induce the recovery of the field potentials. The neuroprotective effect of ALC was stereospecific since the pretreatment with two different carnitine-related compounds did not cause neuroprotection. The choline transporter inhibitor hemicholinium-3 blocked the neuroprotective effect of ALC. ALC-mediated neuroprotection was also prevented either by the non-selective muscarinic antagonist scopolamine, or by the putative M2-like receptor antagonist methoctramine. Conversely, the effect of ALC was not altered by the M1-like receptor antagonist pirenzepine.
In intracellular current-clamp recordings from spiny neurons the application of ALC 100µM didn't affect per se EPSP amplitude. Interestingly, ALC application ten minutes before delivering ischemia was able to prevent post-ischemic-LTP (i-LTP), an abnormal form of synaptic plasticity correlated to apoptotic/necrotic neuronal death. On the contrary, the application of the drug after the ischemic insult didn't show any effect on LTP maintenance.
These findings show that ALC might exert a neuroprotective action against *in vitro* ischemia only when applied before the insult by a mechanism depending on the increased level of Ach in the brain and by the activation of muscarinic receptors.

P10

NEUROPROTECTION BY BERGAMOT ESSENTIAL OIL (BEO) INVOLVES INHIBITION OF EXCITATORY AMINOACID RELEASE IN THE ISCHEMIC RAT BRAIN

Pelle C.[1], Amantea D.[1], Morrone L. A.[1], Rombolà L.[1], Corasaniti M.T.[2] and Bagetta G.[1]

[1]Department of Pharmacobiology, University of Calabria, Cosenza, Italy; [2]Department of Pharmacobiological Sciences, Faculty of Pharmacy, University "Magna Graecia" of Catanzaro, Catanzaro, Italy

Bergamot essential oil (BEO) is obtained from cold press of epicarp and, partly, of the mesocarp of the fresh fruit of Citrus bergamia Risso, a citrus that grows almost exclusively in Calabria (Italy). BEO comprises a volatile fraction (93-96% of total) containing terpenes and oxygenated derivatives and a non volatile fraction (4-7% of total) containing cumarines and furocumarines such as bergamottine, 5-geranoxy-7-metoxycumarine, cipropten and bergaptene (5-methoxy psoralen) (Di Giacomo and Mincione, 1993). Psoralens, e.g. 5-methoxy psoralen (bergaptene) and 8-methoxy-psoralen, have been shown to block voltage-gated K^+ channels in Ranvier node cells maintained in vitro (During et al., 2000), whereas bergamottine (5-geranoxy-psoralen), another important component of the non volatile fraction of BEO, seems to be endowed with Ca^{2+} antagonist properties in vitro (Occhiuto and Circosta, 1996, 1997). Moreover, BEO has recently been demonstrated to reduce NMDA-induced cell death in cultured human SH-SY5Y neuroblastoma cells (Navarra et al., 2005), although less is known about the neuroprotective effects of the phytocomplex in vivo.

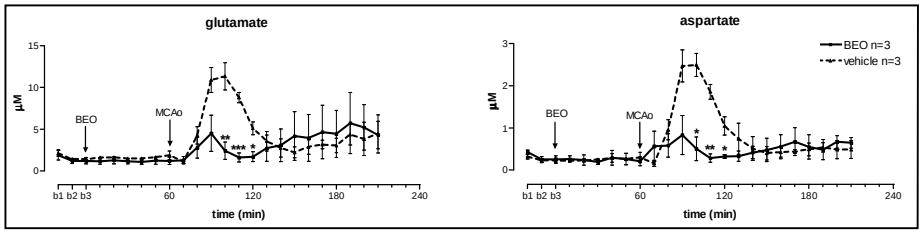
Here we demonstrate that the neuroprotective effect of BEO in a rat model of permanent focal brain ischemia involves modulation of excitatory aminoacid efflux in the ischemic cortex.

Male Wistar rats (250-300g) were anaesthetised with chloral hydrate and a custom made microdialisys probe (concentric design, 2 mm regenerated cellulose membrane) was implanted in the upper frontoparietal cortex. 24 h after surgery, animals were re-anesthetised with urethane and the microdialisys probe perfused with artificial CSF (mM: NaCl, 125; KCl, 2.5; $MgCl_2$, 1.18; $CaCl_2$, 1.26; NaH_2PO_4, 0.2; pH adjusted to 7.0) at a flow-rate of 2 ul/min. After 1 h stabilisation period, dialysate samples were collected every 10 min for 1 h to establish basal amino acid levels. At this point, bergamot essential oil or vehicle (vegetable oil) were administered i.p. and 10-min dialysate samples collected for a further 1 h. At the end of collection, MCAo was performed by insertion of a silicon-coated nylon filament (0.28 mm diameter) through the internal carotid artery and sample collection continued for further 3 h. Concentrations of glutamate, aspartate, glycine, GABA, glutamine, taurine and citrulline were determined by high performance liquid chromatography (HPLC) with fluorescence detection after derivatisation with o-phthalaldehyde/mercaptopropionic acid (Richards et al., 2000).

Administration of BEO, 1 h before MCAo, resulted in a dose-dependent reduction of brain infarct volume as detected after 24 h ischemia by the triphenyltetrazolium chloride (TTC) staining technique (infarct volume in mm^3: vehicle, 543 $\pm$ 18; BEO

0.05 ml/kg, 416 ± 49; BEO 0.1 ml/kg, 397 ± 25*; BEO 0.5 ml/kg, 344 ± 33***; *P<0.05 and ***P<0.001 vs vehicle, ANOVA + Dunnett's, n = 4-6). At the dose that exerted maximal neuroprotection (0.5 ml/kg), BEO did not affect basal aminoacid levels, whereas it significantly reduced excitatory aminoacid, namely aspartate and glutamate, efflux in the frontoparietal cortex typically observed following MCAo (see Figure, *P<0.05, **P<0.01, ***P<0.001 vs vehicle, Student's t-test). By contrast, extracellular levels of glycine, GABA, glutamine, taurine and citrulline were not affected by the phytocomplex.

Thus, inhibition of excitatory aminoacids increase might represent a pivotal mechanism involved in the neuroprotection exerted by BEO against MCAo-induced brain damage.



References

1 Di Giacomo and Mincione (1993). In "Olio Essenziale di Bergamotto, Citrus Bergamia Risso" Laruffa Editore, pp. 1-30
2 During et al. (2000) General Physiology and Biophysics 19, 345-364
3 Navarra et al. (2005) 32° Congresso Nazionale della Società Italiana di Farmacologia, Napoli 1-4 Giugno 2005, p.221
4 Occhiuto and Circosta (1996) Phytotherapy Research 10, 491-496
5 Occhiuto and Circosta (1997) Phytotherapy Research 11, 450 453
6 Richards et al. (2000) Neuropharmacology 39, 2433-2441

Financial support from the University of Calabria and from Calabria Region is gratefully acknowledged.

P11

PROTECTIVE ROLE OF ATP-SENSITIVE POTASSIUM CHANNELS BLOCKERS IN OGD DEPRIVED HIPPOCAMPAL SLICES

Nisticò R. [a,b], Piccirilli S. [b,c], Giampà C. [b], Fusco F. [b] and Mercuri N. B. [b]
[a]Department of Pharmacobiology and University Centre for Adaptive Disorders and Headache (UCHAD), Section of Neuropharmacology of Normal and Pathological Neuronal Plasticity, University of Calabria, Arcavacata di Rende, Italy [b]IRCCS S. Lucia Foundation, Rome Italy [c]IRCCS C. Mondino, Experimental Neurobiology Center Mondino-Tor Vergata, Rome, Italy

Introduction Several studies have shown that oxygen deprivation and energy failure evoke endogenous adaptive mechanisms that can delay neuronal injury and increase neuronal survival (1). One such mechanism represents the activation of K_{ATP} channels, that are thought to be endogenous modulators of the cellular excitability and to represent a link between the metabolic state and the excitability of the cell. These channels are activated when the intracellular level of ATP drops, as it occurs during hypoxia and ischemia. Activation of these channels leads to hyperpolarization of the neuronal membrane and therefore is assumed to be an endogenous defense mechanism against cerebral hypoxia/ischemia by prolonging the period before membrane depolarization occurs (2). Although it has been demonstrated that ATP-sensitive potassium channels openers (KCOs) decrease neuronal injury caused by ischemia (3), in our ischemic model we show that blocking the ATP-sensitive potassium channels has a neuroprotective effect. We assume this could possibly be due to the decreased efflux of K^+, that would therefore preserve the Na^+/K^+ ATPase activity under ischemic conditions.

Materials and methods Experiments were performed on transverse rat hippocampal slices using extracellular recording standard techniques. Slices were perfused with artificial CSF and drugs were applied by bath perfusion. In vitro OGD was obtained by perfusing the slice with aCSF without glucose and gassed with nitrogen (95% N_2– 5% CO_2). After the recording session, the slices were stained with Cresyl Fast Violet solution to evaluate the number of cells irreversibly damaged either by the handling procedures alone (control) or by the putative ischemic insult (untreated and treated).

Results Here we show that: (a) The application of 14 min OGD irreversibly abolishes synaptic neurotransmission whereas synaptic activity is fully reversible after 9 min OGD; (b) Barium chloride (100 μM), tolbutamide (1 mM) and glibenclamide (20 μM) counteract OGD induced fEPSP depression of synaptic transmission; (3) Glibenclamide (20 μM) protects against the cellular damage caused by 14 min OGD in the pyramidal cell layer of the CA1 hippocampal area.

Conclusions

These experiments demonstrate neuroprotective effects of potassium channel blockers, therefore the targeting ATP-sensitive potassium channels may represent a new therapeutical approach for the development of potential neuroprotective compounds.

References

1 Haddad GG and Jiang C (1992) Prog Neurobiol 40, 277–318
2 Miller R (1990) Trends Neurosci 13, 197–199.
3 Wind T (1997) Brain Res 751, 295–299

P12

AKT CONTRIBUTES TO NEUROPROTECTION AFFORDED BY BERGAMOT ESSENTIAL OIL (BEO) AGAINST FOCAL CEREBRAL ISCHEMIA IN RATS

Fratto V.[1], Amantea D.[2], Morrone L. A.[2], Bagetta G.[2] and Corasaniti M. T.[1]
[1]Department of Pharmacobiological Sciences, University "Magna Graecia" of Catanzaro, Catanzaro, Italy; [2]Department of Pharmacobiology, University of Calabria, Cosenza, Italy

Activation of phosphatidylinositol 3–kinase (PI3-K)/Akt pathway plays an important role in rescuing neurones of the ischemic penumbra from delayed cell death (see Fukunaga et al., 2003). The serine/threonine kinase Akt functions as a major downstream target of phosphatidylinositol 3-kinase and after phosphorylation it phosphorylates and inhibits several proteins which promote cell death including Bad, caspase-9 and glycogen synthase kinase-3 (GSK-3β) (Brazil & Hemmings, 2001). In animal models of experimental brain ischemia a dramatic decrease of phospho-Akt (P-Akt) occurs within 24 hours or more after ischemia (Zhao et al., 2005). Under these experimental conditions, a pretreatment with neurotrophic factors able to activate PI3-K/Akt pathway, confers neuroprotection by maintaining the phosphorylation of Akt and thus its activity (see Fukunaga & Kawano, 2003). Recently, interest in natural products regarded as potential source of neuroprotective agents has been renewed. In the frame of a research project aimed at investigating the neuropharmacological profile of the essential oil of bergamot (*Citrus bergamia*, Risso), a citrus growing almost exclusively in a restricted area of the Calabrian coast, in Italy, we addressed the question as to whether the oil is endowed with neuroprotective properties in vivo.

The essential oil of bergamot (BEO), is obtained by cold pressing of the epicarp and, partly, of the mesocarp of the fresh fruit of bergamot. BEO comprises a volatile fraction (93-96% of total) and a non volatile fraction (4-7% of total) containing waxes, polymethoxylated flavones, coumarins and psoralens i.e. bergapten (5-methoxypsoralen) and bergamottine (5-geranyloxypsoralen) (Dugo et al., 2000). The most abundant compounds found in the volatile fraction are the monoterpene hydrocarbons limonene, γ-terpinene, and β-pinene, the monoterpene alcohol, linalool, and the monoterpene ester, linalyl acetate (Verzera et al., 2003).

By using a rat model of focal cerebral ischemia, here we demonstrate that BEO prevents the injury-induced deactivation of Akt and the activation of GSK-3β, thus protecting against neuronal damage caused by 24 hours occlusion of middle cerebral artery (MCA).

MCA occlusion (MCAo) was induced in rats by using a relatively non-invasive technique with an intraluminal filament, according to Longa et al. (1989). Rats were sacrificed by decapitation 24h following MCA occlusion. Brain infarct volume was detected by the 2,3,5-triphenyltetrazolium chloride (TTC) staining techique. Expression of phosphorylated Akt and GSK-3β was studied by means of western blotting analysis of proteins obtained from rat brain cortical tissue homogenates by using polyclonal antibodies specific for Akt phosphorylated at Ser473 and GSK-3β

phosphorylated at Ser9. Nitrocellulose membranes were subsequently immunoblotted with antibodies specific for total Akt and total GSK-3β. Equal protein loading in each lane was confirmed by hybridization with a anti-actin or anti-tubulin antibody.

Intraperitoneal (i.p.) administration of BEO (0.05-0.5 ml/kg, n= 3 rats per group), 1 hour before MCAo, dose-dependently reduced brain infarct volume. Western blot experiments performed using brain cortex homogenates of rats sacrificed 24 hours after ischemia, indicate that P-Akt significantly ($P<0.05$) decreases in the ipsilateral, ischemic, cortex as compared to contralateral, nonischemic, side (n= 3 rats) whereas no changes in P-Akt were detected in sham-operated animals (n=3). Administration of BEO, 1 hour before MCAo, prevents the reduction of P-Akt (n= 3 rats), thus suggesting that the essential oil is able to preserve Akt activity. Akt inactivates GSK-3β by phosphorylating the enzyme at Ser9. Accordingly, under our experimental conditions, deactivation of Akt in the ischemic cortex was associated to a significant ($p<0.05$) reduction of P-GSK-3β levels. Of interest, BEO preserved phosphorylation of GSK-3β by significantly ($P<0.05$) enhancing levels of P-GSK-3β in the ipsilateral cortex of rats underwent 24 hours occlusion of MCA (n= 3). Changes in the phosphorylation levels of both Akt and GSK-3β induced by BEO were not associated to enhancement of Akt and GSK-3β protein expression. Collectively, these observations suggest that BEO prevents damage due to ischemic brain injury and that Akt activation and GSK-3β inactivation by BEO mediate neuroprotection.

References

1 Brazil D.P. & Hemmings B.A. (2001). *Trends Biochem. Sci.*, **26,** 657-664.
2 Dugo P. et al. (2000). *J. Pharm. Biomed. Anal.*, **24,** 147-150.
3 Fukunaga K. & Kawano T. (2003). *J. Pharmacol. Sci.*, **92,** 317-327.
4 Longa E.Z. et al. *Stroke*, **20,** 84-91.
5 Verzera A. et al. (2003). J. Agric. Food Chem. 51: 206-210.
6 Zhao H. et al. (2005). *J. Neurosci.*, **25,** 9794-9806.

P13

NEUROPROTECTIVE EFFECT OF NITROGLYCERIN IN AN ANIMAL MODEL OF ISCHEMIC STROKE: INTERFERENCE WITH BCL2 EXPRESSION

Greco R.[1], Amantea D.[2], Sandrini G.[1,3], Nappi G.[1,3,4], Bagetta G.[2], Corasaniti M. T.[5] and Tassorelli C.[1,3]

[1]IRCCS Neurological Institute "C. Mondino" Foundation, Pavia Italy; [2]Dept. of Pharmacobiology, University of Calabria, Rende (CS) Italy; [3]University Centre for the Study of Adaptive Disorder and Headache (UCADH) - Pavia Italy; [4]Chair of Neurology, University "La Sapienza", Rome Italy; [5]Faculty of Pharmacy, University Magna Graecia of Catanzaro Italy

Nitric oxide (NO) plays a critical role in the control of cerebral circulation and has been involved in cerebral ischemic damage with both deleterious and ameliorative effects. Transient focal ischemia by middle cerebral artery occlusion (MCAO) produces cell death in penumbra through the activation of complex signaling pathways. Bcl2, a proto-oncogene, promotes cell survival in a variety of tissues, including the brain, by preventing caspases activation. It has been suggested that nitroglycerin (NTG), a NO donor, reduces ischemia/reperfusion-induced brain damage via inhibition of activity of caspases and NMDA receptor. In the present study we have evaluated the protective effects of NTG against cerebral damage in transient (2 hours) MCAO, focusing our interest on the expression of Bcl2. Male Wistar rats were injected systemically with NTG (10 mg/Kg) or vehicle (PEG, 1 ml/Kg) 20 minutes before the induction of MCAO by intraluminal silicon-coated filament (0.37 mm diameter). Cerebral infarct volume was measured 24h after reperfusion by the triphenyltetrazolium chloride staining technique. Bcl2 expression was evaluated after 2 hours of MCAO (without reperfusion) and at 5 hours of reperfusion in cortical specimens (ischemic penumbra) ipsilateral and contralateral to damage by western blotting. The results show a significant reduction of the infarct volume in rats pre-injected with NTG compared to the vehicle group. After 2 hours of occlusion, no significant difference was seen in Bcl2 expression in the ipsilateral and contralateral cortex of either experimental groups (NTG and vehicle). However, 5 hours after reperfusion, Bcl2 expression was significantly increased in the cortex ipsilateral to damage in the rats pre-injected with vehicle. By contrast, in NTG-treated rats, Bcl2 expression did not show any significant changes in the cortex ipsilateral to the ischemic damage at the same time-point. These data confirm the neuroprotective effect of NTG in an ischemia/reperfusion model. More importantly, they suggest that the possible target of NTG-mediated neuroprotection lies upstream from Bcl2 activation.

Formattato: Tipo di carattere: Grassetto, Colore carattere: Automatico, Inglese (Regno Unito)

Formattato: Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Formattato: Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Formattato: Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Formattato: Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Formattato: Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Formattato ... [1]

Formattato ... [2]

Formattato ... [3]

Formattato ... [4]

Formattato ... [5]

Formattato ... [6]

Formattato ... [7]

Formattato ... [8]

Formattato ... [9]

Formattato ... [10]

Formattato ... [11]

Formattato ... [12]

Formattato ... [13]

Formattato ... [14]

Formattato ... [15]

Formattato ... [16]

Formattato ... [17]

Formattato ... [18]

Formattato ... [19]

Formattato ... [20]

Formattato ... [21]

P14

NEUROCHEMICAL AND NEUROPATHOLOGICAL EVIDENCE THAT COENZYME Q10 PREVENTS RETINAL DAMAGE CAUSED BY HIGH INTRAOCULAR PRESSURE (IOP)-INDUCED TRANSIENT ISCHEMIA IN RAT

Nucci C.[1], Tartaglione R.[2], Cerulli A.[1], Cavaliere F.[2], Rombolà L.[2], Bagetta G.[2], and Morrone L. A.[2]

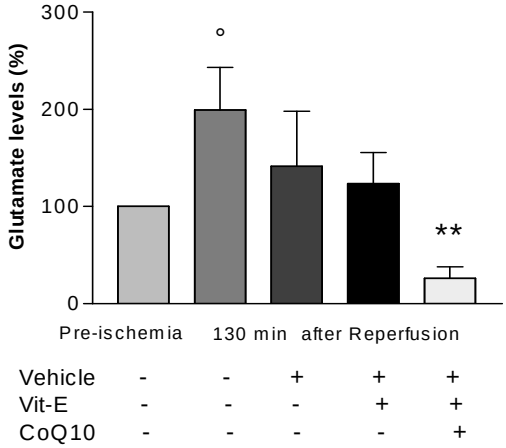
[1]Physiopathological Optics, Department of Biopathology, University of Rome "Tor Vergata" Rome Italy; [2]Department of Pharmacobiology, University of Calabria, 87036 Rende (Italy)

Recent studies support a role for excitotoxicity in the development of retinal ganglion cell (RGC) damage in subjects suffering from glaucoma (Manabe et al., 2005). Excitotoxic, glutamate-mediated, damage implicates oxidative stress and energy failure affecting the properties of the NMDA subtype of glutamate receptors and causing impairment of glutamate transporters (Sandhu et al., 2003). Accordingly, free radical scavengers may improve mitochondrial function and this may contribute to the pharmacological treatment of glaucoma. Coenzyme Q10 (CoQ10), an essential cofactor of the electron transport chain, has been reported to protect against ischemia/reperfusion damage of the heart and to afford neuroprotection in neurodegenerative disorders (Matthews et al., 1998). Neuroprotection by CoQ10 has been attributed in part to its free radical scavenger ability and to a specific regulation of the mitochondrial permeability transition pore (Papucci et al., 2003).

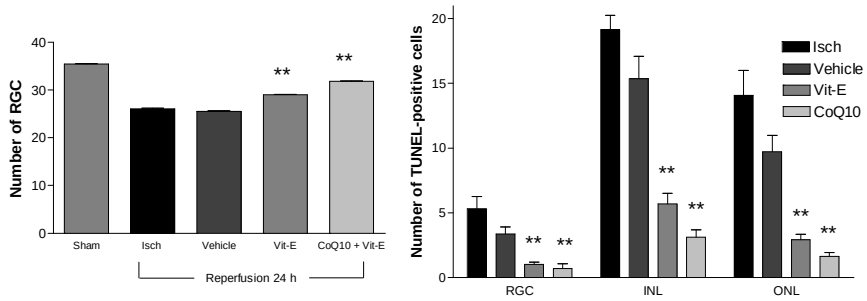
To gain more insight in the neuroprotective profile of CoQ10 this has been assessed here in an animal model of RGC death induced by acute rise of intraocular pressure (IOP, Osborne et al., 2004). To this end, anaesthetized (urethane 1500 mg/kg i.p.) Sprague-Dawley rats, bearing a retinal microdialysis probe to measure extracellular glutamate as detailed elsewhere (Richards et al., 2000), have been used. For test studies, animals received (30 min before ischemia) intravitreal administration (via the microdialysis probe; 2 µl/min rate, 5 min duration of treatment in all instance) of 1) CoQ10 (solution of CoQ10 0.1% + vitamin E 0.5%), 2) vitamin E (Vit-E, 0.5%) or 3) vehicle.

For neuropathological studies, animals have been treated topically with CoQ10 (solution of CoQ10 0.1% + Vit-E 0.5%), Vit-E (0.5% solution) or vehicle; in all instance, rats received eye application of 50µl of solution every 15 min during the 1 hour before and after ischemia had been induced. After 24 h of reperfusion, the animals were sacrificed and tissue sections, cut along the vertical meridian of the eye and passing through the optic nerve head, were stained with haematoxylin and eosin. The number of RGC was counted as previously reported (Nucci et al., 2005). Adjacent tissue sections were processed for in situ detection of DNA fragmentation using the TUNEL technique (Nucci et al., 2005).

The neurochemical (upper panel) and neuropathological (lower panels) results are reported in the figures shown below.



Neurochemical data obtained by intraocular microdialysis experiments in anaesthetized (see Materials and methods) rats demonstrate that ischemia/reperfusion insult increases intraretinal glutamate sensitive to the reversal of Vit-E and CoQ10. The observed increase in glutamate (199.3± 43.7% vs. pre-ischemia levels set to 100%; pre-ischemia glutamate levels = 0.307 ± 0.044 μM, n = 6) peaks at 130 min after beginning of reperfusion. Retinal administration, via the probe, of CoQ10 (n=3) (30 min before ischemia), significantly, prevents glutamate increase (data not shown) and prevents the peak increase seen after 130 min of reperfusion (26.06 ± 12.1% vs. 130 min reperfusion levels). Glutamate values are expressed as mean percentage changes ± S.E.M. Statistical significance was assessed by one-way ANOVA with post-hoc comparisons using Tukey test. °p< 0.05 vs pre-ischemia, **p < 0.001 vs. 130 min after reperfusion levels.



Elevated IOP-induced ischemia for 45 minutes followed by an interval of 24 h reperfusion induces loss of RGC and DNA fragmentation sensitive to the reversal of CoQ10 and Vit-E. Ischemia causes a reduction in the number of RGC (stained with ematoxylin and eosin) per counted area compared to controls (-25.7%, n=9). Topical treatment with Vit-E and CoQ10 significantly prevents RGC loss (-17.8 %, n=6 and -10.3%, n=7, respectively). DNA fragmentation is detected by TdT-dUDP terminal

nick end-labeling (TUNEL) technique in retinal ganglion cell layer (RGC, 5.3 ± 0.9, n=3), inner nuclear layer (INL, 19.1 ± 1.1, n=3); outer nuclear layer (ONL, 14.0 ± 1.9, n=3). No TUNEL positive cells are detected in retinal tissue sections obtained from sham-operated rats (data not shown). Vit-E (n=6) and CoQ10 (n=5) prevent DNA fragmentation in RGC (1.0 ± 0.1, and 0.7 ± 0.3, respectively), INL (5.6 ± 0.8, and 3.1 ± 0.5) and ONL (2.9 ± 0.4 and 1.6 ± 0.2). Data ere expressed as means ± SEM per area and are evaluated statistically for differences using ANOVA followed by Tukey-Kramer's test. **p < 0.001 vs. Isch.

In conclusion, the present data demonstrate that retinal administration of CoQ10 and Vit-E significantly reduces the increase of glutamate levels induced in the retina by high IOP and this may contribute to the neuroprotection afforded by topical application of CoQ10 and Vit-E in rats undergone ischemia/reperfusion. It is conceivable that the underlying mechanism of the observed neuroprotection might implicate control of the energy failure and oxidative stress. In fact, it is well established that increased free radicals production and consequent oxidative stress may damage glutamate transporters, decreases the capacity of astrocytes to metabolize glutamate, leading to increased synaptic glutamate resulting in excitotoxic neuronal cell death.

Financial support from the Ministry of Health (RF 2005) is gratefully acknowledged.

References

1 Manabe *et al*., (2005) Invest. Ophthalmol. Vis. Sci. 46; 4747-53.
2 Matthews et al. (1998) Proc Natl Acad Sci U S A. 95;8892-8897
3 Nucci *et al.,* (2005) NeuroToxicology, 26; 935-941
4 Osborne *et al.,* (2004) Prog. Ret. Eye Res. 23; 91-147.
5 Papucci *et al.,* (2003) J. Biol. Chem. 278; 28220-28228
6 Richards *et al.,* (2000) Neuropharmacology 39; 2433-2441.
7 Sandhu *et al*., (2003) J. Neurosci. Res. 72; 691–703

P15

A ROLE FOR BRAIN COX2 AND PROSTAGLANDINS IN MIGRAINE: INSIGHTS FROM AN ANIMAL MODEL

Tassorelli C.[1,2], Greco R.[1], Armentero M. T.[1], Cappelletti D.[1], Sandrini G.[1,2], Blandini F.[1] and Nappi G.[1-3]

[1]IRCCS Neurological Institute "C. Mondino" Foundation, Pavia, Italy; [2]University Centre for the Study of Adaptive Disorder and Headache (UCADH) Pavia, Italy; [3]Chair of Neurology, University "La Sapienza", Rome, Italy

Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid. The major mechanism of action of non-steroid anti-inflammatory drugs in migraine is the inhibition of COX. COX enzymes have been localized in the brain as well as peripherally. In addition, evidence suggests that the gene expression of the neuronal contingent of the inducible isoform of COX (COX-2) is mainly regulated by the transcription factor nuclear factor kappa-B, another possible player in the determinism of migraine attacks.

In this study we sought to evaluate the possible role of COX2 induction and PGs synthesis within neuronal areas proposed to be involved in migraine genesis in the animal model of migraine based on the administration of systemic nitroglycerin.

Male Sprague-Dawley rats were injected with nitroglycerin (10 mg/kg, i.p.) or vehicle and killed 2 and 4 hours later. The hypothalamus and the lower brainstem were dissected out and utilized for the evaluation of COX-2 expression by means of western blotting and for the determination of PGE2 levels by means of competitive enzyme immunoassay. COX-2 expression increased in the hypothalamus at 2 hours and in the lower brainstem at 4 hours, while no changes were observed in the mesencephalon. PG_{E2} levels showed an opposite pattern of change in the hypothalamus, where a decrease was detected at 2 hours, while an increase was observed in the lower brainstem at 4 hours.

These data support the hypothesis that nitroglycerin administration is capable of activating the COX-2 pathway within cerebral areas. This activity may explain the pro-nociceptive effect of nitroglycerin described in animal and human models of pain. Most importantly, these findings point to mediators and areas that may be relevant for migraine pathogenesis and treatment.

P16

THE HISTAMINE-INDUCED ALLODYNIA IN NEUROPATHIC PAIN MODEL MICE

Sakurada S.[1], Nakayama D.[1], Mizoguchi H.[1], Yonezawa A.[1] and Sakurada T[2].
[1]Department of Physiology and Anatomy, Tohoku Pharmaceutical University, Sendai, Japan; [2]Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan

Aim: Histamine is a widely distributed amine, which plays physiologically important roles in the central nervous system and peripheral tissues. We recently identified that histamine mediates the nociceptive transmission at the spinal cord. In fact, intrathecal administration of histamine elicits pain-related behaviors and thermal hyperalgesia in mice. In the present study, we characterized histamine-induced tactile allodynia in the mouse spinal cord.
Methods: Male ddY mice were used. All drugs were treated intrathecally to mice according to the method described by Hylden and Wilcox. To identify the tactile allodynia, the withdraw threshold of hind paw against von Frey filaments was measured. The sciatic nerve ligation was performed according to the method described by Seltzer and his colleagues.
Results: Intrathecal administration of histamine produced a remarkable tactile allodynia in mice. The histamine-induced tactile allodynia was dose-dependently attenuated by intrathecal co-administration with the H_1 receptor antagonists or the neurokinin 1 (NK_1) receptor antagonists, but not with the H_2 receptor antagonists or NMDA receptor antagonists. In the neuropathic pain model mice whose sciatic nerve was ligated, marked tactile allodynia was observed in the ipsilateral paw. Intrathecal administration of histamine significantly potentiated the tactile allodynia induced by sciatic nerve ligation. Unlike histamine-induced tactile allodynia in naive mice, the potentiation of tactile allodynia by histamine in neuropathic pain model mice was dose-dependently attenuated by the NMDA receptor antagonists, but not with the H_1 receptor antagonists, H_2 receptor antagonists or NK_1 receptor antagonists.
Conclusion: The present results suggest that intrathecal administration of histamine produces the tactile allodynia in mice through the activation of H_1 receptor and NK_1 receptor. In contrast, intrathecally administered histamine potentiates the tactile allodynia induced by sciatic nerve ligation through the activation of NMDA receptor. In the neuropathic pain model mice, histamine may contribute to pain transmission in the spinal cord with different mechanisms from naive mice.

P17

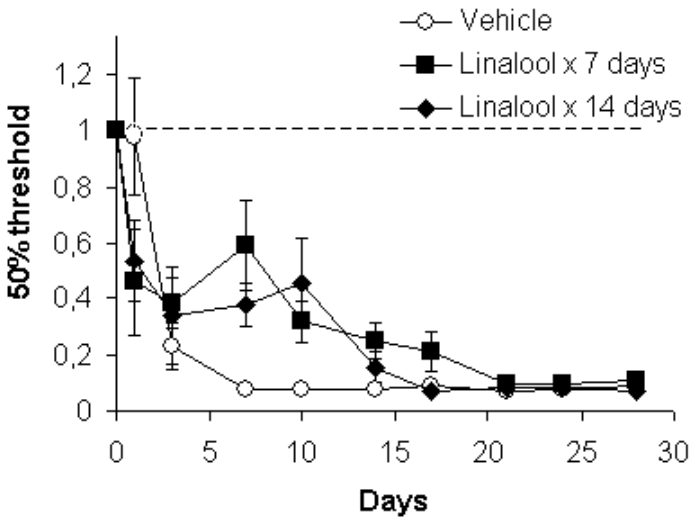
(-)-LINALOOL ATTENUATES ALLODYNIA IN THE SPINAL NERVE LIGATION MODEL OF NEUROPATHIC PAIN IN C57BL6 MICE

Levato A.[1], Rombolà L.[1], Morrone L. A.[1], Corasaniti M. T.[2], Bagetta G.[1] and Berliocchi L.[1,3]

[1]Department of Pharmacobiology and University Centre for Adaptive Disorders and Headache (UCHAD), Section of Neuropharmacology of Normal and Pathological Neuronal Plasticity, University of Calabria, Arcavacata di Rende, Cosenza 87036 (Italy); [2]Department of Pharmacobiological Science, University of Catanzaro "Magna Graecia" 88100 Catanzaro (Italy); [3]London Pain Consortium, University College London, Gower Street WC1E6BT, London, (UK)

(-)-Linalool is the natural occurring enantiomer of the monoterpene compound commonly found as a major volatile component of the essential oil extracted from several aromatic plants such as bergamot, jasmine and lavender. (-)-Linalool has been shown to possess both anti-inflammatory (Peana *et al*., 2002) and antinociceptive properties (see Peana et *al*., 2006) in a series of inflammatory pain models though the mechanism underlying the antinociceptive action remains largely to be elucidated. More importantly, no studies have been carried out to assess the effect of (-)-linalool in behavioural changes typically observed in neuropathic pain states. Accordingly, the aim of the present study was to evaluate the antinociceptive activity of (-)-linalool in the hypersensitivity states induced by nerve injury. To this end we have used the spinal nerve ligation (SNL) model of neuropathic pain (see Kim & Chung, 1992 for methodological details) to study the effects of acute and chronic administration of a dose of this monoterpene known to be active in several models of pain (see Peana et al., 2002, 2006). (-)-Linalool (Sigma-Aldrich, Milan, Italy) was dissolved in a mixture of polyethylene glycol 200 (Sigma-Aldrich, Milan, Italy) and saline solution (1:1) and administered (100 mg/Kg, s.c.) in a single daily injection, 1 hour before the spinal nerve ligation in C57BL6 male mice (20-22 g); control animals were administered an identical volume of vehicle (1ml/Kg, s.c.). Vehicle (n=6 for each group) and linalool (n=9 for each group) injected mice were divided in three groups: 1) one group was treated once, one hour before ligation (day 0); 2) a second group was treated from day 0 to day 7; 3) and a third group was treated for up to day 14. Animals were tested according to the up-down method (Chaplan et al. 1994) for mechanical sensitivity using von Frey filaments (calibrated from 0.04 to 12.75 g) and for thermal sensitivity using the Haregreaves test (Haregreaves et al. 1988). Behavioural tests were performed twice daily at day 3 and day 5 before surgery to evaluate the baseline pain threshold and at the following days 1, 3, 7, 10, 14, 17, 21, 24 and 28 after surgery.

In accordance with the experimental model, all vehicle treated animals fully developed mechanical allodynia at day 7 after SNL and maintained it for up to 28 days (Fig. 1), whereas linalool attenuated this effect. Attenuation of mechanical allodynia was observed starting from day 3 and 7 in the group of mice receiving one single daily injection of linalool for 7 days and at day 3, 7 and 10 in the group treated for up to 14 days (Fig. 1). No changes were observed in the group of mice receiving a single injection of linalool (data not shown).



In conclusion, our original data demonstrate that linalool possesses antiallodynic effects in a mice model of neuropathic pain. The mechanisms through which linalool exerts its antiallodynic effect in the SNL model of neuropathic pain is still not known. However, it has been reported that linalool may acts as a competitive antagonist at the N-Methyl-D-aspartate (NMDA) receptor (see Silva Brum *et al*., 2001) and this may conceivably contribute to its protective role in pain states. Further studies are currently in progress to elucidate these mechanisms.

Partial financial support from Calabria Region is gratefully acknowledged.

References

CHAPLAN, S.R., BACH, F.W., POGREL, J.W., CHUNG, J.M., & YAKSH, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods*, **53**, 55-63.

HAREGREAVES K., DUBNER R., BROWN F., FLORES C. & JORIS J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, **32**, 77-88.

KIM S.H. & CHUNG J.M. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*, **50**, 355-363.

PEANA, A.T., MARZOCCO, S., POPOLO, A., & PINTO, A. (2006). (-)-Linalool inhibits in vitro NO formation: Probable involvement in the antinociceptive activity of this monoterpene compound. *Life Sciences*, **78,** 719 – 723.

PEANA, A.T., D'AQUILA, P.S., PANIN, F., SERRA, G., PIPPIA, P., & MORETTI, D.L. (2002). Anti-inflammatory activity of linalool and linalyl acetate constituents of essential oils. *Phytomed.*, **9**, 721-726.

SILVA BRUM, L.F., ELISABETSKY, E., & SOUZA, D.O. (2001a). Effects of linalool on [3H] MK801 and [3H] muscimol binding in mouse cortical membranes. *Phytother. Res.*, **15**, 422-425.

P18

STUDIES ON THE ROLE OF ENDOTHELIAL NO SYNTHASE IN CELL MIGRATION

Cerullo A.[1], Bulotta S.1, Ierardi V.[1], Clementi E.[2], Rotiroti D.[1], and Borgese N.[1,3]
[1]Faculty of Pharmacy, University of Catanzaro "Magna Graecia" 88063 Roccelletta di Borgia (Catanzaro) Italy; [2]Department of Preclinical Sciences, University of Milan, 20157 Milan Italy; [3]CNR Institute of Neuroscience, 20129 Milan, Italy

1) Endothelial nitric oxide synthase (eNOS), which catalyzes the production of NO from L-arginne, is expressed in endothelial and many other cell types. Its activity is important for the regulation of fundamental physiological processes such as cellular movement, differentiation, proliferation and death. This enzyme is localized in the Golgi complex and at the inner surface of plasma membrane where it was previously thought to be concentrated in caveolae (Michel and Feron, 1997, Ghosh et al., 1998). Indeed, biochemical evidence indicate that eNOS physically interacts with Caveolin-1, which inhibits its enzyme activity (Ju et al., 1997) and studies with mouse models indicate that this interaction is physiologically important (Razani et al., 2001). The aim of the present study is to characterize the sites where eNOS is distributed, where it interacts with Caveolin-1 and to clarify the role of presence of eNOS at in particular subcellular locations.

2) We have used high resolution confocal microscopy to study the distribution of eNOS and Caveolin-1 in HeLa cells lines with inducible expression of the bovine enzyme (Bulotta et al., 2001) and in Human Umbilical Vein Endothelial Cells (HUVEC). In motile cells, eNOS was accumulated at membrane ruffles and at the leading edge, together with actin. In contrast, Caveolin-1 was not detectable in these structures, but was distributed in punctae in the rest of the membrane where it did not colocalize with eNOS (Bulotta et al., 2006). On the other hand, eNOS colocalized with the glycosphingolipid GM1 - a glycolipid enriched in rafts (sphingolipid- and cholesterol- rich clusters in the membrane), suggesting that eNOS is present in a subclass of rafts not containing caveolin. To investigate the role of actin in the localization of eNOS, we disrupted the actin cytoskeleton by serum starvation for 24h or by treatment with cytochalasin D. Both treatments determined a dramatic redistribution of eNOS to the cytosol (Bulotta et al., 2006), indicating a role of the cytoskeleton in determining eNOS subcellular distribution.

3) We investigated the effect of eNOS on cell migration by comparing the migration of eNOS-HeLaTetOff cells induced or not induced to express the enzyme. Contrary to expectation, we observed that the expression of eNOS reduced cell migration when the cells were exposed to gradient serum stimulus (Boyden assay), while no effect of eNOS was noted when the cells were polarized without a gradient stimulus (wound assay). These data suggest that eNOS expression may be blunting the response of the cells to chemotactic factors present in serum.

4) To study the sites of interaction of eNOS and caveolin-1 (not identified with normal immunofluorescence techniques so far), we are constructing fusion proteins between eNOS or caveolin-1 and two fluorescent proteins: Cerulean and Venus. These fluorescent proteins act as donor and acceptor in Fluorescence Resonance Energy

Transfer (FRET), and will allow us to detect sites of eNOS and caveolin-1 interaction. In preliminary FRET experiments in cells co-transfected with Caveolin 1-Venus and Caveolin 1-Cerulean, we have detected FRET, confirming the capacity of caveolin-1 to form homo-oligomers.

5) In conclusion, our studies report novel and unexpected observations on eNOS: its localization to the leading edge of migrating cells, the lack of major colocalization with caveolin 1, and its inhibitory effect on chemotaxis. These findings have important implications for eNOS biology and for its role in the regulation of cell migration and angiogenesis.

1 Ju H. et al.,1997 : *J Biol Chem* ***272***,18522-18525.
2 Michel T. and Feron O., 1997: *J Clin Invest* ***100***, 2146-2152.
3 Gosh et al.,1998: *J Biol Chem* ***273***, 22267-22271.
4 Bulotta et al., 2001: *J. Biol. Chem.,* **276**, 6529-6536.
5 Bulotta et al., 2006 :*Exp Cell Res*., 312, 877-889.
6 Razani et al., 2001, J. Biol. Chem. 276, 38121-38138.

P19

ACID SPHINGOMYELINASE: A NEW BIOLOGICAL TARGET OF THE NITRIC OXIDE/CYCLIC GMP PATHWAY

De Palma C.[1,3], Perrotta C.[2,3], Sciorati C.[2], and Clementi E.[2,3,4]
[1]Dept. Pharmacobiology University of Calabria, 87036, Rende, Italy, [2]Stem Cell Research Institute, H. San Raffaele Scientific Institute, 20132, Milan, Italy; [3]Dept. of Preclinical Sciences, University of Milan, 20157 Milan, Italy; [4]IRCCS E. Medea, 23842 Bosisio Parini, Italy

Acid Sphingomyelinase (A-SMase) hydrolyses sphingomyelin to ceramide and phosphocholine. A-SMase activation may be triggered by agonists of apoptogenic receptors, TNF-RI and CD95, to trigger apoptosis in several cells. Nitric oxide (NO) and its second messenger cyclic GMP (cGMP) are known antiapoptotic molecules. We decided to study whether regulation of A-SMase activity mediated their antiapoptotic action. We used the U937 monocytic and U373 glioma cell lines. We found that the time-course of inhibition of apoptosis by the NO donor DETA-NO (10 μM) correlated with that of A-SMase activation by TNF-α and CD95 in both U937 and U373 cells, with a peak of activity at 5 min. At 5 min, DETA-NO inhibited A-SMase activity by 95 ± 3.5 % and 85 ± 4.5 % in U937 and U373 cells, respectively (*n* = 3). This action by NO was cGMP-dependent since it was mimicked by 8-Br cGMP (1 mM) and inhibited by the guanylyl cyclase inhibitor ODQ (1 μM).
We also found that A-SMase activation was accompanied by translocation of the protein from its internal compartments to the plasma membrane and that NO/cGMP regulated this process. Moreover, we observed the presence of two vesicular pools of ASMase within the cells. A-SMase co-localised with the lysosomal marker cathepsin D, consistent with previous reports suggesting an endolysosomal localisation of the enzyme. Surprisingly, however, we found that this co-localisation was only partial, with some A-SMase staining occurring in cathepsin D-negative vescicular structures.
The identification of A-SMase as a new target of NO might open new vistas to the biological action of these molecules since both NO and A-SMase are involved not only in apoptosis regulation but also in cell growth and differentiation.

P20

ROLE OF HEAT SHOCK PROTEINS 90 AND 70 ON APOPTOTIC PROCESSES IN TELEOST BRAIN FOLLOWING EXPOSURE TO ENVIRONMENTAL STRESSORS

Facciolo R. M., Giusi G., Alò R., Carelli A. and Canonaco M.
Comparative Neuroanatomy Laboratory, Ecology Department, University of Calabria, Arcavacata di Rende 87036, Cosenza, Italy

The complex network of cerebral heat shock proteins (Hsps) is presently known to be involved in the regulation of neuronal signaling events linked to protective mechanisms that assure cellular recovery especially after contact with neurotoxicants such as environmental stressors. Of all contaminants that pose serious risks to behavioral and neuroendocrine functions, heavy metals and pesticides are considered major threats to many aquatic animals. In this context, it is the aim of the present work to evaluate the type of response supplied by the two main hypothalamic, mesencephalic and telencephalic molecular chaperones (Hsp90 and Hsp70) in the teleost *Thalassoma pavo* following exposure to the heavy metal cadmium (Cd) and the pesticide atrazine. For this purpose, *Thalassoma pavo* was treated with different concentrations of Cd (2.26-11.32 mg/L) and atrazine (100-400 μg/L) for one week. Subsequently, application of a specific histological method, such as Fluoro-Jade B, that is capable of displaying neurodegenerative events, showed neuronal damages throughout the various sites of the above brain areas. Almost the entire layer of the optic tectum (+96%) and dorsal telencephalon (+110%) were the principal areas that revealed a very great ($p<0.001$) number of damaged neurons when exposed to Cd. In the case of atrazine-dependent effects, it was the lateral diffuse nucleus of the hypothalamus (+82%) and preoptic nucleus (+75%) of the same brain region that supplied, in this case, a great ($p<0.01$) number of damaged neurons. Interestingly, *in situ* hybridization analyses, via the use of selective antisense Hsp90 and Hsp70 probes, instead demonstrated that both stressors were mainly responsible for up-regulatory effects in some mesencephalic areas such as stratum griseum centralis (Hsp90 = +66%; Hsp70 = +70%) following treatment with Cd, whereas atrazine accounted for an up-regulation of Hsp90 (+62%) and Hsp70 (+72%) in the ventromedial nucleus of the hypothalamus. A condition that was also detected for Cd-dependent up-regulation of Hsp70 in the entopeduncular nucleus of the ventral telencephalon (+74%) plus a moderate ($p<0.05$) up-regulatory influence of the pesticide on Hsp90 (+58%) in the torus longitudinalis of pretectal region. Overall these results show for the first time an anti-apoptotic role of Hsp70/90 in motor behavioral and neuroendocrine sites of *Thalassoma pavo*; an effect that is caused by the synergic interaction of the two molecular chaperones with consequent facilitation of normal neuronal propagating activities in stressful conditions, at least in teleost brain.

P21

DIFFERENTIAL ROLES OF P63 ISOFORMS IN EPIDERMAL DEVELOPMENT: SELECTIVE GENETIC COMPLEMENTATION IN P63 NULL MICE

Rufini A., Candi E., Terrinoni A., Ranalli M. and Melino G.
Biochemistry Laboratory, IDI-IRCCS, c/o University of Rome Tor Vergata, Rome, Italy

Epidermal development requires the transcription factor p63, as p63 Knockout mice (p63-/-) mice are born dead without skin (Yang et al., 1999). The gene expresses two proteins, one with an amino-terminal transactivation domain (TAp63) and one without (ΔNp63). We aim to define the relative contribution of this two isoforms to epidermal development. To address this issue, we developed transgenic mice expressing TA and ΔN p63 under the control of the skin specific promoter of Keratin (K) 5 and crossed them with p63-/- mice (Yang et al., 1999), to get genetic in-vivo complementation. We collected E18.5 embryos and analyzed their skin by immunohistochemistry, western blot and electromicroscopy (Candi et al., 2006). We found that reintroduction of both isoforms resulted in the highest level of rescue, compare with reintroduction of the single isoforms. Anyway, embryos with the ΔNp63 isoform also showed significant epidermal basal layer formation. Furthermore, we developed Saos2 Tet-on inducible cell lines (Gressner et al., 2005) to study the molecular mechanisms allowing p63 regulation of skin formations and we found by real time PCR that p63 regulated genes involved in skin development. In particular ΔNp63 upregulated expression of genes characteristic of the basal layer (K14), while TAp63 transcribed the upper layer's genes (K1, transglutaminase, involucrin) (Candi et al., 2006). So, in conclusion, by in-vivo and in-vitro studies, we showed that TA and ΔN p63 exert different, specific function in skin development.

YANG, A., SCHEITZER, R., SUN D., KAGHAD M., WALKER, N., BRONSON, R.T., TABIN, C., SHARP, A., CAPUT, D., CRUM, C. & McKEON, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature*, **398(6729),** 714-8.

GRESSNER, O., SCHILLING, T., LORENZ, K., SCHULZE SCHLEITHOFF, E., KOCH, A., SCHULZE-BERGKAMEN, H., MARIA LENA, A., CANDI E, TERRINONI, A., CATANI,V., OREN, M., MELINO, G., KRAMMER, PH., STREMMEL, W. & MULLER, M. (2005). TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J.*, **24(13)**:2458-71.

CANDI E, **RUFINI A**, TERRINONI A, DINSDALE D, RANALLI M, PARADISI A, DE LAURENZI V, SPAGNOLI LG, CATANI MV, RAMADAN S, KNIGHT RA, MELINO G. Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. (2006). *Cell Death Differ*., **13(6),** 1033-47.

P22

ALL-*TRANS*-RETINOIC ACID (RA) INDUCES APOPTOSIS IN LEYDIG (TM-3) CELLS

Tucci P., Cione E., Senatore V. and Genchi G.
Department of Pharmacobiology, University of Calabria, Arcavacata di Rende (CS), Italy

Retinoids are natural and synthetic substances structurally related to vitamin A. They exert antiproliferative and differentiation-inducing effects on cancer cells and are used in the prevention and therapy of certain types of human cancer and precancerous lesions. There is strong evidence that retinoids induce apoptosis in different types of normal and neoplastic cells. The aim of this study was to investigate the RA effect on the apoptosis in TM-3 cells and the role of mitochondria in RA-induced apoptosis. TM-3 cells were treated with RA at a concentration range of 0.1-20 μM for 24 hours. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) assay showed that RA exerts a cytotoxic effects on TM-3 cells in a dose- and time-dependent manner. Lactate dehydrogenase (LDH) activity in the culture media was measured as an index of plasma membrane damage and loss of membrane integrity. RA treatment for 24 hours resulted in a dose-dependent induction of LDH release. Since in vitro apoptotic cells cannot be cleared by phagocytes cells, death proceeds to a postapoptotic state in which damage of the plasma membrane becomes evident. The nature of RA-induced cell death has been examined in various ways in this study. RA induces apoptosis in TM-3 cells in a dose-dependent manner. The apoptosis is associated primarily with the mitochondrial pathway involving release of cytochrome c and caspase-3 activation, both revealed by immunoblotting analysis. During the apoptosis it is also known that endonucleases are activated and this activation leads to a cleavage of genomic DNA into well-defined fragments. DNA ladders were visible on agarose gel after staining with ethidium bromide, when the cells were treated with 10 and 20 μM RA for 24 hours. Moreover the ceramide synthase inhibitor, fumonisin B1, did not alter RA-induced apoptosis, suggesting that RA has not effect on ceramide synthesis. We further investigated the effect of cycloeximide, an inhibitor of protein sinthesys. The obtained results indicated that synthesis of anti-apoptotic proteins plays a very important role in the inhibition of apoptosis. In addition, we examined the effects of palmitoyl-CoA on RA-induced apoptosis. Interestingly, palmitoyl-CoA prevents RA-induced apoptosis of TM-3 cells. After 24 hours stimulation by RA, the release of LDH from TM-3 cells and of cytochrome c from mitochondria appeared to decrease in the presence of palmitoyl-CoA. An important concept in the initiation of apoptosis is the opening of a non-specific pore within the inner membrane of the mitochondria, the mitochondrial permeability transition pore (MPTP). Current evidence suggests that there are proteins that may bind to the adenine nucleotide translocase (ANT), modulating MPTP opening and hence cell death. Maybe palmitoyl-CoA prevents pore opening by interacting with the conformational change of ANT. In conclusion, understanding the induction of apoptosis by RA in vitro contribute to understand its mechanism of action and thereby improve its efficacy as therapeutic agent.

P23

LESIONS OF SOME AMYGDALAR SITES INDUCE NEURODEGENERATIVE EVENTS IN HIBERNATING RODENTS

Granata T., Madeo M., Carelli A., Facciolo R. M. and Canonaco M.
Comparative Neuroanatomy Laboratory, Ecology Department, University of Calabria, Arcavacata di Rende (CS), Italy

Lesions generated by the glutamatergic neuro-excitotoxic agonist, kainic acid (KA) represent a good model to study neurological diseases, especially for neurodegenerative paradigms and namely schizophrenia and epilepsy. The telencephalic limbic areas such as the hippocampus (HIP), cortex (COR) and above all the amygdala (AMY) are largely implicated in various motor and mnemonic functions; activities that require constant neuronal reorganization and synaptic plasticity in adults. However, it is not only during these activities that similar processes occur because in some serious disorders such as Alzheimer's disease there is a constant turnover of the different neuronal districts. For this phenomenon, application of KA proved to be a useful substance towards the establishment of cellular neurodegenerative events and eventual neuronal plasticity during the various physiological states such as hibernating cycle. In rodents this physiological state is essentially considered an adaptive neuroplastic mechanism for their survival throughout severe environmental conditions. With this aim, it was our intention to evaluate the role of some amygdalar nuclei during the different hibernating bouts of adult Syrian golden hamsters (90 days old). These animals were stereotaxically implanted with guide cannulae into basolateral (BLA: AP+3.4, ML+3.0, DV-5.1) and central amygdala (Ce: AP+ 3.4, ML+ 3.1, DV- 4.0) and then microinjected with either saline solution (Sham operated: SHAM) and KA solution. Morphological analysis highlighted a reduced neuronal density and a lack of well-defined nuclear borders of the different KA treated brain slices stained with thionin. In addition, Amino Cupric Silver Stain displayed a widespread neuronal degeneration of the other amygdalar areas, which is the case of BLA lesioned hamsters that supplied evident argyrophilic stained neuronal filaments. From a behavioural point of view, it was the BLA lesioned and not Ce lesioned hamsters that showed immediate nest building sessions although of a longer duration during the early stage of their hibernation cycle. Moreover, reduced eating and drinking behaviors were detected when BLA was functionally and structurally separated from the other amygdalar sites. Interestingly, the activation of KA-enriched sites in BLA tend to disynchronize behavioral parameters that seems to be tightly related to the modulation of neuronal death and hence represent a fundamental factor, at least of certain motor activities of our hibernating rodent model. These results seems to be in agreement with KA-dependent promotion of stereotyped behaviors after the inhibition of both NMDA and AMPA receptors, suggesting it to be a "rapid" type of neurosignaling mechanism for the so-called critical physiological activities.

INDEX OF AUTHORS

Pagina 43: [1] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [2] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [3] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [4] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [5] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [6] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [7] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [8] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [9] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [10] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [11] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Pagina 43: [12] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [13] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Pagina 43: [14] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Pagina 43: [15] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Pagina 43: [16] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico

Pagina 43: [17] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [18] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [19] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [20] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato

Pagina 43: [21] Formattato **Mondino** **20/07/2006 18.31.00**

Tipo di carattere: Times New Roman, 11 pt, Non Grassetto, Colore carattere: Automatico, Non Evidenziato