THEMATIC PROJECTS

FAPESP
THE STATE OF SÃO PAULO
RESEARCH FOUNDATION

OPPORTUNITIES FOR HEALTH RESEARCH IN BRAZIL

MORPHOLOGY

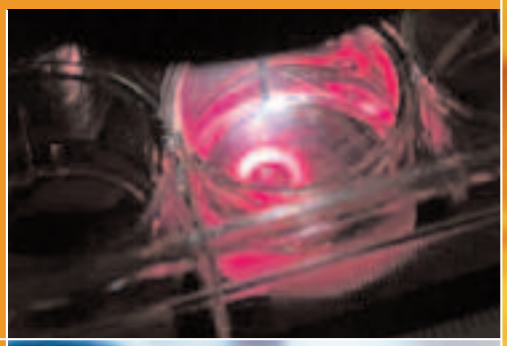
PHYSIOLOGY

PHARMACOLOGY

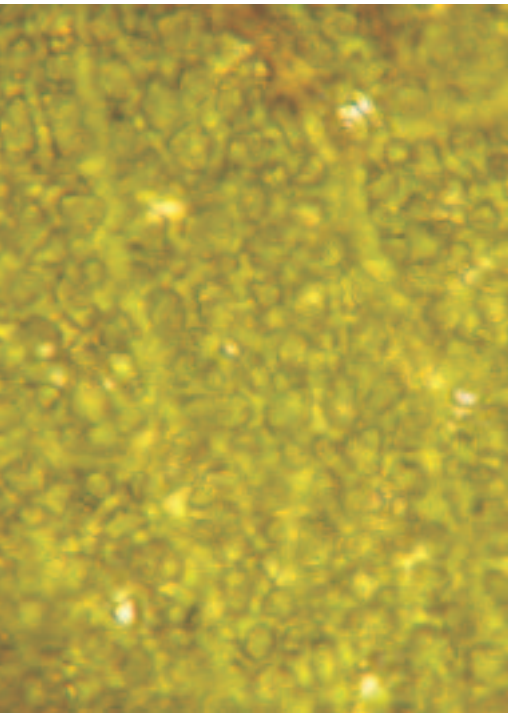
PHARMACY

PUBLIC HEALTH

DENTISTRY



EXPLORATORY RESEARCH RECOGNIZED WORLDWIDE



Cancer, genetics, immunology, biochemistry, tropical diseases, medicine. In these and many other sub-areas of Health science, Brazilian scientistas contributed results recognized worldwide.

FAPESP, the State of São Paulo Research Foundation, is one of the main Brazilian agencies for the promotion of research. The foundation supports the training of human resources and the consolidation and expansion of research in the state of São Paulo.

Thematic Projects are research projects that aim at world class results, usually gathering multidisciplinary teams around a major theme. Because of their exploratory nature, the projects can have a duration of up to four years.

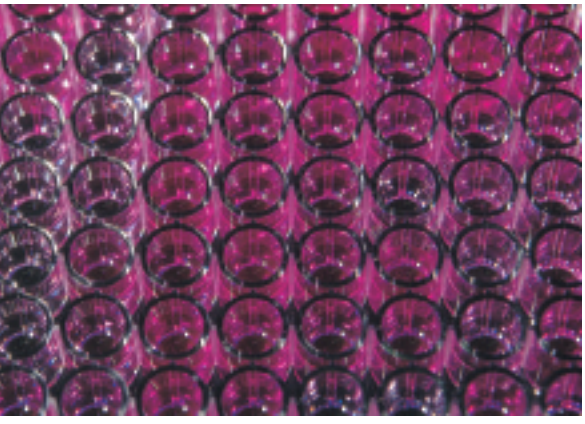
SCIENTIFIC OPPORTUNITIES IN SÃO PAULO, BRAZIL

Brazil is one of the four main emerging nations. More than ten thousand doctorate level scientists are formed yearly and the country ranks 15th in the number of scientific papers published.

The State of São Paulo, with 40 million people and 34% of Brazil's GNP responds for 53% of the science created in Brazil. The state hosts the University of São Paulo (USP) and the State University of Campinas (Unicamp) , both classified among the 200 best in the world by the Times Higher Education Suplement (THES), the growing University of The State of São Paulo (UNESP), Federal University of ABC (ABC is a metropolitan region in São Paulo), Federal University of São Carlos, the Aeronautics Technology Institute (ITA) and the National Space Research Institute (INPE).

Universities in the state of São Paulo have strong graduate programs: the University of São Paulo forms two thousand doctorates every year, the State University of Campinas forms eight hundred and the University of the State of São Paulo six hundred.

In addition to the three state universities the state has 19 research institutes, three federal universities of international research level and most of Brazilian industrial R&D. The state houses more than 10 thousand fulltime faculty and 130 thousand students. São Paulo alone, produces more scientific papers than any country in Latin America, except for Brazil.



FAPESP: SUPPORT FOR RESEARCH IN SÃO PAULO

The State of São Paulo Research Foundation (FAPESP) promotes scientific research in the State of São Paulo, Brazil. Through a robust program of fellowships and research grants it supports fundamental and applied research.

Created in 1962, the foundation is entitled by the State Constitution to 1 per cent of the tax revenues of the state of São Paulo. FAPESP has a sizable endowment and has already supported, over these 46 years, 89,000 fellowships and 80,000 research awards.

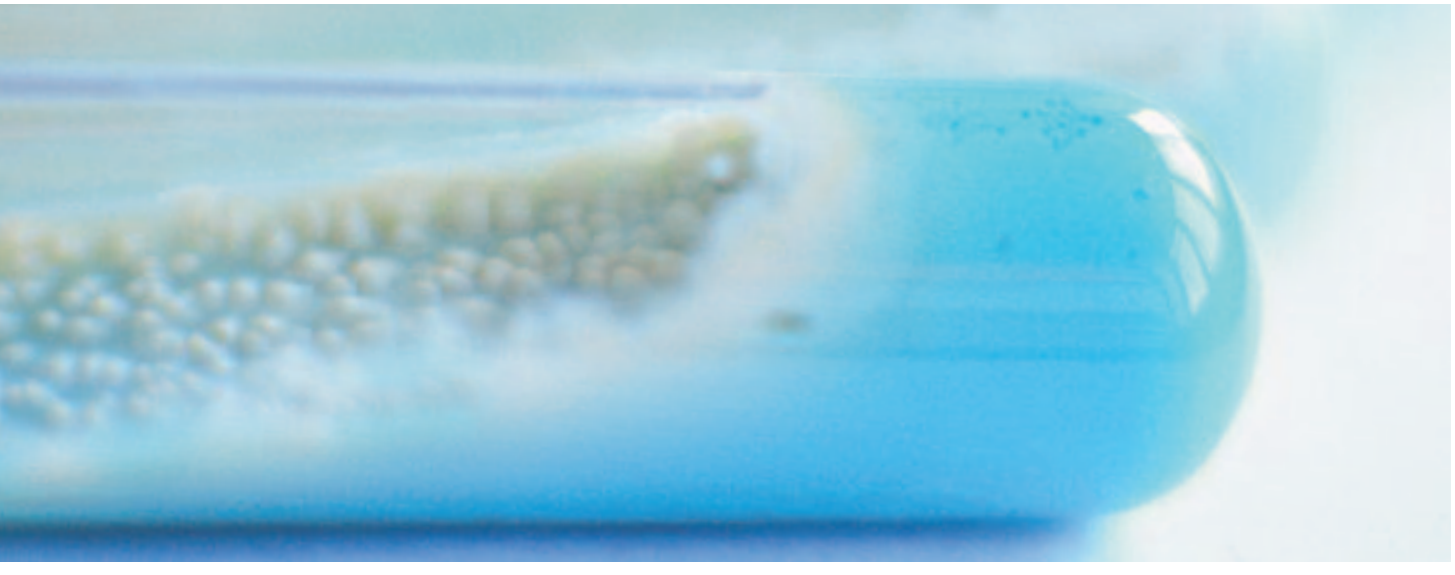
In 2008 FAPESP will invest US$ 388 million in fellowships and research grants. The success rate for proposals in the fellowship programs ranges from 40 per cent to 63 per cent. In the grants programs the proposal success rate ranges from 40 per cent to 60 per cent, depending on the particular type of grant.

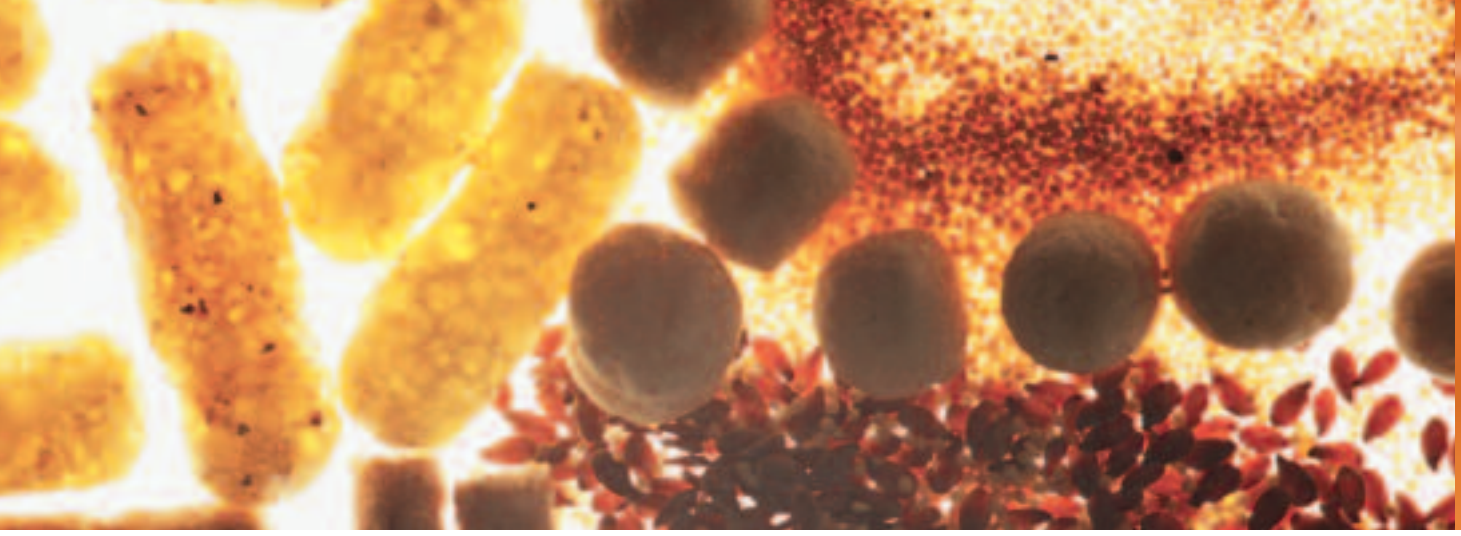
OPPORTUNITIES AND CHALLENGES

One of FAPESP's goals is the broadening and diversification of the research system in the state of São Paulo, strengthening the existing centers of excellence, by supporting their research, and stimulating the creation of new centers or research groups tackling new lines of activity. This is achieved mainly by funding Young Researchers Awards, the Biota-FAPESP Program, RIDC (Research, Innovation and Dissemination Centers) Program and the Thematic Projects.

All of these have in their teams, in addition to experienced scientists, young researchers as post-doctoral fellows, from Brazil and from abroad. FAPESP supports more than one thousand post-doctoral fellowships.

Contact FAPESP (www.oportunidades.fapesp.br) or a coordinator from the Thematic Project which interests you and see how to obtain a post-doctoral internship.





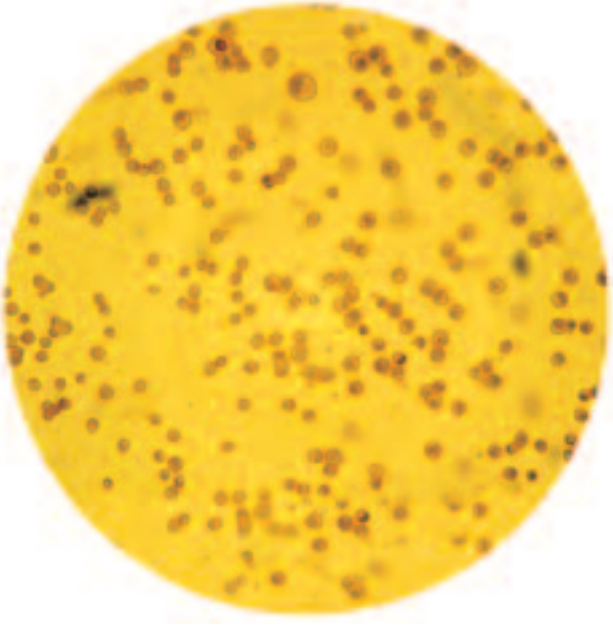
RESULTS OF GREAT IMPACT

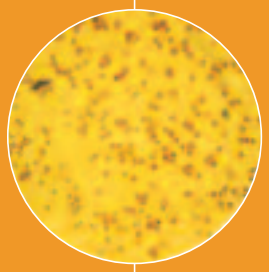
When the research program for Thematic Projects was created, in 1990, FAPESP's objective was to provide a qualitative leap in Brazilian scientific research and meet the state of São Paulo's own particular demands for development. Since then, 1,100 projects in all fields of knowledge have been selected and supported. Selection is through a stringent peer reviewing process, using multiple reviewrs for each proposal.

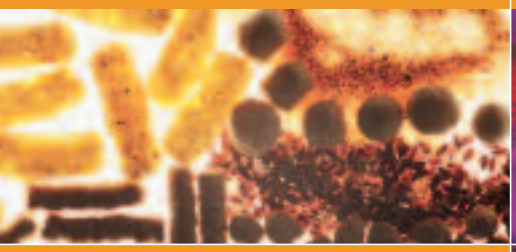
Thematic Projects are characterized by the breadth of their research and the boldness of their objectives. They are supported for four years (as opposed to two years for a regular research grant) and are lead by teams of experienced researchers.

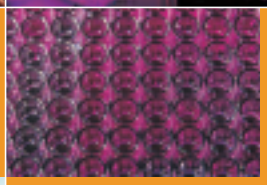
Thematic Projects are funded, on the average, with 450 thousand dollars, plus fellowships. The salaries for the investigators and staff are not included in this amount since in Brazil they are paid by their universities. Each project is lead by 3 PI's and involves several undergraduate and graduate students.

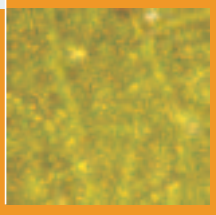
Thematic Projects create opportunities for scientists in São Paulo to advance knowledge by creating internationally competitive science, while, simultaneously, educating a new generation of researchers.











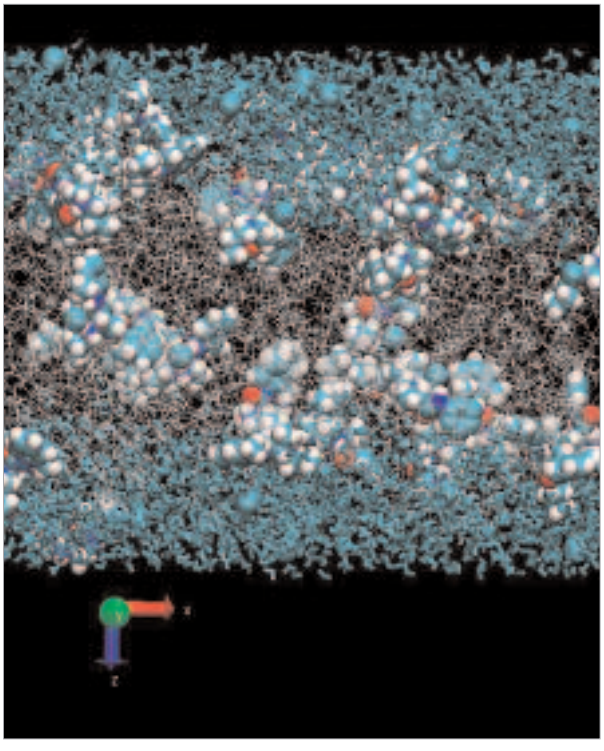
FAPESP
THE STATE OF SÃO PAULO
RESEARCH FOUNDATION

RUA PIO XI, 1500, ALTO DA LAPA
CEP: 05468-901 - SÃO PAULO - SP - BRASIL
FONE: 55.11.3838-4000

NEW FORMULATIONS FOR THE CONTROLED LOCAL ANESTHETICS IN DENTISTRY: FROM DEVELOPMENT TO CLINICAL TESTS

Eneida de PAULA

Institute of Biology / State University of Campinas (Unicamp)



Molecular modeling simulation showing the insertion of Prilocaine molecules (full representation) 1:3 anesthetic:lipid, mole%, in a phospholipid bilayer composed of 120 POPC molecules. NAMD program (Pickholz et al., 2008)

The structure and physicochemical features of local anesthetics (LA) are determinant for their potency and toxicity. Water solubility is, for instance, an essential parameter for the transport of the anesthetic molecule to the nerve fibers as well as to the ionization equilibrium that guarantees the existence of both ionized and non-ionized LA forms on the site of action. On the other hand lipid solubility is also crucial for drug partitioning into the axon, so that enough amounts of LA molecules will sit inside that membrane in order to keep the sodium channel in its non-conducting state. The development of new LA formulations is nowadays a challenge in dentistry. Liposomes are interesting drug-delivery systems for LA since they enhance the availability of compounds, reduce their systemic toxicity and increase their half-life *in vivo*. Similar advantages have been claimed for cyclodextrin formulations of poorly water-soluble anesthetics, for hydrogel formulations and, more recently, for LA in polymers. We intend in this project to develop new pharmaceutical forms for the controlled release of classic LA molecules, in vehicles such as liposomes, cyclodextrins, gels and polymers. Our aim is to enhance the pharmacological actions and to reduce the local and systemic toxicity of LA, looking forward to a future application in dentistry. The potentiality of this kind of project can be understood by the dimensions of the local anesthetics market in Brazil: ca. US$ 12.5 billion per year, according to the Brazilian Pharmaceutical Industry Association. Researches designed to develop new pharmaceutical forms have, necessarily, an interdisciplinary approach such as ours, due to the different stages of the research involved: technological development, physicochemical characterization, scale up and clinical steps. The development of these stages is the focal point of this thematic research.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

In this first year of the thematic research project we have obtained results with different approaches, reflecting the multi-disciplinary vocation of the project: molecular details on the interaction of local anesthetics and membranes; development (preparation, physicochemical characterization); *in vitro* toxicity tests, biologic activity tests in animals and clinical tests; all of which were carried out with new pharmaceutical forms substitute for classic local anesthetics.

a) On the mecanism of action of local anesthetics – By using Nuclear Magnetic Resonance (NMR) we have demonstrated the interaction of benzocaine and lidocaine with a peptide belonging to the inner cytoplasm linker between helices S4-S5, domain IV, of the voltage-gated sodium channel. Those LA interact with specific residues of the linker are known to be important for the stabilization of the protein in its inactivated (non-conducting) state. In a similar approach we have employed different spectroscopic techniques such as NMR, Electron Paramagnetic Resonance and Fluorescence to detect changes in the organization and dynamics of the lipid phase of model membranes after treatment with LA. The results allowed us to propose the existence of transient sites, specific for each LA molecule (lidocaine, etidocaine, mepivacaine, bupivacaine), that should determine their access to the sodium channel site.

b) Development and physico-chemical characterization of drug-delivery systems (liposomes, cyclodextrin, liposome gels) of local anesthetics – We have developed liposome local anesthetic formulations for mepivacaine, lidocaine and prilocaine. In this period we have also characterized the formation of supramolecular complexes between beta-cyclodextrins and LA such as bupivacaine, ropivacaine, lidocaine, tetracaine and benzocaine by employing diverse techniques. We have also developed gel formulations of local anesthetics (within or without liposomes) for topical use (pre-anesthesia) in dentistry, which resulted in the application of a patent report to the INPI/Brazil. Finally, we have also started studies for the future development of polymeric nanoparticles to carry local anesthetics.

c) Biologic activity tests – The anesthetic effect of the new formulations was evaluated through the infraorbital nerve blockage test in animals. The results for lidocaine, mepivacaine, prilocaine and ropivacaine revealed an increase in the time of anesthesia for all the liposome formulations. The *in vitro* toxicity of the formulations was tested in cell cultures. The *in vivo* local toxicity was evaluated through histological analysis in muscles and nerve tissues (Cereda et al, submitted).

d) Pre-clinical / clinical trials – With the approval of the Ethical Committee of Piracicaba School of Dentistry, University of Campinas, Brazil, up to now we have finished two clinical trials with healthy volunteers, showing an increase in time of anesthesia with liposomal formulations of ropivacaine and benzocaine (Silva et al, submitted), for topic use.

MAIN PUBLICATIONS

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Eneida de PAULA

Instituto de Biologia
Universidade Estadual de Campinas (Unicamp)
Departamento de Bioquímica
Laboratório Biomembranas
Caixa-Postal: 6109 – Barão Geraldo
CEP 13083-970 – Campinas, SP – Brasil

+55-19-3521-6143
depaula@unicamp.br

DEFECTS IN THE FORMATION OF THE DENTAL ORGAN

Sérgio Roberto Peres LINE

Piracicaba School of Dentistry / State University of Campinas (Unicamp)

Defects in tooth development are among the most common alterations in humans. Although enamel defects are not a threat to the patients' health, they may cause serious esthetical problems and interfere with masticatory and speech functions. Tooth agenesis and defects in enamel formation are among the most common alterations in human dentition. The importance of several genes in tooth development was evidenced by the lack of teeth in mutant knockout mice models and mutations in human families. Mutations in PAX9 coding sequences have been implicated in autosomal dominant oligodontia (the lack of more than 6 teeth) affecting predominantly permanent molars and second premolar. The origin of hydopontia (the lack of one to 6 teeth) is not well understood. In a previous study we have found that two polymorphisms in the promoter region of PAX9 gene are associated with hypodontia in humans (submitted for publication). These results led us to focus our analysis on the 5' region of PAX9 gene by studying: (1) the association of two other polymorphisms present in this region with hypodontia; (2) the influence of these (in spite of many studies being conducted, much remains to be unveiled) polymorphisms in the transcriptional activity of PAX9 gene by the use of reporter gene systems and gel shift analysis; (3) the comparison of the human promoter sequence with other primates from Africa (which have the same number of teeth as humans) and Brazil (which have three premolars in each hemi arc); (4) analyzis of the pattern of methylation in the CpG rich regions of the PAX9 promoter; (5) the influence of vitamin A, and dexamethasone on the activity of PAX9 promoter. Enamel defects are caused by genetic or environmental factors that interfere in the formation of this tissue. These factors can interfere with the metabolism of ameloblasts or interfere directly with the formation of enamel matrix. Mutations in the amelogenin, enamelin, amelobasltin and MM-20 genes were shown to cause severe enamel malformations known as amelogenesis imperfecta in humans and mice. However, the major causes of enamel defects in human population are environmental factors. Enamel defects can be caused by fluoride, lead, biphosphonates, virus infections and high fever. In spite of many studies being conducted, much remains about the role of the organic matrix in the mineralization of tooth enamel as well as how genetic and environmental factors will influence the formation of this structure. The aims of the present project are: (1) to study the birrefringency of the enamel matrix in the diverse phases of amelogenesis; (2) to study the effect of fluoride in the supramolecular organization of enamel matrix; (3) in collaboration with Dr. John D. Bartlett from Harvard Medical School and Dr. Ashok Kulkarni NIH-USA we intend to study the birrefringency of the enamel matrix in MMP 20 and amelogenin knockout mice (homozygous and heterozygous mice; (4) to study the effect of protease inhibitors *in vitro* on the birrefringency of enamel matrix.

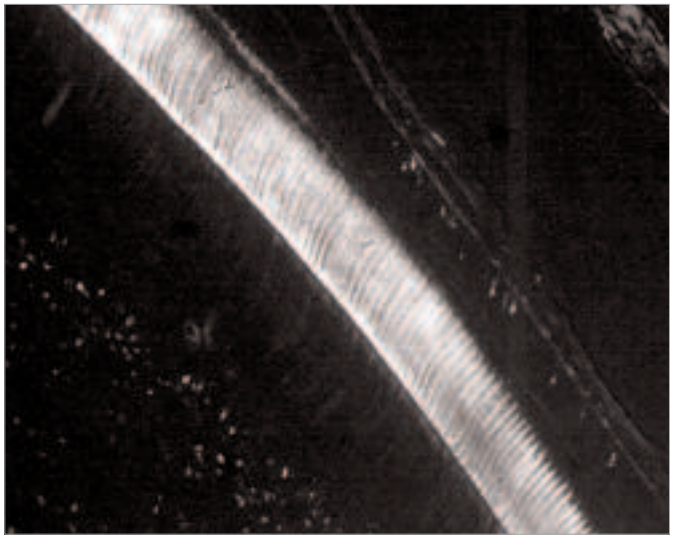

Figure showing birefringence of the organic matrix of mouse dental enamel

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

To date we have demonstrated that the organic matrix of dental enamel is highly organized and the level of organization can be studied by polarized light microscopy. Through this method, we showed that the supermolecular organization of the matrix can be altered by genetic mutations and environmental alterations.

We also found a region in the genes related with dental agenesis which appears to be related to the number of teeth present in mammal species (results not yet published).

MAIN PUBLICATIONS

Espírito Santo AR, Bartlett JD, Gibson CW, Li Y, Kulkarni AB, Line SR. 2007. Amelogenin- and enamelysin (Mmp-20)-deficient mice display altered birefringence in the secretory-stage enamel organic extracellular matrix. *Connect Tissue Res*. **48(1)**:39-45.

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do Espírito Santo AR, Novaes PD, Line SR. 2006. Anisotropic properties of the enamel organic extracellular matrix. *Eur J Oral Sci*. **114 Suppl 1**: 333-7; discussion 349-50, 382.

Sérgio Roberto Peres LINE

Faculdade de Odontologia de Piracicaba
Universidade Estadual de Campinas (Unicamp)
Caixa Postal 52, Areião
13414-903 – Piracicaba, SP – Brasil

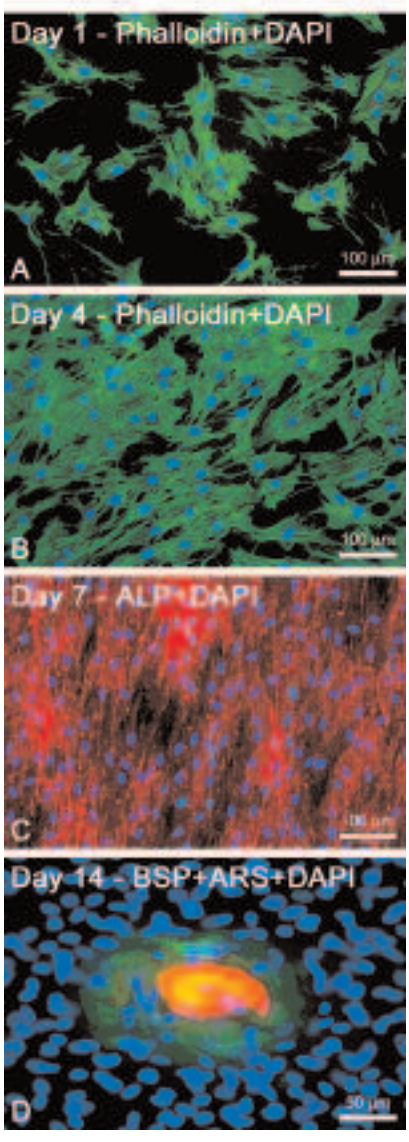
+55-19-2106-5333
serglin@fop.unicamp.br

OSTOGENESIS ON TITANIUM: *IN VITRO* EVALUATION OF THE EFFECTS OF DIFFERENTS STIMULATORY METHODS

Adalberto ROSA

Ribeirão Preto School of Dentistry / University of São Paulo (USP)

Human Osteoblastic Cells on Titanium Surfaces



Epifluorescence of human alveolar bone-derived osteoblastic cells grown on Ti surface. Phalloidin labelling indicates actin cytoskeleton (A,B) whereas blue flourescence reveals cell nuclei stained with DAPI (A-D). The development of the osteogenic phenotype is demonstrated by a strong alkaline phosphatase labeling (ALP, red fluorescence) at day 7 (C) and the formation of Alizarin red S (ARS, red fluorescence) stained-/bone sialoprotein (BSP, green fluorescence) labelled- bone-like nodules at day 14 (D)

In oral implantology, one of the biggest challenges has been to increase and/or accelerate osteogenesis on titanium (Ti) surfaces. Regarding the bone-implant interface and possible ways of stimulating osseointegration of Ti, it is possible to act on both sides. In this way, chemical and morphological modifications of implant surfaces and stimulation of bone formation seem to be the most suitable strategies. Therefore, the aim of this study has been to evaluate methods that could contribute to stimulate osteogenesis on Ti surfaces by using osteoblastic cell culture models, and to gain additional information about bone biology. In this project the effect of chemical modifications of Ti surfaces by Ca/P and collagen deposition on *in vitro* osteogenesis were evaluated. Moreover, the role of alkaline phosphatase and biomimetic systems on matrix mineralization, the expression pattern of some noncollagenous proteins, and the effect of a mixture of growth factors, growth hormone, and laser therapy on osteogenesis have been investigated.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This project allows us to study the behavior of bone-derived cells cultured on Ti during the period necessary for the osteoblastic phenotype to develop. The results showed that the chemical modifications of Ti surfaces stimulate *in vitro* osteogenesis. Human osteoblastic cells are sensitive to the Ca/P modified Ti surface during the transitional stage between the end of the proliferative phase and the onset of the differentiation/matrix maturation phase. Proliferation and differentiation of these cells are enhanced by collagen deposition. It means that both surface modifications could represent useful approaches for producing new Ti implants.

The effect of growth hormone on osteogenesis and on gene expression of osteoblastic markers is donor-age-dependent, being more pronounced on alveolar bone-derived cells from adolescents. Interestingly, its effect on cells derived from bone marrow seems to be somewhat different. It precludes the therapeutic use of growth hormone in combination with Ti implant placement but at the same time opens many interesting opportunities to investigate its role in bone biology. The mixture of growth factors plus proteins similar to the platelet rich plasma affected the development of the osteogenic phenotype both in human and rat cultures, leading to an increase in the number of cells. Despite such increase, the latter express a less differentiated state. The stimulation of osteoblastic cells using laser therapy was evaluated under several different conditions, i.e. laser dose-response, different time of laser exposition and in serum privation condition. None of them seemed to affect cells in any way. The expression of heat shock protein HSP70 is increased after submitting osteoblastic cells to stressful condition represented by thermal treatment. However, it remains to be determined whether Ti could represent a stressful environment capable of increasing HSP70 expression. A method to obtain purified alkaline phosphatase from human bone marrow cells differentiated into osteoblasts was developed. That enzyme can be incorporated into vesicular systems that mimic the matrix vesicles enrolled in the calcification of extracellular matrix. Such approach could be used to increase and/or accelerate the osseointegration of Ti implants.

MAIN PUBLICATIONS

Assis AF, Beloti MM, Crippa GE, Oliveira PT, Rosa AL. Development of osteoblastic phenotype in human alveolar bone-derived cells grown on collagen type I-coated titanium surface. *Clinical Oral Implants Research*, in press.

Crippa GE, Beloti MM, Cardoso CR, Silva JS, Rosa AL. Effect of growth hormone on *in vitro* osteogenesis and gene expression of human osteoblastic cells is donor-age-dependent. *Journal of Cellular Biochemistry*, in press.

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Adalberto Luiz ROSA

Faculdade de Odontologia de Ribeirão Preto
Universidade de São Paulo (USP)
Departamento de Cirurgia
Avenida do Café, s/n – Campus da USP
14040-904 – Ribeirão Preto, SP – Brasil

+55-16-3602-3980
adalrosa@forp.usp.br

CELLULAR AND MOLECULAR ASPECTS OF MUSCLE PLASTICITY

Anselmo Sigari MORISCOT

Institute of Biomedical Sciences / University of São Paulo (USP)

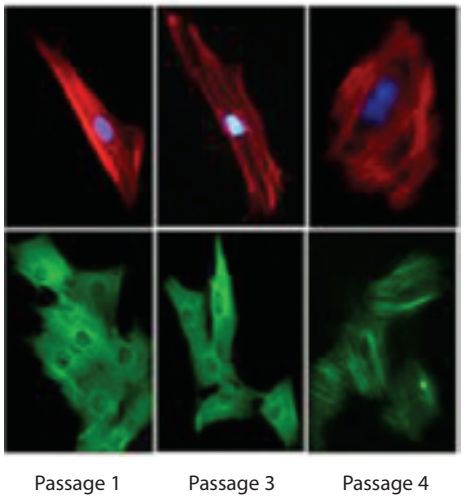


Figure 1. Representative immunostaining positive for SM α-actin (top) and calponin (bottom), magnification x200. Intense immunostaining for both markers was seen in passages 1-3. Note the elongation and assembly of actin filaments, and the spindle-shaped VSMCs from passage 1 through 3. Marker intensity decreased progressively from passage 4 on while VSMC morphology altered from spindle-shaped to polygonal, also seen at passage 5. At passage 10 VSMCs showed no marking for SM α-actin, which was seen only at the cell periphery, or for calponin, seen only at isolated points throughout the cytoplasm. Both markers decrease significantly in VSMCs submitted to freezing.

Muscles are tension generators that play a key role in 1) skeleton position, 2) moving blood along the circulatory system allowing tissue perfusion, 3) venous return, 4) peripheral vascular resistance control, 5) visceral movements and 6) ocular movements. Muscle tissues are highly plastic and respond quickly to injury and hormonal stimuli. Conditions in which muscle tissues are debilitated highlight their role in homeostatic control. For example, 1) a major cause of death in developed and developing countries is heart failure; 2) loss of skeletal muscle mass in severe cardiac failure is related to poor prognostic; 3) loss of skeletal muscle mass over aging is an important element of the senile syndrome. The development of new strategies aiming for a better outcome of muscle function necessarily relies upon a deeper knowledge of cellular and molecular biology of tissue responses. Therefore, the aim of this study is to gain further insight on cellular and molecular mechanisms underlying muscle plasticity. In subproject 1 the effect of certain mechanical stimuli will be stressed, to further investigate the role of Akt/mTOR on skeletal muscle mass control. In subproject 2, we will address the effects of increased skeletal muscle mass upon energy balance control. This will be achieved by using muscular IGF-1 transgenic mice. In subprojects 3 and 4 we will evaluate cellular and molecular effects of hormones extremely important to the homeostasis of muscle tissues: thyroid hormone (T3) and Angiotensin II. In subproject 5 a molecular approach of skeletal muscle proteolysis triggered by T3 will be performed. In subprojects 6 and 7, the activity of Akt/mTOR and calcineurin will be investigated in skeletal muscle of mice undergoing cardiac failure. Finally, in subproject 8, we will determine the effects of thyroid hormone and GC-24 (a thyroid hormone receptor , selective agonist) upon the global gene expression pattern in all three muscle tissues.

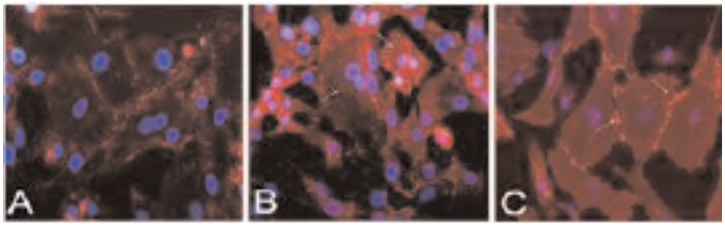
SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Subproject 4. VSMCs obtained from rat thoracic aortas by explant method were evaluated at passages 1 until 10 (fig. 1). From the 4th passage on, VSMCs underwent significant morphological changes from spindle- to polygonal-shaped and decreased differentiation markers. These data suggest that VSMCs until passage 3 may be employed as a model of differentiated phenotype but during later passages represent a model of dedifferentiated/proliferative phenotype. These findings highlight the importance of adequate manipulation of VSMCs since they undergo phenotypic modulation as a result of serial passages and freezing processes.

Subproject 6. By using KO mice which have sympathetic hyperactivity as a model for heart failure (HF), we showed that at 7 months of age HF mice displayed systolic dysfunction (32% vs. 24%, $p<0.05$) and exercise intolerance. Cross-sectional area of soleus and plantaris muscles was lower in 7 month-old HF mice with severe cardiomyopathy. Exercise training prevented soleus and plantaris muscles atrophy, since it significantly increased cross-sectional area in all fibers studied of both muscles. Analysis of reduced to oxidized glutathione ratio (GSH:GSSG) in WT and HF mice revealed that at 7 months HF mice displayed reduced GSH:GSSG ratio. Exercise training restored redox status of HF mice to age-matched WT mice levels. Taken together, these results suggest that exercise training by restoring skeletal muscle mass and redox state can be considered an important therapeutic strategy for preventing or reversing skeletal muscle myopathy in HF.

Subproject 8. Microarray analysis of cardiac tissue from rats submitted to a experimental hyperthyroidism revealed that out of the 30,000 sequences addressed, as many as ~8,000 are modulated 12hs, 24hs and 7 days after the onset of T3 treatment. Interestingly, about half of these genes were down-regulated. Genes associated with the extracellular matrix showed the highest Z score (percentage of genes regulated) in the array, reinforcing the broad impact of T3 in heart matrix remodeling. Aditional information in the array prompted us to investigate the effects of T3 upon B-catenin in the cardiomyocytes. T3 rapidly (30 min) increases B catenin protein levels in cardiomyocytes (fig. 2) but not in cardiac fibroblasts. In addition we found that pharmacological inhibition of PI3K severely decreases T3 dependent B catenin response. Considering that B catenin is a key regulator of cardiac hypertrophy, our results suggest that at least part of the hypertrophic effect of T3 might be mediated by B catenin throughout PI3K.

Figure 2 – Immunofluorescent staining against β-catenin (Red) and Cell Nuclei (DAPI-Blue), 200x. A- Control Cardiomyocytes, B-Cardiomyocytes treated with T3 for 30 minutes, C-Cardiomyocytes treated with T3 for 24 hours



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Anselmo Sigari Moriscot

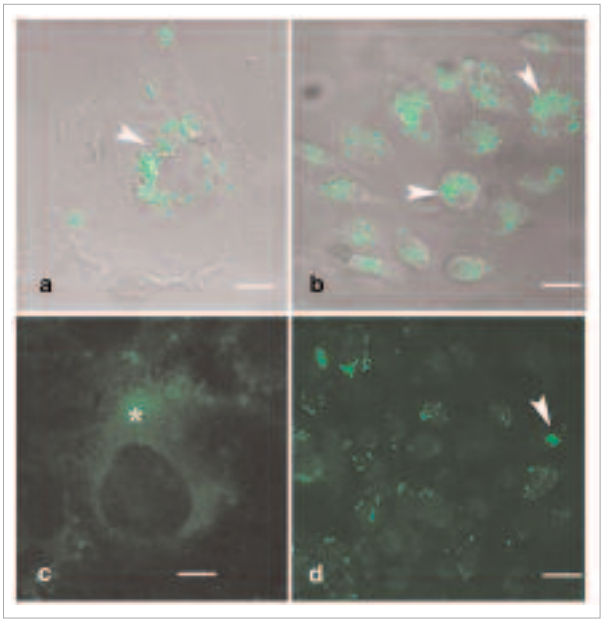
Instituto de Ciências Biomédicas
Departamento de Histologia e Embriologia
Universidade de São Paulo (USP)
Avenida Prof. Lineu Prestes, 1524 – C. Universitária
CEP 08500-900 – São Paulo, SP – Brasil

+55-11-3091-7311
moriscot@usp.br

PHAGOCYTOSIS AND IFN-γ-MEDIATED SIGNALING AT MATERNAL FETAL INTERFACE

Estela Maris Andrade Forell BEVILACQUA

Institute of Biomedical Sciences / University of São Paulo (USP)



Trophoblast and GFP-E. coli in IFN-γ-treated cultures

In many species, phagocytosis is a remarkable characteristic exhibited by implanting and post-implanting trophoblast cells. Particularly in mice, during the first half of the gestation, giant trophoblast cells phagocytize maternal endometrial cells and among them, blood cells, which may have a role in nutrition and acquisition of space for embryo development. Throughout gestation, however, phagocytosis of microorganisms can also be experimentally observed. In relation to the phagocytic activity, trophoblast cells and macrophages share in common several characteristics, such as reactive oxygen species production, C3b-mediated phagocytosis, production of nitric oxide, and both those cell types increase their potential for phagocytosis in the presence of IFN-γ. In macrophages, IFN-γ is a potent regulatory molecule for phagocytosis and able to suppress the synthesis of cytoplasmic proteins involved in viral replication, to activate the transcription factor NF-KB involved in the inflammatory defense response and to induce the production of nitric oxide and oxidases responsible for reactive oxygen species formation. On the other hand, IFN-γ produced by immune and non-immune cells is present in the materno-fetal interface and is considered a physiological component of gestation. However, under conditions in which the immune/inflammatory response against pathogens prolongs non-physiological concentrations of IFN-γ in the maternal organism, pregnancy may be affected. So, considering that phagocytosis is an inherent activity of trophoblast cells, which can be related to defense functions, this study aims to determine the concentrations of IFN-γ in normal gestation and in pregnant remates challenged with LPS; to determine the maximum IFN-γ concentration that does not interfere with the gestation progression; to establish *in vivo* and *in vitro* models that allow the study of the process of phagocytosis of microorganisms or part of them by trophoblast cells; to evaluate the action of IFN-y on the gene expression of trophoblast cells and mainly, on the expression of nitric oxide synthase, by using DNA macroarray approaches.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Trophoblast phagocytosis is an event of fundamental importance for the pregnancy. It takes place in early gestation, and is crucial for the embryo nutrition, iron uptake, acquisition of space, and protection against microorganisms and, very likely, is also crucial for the elaboration of a specific immune response at the maternal-placental interface rather than for directly destroying pathogens. In macrophages, IFN-γ is able to mediate antiviral effects through the expression of a large panel of cytokines directly associated with the innate and adaptive immunity and to induce a number of processes to activate phagocytosis. Recently it has been found that IFN-γ is produced by uterine NK cells, coincidently with the period, in which the mouse trophoblast exhibits high phagocytic activity. Our hypothesis is that physiological IFN-γ is one of the central regulators of the gestational homeostasis. Exposure of mouse embryos to IFN-γ definitively showed the potential of this cytokine for inducing phagocytosis of microorganisms in trophoblast cells. IFN-γ-mediated response involved the expression of nitric oxide synthase (NOS) and NADH-oxidase enzymatic complexes and, respectively, the release of nitric oxide and reactive oxygen species, all of which are able to damage various biological molecules. The release of NO may also indicate a relevant role in the pro-inflammatory activity at the maternal fetal interface. The expression of inducible NOS at the transcriptional level was a JAK/STAT1-dependent pathway that significantly decreased upon pharmacological inhibition of IFN-γ receptor phosphorylation. The antioxidant balance in stimulated trophoblast was also verified. Through gene and protein expression, the activity of trophoblast oxidative enzymes was shown to be able to promptly compensate the secretion of reactive species. Furthermore, cDNA macroarray and RT-PCR showed that at least 7 genes are prominently upregulated in the presence of IFN-γ. These results not only suggest that IFN-γ for long considered as an abortifacient molecule may play physiological functions during the gestation by regulating trophoblast phagocytosis and expression of cytokines, but also contribute to understanding important roles of trophoblasts.

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Estela Maris Andrade Forell BEVILACQUA

Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Avenida Prof. Lineu Prestes, 1524 – C. Universitária
CEP 05508-900 – São Paulo, SP – Brasil

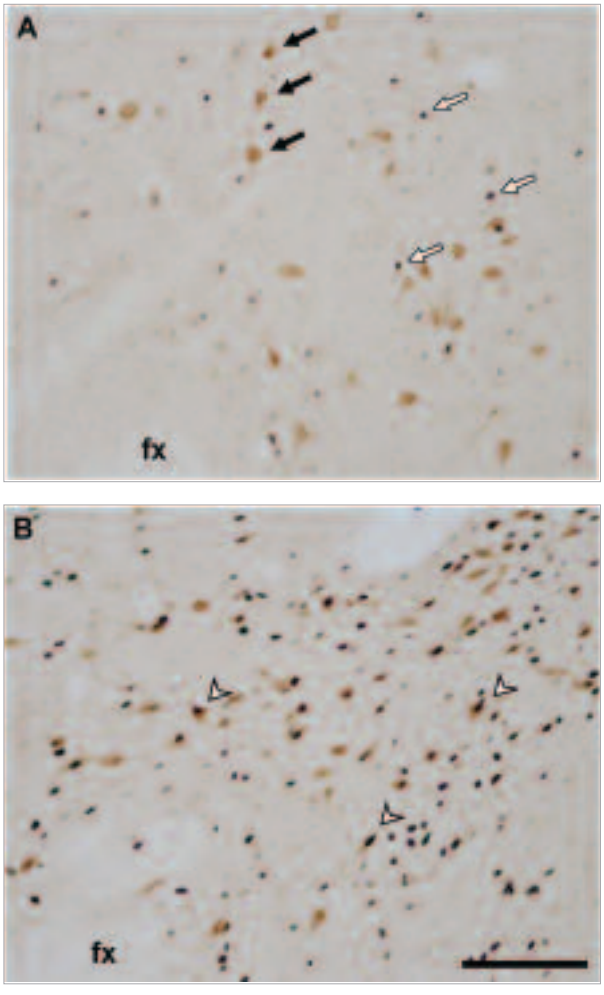
+55-11-3091-7307
bevilacq@usp.br

PEPTIDERGIC PATHWAYS INVOLVED IN THE ORGANIZATION OF FEEDING BEHAVIOR

Jackson Cioni BITTENCOURT

Institute of Biomedical Sciences / University of São Paulo (USP)

Feeding behavior is an essential part of the energy balance control. It allows the maintenance of health and an adequate body weight. In the last years, the description of leptin action as a lipostatic or satiety factor in the hypothalamus originated a burst of information about its action in food intake control and related disorders (obesity and anorexia). In parallel, an increasing number of peptides and receptors implicated in energy homeostasis have invaded the field. In this regard, melanin-concentrating hormone (MCH) has been related to the tonic control of feeding. MCH neurons are located in the hypothalamus, which made it a natural candidate for neuroendocrine, autonomic and behavioral control of the homeostasis and species subsistence. Accordingly, various findings support MCH action on feeding behavior: 1) MCH mRNA is abundant in fasted animals or obese (ob/ob) mice; 2) MCH administration in the cerebral ventricles or in specific hypothalamic nuclei that express MCH receptor (MCH -IR) induces hyperphagia and increases body weight; 3) MCH knockout mice shows decreased body weight due to hypophagia and rise in energy expenditure; 4) MCH-IR knockout mice are lean albeit hyperphagic; 5) administration of MCH-IR specific antagonists decreases body weight gain. Intriguingly however, the brain sites where MCH may act by inducing feeding, and the cell biology of these putative systems are not completely understood. It is important to point out that feeding is a complex behavior that includes homeostatic and hedonistic mechanisms, embracing from hunger sensation to motor activity, exploratory behavior, emotional responses, anxiety, memory and learning processes. Therefore, this study attempts to investigate MCH action in various feeding aspects and the related circuitry. Initially, we plain to analyze MCH participation in spatial orientation in the search for food, using a maze paradigm and different types of food: only nutritional or with hedonic value. In addition, by using the same paradigm we propose to investigate the animal performance following lesion, or injection of MCH or anti-MCH in specific brain nuclei previously identified. In these regions, we intend to investigate the neurochemistry, the occurrence of synaptic terminals by using electron microscopy, and describe the synaptic characterization. Finally, we aim to look into MCH's role in complex systems of search for food that include predatory behavior.



Colocalization of ƒos and orexin immunoreactivity (ƒos-ir and orx-ir) in diencephalic territory. Brightfield photomicrography of immunoperoxidase material stained for ƒos protein and orexin. A, ƒos-ir cells localized in the lateral hypothalamic area (LHA) (white arrows) and orx-ir cells (black arrows) in the control animals; B, cells colocalizing ƒos-ir and orx-ir (arrowheads) in the LHA region of animals during the predatory hunting behavior. Abbreviations: fx, fornix. Bar = 200µm

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The participation of the lateral hypothalamic area (LHA) and the neuropeptide orexin in the predatory hunting behavior: for the first time, orexin was shown to be co-localized with *fos* protein in the neurons of the LHA after rats had been exposed to free cockroaches in the cage. The number of neurons in the LHA presenting the *fos* protein (a protein that is used as a marker for activated cells) significantly increased in the experimental model, as compared to the control animals, for which we did not observe the same results. This finding suggests that the LHA functions as an integrative center, also participating in the arousal system for this kind of behavior, and also indicates orexin as a neuromodulator.

In order to study the spatial memory participating in the feeding behavior and the involvement of some of the orexigenic peptides, such as the melanin-concentrating hormone and orexin (both present at hypothalamic territories), we were developed an experimental model that makes use of the already known "dry maze". To our knowledge, this is the first time such an apparatus is employed for studying the spatial memory participating in this type of behavior. Despite the fact that we have not yet concluded these experiments, we have already established a protocol to study the feeding behavior. The validation of such method is under way.

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Jackson Cioni BITTENCOURT

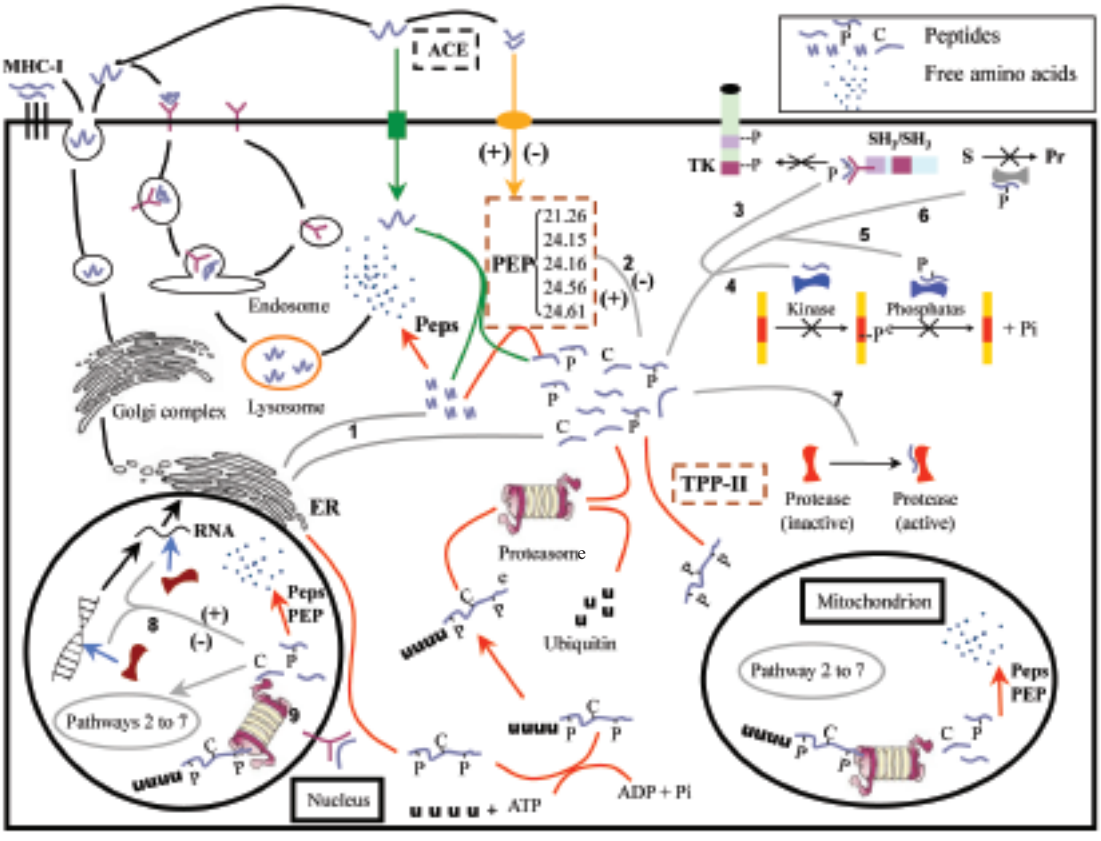
Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Departamento de Anatomia
Av. Prof. Lineu Prestes, 2415 Ed. B-III Lab 105
Cidade Universitaria
CEP 05508-900 – São Paulo, SP – Brasil

+55-11-3091-7300
jcbitten@icb.usp.br

MOLECULAR CELL BIOLOGY OF OLIGOPEPTIDASES

Emer Suavinho FERRO

Institute of Biomedical Sciences / University of São Paulo (USP)

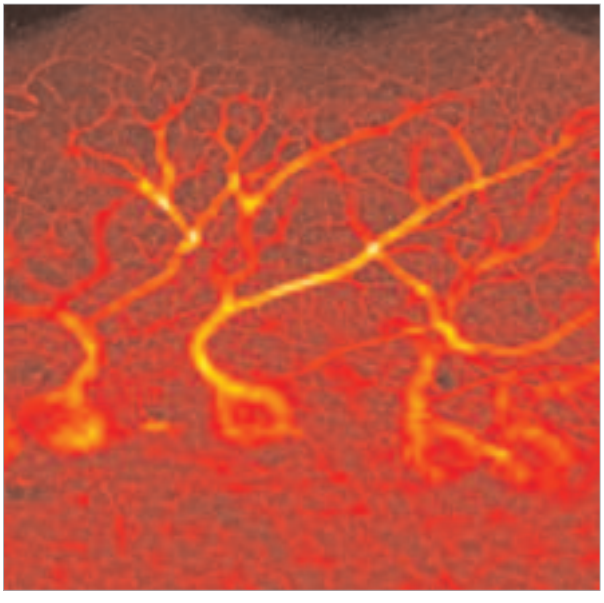


Diagramatic scheme showing oligopeptidases in the cell

Endo-oligopeptidases is a denomination coined by Camargo and cols. (Oliveira, et al., 1976) to describe the substrate specificity of two enzymes named endo-oligopeptidases A and B, which cleave only short peptides (from 5 to 17 amino acids). At this time we intend to keep on investigating the cell biology and function of endo-oligopeptidases EP24.15 (EC 3.4.24.15) and EP24.16 (EC 3.4.24.16). The primarily intracellular location (e.g., cytosolic, nuclear, mitochondrial) of peptidases such as EP24.15 (EC 3.4.24.15) and EP24.16 (EC 3.4.24.16) suggests additional functions besides extracellular neuropeptide/hormone metabolism/processing. In collaboration with the laboratories of Professors Antonio C.M. Camargo (Butantan Institute) and Célio Silva (FMRP, USP), we have shown that oligopeptidases such as EP24.15 play an important intracellular role in degrading peptides released by the 268 proteasome. Thus, the aim of the present project is to investigate the molecular cell biology of endo-oligopeptidases EP24.15 and EP24.16.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We have developed knockout animals for the neurolysin endopeptidase, and we are in the final phase of obtaining knockout animals for the thimet oligopeptidase. We are concluding studies which suggest high concentration of intracellular peptides, with biological activity capable of altering the signaling of receptors coupled with G proteins (GPCRs) and tyrosine kinases. We have obtained specific conformation anti-GPCR antibodies which were used for the identification of hemopressin as an inverse agonist of type-1 cannabinoid (CB1) receptors. Another 25 peptides had their GPCRs identified, thus composing a new group of molecules with potential therapeutical use.



Oligopeptidases: presence in rat brain neurons is shown by immunohistochemistry suggesting a physiological role in brain peptide degradation (Massarelli et al., 1999)

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Emer Suavinho FERRO

Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Av. Prof. Lineu Prestes 1524, Sala 431-435 – Butantã
05508-900 – São Paulo, SP – Brasil

+55-11-3091-7310
eferro@usp.br

PSYCHOBIOLOGY OF FEAR AND ANXIETY

Marcus Lira BRANDÃO

Faculty of Philosophy, Sciences and Languages of Ribeirão Preto / University of São Paulo (USP)

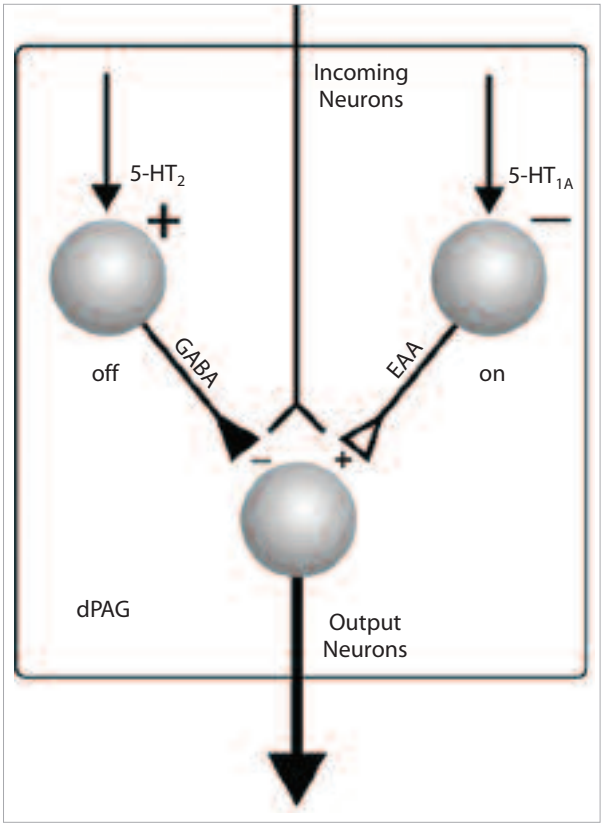

Figure depicting the defense modulating neurons in the dorsal periaqueductal gray (dPAG)). Both off- and on-cells are intrinsic neurons of the dPAG, where they exert a dual control over output neurons; on-cells excite and off-cells inhibit these neurons. Excitatory amino acids and GABA could be the neurotransmitter of on- and off-cells, respectively. While on-cells are excited by 5-HT2A agonists, off-cells are inhibited by 5-HT1A agonists. As a result both 5-HT1A and 5-HT2A mechanisms cooperate in the regulation of the neural substrates of fear in the dPAG. EAA= excitatory amino acids. 5-HT= serotonin.

The medial hypothalamus, amygdala and dorsal periaqueductal gray (dPAG) have been traditionally grouped together as a "brain aversion system". More recently, a continuos strip of midbrain structures composed of superior and inferior colliculi have also been proposed as parts of this "system". In this project we will focus on the neural substrates of defensive behavior in the midbrain tectum (dPAG, superior and inferior colliculi), and their relevance for understanding fear and anxiety. The proposed link between the defense behavior, fear and anxiety is consistent with many behavioral, electrophysiological and immunohistochemical studies showing expressive activation of these regions by threatening stimuli or conditions. The present project further investigates general principles that regulate the sensory information input and the behavioral output that animals present in fearful situations as well as the neurochemical mechanisms underlying the aversive responses associated with fear and anxiety. The presentation of these studies is organized in nine groups representing the behavioral (I, II and III), immunohistochemical (IV), sensorimotor (V and VI), electrophysiological (VII) and neurochemical (VIII) approaches to the defense reaction. The last subproject (IX) is an atempt to make a multifaceted approach to the neurobiology of fear so as to produce scientific material with enough impact to contribute to this field of enquiry.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Freezing defined as the complete absence of body movements is a normal response of animals to unavoidable fear stimuli. In the present project we have obtained a series of evidence relating different defensive patterns with specific anxiety disorders. There are at least four different kinds of freezing with specific neural substrates. The immobility induced by stimulation of the ventral column of the periaqueductal gray (vPAG) has been considered a quiescence characteristic of the recovery component of defense–recuperative processes. There is an isomorphism between freezing response to contextual stimuli paired with electrical shocks and generalized anxiety disorder. Besides, two types of freezing emerge with the electrical stimulation of the dorsal aspects of the periaqueductal gray (dPAG): the dPAG-evoked freezing and the dPAG post-stimulation freezing. Evidence is presented in support of the hypothesis that whereas dPAG-evoked freezing would serve as a model of panic attacks, the dPAG post-stimulation freezing appears to be a model of panic disorder. It is also proposed that conditioned freezing plus dPAG electrical stimulation might also mimic panic disorder with agoraphobia. It has also been possible to present a model of serotoninergic modulation through on- and off-cells of the defense reaction generated in the dPAG. The understanding of how the periaqueductal gray generates and elaborates different types of freezing is of relevance for our better knowledge of distinct types of anxiety such as panic disorder or generalized anxiety disorder.

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Marcus Lira BRANDÃO

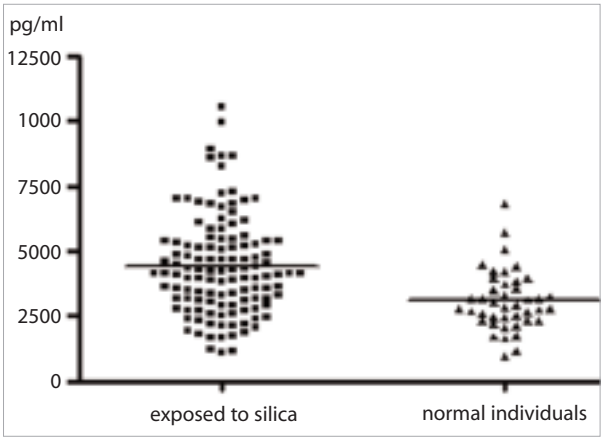
Faculdade de Filosofia, Ciências e Letras
de Ribeirão Preto / Universidade de São Paulo (USP)
Avenida Bandeirantes, 3900 – Monte Alegre
CEP 14049-901 – Ribeirão Preto, SP – Brasil

+55-16-3602-3638
mbrandao@usp.br

IMMUNOTOXICOLOGICAL ALTERATIONS IN WORKERS EXPOSED TO SILICA

Mary Luci de Souza QUEIROZ

School of Medical Sciences / State University of Campinas (Unicamp)



Soluble interleukin 2 Receptor (IL2R): Significant increases in IL2R were observed in the exposed population (exposed = 4395 ± 1893 vs control = 3090 ± 1150 p<0.0001)

The present study was organized in association with prominent leading researchers in the area of epidemiology and clinical immunotoxicology of silica exposure. Studies of occupational groups with high-level silica exposures (e.g. miners) have shown increased rates of autoimmune diseases compared to the expected rates in the general population. Pulmonary deposition of crystaline silica can result in a cycle of lung damage, fibroblast proliferation, and excess collagen production in the lung causing lung fibrosis or silicosis. There have been a number of epidemiological studies examining the relationship between exposure to silica and autoimmune disease and strong associations have been made between systemic lupus erytematosus, rheumatoid arthritis, ANCA associated vasculitis and glomerulonephritis, and scleroderma. However, a significant number of questions remain with regard to the pathophysiology, etiology, mechanisms and multiplicity of effects following silica exposure. For example, silicosis and mineral dust pneumoconiosis have been linked to increased levels of auto-antibodies, immune complexes, and excess production of immunoglobulins, even in the absence of clinical features of specific autoimmune diseases. Many cases of autoimmune diseases in silica-exposed individuals have been identified during screening or treatment for silicosis. In addition, in a number of silica exposed individuals, autoimmune disease develops prior to or without overt manifestations of silicosis. It is therefore unclear whether silicosis is simply a marker for high-level silica dust exposure or whether it represents a pathologic process that may predispose some individuals to the development of autoimmune disease. To date, a majority of studies have used disease as an endpoint, and systemic examination of changes in autoimmune parameters in silica-exposed individuals are lacking. Thus we have little information on what types of immunologic changes occur, what the persistence of these changes is, how these changes relate to the progression and development of autoimmune disease, and whether these changes relate to the dose of silica received. In the present study we intend to advance the understanding of autoimmunity associated with occupational exposure to environmental factors by evaluating immunotoxicological changes in silica-exposed workers.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Consistently higher incidence of immunological alterations were found in silica-expose workers (n=130), as compared with non-exposed population (n=118). Increased incidence of antinuclear antibody and rheumatoid factor (20% and 15%, respectively) were observed in the exposed population, as compared to 3% and 2% in the non-exposed group. IgE levels were increased in more than 48% of the exposed population (controls=29%).

Significant increase in the two subpopulations of NK cells (CD16+ and CD56+) were also found in the exposed population (59% and 42%, respectively). These findings are relevant in view of the importance of NK cells in promoting autoimmunity and the development of fibrosis. NK cells are able to induce a population of lymphoid cells expressing CD8+, which is responsible for auto-reactive responses, as opposed to CD4+ cells, which inhibit the development of autoimmunity. Increased T CD8+ cells, associated to reduced T CD4+, constitutes an important indicator of increased susceptibility to autoimmunity. In this respect, CD3+ CD8+ was increased in 17%, and CD3+ CD4+ was reduced in 12% of the exposed population. Of these, 13% presented a CD4/CD8 ratio lower than l.2, thus demonstrating a reduction in the population of CD4+ lymphocytes. NK and T cells depend on IL-2 for their development and maintenance of cytotoxic responses. In addition, IL-2 is important for the prevention of autoimmune diseases due to its involvement in the differentiation and function of T regulatory CD4+ CD25+ cells expressing the α chain of IL-2 receptor. Reduced frequency of these cells are being observed in certain autoimmune diseases, associated to increased levels of soluble IL-2α receptors (sIL-2Rα). These receptors are liberated in the circulation by activated T lymphocytes, thus functioning as a measure of immune activation. Concurrently, the increased levels of sIL-2Rα might lead to competition with the same receptor present in the surface of regulatory T CD4+ CD25+, thus preventing the normal development of these cells and predisposing to the development of autoimmune diseases. Corroborating these findings, our results demonstrated a significant increase in the levels of sIL-2Rα in the serum of silica-expose workers compared to non-exposed controls (exposed = 4395±1893 *vs* non-exposed = 3090 ± 1150, $p<0.0001$).

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Mary Luci de Souza QUEIROZ

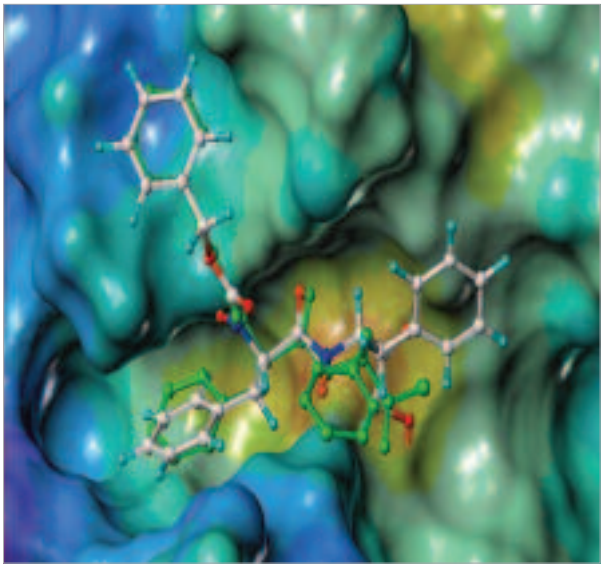
Faculdade de Ciências Médicas
Universidade Estadual de Campinas (Unicamp)
Departamento de Farmacologia
Caixa Postal 6111 – Barão Geraldo
13083-970 – Campinas, SP – Brasil

+55-19-3521-8751
mlsq@fcm.unicamp.br

POTENTIAL ANTITRIPANOSOMAL DERIVED FROM NITRO-HETEROCYCLIC COMPOUNDS

Elizabeth Igne FERREIRA

School of Pharmaceutical Sciences / University of São Paulo (USP)



Cruzain inhibitor identification by virtual screening

Chagas' disease is endemic for most of Latin America, serioulsy affectinq the health of infected people and shortening their lives. Around 16 to 18 million people are estimated to be infected and about 50 thousands of deaths are registered each year in the 21 endemic countries. The therapeutic armamentarium against he parasitosis is scarce, s only two drugs have been used, none of them sufficiently effective at the chronic stage of this disease. Considering that only people from underdeveloped countries are inffected or under the risk of contracting the parasitosis, the interest is relatively low to the countries responsible for developing most of the therapeutic drugs in use to date. Thus, searching for new drugs against the disease is mainly a task for those underdeveloped countries, among the above. Taking into account the high activity showed by the nitro-heterocyclic derivatives that have been already synthesized by our group, and/or others using diverse approaches, our main goal is to find new and effective drug candidates among this class of compounds. This objective will necessarily be supported by *in vitro* and *in vivo* assays, as well as by mutagenicity tests. The study of the mechanism of action of those candidates through electrochemical methodologies, by using biosensors with immobilized nucleic acids and enzymes, and the elucidation of the chemical structure-biological activity (QSAR), especially by molecular modeling – assisted 3D-QSAR, are the rational bases for the design of new and more effective nitro-heterocyclic derivatives. We hope to contribute in an integrated way to the search for better antitripanosomal agents.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The results obtained by using computational and electrochemical methods indicate the advances not only in the synthesis of prodrugs, but also in the understanding of their mechanisms of action at the molecular level. Such synthesized prodrugs, polymeric or not, comprise bioisosteric analogues of the nitro-heterocyclic derivatives originally proposed – nitrofural and hydroxymethylnitrofural – and of structures of congenerous compounds, such as benzhydrazides, and others selected by virtual screening. Application has been made for the invention patent "Dendrimeric prodrug, process for its preparation and compositions containing it", P.I. 0.705.122-0, published on February 6th, 2008, in Revista da Propriedade Industrial Nº 1935, p. 87, item 2.1. Similarly, we applied modern methodologies antichagasic compounds planning to a series of semicarbazone analogues taken from the literature, and as results we generated QSAR models with a high prediction, and a restricted series of synthesized and substituted phenylhydrazones, all of which demonstrated a "promiscuous" mechanism of cruzain inhibition. Also, we proposed models of virtual screening, applied them to a library of 3,294,714 compounds, and one of them permitted the discovery of a compound with a promising inhibitory activity (K_i= 21 ÌM). In addition, we demonstrated the importance of the experimental validation of the models obtained by calculation. The application of electrochemical methodologies allowed for the stabilization study in aqueous medium of the anionic nitro-radicals derived from nitrofural, as well as the study of their interaction with natural biological electron acceptors, oxygen, cysteine and glutathione. In addition, the electrochemical characterization of the prodrug hydroxymethylnitrofural was also conducted. Molecular modeling data were then consolidated with the voltametric results obtained from the oxidation of primaquine and prodrugs (both their succinyl and maleyl derivatives), which thus allowed for determining their respective molar volumes based on their estimated coefficients of diffusion and electrophoretic mobility in aqueous medium. We also characterized in aqueous medium the redox properties of 5-nitro-2-thiofilidene-4- and 5-nitro-2-furfurilidene-4-R-benzhydrazides, barely soluble in water, by using modified carbon paste electrodes, in which the modifying agent was the same as the compound being studied.

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Elizabeth Igne FERREIRA

Faculdade de Ciências Farmacêuticas
Universidade de São Paulo (USP)
Av. Prof. Lineu Prestes, 580 – Bloco 15
Caixa Postal 66083 – Cidade Universitária
05508-900 – São Paulo, SP – Brasil

+55-11-3091-3793
hajudan@usp.br

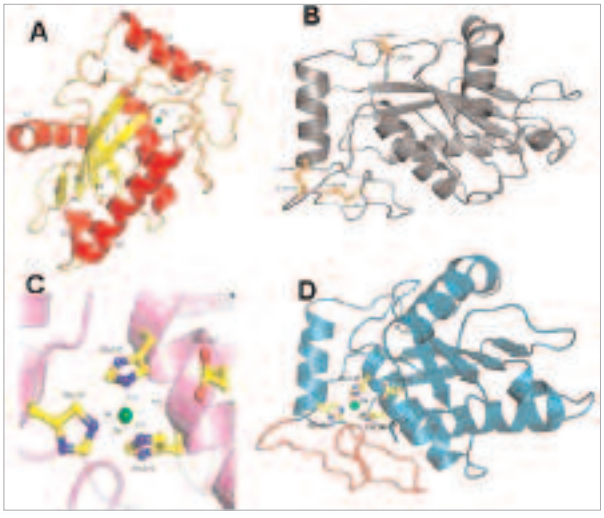
ANIMAL TOXINS: STRUCTURE, FUNCTION AND BIOTECHNOLOGICAL APPLICATIONS

Suely VILELA

Ribeirão Preto School of Pharmaceutical Sciences / University of São Paulo (USP)

Animal toxins have contributed significantly for the development of Biological and Biomedical Sciences. These molecules, used as important tools in the investigation of cellular and molecular mechanisms, are involved in immunological, pharmacological and toxicological processes. In addition, they constitute interesting molecular models for the development of biotechnological strategies to generate therapeutic agents and/or experimental tools for basic and applied research. However, animal venoms/toxins still lack additional biological and/or functional characterization, including those from snakes, toads and scorpions. Within this purpose, the isolation and biochemical structural and functional characterization of biologically active proteins/components of these venoms will be able to provide important information for a better understanding of the composition and physiopathological effects of these toxins. This project aims at the functional and/or structural analyses of new toxins and model toxins (already described) from snakes (*Bothrops jararacussu*, *B. pirajai*, *B. alternatus*, *B. atrox* e *Crotalus d. terrificus*), scorpion (*Tityus serrulatus*) and toad (*Bufo paracnemis*). Isolation of biolocally active components will make use of classical chromatographic techniques, such as gel filtration, ionic exchange, hydrophobic interaction, bioaffinity and HPLC (reversed phase). This phase of the project is fundamental for all the following ones to be developed, since it will provide the active components which, along with the crude venom, will be objects of study of this project. The investigation of the biological activities of venoms and toxins will be multiparametric, considering the adequation of the assays to the character of toxins (new or models). Effects still roughly explored of these toxins will be evaluated on the immune system (complement, apoptosis and inflammation), microorganisms (leishmanicide, trypanocide, bactericide, fungicide and antiviral activities) and cell lines (cytotoxicity, antitumoral effect and apoptosis).

Actions upon Ca^{2+} and Na^{+} channels and upon receptor and transporter systems for L-glutamate and GABA will be explored as well. In addition, activities as hyaluronidase, proteolytic, PLA2, L-amino acid oxidase, hemorrhagic, myotoxic, edema inducing, coagulant, lectinic and anticoagulant will also be evaluated. These activities were chosen since they are target of the venoms actions and highly relevant in physiological processes. Within the different systems to be evaluated, the structure of the isolated toxins will be the basis for the possible elucidation of the structure – function relationship.

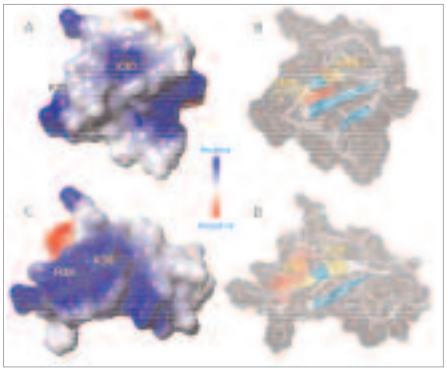


Structure of B. jararacussu *metalloprotease BJUSSUMP-II. (A): Secondary and tertiary structure of the BJUSSUMP-II model. The ion Zn^{2+} is shown as a green sphere. (B): Disulfide bridges (represented as sticks) present in the BJUSSUMP-II model. (C): Distances between the ion Zn^{2+} (green sphere) and the residues from the catalytic site of the BJUSSUMP-II model. (D): Cartoon representation of BJUSSUMP-II model highlighting the flexible region 153-176 (in red). (MARCUSSI et al., 2007).*

The structural characterization of the toxins will be achieved by automatic sequencing, X-ray crystallography and molecular modeling. Considering the number of venoms and toxins to be explored and the proposed assays for characterizations of their effects and structures, we believe that this project is multidisciplinary, comprehensive and promising. The results of the proposed project will broaden the understanding of the structure/function relationship of toxins in biological systems, which will contribute for the generation of new tools for basic or applied research.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

L-Aminoacido oxidases (LAAOs) isolated from the venom of *B. jararacussu*, *B. moojeni* and *B. pirajai*, were characterized biochemically, functionally and structurally. From the venom of *B. jararacussu*, two metalloproteases were isolated: BJUSSUMP-I, Mr ~ 60,000 and pI 5.6; and BJUSSUMP-II, Mr ~ 24,000 and pI 6,43. Both metalloproteases have been proved potent αβ-fibrinogenases, and inhibit platelet aggregation. A serinoprotease, BjussuSP-I, was also isolated, and exhibits thrombin-like proteolytic activity. It also presented pro-coagulant and potential calicrein-like dysfibrinogening activities, the latter likely to be of clinical relevance. Also isolated were: phospholipases A2 called Bmoo-I-PLA2 and Bmoo-II-PLA2 from *B. moojeni*; acidic Bp-PLA2 from *B. pauloensis*; and two basic neurotoxic isoforms from *B. neuwiedi pauloensis*. MjTX-II from *B. moojeni* complexed with fatty acid had both its tertiary and quaternary structures elucitadated. The PLA2 (CB)-crotoxin complex from the venom of *C. d. terrificus* was crystallized. NMR studies with the venom of scorpion *Tityus serrulatus* allowed for the structure determination of alpha-KTx12.1, a toxin that blocks potassium channels. They were also tested with crude venom, and the toxins TsTX-I and TsTX-V, with resulting data showing that they induce a marked increase both in blood pressure and in plasmatic catecholamines. Also from the venom of *Tityus serrulatus*, proteases that are able to activate the complement system could be identified. An acidic protein able to activate the complement system has also been isolated from the venom of toad *Bufo paracnemis*. The results obtained to date should contribute significantly to the development of Toxinology in Brazil, improving the understanding of the mechanism of action of proteins and toxins from snake venoms, thus being valuable for searching pharmacologically active new molecules of interest in the clinical-medical and scientific fields of knowledge.



Accessible surfaces of the last structures of each MD simulation of the pH 7.0 (A and B) and 4.0 (C and D). Areas represented in A and C are colored according to the electrostatic potential. Waste His28 and Lys30, are highlighted in yellow (B and D)

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Suely VILELA

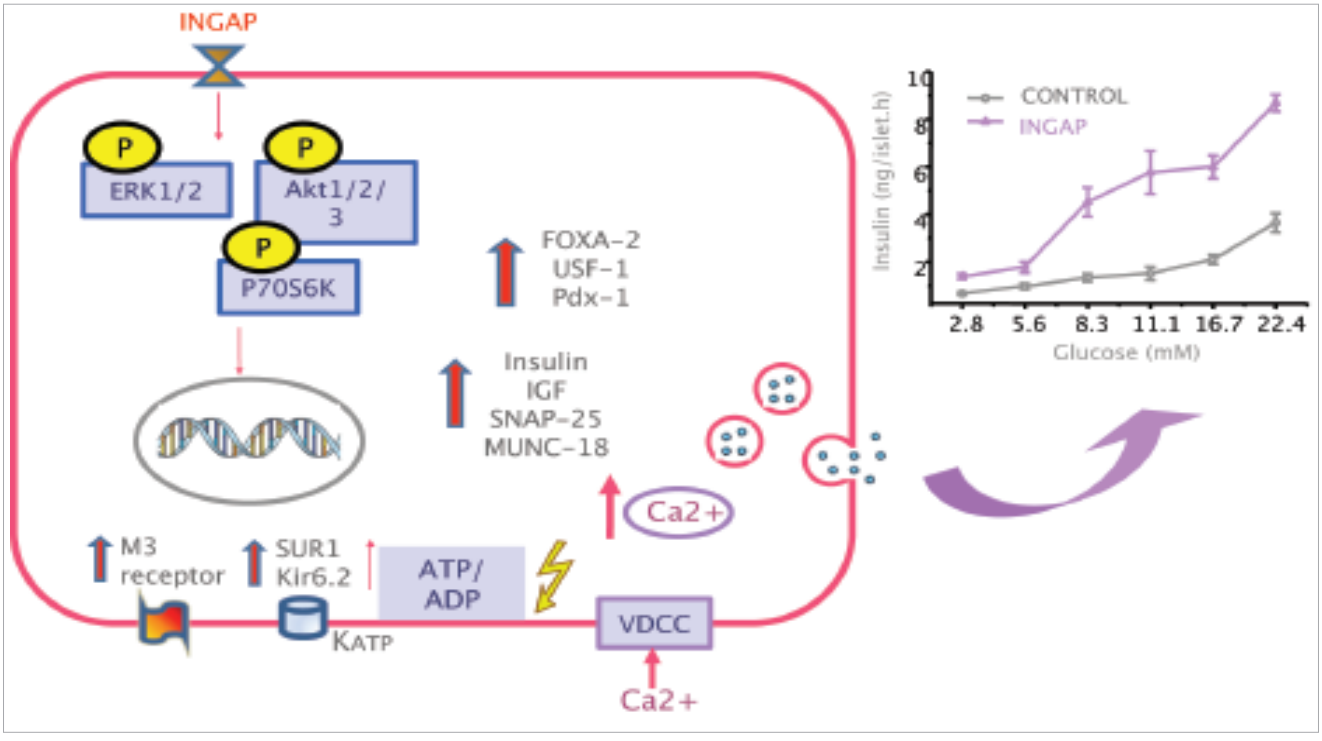
Reitoria
Universidade de São Paulo (USP)
Rua da Reitoria, nº 109 – Cidade Universitária
CEP 05508-900 – São Paulo, SP – Brasil

+55-11-3091-3266
suvilela@usp.br

STUDY OF THE DESTRUCTION MECHANISM OF BETA PANCREATIC CELLS DURING THE ONSET OF DIABETES MELLITUS (DM2): SEARCH FOR INHIBITION STRATEGIES OF THIS PROCESS AS WELL AS FOR THE RECOVERY OF INSULAR MASS IN DIFFERENT ANIMAL MODELS

Antonio Carlos BOSCHERO

Institute of Biology / State University of Campinas (Unicamp)



INGAP (islet neogenesis associated protein) is related to islet neogenesis and beta cell mass increase in rodents and dogs. INGAP-PP, the active part of INGAP, promotes maturation and improves insulin secretion in response to glucose in pancreatic islets. The expression of several genes is modulated by INGAP in pancreatic islets of neonatal rats, as well as insulin secretion increases in response to different concentrations of glucose. Beta cell transcription factors, insulin granules extrusion machinery proteins, insulin, insulin-like growth factor, type 3 muscarinic receptor , and KATP channel subunits genes are examples of genes modulated by INGAP in pancreatic

The most frequent Diabetes mellitus (DM) is DM2 which, generally, results from an increase in resistance to the action of insulin followed by the inability of the pancreatic B cells to secrete sufficient quantities of the hormone to compensate for hyperglycemia. It became evident that the presence of an adequate and renewable mass of pancreatic B cells during various stages of life is fundamental for the maintenance of the normoglycemia. The alterations in the mass and sensitivity of the secretory cells of insulin to glucose are commanded by several hormones at different periods in life. In this project we studied the destruction mechanism of the beta cells in several experimental models, *in vivo* and *in vitro*, as well as different strategies for the inhibition of this process and the recovery of insular mass.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Signaling paths of prolactin (PRL) and effects of the "Islet Neogenesis Associated Protein" and of the "Ciliary Neurotrophic Factor" (CNTF) on pancreatic islets

PRL modulated the expression of the CERCA (responsible for the control of Ca^{2+} in the reticule), a mechanism dependent on the STAT3. As the effects of the PRL were antagonized by dexametasone, we suggest that glycocorticoids participate in the readaptation of the endocrine pancreas in the postpartum. PRL also increased the expression of proteins that participate in the extrusion of granules of insulin. Acutely, PRL increased phosphoryrilation/association of proteins implicated in the secretory machinery indicating that the hormone prepares the pancreatic β cells for secretion, probably through the MAPK/PKC path. The chronic treatment of new-born or adult mice islets with INGAP increased the mass of the islets and their secretory capacity in response to different stimulators. The INGAP modified the expression of two hundred genes in islets of new-borns after 4 days' culture. Among those genes, the ones that express proteins forming the KATP (*Sur*1 and *Kir*6.2 and *Fox*A2) channels making the islets more sensitive to glucose, indicating that the INGAP improved secretion through the increase in the number of KATP channels associated with a better handling of the Ca^{2+} by the pancreatic islets. Finally we also showed that in islets of newborn mice treated with CNTF, a decrease occurred in the activation of caspase-3 (which is one of the main caspases effecting and promoting apoptosis). Associated with this, we observed a decrease in the production of NAD(P)H and reduction in the secretion of insulin.

Participation of UCP2 in the process of the insulin secretion

This project involved diabetic mice with "antisense oligonucleotides" to *Ucp*2 and we evaluated the effects of treatment on the secretion and action of insulin. Swiss mice, made obese through a hyperlipidic diet as well as obese mice ob/ob, treated with the above-mentioned antisense showed a significant improvement in the hyperglycemic syndrome. This improvement was due to an increase in the peripheral sensitivity to insulin, associated with a better secretory response to glucose.

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Antonio Carlos BOSCHERO

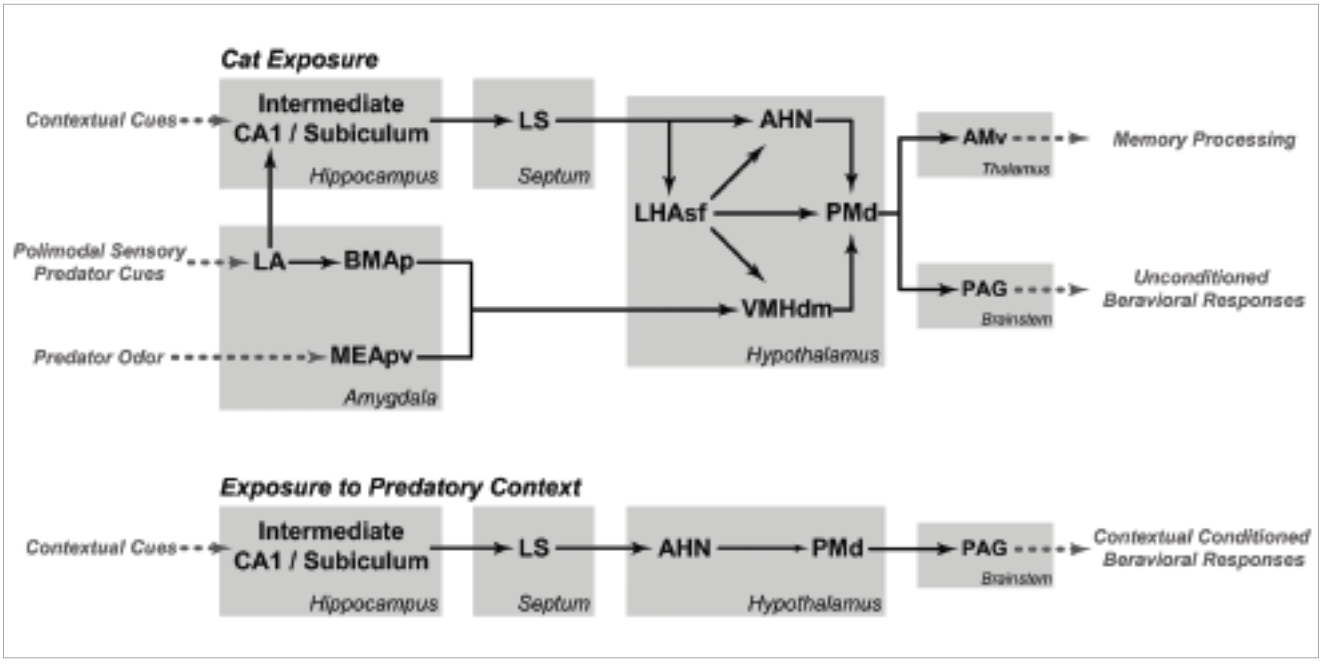
Instituto de Biologia
Universidade Estadual de Campinas
Departamento de Fisiologia e Biofísica
Caixa Postal 6109 – Barão Geraldo
CEP 13084-970 – Campinas, SP – Brasil

+55-19-3521-6202
boschero@unicamp.br

NEURAL BASIS OF MOTIVATED BEHAVIORS

Newton Sabino CANTERAS

Institute of Biomedical Sciences / University of São Paulo (USP)



Schematic diagram showing the putative brain systems involved in processing unconditioned and contextual-conditioned predatory threats, as well as in organizing unconditioned and contextual-conditioned behavioral responses. Cezario et al., 2008

We have proposed a series of studies to deepen our understanding about the neural control of a number of motivated responses, such as defensive behavior, predatory hunting, and social aggression.

These studies include:

1. The study of the neural basis of the anti-predator defensive behavior. We will investigate the cortico-amygdalar paths involved in predator detection. Combining functional mapping and neuronal tract-tracing, we will identify the cortico-amygdalar paths putatively involved in predator detection. We will next determine how these cortical and amygdalar sites contribute to the defensive responses to a predator, by examining behavioral responses during direct exposure to the predator in animals bearing selective NMDA lesions in different elements of this pathway.

2. The role of the main thalamic and sub thalamic targets of the hypothalamic defensive circuit (i.e., nucleus reuniens, ventral part of anteromedial nucleus, and rostral part of zona incerta) in both unconditioned and contextual-conditioned responses to a predatory threat.

3. A comprehensive analysis of the projections from the dorsolateral part of the periaqueductal gray and the cuneiform nucleus, critical sites related to both unconditioned and conditioned responses to predatory threat.

4. The evaluation of the role of the ventrolateral part of caudoputamen in the motor pattern display seen during insect hunting.

5. Using the intruder x resident paradigm, we will study the hypothalamic pattern of activation during the agonistic encounter. We will next evaluate how NMDA lesions placed in the PMv or in the hypothalamic attack area interfere with agonistic responses in both intruder and resident.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our group has made considerable progress in the understanding of defensive behavior and predatory hunting through the development of this thematic project. For the defensive behavior, we have finished a series of studies investigating the role of the hypothalamic systems in organizing anti-predatory defensive responses.

For the systems involved in anti-predatory defensive behavior, we are now investigating the cortico-amygdalar paths involved in predator detection. In addition, we are also evaluating the role of the main thalamic and subthalamic targets of the hypothalamic defensive circuit (i.e., nucleus reuniens, ventral part of anteromedial nucleus, and rostral part of zona incerta) in anti-predatory defensive behavior.

During the study of agonistic interaction, using the intruder x resident paradigm, we had the chance to examine the neural basis of defensive response of the intruder, and found that the hypothalamus has a critical role in fear expression to rival conspecifics. We are currently working on a number of studies to investigate the neural circuits underlying social defensive responses.

We have started this project by evaluating the role of the ventrolateral part of caudoputamen in the motor pattern display seen during insect hunting. According to our observations, the ventrolateral part of caudoputamen is a possible candidate to organize the stereotyped sequence of actions – action syntax – observed during predatory hunting.

For the investigation of predatory hunting, we are currently improving our data on the role of the ventrolateral striatum, making NMDA lesions and observing the pattern of capture during roach hunting. In this project, we have also investigated the role of the Superior Colliculus during the insect hunting. We have found that the lateral part of the CS is critical for prey detection and influences the direction and speed of the movement during prey capture. In addition, the CS also influences motivation to start pursuing the prey.

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Newton Sabino CANTERAS

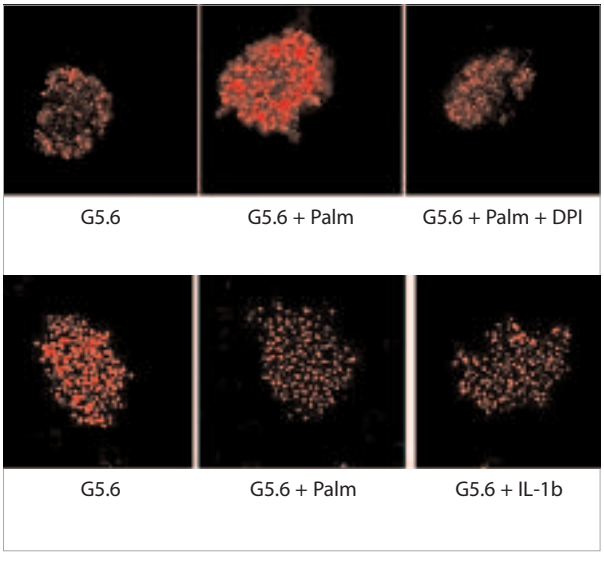
Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Departamento de Anatomia
Av. Professor Lineu Prestes, 2415 – C. Universitária
05508-900 – São Paulo, SP – Brasil

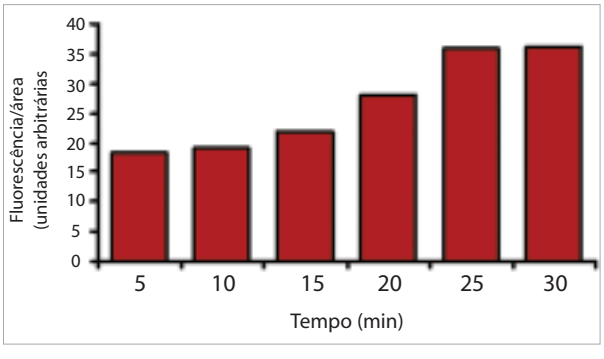
+55-11-3091-7628
newton@icb.usp.br

MOLECULAR MECHANISMS OF THE REGULATION OF THE FUNCTION OF PANCREATIC B CELLS

Ângelo Rafael CARPINELLI

Institute of Biomedical Sciences / University of São Paulo (USP)





Effect of palmitate or IL-1b on ROS production assessed by ethidium fluorescence in isolated pancreatic islets. The production of ROS by isolated rat pancreatic islets was determined by using a hydroethidine oxidation assay. The fluorescence intensity of islets was analyzed by Zeiss confocal microscopy

We first demonstrated that pancreatic B cells express NAD(P)H oxidase, and that this enzyme seems to be involved in superoxide generation during the process of insulin secretion. This study started after our observation that glucose controls antioxidant enzyme activities such as superoxide dismutase (SOD), catalase and glutathione peroxidase. We found that the increase in SOD activity is directly correlated with increasing glucose concentration. Considering that SOD activity increases concomitantly with raises in superoxide generation in several tissues, we examined the same phenomenon in pancreatic B cells. We found not only a direct correlation between the increase of glucose concentration and superoxide generation by isolated rat islets, but also that the production of these compounds was dependent on NAD(P)H. Unpublished results from our laboratory indicate that glucose, palmitate and interleukin-1B increase superoxide generation through NAD(P)H oxidase activity. These compounds also increase the expression of some NAD(P)H oxidase components. The function of NAD(P)H oxidase in the process of insulin secretion and the action of other endogenous substances on the activity of this enzyme were then investigated. Isolated pancreatic islets chronically exposed to high glucose concentration, free fatty acids, or interleukins have shown impaired insulin secretion. These changes are, at least in part, due to the production of reactive oxygen species. We also investigated whether NAD(P)H oxidase could be involved in these process as well. This project will initially study the participation of NAD(P)H oxidase in the molecular mechanisms that regulate the glucose – and palmitate-induced insulin secretion. Among the objectives of this project are the study and the evaluation of the effect of oxidative stress in RINm5F in order to clarify the participation of this enzyme in the impairment of secretory events in pancreatic B cells.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We are evaluating the role of NAD(P)H oxidase in the regulation of the secretion of insulin in isolated islets. We demonstrated that NAD(P)H oxidase is modulated by glucose, palmitate and interleukin -1β which increased the proteic expression of the subunit $p47^{PHOX}$ and the production of superoxide. Hydrogen peroxide (H_2O_2) formed from the superoxide decreased in the presence of high concentration of glucose due to the activation of the pentose path, an important metabolic path responsible largely for the maintenance of the antioxidant activity of the B cells of the pancreatic islet. The palmitate induced the activation of the NAD(P)H oxidase through the membrane receptor GPR40 and/or through its metabolization. Melatonin decreased the production of ROS by pancreatic islets without promoting alteration in the subunits of the NAD(P)H oxidase thus suggesting that the hormone acts by different routes stimulating antioxidant enzymes. In another series of experiments, palmitate and glucose interacted, and altered the expression of some early genes (*C-fos* and *C-jun*) and proteins of the mitogenic paths (ERK1/2, AKT and SAPK/JNK), which could also be related to long term alterations in the secretion of insulin. Oleate in turn increased the expression of insulin induced by 5.6 e 16.7mM of glucose without altering the proteic expression of the *Pkc* and $Gp91^{PHOX}$. In the presence of 16.7 mM of glucose, oleate increased the expression of GPR40, although its expression and the expression the pro-insulin had decreased. Exact like the palmitate, oleate may be acting through its own metabolization and/or via GPR40. Mice fed with a diet rich in medium-chain fatty acids present peripheral resistance to insulin through isolated islets, which however decrease the expression of the hormone and increase the cell death percentage.

Our research has brought important discoveries related to the oxidative stress involved in the initial phases of the onset of Diabetes mellitus.

MAIN PUBLICATIONS

As the project has just commenced, data obtained will lead to publications on due course.

Ângelo Rafael CARPINELLI

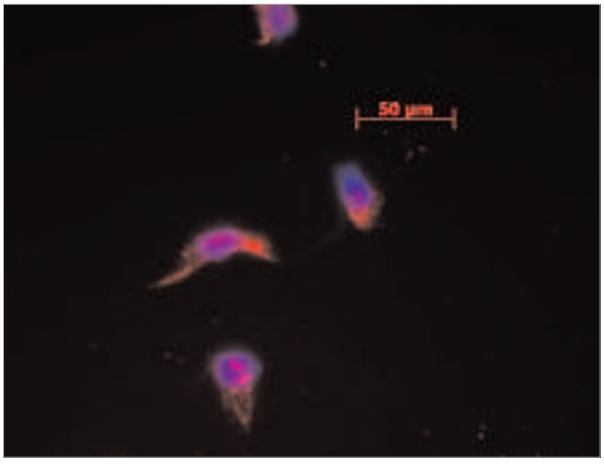
Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Departamento de Fisiologia e Biofísica
Avenida Prof. Lineu Prestes, 1524 – C. Universitária
05508-900 – São Paulo, SP – Brasil

+55-11-3091-7363
angelo@icb.usp.br

COMPARATIVE PHYSIOLOGY OF PERIPHERAL CLOCKS. CLOCK GENES (*Clock, Per*1, *Per*2, *Cry*1 AND *Bmal* 1) AND THEIR MODULATION BY LIGHT AND HORMONES IN FISH, AMPHIBIANS AND MAMMALS

Ana Maria de Lauro CASTRUCCI

Institute of Biosciences / University of São Paulo (USP)



Immunolabeling of ZEM-2S embryonic cells with rabbit anti-serum UF061 (1:2,000) against Danio rerio *melanopsin (red, Cy3-labeled secondary antibody). In blue, nuclear staining with DAPI*

Melanopsin can be expressed, as well as other opsins, in skin pigment cells, to mediate photoresponses. Having in mind that cultured cells may constitute peripheral clocks and respond to visible light, we will analyze: 1. the mechanisms to adjust biological clocks in single cells of teleosts, amphibians and mammals; 2. their regulation by varying photoperiod regimes and hormones. We will then investigate: 1. whether the ZEM-2S embryonic cell line of *Danio rerio*, melanophores of *Xenopus laevis*, and B-16 F10 murine melanoma cells are able to cycle genes such as Per1, independently of light:dark cycles; 2. whether opsin expression is rhythmic, dependent on the integrity of the cellular clock and independent of the light:dark cycle; 3. whether opsin and clock gene expressions may be modified by hormones. These hypotheses will be tested by the quantification of luciferin bioluminescence originated from the activation of luciferase located in *Per*1 promoter, of mRNA (and of proteins whenever possible) of opsins and clock genes, under light:dark cycles, or constant darkness, in the presence of increasing hormone (melatonin, endothelin and α-MSH) concentrations, for increasing periods of time.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

To investigate the photosensitivity of ZEM-2S cells of the teleost *Danio rerio*, we accompanied the proliferation of cells maintained for 5 days on a regime of 14 hours of light by 10 hours of dark (14L:10D) and then transferred into constant darkness (DD), 14L:10D, 10L:14D or constant light (LL), which the rate of cell proliferation observed in cells submitted to constant light being lower. The expression of a photopigment is essential for this photo-sensibility and, in fact, we demonstrated the presence of RNA messenger for melanopsin (*Opn4x*) and for six *Crys* genes. The presence of the melanopsin protein was also demonstrated by immunocytochemistry. We then studied the temporal expression patterns of the genes *Per*1, *Cry*1*b*, *Clock* and *Opn4x* in ZEM-2S cells maintained for 5 days in 12L:12D or DD. In 12L:12D, the expression of *Opn4x* exhibited 2 peaks: at the start of the light phase and at the start of the dark phase. These peaks are also present in cells maintained in constant dark, during which the expression of *Opn4x* was significantly increased at all times, when compared to that observed in cells maintained in light: dark cycle. Although the expression of *Clock* does not vary over the 24 hour period, whether in 12L:12D or DD, it tends to increase during the dark phase and the subjective night, respectively. The clock genes *Per*1 and *Cry*1*b* exhibit robust circadian oscillation, with a significant increase 3h before the light phase, which persists during the entire photo-phase and declines abruptly in the dark phase. In constant dark, the amplitudes of temporal variation of *Per*1 and *Cry*1*b* attenuate, but the circadian rhythm remains significant. However, the peaks of expression appear shifted for the times of transition between subjective day and night. These results demonstrate that the ZEM-2S cells possess an intrinsic clock, since the rhythmicity of expression of the genes of the clock is maintained in constant conditions. Because such cells possess a functional photopigment, melanopsin, the adjustment of this clock can be effected by light. These cells, therefore, constitute an excellent model for the study of regulation mechanisms of peripheral clocks by light and hormones.

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Ana Maria de Lauro CASTRUCCI

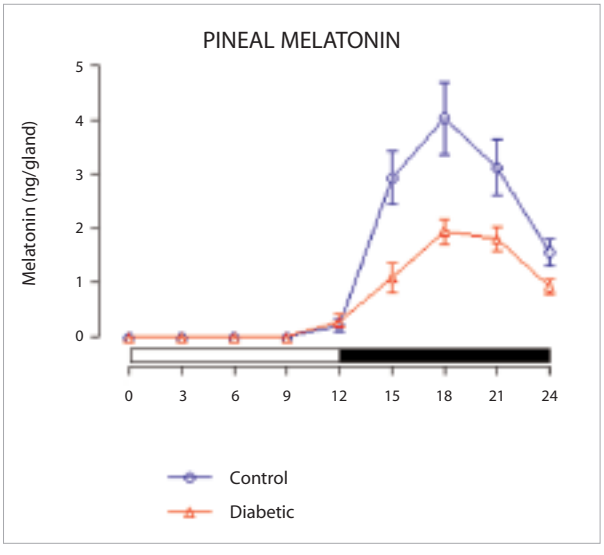
Instituto de Biociências
Universidade de São Paulo (USP)
Departamento de Fisiologia, sala 318
Rua do Matão, travessa 14, 321 – C. Universitária
05508-900 – São Paulo, SP – Brasil

+55-11-3091-7610
amdlcast@ib.usp.br

MELATONIN AND THE CONTROL OF ENERGY METABOLISM: CENTRAL AND PERIPHERAL ACTIONS AND ITS INTERACTION WITH OTHER HORMONES

José CIPOLLA Neto

Institute of Biomedical Sciences / University of São Paulo (USP)



Pineal melatonin contentin control andstreptozotocin-induced diabetic rats

The aim of this work is to study the role played by melatonin in the control of energy metabolism. By using *in vivo* and *in vitro* experiments, in intact or pinealectomized young or old animais, treated or not with melatonin, we are proposing to study the metabolic function and gene expression in the white adipose tissue, skeletal muscular system and the pancreatic B cells and the apoptosis phenomenon induced by fatty acids. Moreover, we intend to study the action of melatonin in association or not with insulin and/or leptin on cellular metabolism and gene expression in specific hypothalamic nuclei or in neurons and/or glia cells maintained in culture. The central nervous system structures to be studied are those mainly involved in the control of energy metabolism and circadian rhythms.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Due to its special characteristics of production and secretion, melatonin is considered the darkness hormone. Its unique feature of being synthesized exclusively at night, regardless of the organism activity pattern and the fact that the duration of the daily secretory episode follows exactly the duration of the night confer to melatonin the very important role of timing the circadian and seasonal biological rhythms of the organism in order to adapt it to the regular daily and annual environmental fluctuations.

Therefore, it is not surprising that it is possible to find scientific data demonstrating the effect of melatonin on almost all physiological processes, such as sleep-wakefulness, reproduction, aging, immune and inflammatory responses, cardiovascular reactions, energy metabolism including insulin secretion and action on adipose tissue function and weight regulation, among others.

The aim of this project is to study the melatonin effects on the regulation of energy metabolism and its implication on diabetes and obesity control and aging.

Pinealectomized animals develop a diabetogenic syndrome characterized by insulin resistance and 50 % reduction of GLUT4 in adipose and muscular tissue. This dramatic picture can be partially or totally restored by melatonin reposition or restricted feeding.

Melatonin, in addition, by acting through MT1 membrane receptors, is able to induce insulin receptor phosphorylation, at the same time as it mobilizes several intracellular transduction steps that are common to insulin signaling. Moreover, it was demonstrated that the absence of melatonin in pinealectomized animals impairs the temporal organization of several metabolic functions associated to the carbohydrate metabolism, such as daily insulin secretion, adaptation to starvation and exercise. The same melatonin timing action was demonstrated *in vitro*, in adipocyte cultures synchronized to 24h cycle of melatonin administration. In this experimental condition, the expression of some clock genes, particularly *Bmal*1 and *Clock*, and the lipogenic and lipolytic functions were synchronized to a particular phase of the *in vitro* daily melatonin cycle. In addition, it was demonstrated that melatonin given to old animals is able to reduce the insulin resistence in several tissues and to reduce body weight.

As a corollary of the above described actions, it was demonstrated that insulin can act on *in vitro* pineal glands by potentiating the noradrenergic-induced melatonin synthesis, and regulating the activity of the enzymes tryptophan hydroxylase and N-acetyltransferase through post-transcriptional mechanisms.

Most importantly, it was demonstrated, by using the pineal microdialysis technique, that streptozotocin-diabetic rats show a 50% reduction in the nocturnal melatonin production, which contributes for aggravating the diabetic syndrome.

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José CIPOLLA Neto

Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Departamento de Fisiologia e Biofísica
Avenida Prof. Lineu Prestes,1524 – C. Universitaria
CEP 05508-000 – São Paulo, SP – Brasil

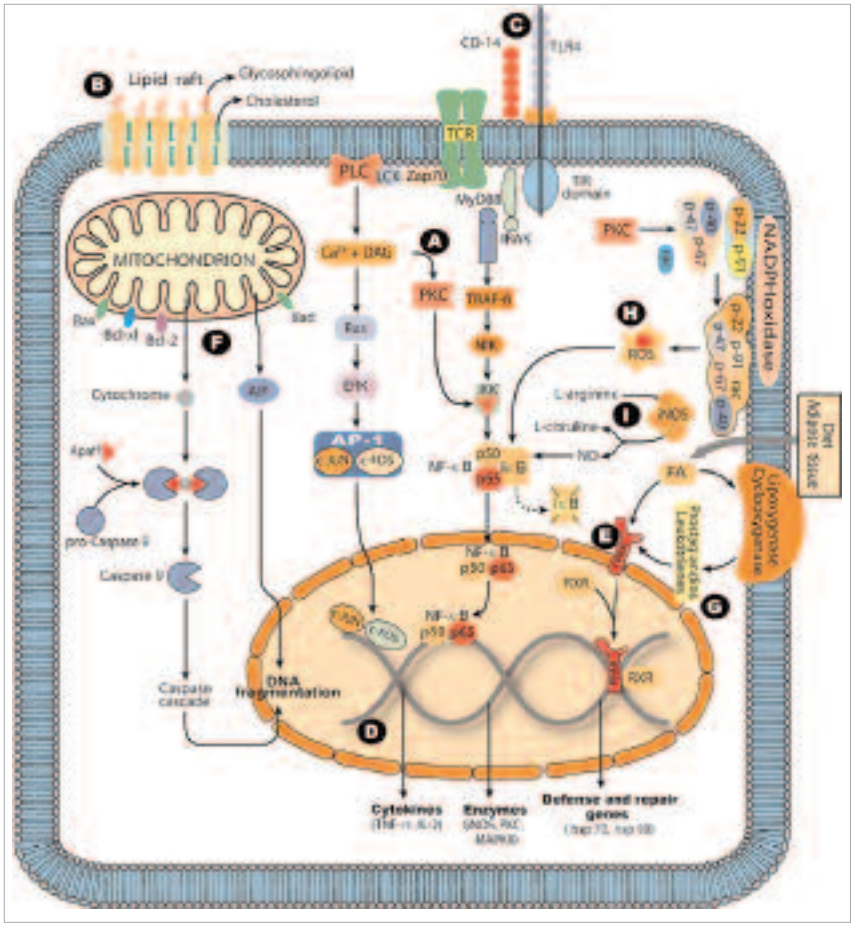
+55-11-3091-7466
cipolla@icb.usp.br

ROLE OF FATTY ACIDS IN THE CONTROL OF LEUKOCYTE FUNCTION AND IN THE ESTABLISHMENT OF DIABETES

Rui CURI

Institute of Biomedical Sciences / University of São Paulo (USP)

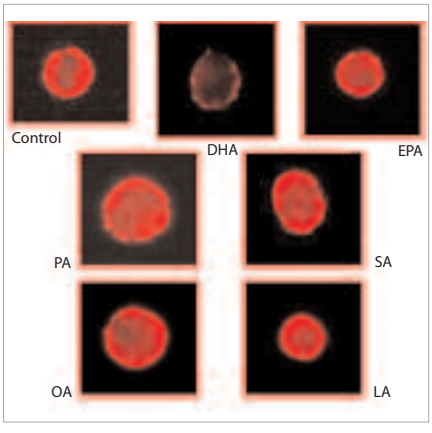
Previous studies of our group have determined that fatty acids (FA) can function as signaling factors in intercellular interactions, regulating several aspects of leukocyte function (mainly lymphocytes and macrophages) and insulin secretion by beta cells of pancreatic islets. In this project we focus the investigation on the mechanisms of action of fatty acids. The first site of interaction of fatty acids in the cell is the plasma membrane which leads us to investigate the interactions between FA and the lipid bilayer in model membranes and isolated cells. The effects of FA in the intracellular signaling pathways will be investigated as well as the involvement of receptors (like the PPAR) and transcription factors (NFÎB and AP1). In Langerhans islets the effects of FA on the insulin signaling pathways and on the activation of the enzymatic complex NADPH oxidase (that was identified by us in rat Langerhans islets) will be investigated. The toxicity of FA has been clearly evidenced by our group in lymphocytes and macrophages and will be now studied in neutrophils, by using *in vivo* and *in vitro* models. The persistence (or exacerbation) of the effects of FA administration, particularly fish oil, will be assessed by its administration during two generations of rats. These issues complete our investigation on the mechanisms of FA action. It is necessary to determine whether the toxicity of FA occurs indistinctly for all leukocytes and whether the use of fish oil (as alimentary supplement) can modify the immune function and ensure a better life quality to our descendents.



SUMMARY OF THE POSSIBLE MECHANISMS BY WHICH FATTY ACIDS MODULATE LEUCOCYTE FUNCTION
(A) Activation of intracellular signalling pathways; (B) activation of lipid-raft-associated proteins; (C) binding to Toll Like Receptors; (D) regulation of gene expression; (E) activation of transcription factors; (F) induction of cell death; (G) production of eicosanoids; (H) production of reactive oxygen species; and (I) production of reactive nitrogen species. Apaf-1, apoptotic protease-activating factor-1; AIF, apoptosis-inducing factor; Bax, Bcl-2 associated X protein; Bad, Bcl-2-associated death promoter; Bcl-xl, B-cell lymphoma X (long form); IKK, IÎB kinase; IRAK, IL-1 receptor-associated kinase; LK, leucocyte-specific protein tyrosine kinase; MyD88, myeloid differential factor 88; NIK, NFÎB-inducing kinase; TRAF-6, TNFR-associated factor 6; TIR, Toll/IL-I receptor/resistance domain; Zap70, zeta-chain-associated protein kinase 70

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Macrophage and lymphocyte convert glucose and glutamine into lipids (fatty acids, phospholipids and cholesterol). These lipid molecules are accumulated inside the cells, released to the medium or transferred to leukocytes and other cell types such as insulin-secreting cells. This phenomenon regulates several functions of the acceptor cells, such as lymphocyte proliferation and macrophage phagocytosis. This is a new intercellular communication mechanism that may play an important role in certain tissue microenvironments.



Effect of docosahexaenoic (DHA), eicosapentaenoic (EPA), stearic (SA), palmitic (PA), oleic (OA) and linoleic (LA) acids on the distribuition of lipid rafts in the membrane. Lymphocytes were marked with CT-B conjugated to 594-Alexa. Fluorescence was then monitored by fluorescence microscopy under 100x magnification. Cells were evaluated by fluorescence microscopy. All images are from a representative experiment involving three different assays with similar results

Dietary lipids regulate various leukocyte functions such as lymphocyte proliferation, macrophage and neutrophil phagocytosis, and production of nitric oxide, reactive oxygen species and cytokines. The fatty acids also control gene expression and phosphorylation of proteins as those of the interleukin-2 signaling pathway in lymphocytes.

High plasma levels of fatty acids (as observed in prolonged exercise, fasting and diabetes) are associated with the occurrence of leukocyte death. In critically ill patients, the lipid content of parenteral diet also causes leukocyte death. In turn, linoleic and oleic acids accelerate the wound healing process and ω-3 fatty acids present beneficial effects on tumor growth and cachexia.

Saturated fatty acids decrease the activity of the insulin signaling pathway, cause oxidative stress, raise nitric oxide production, and impair mitochondrial function in skeletal muscle. On the other hand, high levels of fatty acids lead insulin-secreting cells to death. This effect involves changes in protein phosphorylation and production of reactive oxygen species. The presence of NADPH oxidase activity in pancreatic beta cells was shown for the first time as well as its regulation by glucose, fatty acids and cytokines. These effects of the fatty acids might be involved in the establishment of diabetes (types I and II).

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Rui CURI

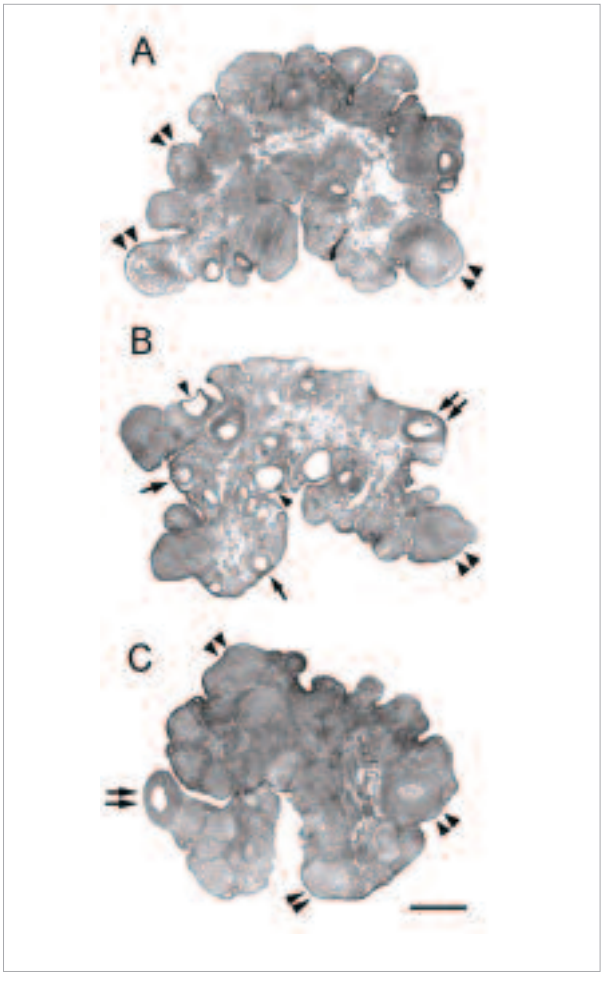
Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Avenida Professor Lineu Prestes, 1524 – Butantã
05508-900 – São Paulo, SP – Brasil

+55-11-3091-7245
ruicuri@icb.usp.br

NEUROENDOCRINE REGULATION AND EFFECTS OF STRESS ON THE REPRODUCTIVE FUNCTION OF FEMALE RATS

Janete Aparecida Anselmo FRANCI

Ribeirão Preto School of Dentistry / University of São Paulo (USP)

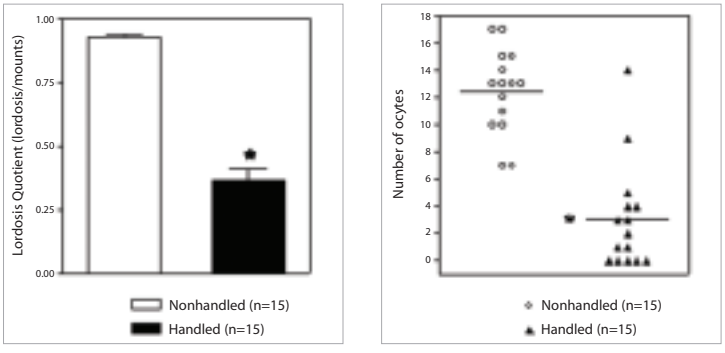


Cold stress effect on ovarian morphology. Ovarian morphology on estrus of rat maintained at ambient temperature (A). Exposed to 8 weeks of cold stress with no surgical treatment (B) or with previous LC lesion (C). Arrow: antral follicle with hyperthecosis. Double arrow: type III follicle. Arrowhead: follicular cyst. Double arrowhead: corpus luteum. Scale bar = 1 mm. Bernuci et al, Endocrinology, in press.

The knowledge of the control mechanisms of fertility has implications in social (e.g., the option of conception or anti-conception in humans, populational control, and therapeutic development), economic (e.g., improvement of the fertility of domestic or captive wild species to provide food and clothing) and environmental (e.g., maintenance or reestablishment of ecosystems equilibrium, environment repopulation) aspects. Natural reproduction depends on complex interactions of hormones produced in the brain, pituitary, gonads and other organs and tissues in order to prepare the gametes and induce sexual behavior to ensure fertilization. It is well known that fertility can be markedly influenced by stress, however the interaction between the hypothalamus-pituitary-gonadal axis (HPG) and stress system (hypothalamus-pituitary-adrenal axis, sympathetic system and neurotransmitters brain systems) remains poorly understood. Gonadotropin-releasing hormone (GnRH) neurones constitute the final output pathway of the neuronal network controling gonadotropins preovulatory surges and ovulation. This neuronal network depends on direct and indirect actions of several neuromediators whose specific roles are little known, and it is also a pathway through which the stressful stimuli may interfere on gonadotropins release and ovulation. This project investigates: 1) neural circuits and neuromediators (norepinephrine, angiotensin II, neuropeptide Y, nitric oxide, leptin, serotonin, oxytocin) involved in tonic and cyclic control of gonadotropins and prolactin secretion as well as their modulation by ovarian steroids; 2) the neuroendocrine and sympathetic control of ovarian function; and 3) the effects of stressor stimuli in the neonatal period or in adulthood on the control of reproductive functions.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We have identified important components of the GnRH neuronal network that regulate gonadotropin release as well as mediate stress effects on reproductive function. It was shown that receptors for neuromediators are expressed in neurons of the GnRH network, and that estradiol and progesterone modulate the ovulation process through their receptors in neurons by producing neurotransmitters such as norepinephrine, serotonin, neuropeptide Y, oxytocin and angiotensin. Noradrenergic neurons of locus coeruleus (LC), classically implicated in the response to stress, are also related to the reproductive function. Thus, the control of both functions by the same neurons provides a link between stress and reproduction. We have found that brief maternal separations during the first 10 postnatal days induce definitive and stable marks in few specific areas of the central nervous system as an important reduction in the number of LC neurons, which, in turn, lead to infertility during their adult life, characterized by a reduction in sexual behavior, in the preovulatory surge of luteinizing hormone (LH), hormone responsible for ovulation, and therefore, a decrease in the ovulation rate. In addition, chronic cold stress induced activation of the LC neurons and a polycystic ovary syndrome (PCOS) condition characterized by the presence of follicular cysts in the ovary, increased estradiol and testosterone plasma levels, irregular estrous cyclicity and reduced ovulation. The involvement of the central norepinephrine in the development of PCOS was proven since rats with LC lesion did not develop PCOS in response to cold stress. These data open a new field to investigate the etiology of the PCOS and suggest that attenuation of the stress effects, such as decrease in the central noradrenergic tonus, could help diminish the symptoms of PCOS and increase fertility in women presenting this syndrome. We also demonstrated that ovarian steroids modulate AT1 receptor expression in the brain which may mediate the deleterious effects of stress.



Neonatal handling decreases sexual behavior and ovulation rate. Lordosis quotient was measured on the evening of proestrus day. The number of oocytes was counted on the estrus day and e symbol represents one animal. Significantly different from the nonhandled group. Raineki et al, Neuroendocrinology in press*

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Janete Aparecida Anselmo FRANCI

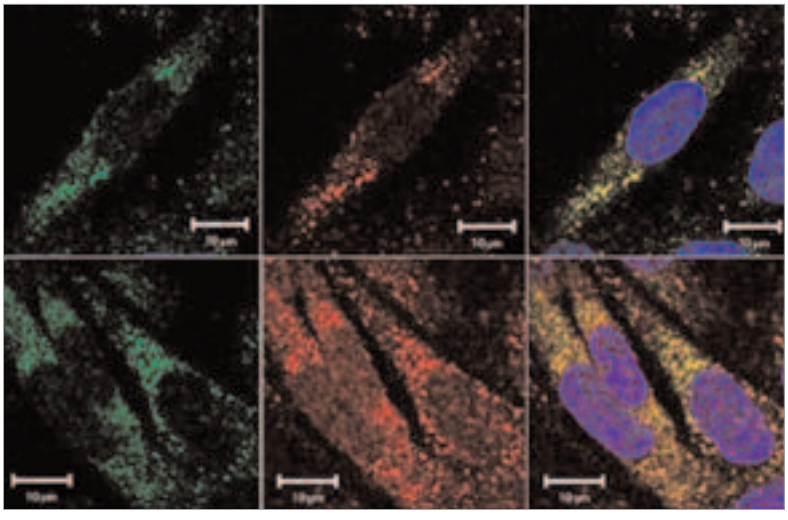
Faculdade de Odontologia de Ribeirão Preto
Universidade de São Paulo (USP)
Laboratório de Neuroendocrinologia I
Avenida do Café, s/n
CEP 14040-904 – Ribeirão Preto, SP – Brasil

+55-16-602-4032
jaafranc@usp.br

COUPLING OF ENDOPLASMIC RETICULUM STRESS TO OXIDATIVE STRESS IN VASCULAR CELLS VIA INTERACTION BETWEEN PROTEIN DISULFIDE ISOMERASE AND NAD(P)H OXIDASE: ROLE OF THIOL OXIDOREDUCTASES IN REDOX SIGNALING

Francisco Rafael Martins LAURINDO

Heart Institute (Incor)



PDI co-localizes with Nox1 and Nox4 in vascular smooth muscle cells (Janisziewski et al.: J. Biol. Chem. 2005)

Our laboratory has focused over the last several years on studying aspects of the molecular physiology of vascular redox processes. Our studies brought us to vascular NAD(P)H oxidase, a major source of reactive oxygen species (ROS). Search for putative regulatory mechanisms of this enzyme led us to the identification of protein disulfide isomerase (PDI), a thiol oxidoreductase chaperone of the endoplasmic reticulum (ER), which closely associates with vascular smooth muscle cell NAD(P)H oxidase and regulates its activation secondary to angiotensin II. Given the combination of PDI redox sensitivity with its known role in the control of protein traffic and secretion, our results provide a novel model for understanding the NAD(P)H oxidase regulation, and consequently the cell redox status. The overall aim of our project is to further our mechanistic understanding and to explore more thoroughly some consequences of the interaction between PDI and NAD(P)H oxidase, in connection with relevant pathophysiological phenomena linked to atherosclerosis and tobacco exposure. A general hypothesis that is central to this proposal is that the interaction between PDI and NAD(P)H oxidase provides an integrative pathway for coupling between ER stress – a frequent condition in which PDI undergoes membrane traffic and may be overexpressed – with oxidative stress linked to NAD(P)H oxidase activation. The proposed investigations may reveal an innovative approach to understand why, how and where oxidative stress occurs in the vascular system. Accordingly, in addition to protocols aimed at defining basic mechanisms underlying PDI-oxidase interaction, our hypothesis will also be tested in different models that are relevant to the pathogenesis and clinical manifestations of atherosclerosis, with emphasis on vascular remodeling. Thus, we will investigate the characteristics of NAD(P)H oxidase and PDI-dependent oxidative and ER stress in models of oscillatory shear stress, vascular response to injury, and vascular and aortic valve calcification.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We showed that protein disulfide isomerase, a dithiol disulfide oxidoreductase chaperone form in the endoplasmic reticulum (ER), displays physical and spatial interaction with the NADPH oxidase complex, assisting in its activity. This led us to assess whether oxidative stress integrates with ER stress through this pathway. Our findings indicate that: a) ER stress promotes oxidative stress; b) oxidative stress sustains both antiapoptotic and proapoptotic branches of ER stress signaling; c) ER stress promotes transcription of the Nox4 NADPH oxidase isoform, which contributes to apoptosis; d) PDI is a key integrator of oxidative and ER stress, at least in part due to its interaction with Nox4; e) vascular response to injury carries an important ER stress. These results opened many further investigations related to molecular mechanisms of PDI/NADPH oxidase interaction and the role of ER stress-associated redox processes in cell senescence. Moreover, those mechanisms integrate to ongoing and prior studies from our laboratory showing that oxidative stress is an important feature of vascular response to injury, which itself is a basic process of atherosclerosis and restenosis post-intervention.

We recently provided novel evidence indicating that a similar process occurs in degenerative aortic valve stenosis, a common disease in the elderly. Our results, both in human valves and from a rabbit model, showed important generation of oxidant species around calcifying foci in stenotic valves, by cells displaying phenotypic markers of osteoblasts/osteoclasts. Importantly, administration to rabbits of the antioxidant lipoic acid prevented valve calcification, but the unrelated antioxidant tempol led to increase in this process, indicating a complex interplay of redox events. One such level of complexity is redox compartmentation.

We have also provided several evidences for the contribution of specific microparticles derived from platelets and endothelial cells to redox processes. Also, we have now results showing a cross-talk between mitochondria, the major source of reactive species and the NADPH oxidase complex, the main dedicated source of signaling reactive species.

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Francisco Rafael Martins LAURINDO

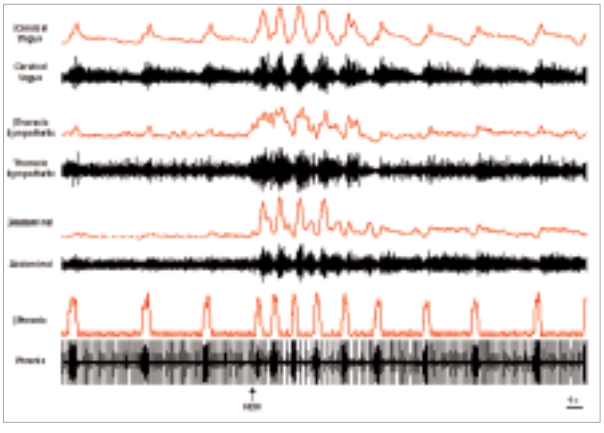
Faculdade de Medicina
Universidade de São Paulo (USP)
Instituto do Coração - Lab. de Biologia Vascular
Av. Dr. Enéas de Carvalho Aguiar, 44
9º andar, bloco II – Cerqueira César
CEP 05403-000 – São Paulo, SP – Brasil

+55-11-3069-5185
expfrancisco@incor.usp.br

CENTRAL MECHANISMS INVOLVED IN THE SYMPATHOEXCITATION IN RESPONSE TO HYPOXIA

Benedito Honório MACHADO

Ribeirão Preto School of Medicine / University of São Paulo (USP)



Raw and integrated (f) simultaneous recordings of the activity of cervical vagus, thoracic sympathetic, abdominal and phrenic nerves in the working-heart brainstem preparation during the activation of peripheral chemoreflex with potassium cyanide (arrow) in a rat previously submitted to chronic intermittent hypoxia

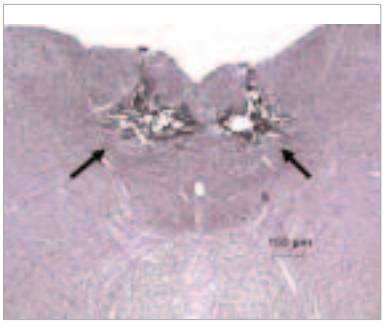
Hypoxia is a possible physiological and pathophysiological situation that plays a major role in the activation of the peripheral chemoreceptors, which produce the correspondent autonomic, respiratory and behavioral responses in order to provide the system with the appropriated level of oxygen in the arterial blood. The acute chemoreflex activation produces the necessary increased activity in the sympathetic nerve to provide the cardiovascular system with the level of vascular resistance required to increase the arterial blood flow to the upper part of the body and preserve the central nervous system from any hypoxic situation. However, the chronic activation of the peripheral chemoreceptors in physiopathological circumstances, such as the chronic intermittent hypoxia, may result in a persistent increase in the level of the sympathetic outflow, which, in turn, may result in arterial hypertension, i.e., another physiopathological situation.

The main focus of this project is the study of several aspects of the neurotransmission of the chemoreflex in different areas of the brain involved in the generation and modulation of the sympathetic nerve activity. Among several neurotransmitter systems, we will evaluated the possible role of the glutamatergic and purinergic systems in the processing of the sympathoexcitatory component of the chemoreflex in the nucleus tractus solitary (NTS), rostral ventrolateral medulla (RVLM) and the paraventricular nucleus of the hypothalamus (PVN), due to a series of previous experimental evidences about the possible involvement of these systems.

The work to be conducted is based upon our previous experience with the pharmacological studies of the brainstem areas in awake and anesthetized rats as well as our more recent experience with electrophysiology and immunocytochemistry. Two major experimental models are envisaged: the acute (KCN) and the chronic activation of the peripheral chemoreceptors. The experiments to be performed as well as the different experimental protocols to be used are divided in 13 sub-projects: functional and pharmacological (7), electrophysiological (4) and immunohistochemical approaches (2).

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The double antagonism of L-glutamate and ATP receptors in the NTS of awake rats produced a large increase in the baseline MAP and we used sodium nitroprusside infusion to normalize MAP. Under this experimental condition, chemoreflex was activated, and we verified that the double antagonism of L-glutamate and ATP receptors almost abolished the pressor response to chemoreflex activation, an antagonism that was reversible. Considering that the record of the sympathetic nerve activity in awake rats, combined with microinjections into the NTS, is not a simple task, we decided to use the working heart-brainstem preparations (WHBP) to verify the effect of bilateral microinjections of PPADS into the commissural NTS on the autonomic and respiratory responses to chemoreflex activation. The data obtained showed that the chemoreflex responses were not affected by bilateral microinjection of PPADS, an antagonism of P2X receptors. On the next experimental protocols in the WHBP, we used the double antagonism with kynurenic acid and PPADS. This combination, similarly to the findings in awake rats, was effective in blocking the sympathoexcitatory response to chemoreflex activation. Therefore, the data obtained in the WHBP not only confirmed he previous observation in awake rats, but also extended it to the concept that the effective antagonism of the sympathoexcitatory component of the chemoreflex was possible only when we combined the antagonism of L-glutamate and ATP receptors in the caudal commissural NTS. The findings that only the double antagonism was effective in blocking the sympathoexcitatory component of the chemoreflex open several interesting perspectives for further studies to better evaluate the central mechanisms involved in hypertension induction.



Example of a coronal section of the brainstem showing the microinjection sites (arrowheads) at the caudal portion of the rat solitary tract nucleus

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Benedito Honório MACHADO

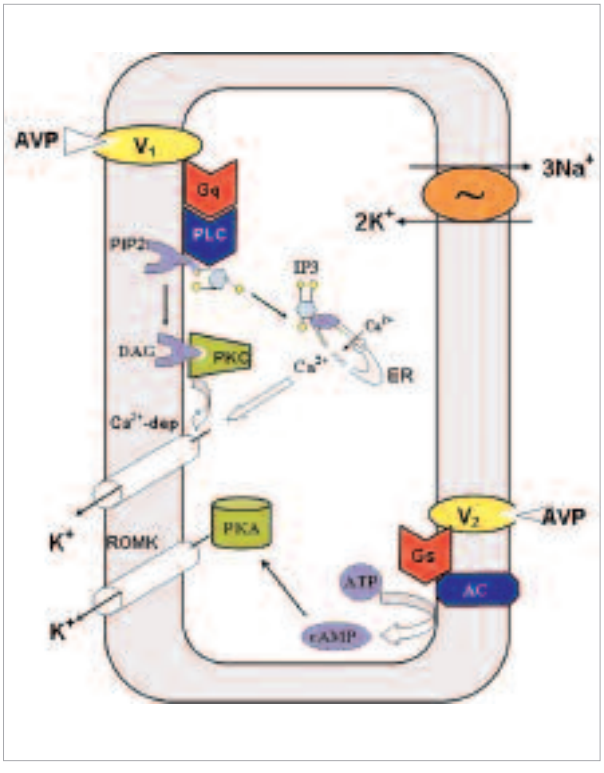
Faculdade de Medicina de Ribeirão Preto
Universidade de São Paulo (USP)
Departamento de Fisiologia
Avenida Bandeirantes, 3.900 – Monte Alegre
CEP 14049-900 – Ribeirão Preto, SP – Brasil

+55-16-602-3015
bhmachad@fmrp.usp.br

MOLECULAR AND FUNCTIONAL STUDIES OF MEMBRANE ION TRANSPORTERS

Gerhard MALNIC

Institute of Biomedical Sciences / University of São Paulo (USP)



Schematic drawing of the action of vasopressin on a principal cell of the collecting duct or of the connecting segment. V1 and V2, vasopressin receptors; G, G-proteins; PKA, protein kinase A; PKC, protein kinase C; AC, adenylate cyclase; ER, endoplasmic reticulum; AVP, arginine-vasopressin

The general objective of this project is the investigation of the molecular and functional mechanisms of ion transport in cells, particularly of the epithelial type, originating from renal and other tissues. Among the methods that will be used for this purpose are renal micropuncture and microperfusion, molecular biology including transfection of wild type and mutant transporters into cultured cells, electrophysiology ("patch-clamp") for the analysis of individual ion channels, determination of cell volume regulation and the role of ion transporters in this regulation, measurement of cell ion activities by fluorescence microscopy allowing for the determination of cell pH and calcium levels. These studies will be performed in mammalian kidney, intestine, colon crypts, cells in primary and permanent cultures such as MDCK, T84, IRPTC and others. Transporters of H^+, HCO3- and K^+ will be investigated, including Na^+/H^+ exchangers, H^+ and H^+/K^+ ATPases, K^+ channels, $Cl^-/HCO3^-$ exchangers, and $Na^+/HCO3^-$ cotransporters, passive mechanisms, and the role of hormones (aldosterone, angiotensin, vasopressin, atrial natriuretic factor, parathyroid hormone) in the regulation of these mechanisms will be studied. Techniques for the determination of transepithelial and transmembrane (apical and basolateral) ion fluxes using microelectrodes or cell fluorescence will be used. Molecular properties of transporters such as the isoforms of the Na^+/H^+ exchanger and of protein kinase C, their genetic modulation and the role of protein regulators (NHERF) in the regulation of the transfer of H^+ will be investigated. Cell signaling of the regulation of H^+ and K^+ ion transport will be studied in different experimental conditions.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Fluorescence and confocal microscopy: Cell pH and calcium level were determined by fluorescence techniques (BCECF for pH and Fluo 4 for Ca) in cultured renal and intestinal cells in order to study the signaling mechanisms of H^+ and Ca^{2+} transport, as well as other regulation mechanisms by angiotensin and vasopressin.

The effects of aldosterone on the intracellular pH recovery rate (pHirr) via Na^+/H^+ exchanger and on the cytosolic free calcium ($[Ca^{2+}]i$) were investigated in rat S3 segment *in vitro*. Aldosterone [10^{-12}, 10^{-10} or 10^{-8} M with 1 h, 15 or 2 min preincubation (pi)] caused a dose dependent increase in the pHirr, but aldosterone (10^{-6} M with 1 h, 15 or 2 min pi) decreased it.

Microperfusion of renal tubules *in vivo*: The direct action of aldosterone (10^{-12} M) on net bicarbonate reabsorption ($JHCO_3^-$) was evaluated by stationary microperfusion of *in vivo* middle proximal tubule (S2) of rat kidney, by using H^+ ion-sensitive microelectrodes. Aldosterone in luminally perfused tubules caused a significant increase in $JHCO_3^-$. Aldosterone perfused into peritubular capillaries also increased $JHCO_3^-$ when compared with basal levels during intact capillary perfusion with blood.

Studies in potassium channel (ROMK) knockout mice were performed, showing that the loss of these channels was largely compensated by maxi-K channels, which are calcium and PD dependent. In rat studies it was shown that vasopressin acts on K^+ transport by V1 (luminal) and V2 (basolateral) receptors, the former mediated by maxi-K channels.

Studies are focused on the Na^+/H^+ exchanger NHE3: We have investigated the mechanisms of chronic regulation of NHE3 by analyzing its promoter activity under influence of changes in pH, and at the parathyroid (PTH) and angiotensin II (AII) levels.

Role of dipeptidyl peptidase IV (DPPIV) in the function of NHE3: Rats were fed for seven days a specific inhibitor of DPPIV. NHE3 activity was depressed due to lower expression of this transporter in the microvilosities of the proximal tubule brush-border, causing natriuresis, diuresis and a more alkaline urine compatible with NHE3 inhibition.

Phenylpropenes are a class of substances produced by angiosperm plants, among which eugenol is included, with a variety of biological functions. In electrophysiological experiments we have demonstrated that eugenol and a series of analog compounds block the action potential firing in mammalian nerves through a reversible, fast inhibitory action on voltage-gated sodium channels.

Purinergic receptors: Fluctuations in the intracellular calcium concentration of satellite cells kept in fresh cultures of cells isolated from the dorsal root ganglion of rats, as measured by fluorescence microscopy, indicate the presence of purinergic receptors in the satellite cells. In addition, our data are consistent with such receptors being of the P2Y, metabotropic type.

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Gerhard MALNIC

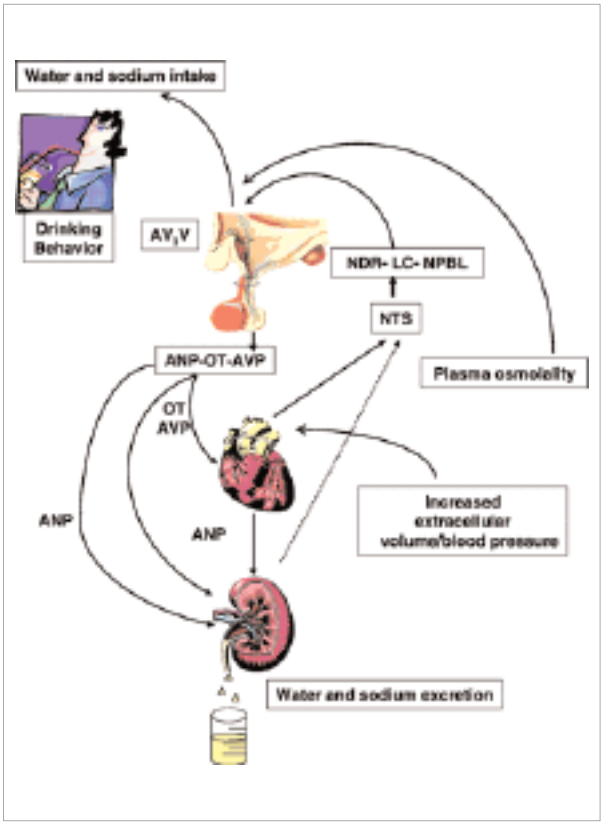
Instituto de Ciências Biomédicas
Universidade de São Paulo (USP)
Departamento de Fisiologia e Biofísica
Av. Prof. Lineu Prestes 1524 – Butantã
05508900 – São Paulo, SP – Brasil

+55-11-3091-7280
gemalnic@usp.br

INTEGRATIVE STUDIES OF THE BODY FLUID HOMEOSTASIS: PHYSIOLOGICAL AND MOLECULAR ASPECTS OF THE NEUROENDOCRINE CONTROL AND CLINICAL AND EXPERIMENTAL EVALUATION

José Antunes RODRIGUES

Ribeirão Preto School of Medicine / University of São Paulo (USP)



Schematic diagram of the atrial natriuretic peptide (ANP) neuroendocrine control of ANP release. For explanation, see text. OT, oxytocin; AVP, arginine vasopressin; NTS, nucleus tractus solitarius

The hypothalamo-neurohypophysial system plays a fundamental role in the maintenance of body fluid homeostasis by secreting vasopressin and oxytocin in response to osmotic and volume changes of the extracellular volume. ANP (atrial natriuretic peptide) is mostly localized in the heart, but ANP and its receptor are also found in hypothalamic and brainstem areas involved in body fluid volume and blood pressure regulation. Blood volume expansion acts not only directly on the heart, by stretch of atrial myocytes to increase the release of ANP, but also on the brain ANPergic neurons through afferent inputs from baroreceptors. The activation of the neuroendocrine pathways involved in the control of body fluid homeostasis induce: 1) modifications in the water (thirst) and salt intake; 2) alterations of the autonomic nervous system; 3) activation of the rennin-angiotensin-aldosterone system (SRAA); 4) Vasopressina (AVP) and Oxytocin (OT) secretion from neural lobe; 5) and of the atrial natriuretic peptide (ANP) from the heart.

We are interested in the determination of the neuroendocrine pathways, as well as the main phenotypes, the participation of the hypothalamus-hypophyseal-adrenal axis, both in normal conditions and under endotoxic shock, involved in the control of body fluid homeostasis. We will also use an experimental model of diabetes insipidus in rats to evaluate the HPA axis in the absence of magnocellular AVP. Molecular studies and the interaction of ANP and the rennin angiotensin system will be conducted in patients with congenital adrenal hyperplasia under basal conditions and after head down tilting.

This thematic project emphasizes the role played by brain ANP and its interaction with neurohypophyseal hormones in the control of body fluid homeostasis and includes:

- Project 1: The neuroendocrine control of hydroelectrolytic balance
- Project 2: Regulation of Oxytocin (OT), vasopressin (AVP) and prolactin release during the experimental septic shock
- Project 3: Regulation of hypothalamus-hypophyseal-adrenal axis (HHA)
- Project 4: Central diabetes insipidus
- Project 5: Congenital adrenal hyperplasia and the hydroelectrolytic homeostasis.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This thematic project emphasizes the role played by brain ANP and its interaction with neurohypophyseal hormones in the control of body fluid homeostasis and includes:

-Following isotonic blood volume expansion (EVEC), the expression of FOS protein in oxytocinergic neurons (FOS-OT) indicates their activation, whereas vasopressinergic neurons (Fos-AVP) are inhibited, which correlates with plasma OT and AVP levels. These effects were inhibited by dexamethasone pretreatment.

The FOS expression in different cell populations of the PVN can be differentially regulated by short- and long-term absence of glucocorticoid negative feedback and also by stress-related excitatory and/or inhibitory neural inputs.

Under stress conditions, there is an activation of several systems, including the autonomic, neuroendocrine system (hypothalamic-pituitary-adrenal axis) and cardiovascular system. Nitric oxide has been implicated in the variations of plasma concentrations of several hormones (prolactin, AVP, and OT) in response to stress induced by lypopolysacharide. The release of AVP induced by endotoxemia involved the production of NO from iNOS that inhibit AVP secretion, and consequently fall of the mean arterial pressure.

High ACTH and corticosterone levels found in rats with pituitary stalk compression under water intake and salt loading conditions suggest an upregulation of the HHA axis, with a preserved adaptive mechanism to chronic stress.

Mineralocorticoid deficiency in 21-OH deficiency is counteracted by a decreased ANP secretion in order to preserve fluid and electrolyte homeostasis.

Mutations in the Hsd3b2 gene induce elevated basal and ACTH-stimulated delta 5-17P levels and delta5-17P/cortisol ratios. Therefore, these data refine the hormonal criteria proposed to predict more accurately 3beta HSD2 deficiency.

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José Antunes RODRIGUES

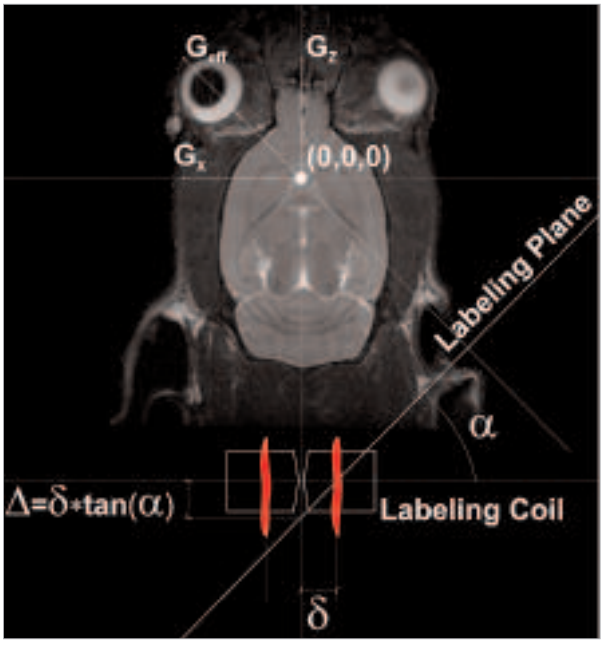
Faculdade de Medicina de Ribeirão Preto
Universidade de São Paulo (USP)
Departamento de Fisiologia – Monte Alegre
CEP 14049-900 – Ribeirão Preto, SP – Brasil

+55-16-3602-3012
antunes@fmrp.usp.br

CENTER FOR IMAGING AND *IN VIVO* MAGNETIC RESSONANCE SPECTROSCOPY FOR STUDYING ANIMAL MODELS

Alberto TANNUS

São Carlos Physics Institute / University of São Paulo (USP)



Example of technological interface: Arterial Spin Labeling measurement of Arterial Perfusion Territories by using a localized labeling RF coil. The figure shows the diagram of the labeling scheme used to label blood flowing in just one of the carotid arteries of a Sprague-Dawley rat

CInAPCe is an abbreviation for the Portuguese expression *Cooperação Interinstitucional de Apoio a Pesquisas sobre o Cérebro* (Inter-institutional Cooperation to Support Brain Research).

The central biological question of our proposal is the investigation of basic mechanisms that lead to epilepsy and related seizure disorders. Our goals are to develop new methods and techniques to improve the understanding of mechanisms of damage, plasticity and repair in epilepsy; and to apply these results to improve diagnosis, prevention and treatment of patients with epilepsy. The main motivation to constitute the CInAPCe Project came from the necessity of approaching this relevant and complex biological problem by combining the expertise of research groups with distinct and complementary backgrounds.

Our mission is to host the center for Animal Model studies as a Main Research Center (MRC) of the Fapesp/ CInAPCe program at the São Carlos Physics Institute – University of São Paulo (IFSC – USP). The proponent group of researchers and collaborators will be responsible for the definition of requirements of this high field Magnetic Resonance animal system, its operation and the hardware and software developments necessary to carry out the experiments for animal studies. Also under the responsibility of the researchers at this center development and adaptation of MRI/MRS methodologies to be used on the other centers of the CInAPCe network where human studies are being conducted.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The Center associated to this project is on its very beginning. All necessary equipment to perform most of the proposed experiments is still in the process of acquisition, and the main impact is expected upon the installation of the new MRI/MRS spectrometer electronics, expected for August 2008. Our team of researchers is already running preliminary tests of the experimental methods using the existing, limited performance equipment that is installed in our laboratory. Preliminary results were obtained on two of our main lines of research: understanding vascular territories by using Arterial Spin Labeling and NMR Instrumentation for MRI/MRS experiments. Our interest on Arterial Spin Labeling (ASL) started early in 2003 as the CInAPCe network began to take form. To perform studies on ASL we found necessary a "turn key" MRI spectrometer, different from all others developed locally at our Institute. Following the period between our proposal and the effective contract of the project by FAPESP, we decided to seek an alternative path to fulfill both our urge for results in this very competitive field and the necessary material and data for one of our students. Most of the results on this line of research are related to the application of the concepts of ASL and its continuous variant CASL to study vascular territories mapping by using a dedicated labeling coil. Another line of research was on NMR Instrumentation for MRI/MRS experiments. Since we intend to use the acquired spectrometer, initially with an existing magnet at our laboratory (31cm/2.0 Tesla) and later on with the recently acquired one (33cm/4.7 Tesla), and since we were granted with resources that allow us to purchase only the electronics for this spectrometer, we spend a great effort on the development of resonating structures to be used as NMR probes for animal studies. Our group has a wide and long time experience on NMR instrumentation, and one of the main missions of this center is its contribution to the CInAPCe network with technologically oriented lines of research. All important results generated by these research topics are described on the papers and conference communications as listed below.

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Queiroz CM, Mello LE. 2007. Synaptic plasticity of the CA3 commissural projection in epileptic rats: an in vivo electrophysiological study. *Eur J Neurosci*. **25(10)**:3071-9.

Alberto TANNUS

Instituto de Física de São Carlos
Universidade de São Paulo (USP)
Departamento de Física e Informática
Avenida Trabalhador São-carlense, 400 - Centro
13566-590 - Sao Carlos, SP - Brasil – Caixa Postal: 369

+55-16-3373-9876
goiano@if.sc.usp.br

ESTIMABILITY OF MEASURES OF ASSOCIATION AND EFFECTS IN SPATIAL CASE-CONTROL STUDIES

Ricardo Carlos CORDEIRO

School of Medical Sciences / State University of Campinas (Unicamp)

This project's theme is to investigate a method: the spatial case-control study, understood as a case-control study which explicitly incorporates spatial location as a covariate of interest. This, given the non linearity of spatial distribution for most occurrences of epidemiological interest, implies a sweeping change to the ordinary analytical instrument used in case-control studies. The objectives of this project are in case-control studies: a) To verify the behavior and epidemiological significance of the spatial relative risk function in function of "case-base sampling", "risk-set sampling" sample designs and sampling from "survivors" of the studied disease. b) To develop a computer model which gives a set of predefined demographic, geographic, and epidemiological parameters, simulating the execution of spatial case-control studies in different epidemic configurations, more as an approach for a study of behavior and precision of spatial relative risk function estimator in function of the above cited sample designs. c) To apply a multinomial logistic regression model to estimate spatial distribution of occurrence risk when occurrences are classified according to their "severity", this is in contraposition to the binary model which classifies studied individuals as either cases or controls, developing a multinomial treatment, which enables estimation of the spatial distribution of occurrence risk for cases in function of their severity. d) To develop significance tests for parameters of the non-linear component from surface estimates of risk in spatial case-control studies, which can be used in parallel or in place of the currently used Monte-Carlo procedure. e) To test the methodological results obtained, particularly the one in item c) above, in the reanalysis of two case-control studies: "Spatial distribution of work accident risk in the informal Piracicaba work market and "Spatial distribution of dengue fever risk in the south region of Campinas Municipal Area, both of which supported by FAPESP.

As the project has commenced, data obtained will lead to publications on due course.

Ricardo Carlos CORDEIRO

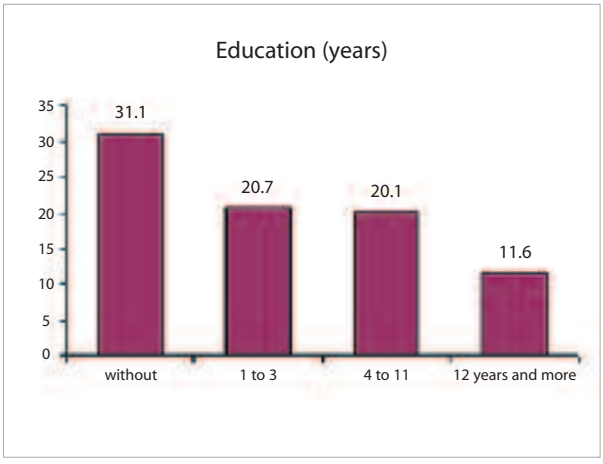
Faculdade de Ciências Médicas
Universidade Estadual de Campinas
Departamento de Medicina Preventiva e Social.
Caixa Postal 6111 - Barão Geraldo
CEP 13083-970 - Campinas, SP - Brasil

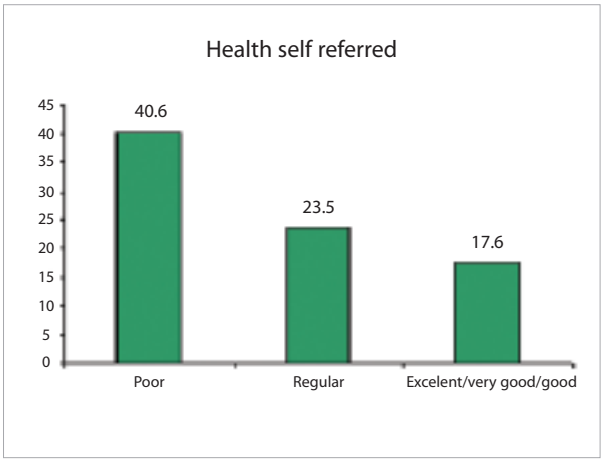
+55-19-3788-8036
ricacordeiro@gmail.com

SABE STUDY– HEALTH, WELL BEING AND AGEING. LONGITUDINAL STUDY ON LIVING AND HEALTH CONDITIONS OF THE ELDERLY IN SÃO PAULO CITY

Ruy LAURENTI

Public Health School / University of São Paulo (USP)





Mortality rates (per 100 individuals aged 60 or above) according to some variables, city of São Paulo, 2000/2006

The health of the elderly is a key element for the social and economic development of Latin American and Caribbean countries. In the 1950's, the average life expectancy in this region was 51 years. Nowadays, it is more than 68 years, and in many countries, nearly 75. This kind of change represents a major public health challenge. The Pan American Health Organization carried out in 2000 a multicentric study on the health and well-being of people aged 60 or above in seven Latin American and Caribbean capitals and major cities, including São Paulo. Five years later, a follow-up study is proposed, which is comprised of two sub-projects: the first aims to interview the elderly who participated in the first wave of the study to collect data on their present health and living conditions; the second sub-project aims to collect information on the new cohort, which is comprised of elderly whose ages range from 60 to 64 years in 2005. The sample will be composed of 400 individuals, which defines a sampling fraction of 400/310694=0.001287. A sampling error of 7%, a design effect equal to 1.5 and prevalence of individuals with hypertension equal to 50% were chosen to calculate the sample size, which was corrected, by considering a mortality rate of 2%, and a response rate of 75%.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The "SABE Study - health, well being and ageing, longitudinal study on living and health conditions of the elderly in São Paulo city" has been in progress for two years, and represents a continuation of a previous project started in 2000. The study's methodology is innovative in Brazil since it is a study of complex cohorts, i.e., in addition to following up the initial cohort, new cohorts will be added to each wave. This kind of study is important in aging studies since each generation is different from the previous one, because of economic, cultural and social reasons and there is no detailed information on which is or will be the behavior of the cohort who were born before, during or after the Second World War. From the initial sample of 2,143 analyzed persons (2000) was done, an extensive search, and 1,115 persons were located and re-interviewed. The difference between those numbers arose from the fact that there were 649 deaths, 379 changes of address (or city) or refusals. Also another 299 persons aged 60-64 were interviewed and added to the new cohort. The data showed that the probability of death is higher with the advancement of age, among elder male, without higher education, with high number of diseases and disabilities, presenting of cognitive decline, history of hospitalization and fall, with self-perception of health referred as bad, and who were not able to perform the tests of mobility, flexibility and balance. Among the survivors, increase in the proportion of elderly people living alone, was noted from 13% in 2000 to 15.9% in 2006. Furthermore, there was an increase in the number of those who feel that their money is sufficient for their expenses. The comparison between the groups age 60-64 of both cohorts showed that there are fewer people living alone in the second cohort, and that they have a good perception that their money is enough for their daily needs. However, the same cohort considered their health worse and reported greater number of diseases than the first cohort.

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Ruy LAURENTI

Faculdade de Saúde Pública
Universidade de São Paulo (USP)
Departamento de Epidemiologia
Avenida Dr. Arnaldo, 715 – Cerqueira César
01246-904 – São Paulo, SP – Brasil

+55-11-3082-3886
laurenti@usp.br