National Institute for Translational Neuroscience

Converging neuroscience with brain health and disease

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1- INTRODUCTION

The magnitude, scope, sophistication and funding support for biomedical research are unprecedented and continue to increase dramatically. In response to these recent changes, we have observed that discoveries in general science have rapidly accelerated and continue to expand.

As a general rule, health research has been traditionally broken into two categories: basic and applied research. Basic research is necessary to further our understanding on normal vs. disease states but does not directly translate this knowledge into clinically useful applications. Applied research advances the development of new diagnostic tests or drugs for patients based on our understanding of disease development and progression. The term "translational research" recently incorporated in the dictionary of health sciences, indicates the integration of the advancements in basic science with clinical trials with the objective to lead the research "from the bench to the bedside". Understanding and interpreting the scientific information obtained from different laboratory methodologies require that information be shared between both, the laboratory and clinic researchers. Furthermore, it is important that the observations made by clinical researchers about the nature and progression of the disease be able to specifically stimulate the basic research.

The clinical investigator analyzes tissue samples, studies the differences between the normal and the pathological state and compares the responses to various therapeutic agents in order to achieve the best possible diagnosis. Basic scientist then provides clinicians with new treatment strategies based on laboratory data. This constant feedback promotes the discovery of new disease biomarkers and drug targets, resulting in more rational drug design, improving the efficacy of therapeutic agents, and the faster optimization of leading compounds for clinical use. Following this context, translational research may also allow more rapid development of new therapeutic agents, by reducing the time between drug target identification and the clinically relevant therapeutic options. Currently, completion of all phases of preclinical and clinical testing of a single drug lasts between 7-12 years, but the vast amount of translational research being conducted around the world bodes well for more rapid advancements. Furthermore, it is very important to note at the present moment that successful health interventions in hospitals and health centers require the translation of other "basic sciences"— such as epidemiology, behavioral science, psychology, communication, cognition, social marketing, economics, political science - besides the translation of biotechnological insights in new diagnostic kits and therapies. These disciplines deserve their place not only in definitions of basic science but also in funding priorities. Discovering better ways to ensure that patients receive the care they need—safely, compassionately, and when they need it — is not easy and poses formidable methodological challenges.

On the whole, the research groups of the National Institute for Translational Neuroscience are "crossing the bridge" between idea and cure, as we are convinced that the effective translation of the new knowledge,

mechanisms, and techniques generated by advances in basic science research into new approaches for prevention, diagnosis, and treatment of disease is essential for improving human health.

2- HIGHLIGHTS

Development and evolution of the nervous system

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Diversity in the nervous system is enormous: among mammals alone, the brain varies in size over 100,000-fold. We use quantitative morphological approaches to investigate the diversity of the nervous system across animals, its evolution and developmental origins.

Most of our studies apply the *Isotropic Fractionator*, a non-stereological method developed in our laboratories (*Herculano-Houzel and Lent, 2005*) that allows the fast, simple and reliable determination of numbers of neuronal and non-neuronal cells in any dissectable brain structure. To assess the scaling rules that underlie diversity and its evolution, we compare the cellular composition of brain structures across adult individuals of various species across several orders (primates, rodents and insectivores, so far). To investigate the developmental origins of diversity, we compare the cellular composition of the brain of a given species across multiple pre- and postnatal developmental time points.

Regarding the diversity in brain size, an unspoken assumption in the field has been that the same scaling rules apply to all mammalian brains, implying that large brains are larger versions of small brains, regardless of whether they were carnivore, insectivore, primate, cetacean or rodent brains. Given the methods available, comparative studies up until the 21st century analyzed neuronal density, the glia/neuron ratio, structure volume and surface area across species of all different mammalian orders as if they were all variations of the same model. According to these studies, larger brains were made of larger numbers of larger neurons**,** with ever increasing glia/neuron ratios (presumably because larger neurons need more metabolic support, provided by a larger number of glial cells).

Another common notion was that evolution often entailed an expansion of the cerebral cortex - which would have happened through the addition of columnar modules, each with a fixed number of neurons. Based on this notion, and in the absence of direct measurements of the number of neurons in the

cortex, scaling models assumed, as a rule, that the number of cortical neurons could be estimated as the product of cortical surface area and the number of neurons underneath 1mm^2 of the cortex - which was believed to be uniform across species.

With the development of the isotropic fractionator, however, this scenario is changing. We have shown that all mammalian brains can no longer be considered equal, as different cellular scaling rules apply to rodents, primates, and insectivore brains. For instance, while larger rodent brains are indeed composed of larger numbers of larger neurons, primate brains grow with the addition of neurons whose average size does not increase, and insectivore brains combine these two strategies. We have also shown that the number of neurons underneath a unit surface area of the cerebral cortex is not uniform across species, a finding that calls into question the validity of many scaling models. We are now examining how cortical folding, the most evident feature or cortical neuroanatomy, is related to changing numbers of neurons in the brain.

Rodent brains of different sizes. From left to right: mouse, hamster, rat, agouti and capybara.

Our goal, therefore, is to use quantitative comparative studies of neuroanatomy to determine what different brains have in common, and also how it is that they differ, in order to unravel the mechanisms that, operating in evolution, give rise to the ordered diversity found in the nervous system.

Changing numbers of neurons in the postnatal rat brain. The scientific literature sustains that, when the mammalian cerebral cortex is born, it already holds all the neurons it will have as an adult. The main evidence for this notion is that while prenatal injections of DNA precursors label copious numbers of newborn neurons, postnatal injections one or two days after birth label only a few neurons. All neurons are therefore presumed to be born with the animal, and any remaining postnatal neurogenesis in the cerebral cortex would thus be residual.

In contrast to this view, we showed (*Bandeira et al., 2009*) that the number of neurons in the rat cerebral cortex actually doubles during the first postnatal week - but only after a quiescent period of about 3 days following birth, which explains why it was not found by previous studies. A similar addition of large numbers of neurons occurs in the hippocampus and remaining areas (the ensemble of brainstem, diencephalon and basal ganglia).

By the end of the first postnatal week, massive numbers of neurons are eliminated from the cortex, hippocampus and remaining areas, simultaneously with the addition of large numbers of nonneuronal cells to the brain. In addition,

by the end of the first postnatal month a new surge of postnatal neurogenesis takes place in the cerebral cortex, adding about 0.6 million neurons per day to this important cerebral structure.

Cellular scaling rules of insectivore brains. They are the smallest living mammals, smaller than what was supposedly the common ancestor that all current mammals share. Hence the question: are their miniaturized brains off the scales? Do they vary in size according to special scaling rules?

We determined the cellular composition of five insectivore species (smoky shrew, short-tailed shrew, star-nosed mole, hairy-tailed mole and eastern mole) by using the isotropic fractionators (*Sarko et al., 2009*). Comparative analysis of the average cell numbers obtained for each species demonstrates that the insectivore cellular scaling rules overlap somewhat with those for rodents and primates.

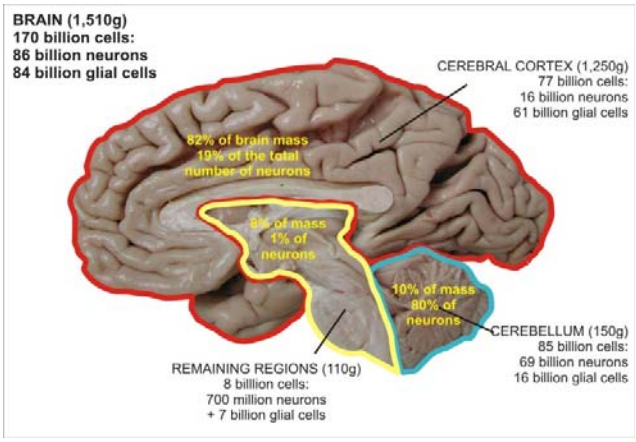
The insectivore cortex shares scaling rules with rodents (increasing faster in size than in numbers of neurons), but the insectivore cerebellum shares scaling rules with primates (increasing isometrically). Brain structures pooled as "remaining areas" appear to scale similarly across all three mammalian orders with respect to numbers of neurons, and the numbers of non-neurons appear to scale similarly across all brain structures for all three orders.

Therefore, common scaling rules exist, to different extents, between insectivore, rodent and primate brain regions, and it is hypothesized that insectivores represent the common aspects of each order.

The human brain as a linearly scaled-up primate brain. The human brain is often considered to be 5-7x larger than expected for a mammal of our body size, with an especially enlarged cerebral cortex that represents over 80% of brain mass. Although the number of neurons is generally assumed to be a determinant of computational power across species, and despite the widespread quotes that the human brain contains 100 billion neurons and 10x more glial cells, the absolute numbers of neurons and glial cells in the human brain remained unknown up to this study. We determined these numbers by using the isotropic fractionator and compared them with the expected values for a human-sized primate (*Azevedo et al., 2009*).

We found that the adult male human brain, weighing on average 1.5 kg, contains on average 86 billion neurons and 85 billion non-neuronal cells: the maximal proportion of glial to neuronal cells in the human brain, therefore, is of 1:1.

According to the linear cellular scaling rules that were previously found to apply to primate brains (*Herculano-Houzel et al., 2007; Herculano-Houzel, 2009*), a primate of approximately 75 kg would be expected to have a brain weight of 1.3 kg, with 78 billion neurons and 94 billion non-neuronal cells. The values we found, of 86 and 84 billion respectively, fall within 10% of the expected from body mass.



Absolute and relative numbers of neurons and nonneuronal cells in the human brain.

According to brain mass, a primate brain weighing 1.5 kg should be composed of 94 billion neurons, with 22 billion in the cerebral cortex, 78 billion in the cerebellum, and 0.6 billion in the remaining areas. The numbers of cells found in the cerebral cortex (16 billion), cerebellum (69 billion) and remaining areas (0.7 billion) are therefore very close to the numbers expected for a generic primate of our brain size. This proximity indicates that the human brain conforms to the primate cellular scaling rules.

For the sake of comparison, a brain with 86 billion neurons built according to the cellular scaling rules that apply to rodents (*Herculano-Houzel et al., 2006*) would be expected to weigh 35 kg and to inhabit a body of 50 tons! Conversely, with our 70 kg of body mass, our brain - if built as a rodent brain - would weigh only 145 g and hold a meager 12 billion neurons…

These comparisons show that the human brain is, indeed, almost 10x larger and richer in neurons than expected for a rodent of our body size - but has just as many neurons as expected for the primate that it is. The human brain, therefore, is a linearly scaled-up primate brain.

Coordinate scaling of cortical and cerebellar numbers of neurons. While larger brains possess concertedly larger cerebral cortices and cerebella, the relative size of the cerebral cortex increases with brain size, but relative cerebellar size does not. In the absence of data on numbers of neurons in these structures, this discrepancy has been used to dispute the hypothesis that the cerebral cortex and cerebellum function and have evolved in concert and to support a trend towards neocorticalization in evolution. However, the rationale for interpreting changes in absolute and relative size of the cerebral cortex and cerebellum relies on the assumption that they reflect absolute and relative numbers of neurons in these structures across all species – an assumption that our recent studies have shown to be flawed. We showed for the first time (*Herculano-Houzel, 2010*) that the numbers of neurons in the cerebral cortex and cerebellum are directly correlated across 19 mammalian species of 4 different orders, including humans, and increase concertedly in a similar fashion both within and across the orders *Eulipotyphla* (*Insectivora)*, *Rodentia, Scandentia*

and *Primata*, such that on average a ratio of 3.6 neurons in the cerebellum to every neuron in the cerebral cortex is maintained across species. This coordinated scaling of cortical and cerebellar numbers of neurons provides direct evidence in favor of concerted function, scaling and evolution of these brain structures, and suggests that the common notion that equates cognitive advancement with neocortical expansion should be revisited to consider instead the coordinated scaling of neocortex and cerebellum as a functional ensemble.

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Development and plasticity of the nervous system

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The period of embryonic and postnatal development of the nervous system, particularly of the cerebral cortex, is strongly characterized by its changing, flexible, plastic "mood". This means that it is during this period that the environment is able to influence developmental pathways, leading to variability among individuals, and building the unique characteristics of each human being. For each functional and morphological aspect of development, plasticity is higher during a particular window of time – the critical period. However, it is well known that a good deal of changes may take place out of the critical period limits, during adulthood.

It is the main objective of our group to investigate – using human subjects and animal models – both the normal development of the cerebral cortex (*Lent, 2010a*) and its neuroplastic properties, that is, the way development is deviated to either a compensatory or a maladaptive track (*Lent, 2010b*). We concentrate our work on two different phenomena: (1) the migratory streams of precursor neurons toward their definitive loci in the brain; and (2) the formation of connections between the cerebral hemispheres, under normal developmental conditions and after manipulations of either the internal brain environment or the external periphery of the body.

Migrating interneurons invade the cortex. In a first set of experiments, we have studied some of the phenomena and mechanisms that orient the migration of inhibitory interneurons from their site of origin at the ventral telencephalon of the embryo, towards their final position in the cortical layers. We determined that there is no topographic match between the origin and the final destination of these important cortical neurons (*Lourenço et al., 2010*). This means that the inhibitory machinery of the cortex takes position within the circuitry without any spatial constraints, different from the strict order of the excitatory complement of neurons.

We have also performed a sequence of experiments to demonstrate the joint participation of molecules of the extracellular matrix and other, smaller, signaling molecules, on pathway guidance of interneuronal tangential migration. We showed that a concert of inhibitory molecules distribute strategically along the pathways to be covered by migrating interneurons, "forcing" them to remain on the right track until they reach their final place at the cortex. We first showed that *ephrins* are one family of these molecules (*Zimmer et al., 2008*), and that *chondroitin sulfate proteoglycans* associated with *semaphorins*, are another example (*Zimmer et al., 2010*). In both cases, these signaling molecules form

inhibitory borders that guide the movement of migrating interneurons towards the cortex (Figure 1).

Figure 1. The three pictures at left represent the same coronal section (illuminated with different filters at the microscope) through the left hemisphere of an embryo, showing the overlap of territories occupied by semaphorin 3A (green), and chondroitin sulfate proteoglycan 4 (red). The picture at right shows that interneuronal precursors (green dots) migrate *in vitro* from the ventral telencephalon (heavy green spot at far right), towards the dorsal telencephalon (at upper left), avoiding the proteoglycan field (in red).

Callosal axons form the interhemispheric circuit under environmental control. The corpus callosum is the main fiber tract connecting the cerebral hemispheres, making them able to functionally interact, what allows the integrated performance of both sides of the body and both sides of the brain (*Lent, 2010c*). The callosal fibers emerge from a selected population of cortical neurons around the 12th prenatal week in humans, and around the 3rd prenatal week in rodents. These juvenile fibers grow towards the midline following molecular cues (*Donahoo and Richards, 2009*) and developmental strategies that allow them to cross the midline at the right spot, and arrive at the correct target in the opposite hemisphere (*Garcez et al., 2007*).

The influence of the local microenvironment on fibers crossing the midline successfully can be illustrated by genetic and surgical interference with midline cues. The result is dysgenesis of the corpus callosum, a condition that afflicts humans and rodents as well. We approach this condition translationally, by using modern neuroimaging tools both in patients and in animal models to identify aberrant callosal projections in cases where the corpus callosum formation is hindered perinatally (Figure 2). The work in humans is well advanced: we could show for the first time an aberrant callosal tract (the *sigmoid bundle*) connecting the frontal pole of one side with the opposite occipital cortex (*Tovar-Moll et al., 2007*). The work in rodents is just beginning, taking advantage of a new facility recently inaugurated at UFRJ (the National Center of Bioimaging), that is able to capture high resolution magnetic resonance images from rodent brains. In addition, in rodents we were be able to perform experiments using neurotracers, molecules that are uptaken by neurons and axons, label their whole profile, and thus allow their computerized reconstruction at the microscope.

An important conclusion from this work is that the interhemispheric circuit reorganizes in the brain of dyscallosal patients, forming abnormal bundles whose function is so far unknown. It is possible that some of these bundles be

compensatory (the longitudinal bundles?), while other be maladaptive (the sigmoid bundles?). This is the next step to follow.

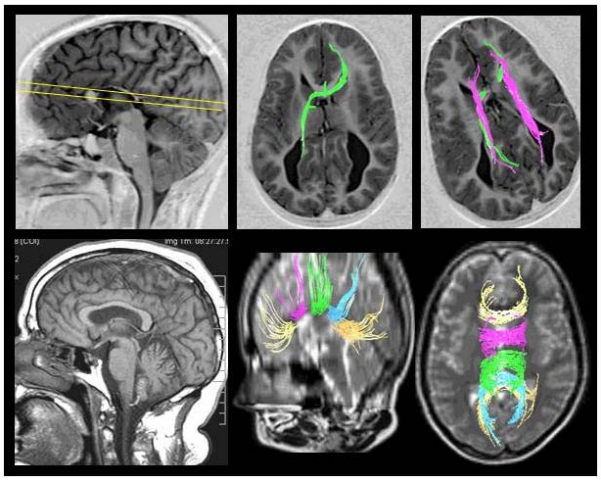

Figure 2. The upper row of images show the medial view of the brain of a dyscallosal patient (left) and two aberrant bundles viewed at a horizontal plane (sigmoid bundle in green, longitudinal bundle in violet). The lower row shows a normal brain with a representation of the topographic distribution of callosal fibers viewed sagittally (middle) and transversely (right).

Another example of plasticity after environmental changes is illustrated by functional cortical maps in patients who suffered amputation of a lower limb. This is being investigated by using neuroimaging tools, and will be followed by experimental techniques in rodents. Preliminary findings (*Lannes et al., 2010*) indicate an enlargement of the representation of the upper parts of the amputated limb in the somatosensory cortex (Figure 3). We have also evidence that corresponding changes take place in the callosal circuit.

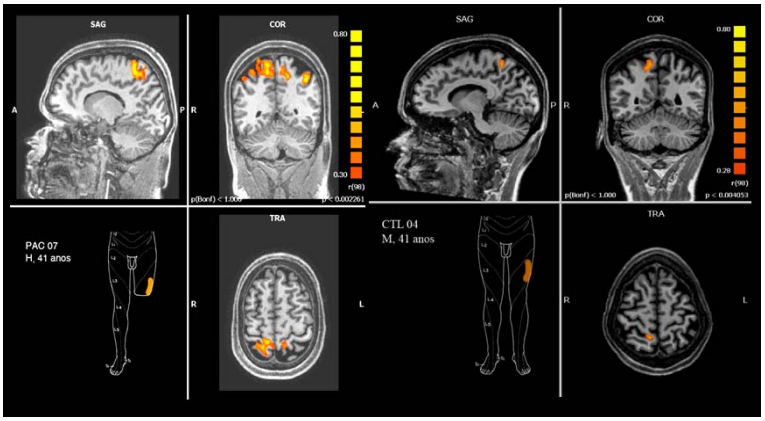

Figure 3. The panel at left shows the extended, bilateral somatosensory representation of the lateral side of the thigh in a transtibial amputee, as compared with the unilateral representation typical of a normal subject (right).

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Investigating the neural underpinnings of human moral emotional experience and behavior with structural and functional neuroimaging

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We are essentially social beings, and the organization of our brains naturally reflects this aspect. Human societies are exceedingly complex and, like in ants, social organization depends on a high degree of specialization and interdependence. Unlike ants, however, our societies are flexible and ever changing. In a lifetime, an average person is likely to witness major societal transformations, for the good and for the bad. Adapting to partaking on these changes involves complex adjustments of individual goals to societal contingencies.

Human sociability finds some parallels, however, in neurobehavioral mechanisms that are discernible in other species, particularly, but not exclusively, in the higher primates. What distinguishes humans from other species is, therefore, how each culture - and for that matter, "morals" (the sets of customs and values adopted by a given culture to guide "proper" behavior) - interacts with these basic motivations, giving rise to conceptually rich social motivations, values and preferences. The cultural mediation of human interactions with the social environment is therefore the biological hallmark of our species (*Baumeister, 2005*). Importantly, in contrast with a commonly held belief, culturally-shaped motivations do not rely exclusively on cortical systems, but critically require the engagement of subcortical-limbic structures, such as the ventral striatum, septal region and amygdala (*Andy and Stephan, 1968*).

Building on the first clues linking morality and brain function (*Welt, 1888*), modern neuroscience employed sophisticated cognitive probes along with structural and functional neuroimaging (*Moll, Zahn et al. 2005*) to address the neural underpinnings of moral judgments and moral emotions (*de Oliveira-Souza and Moll 2000; Moll, Eslinger et al. 2001; Moll, de Oliveira-Souza et al. 2002; Eslinger, Flaherty-Craig et al. 2004*), social concepts (*Zahn, Moll et al. 2007*) and attitudes (*Cunningham, Raye et al. 2004*). A picture that emerges from these recent studies is that there is large agreement about the brain regions supporting moral cognition, pointing to a reliable involvement of cortical (anterior and medial prefrontal and anterior temporal cortex) and subcortical-limbic (ventral striatum, hypothalamus, amygdala, basal forebrain) structures in morality (*Moll, Zahn et al. 2005*) (Figure 1).

During the past few years, a number of functional magnetic resonance imaging (fMRI) studies in normal volunteers have contributed to our

understanding of the moral brain. In an early experiment, normal volunteers were scanned during the auditory presentation of short statements and instructed to silently make categorical judgments (right vs. wrong) on each (*de Oliveira-Souza and Moll 2000; Moll, Eslinger et al. 2001*). Some statements had an explicit

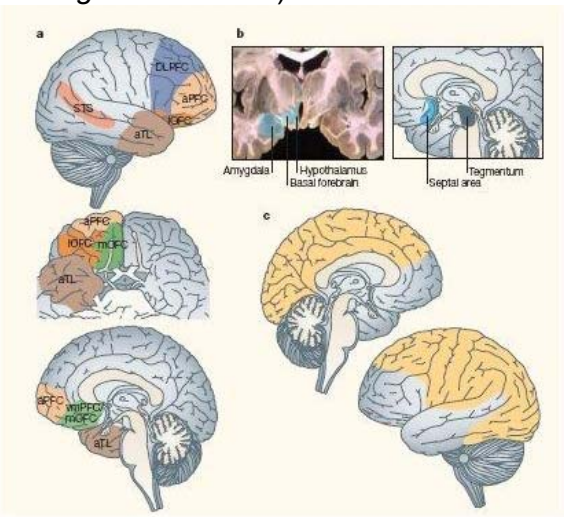

Figure 1. Cortical (a) and subcortical (b) regions consistently involved in human moral cognition and behavior based on converging evidence from lesion and functional neuroimaging studies. In (c), areas which have not been consistently involved in moral cognition and behavior.

moral content (*The judge condemned an innocent man*), while others were factual statements without moral content (*Telephones never ring*). Moral judgments activated the medial frontal gyrus and medial and lateral sectors of the FPC, as well as the anterior temporal lobe (aTL) and the superior temporal sulcus (STS) region. These areas are especially evolved in humans as compared to other primates. A subsequent fMRI study using passive exposure of moral stimuli to participants extended these results and ruled out executive function effects as the main cause of these effects (*Moll, de Oliveira-Souza et al. 2002*). The engagement of the same brain networks by moral appraisals independently of task demands originated the idea of moral sensitivity as a mechanism by which moral significance is automatically attributed to ordinary events. Such mechanism crucially depends on moral sentiments (*Moll, Oliveira-Souza et al. 2007*), and we have proposed a neurocognitive model to explain how these complex sentiments emerge from distributed brain representations (*Moll, Zahn et al, 2005*).

A recent fMRI experiment provided evidence for a direct link between altruistic decision-making in cultural settings and the structures found in studies of moral judgments. Subjects were scanned using functional MRI while they made real-life anonymous decisions about whether to donate to or to oppose a number of charities (*Moll, Krueger et al. 2006*). Decisions, depending on trial type, could be either financially costly or non-costly to the participant. In other trials, participants were able to receive 'pure' monetary rewards (without consequences for the charities). The charities were associated to causes with important societal implications, such as abortion, children's rights, nuclear energy, war and euthanasia. Both pure monetary rewards and decisions to

donate activated the mesolimbic reward system. In comparison to the pure monetary reward condition, decisions to donate selectively activated subgenual-septal area, which is intimately related to social attachment in other species. When participants disliked the charities, the lateral OFC, an area more readily activated by experience of anger and disgust, was engaged. These findings extended the role of fronto-limbic networks in social cooperation from interpersonal economic interactions (e.g., *Sanfey, Rilling et al. 2003; de Quervain, Fischbacher et al. 2004*), to the realm of decisions based on internalized values and preferences shaped by culture (Figure 2).

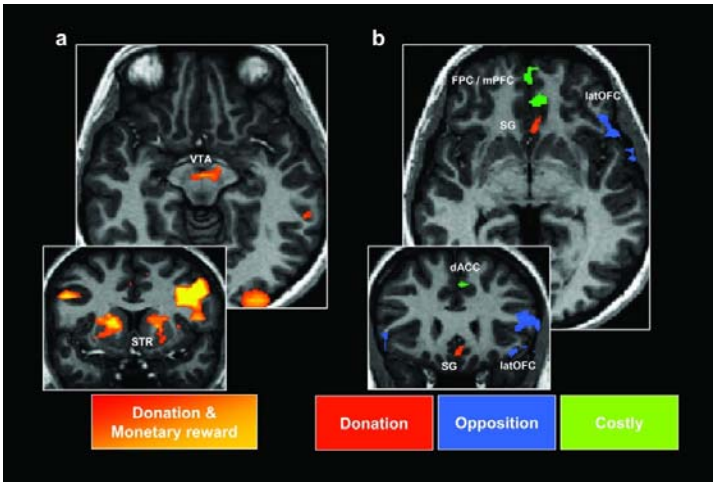

Figure 2. a: activation of the human reward system (ventral tegmental area, VTA; and striatum, STR) both by winning money and by donating money to worthy causes. **b**: specific responses for donation (subgenual and septal area, SG), opposition (lateral orbitofrontal cortex, latOFC) and for costly decisions (frontopolar and medial prefrontal cortex, FPC/mPFC).

Structural assessment of cortical thickness and/or volume is an *in vivo* magnetic resonance imaging method of regional volumetric or thickness analysis that reduces biases involved in arbitrary definitions of anatomical boundaries through the assessment of quantitative differences on a voxel-wise basis (*Good et al., 2001*). This method allows the assessment of cortical thickness and/or volume with great accuracy, representing a revolutionary step for the investigation of several neurological and psychiatric disorders, as well as of normal brain development and aging (*Good et al., 2001; de Oliveira-Souza et al., 2008*). These methods have been recently implemented in our lab. Illustrative results of quantitative assessment of cortical thickness are shown in the Figure 3, which demonstrates cortical thinning in elderly, as compared to young adults.

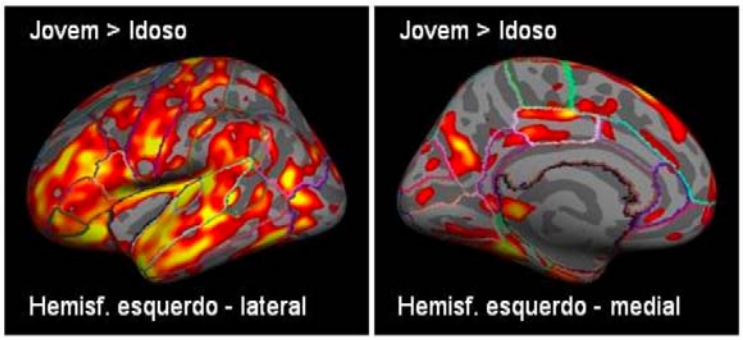

Figure 3. Reduced cortical thickness in normal aging. Areas in yellow and red show reduction in cortical thickness in elderly subjects (n = 10, mean age = 70 +/- 5 years) compared to young (n = 10, mean age = 27 +/- 12 years). Data collected with a 3T MRI.

Abnormal social behaviour that may be attributable to changes in moral motivations was also observed in patients with subcortical mesolimbic lesions and there is evidence for an extended frontotemporal-mesolimbic network of brain regions necessary to support moral behavior. We have recently demonstrated that individuals with high degree of psychopathy show reduced grey-matter volumes in key areas of this brain network (bilateral frontopolar, medial orbitofrontal cortex, subgenual frontal region and bilateral posterior superior temporal sulcus). Grey matter decreases in some of these regions were associated with the degree of callousness in individuals with developmental psychopathy (Figure 4), known to show reduced compassion and guilt (*de Oliveira-Souza et al., 2008*).

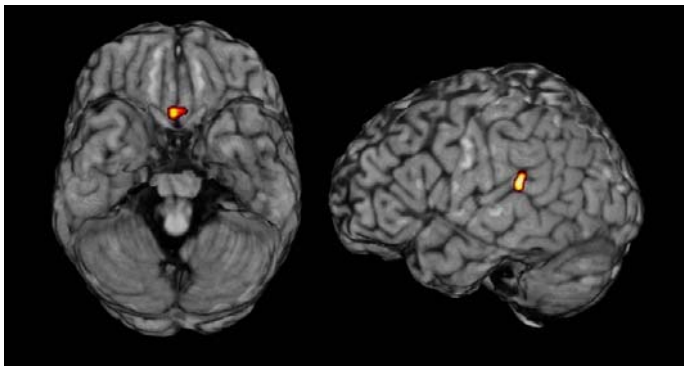

Figure 4. Brain regions showing decreases in grey matter density in individuals with psychopathy, as compared to normal controls, using “voxel-based morphometry”.

As pointed out by Darwin (*Darwin, 1871/1982*) and Adam Smith (*Smith, 1759/1966*), among others, moral sentiments are central for the regulation of social behaviors, enforcing the observance of social norms according to internalized values. Clinical and experimental studies have started to provide evidence on the influence of cultural and biological factors on human morality. There is strong evidence for the engagement of a stable network of brain regions in moral cognition and behavior, with distinct functional roles of components of this network. The complexity and entanglement of cognitive components that

make up moral cognition provides a challenge to future experimental designs in this field. We believe that unveiling the secrets of this unique system can help us not only to understand our own social nature, but to act to promote healthier societies and individuals through enlightened social policies and medical treatment of cognitive and behavioral disorders.

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Blocking axonal degeneration: DNP as a neuroprotective compound

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Preventing the harm caused by nerve degeneration is a major challenge in neurodegenerative diseases and in various forms of trauma to the nervous system. The aim of the current work was to investigate the effects of systemic administration of 2,4-dinitrophenol (DNP), a compound with newly recognized neuroprotective properties, on sciatic nerve degeneration following a crushing injury. Morphometric, ultrastructural and immunohistochemical analysis of injured nerves established that DNP prevented axonal degeneration, blocked cytoskeletal disintegration and preserved the immunoreactivities of amyloid precursor protein and neuregulin 1, proteins implicated in neuronal survival and myelination. Functional tests revealed preservation of limb function following injury in DNP-treated animals. Results indicate that DNP prevents nerve degeneration and suggest that it may be a useful small-molecule adjuvant in the development of novel therapeutic approaches in nerve injury.

Axon degeneration is a common denominator in several types of traumatic injury to the nervous system and in neurodegenerative diseases (Coleman and Perry, 2002). It results in impairment of anterograde and retrograde transport and loss of neuronal connectivity, which culminate in nerve cell dysfunction and death. The loss of connection between the cell body and the axon terminal is damaging not only to the neuron itself but also to the corresponding innervated muscle and, therefore, affects both motor neuron integrity and limb function. Surprisingly, few studies so far have effectively explored anti-degenerative approaches in the development of treatments for nerve trauma. Blocking axonal degeneration should be an integral part of the development of novel approaches to preserve nerve structure and to optimize conditions for regeneration following trauma.

We recently demonstrated that DNP, a classical uncoupler of mitochondrial oxidative phosphorylation with newly recognized neuroprotective actions (for reviews, see De Felice and Ferreira, 2006; De Felice et al., 2007), stimulates neuronal differentiation and neurite outgrowth in primary cortical and hippocampal neuronal cultures, as well as in a neuroblastoma cell line, and that this effect is at least in part related to increased neuronal levels of cAMP (Wasilewska-Sampaio et al., 2005). Other recent studies have shown neuroprotective actions of DNP against different types of neuronal injury (De Felice et al., 2001, 2004). Taken together, these previous findings suggested that DNP might exert a protective effect against degeneration of peripheral nerve.

The current work was driven by the hypothesis that DNP could block axon degeneration *in vivo* and rescue neurons from death following a crushing injury to peripheral nerve.

Results showed that DNP blocks axon degeneration following a crushing injury to the sciatic nerve by preserving myelinated nerve structure. As shown in Figure 1, fiber and axonal areas and G ratios (an index that reflects morphofunctional integrity of the nerve) of DNP-treated animals were preserved at control levels while sciatic nerves from untreated animals presented many degenerated fibers.

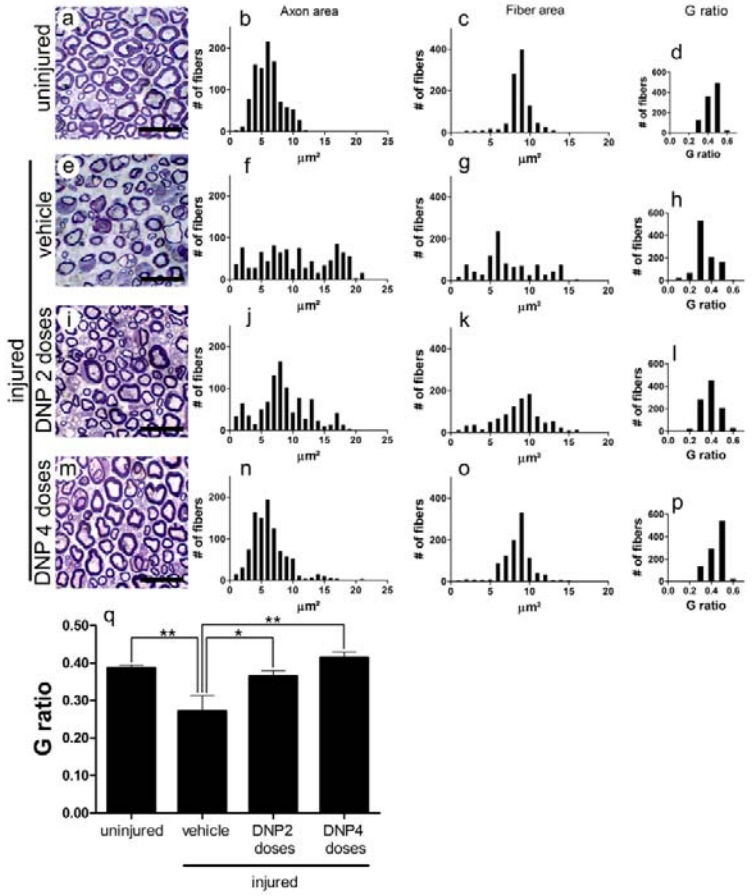
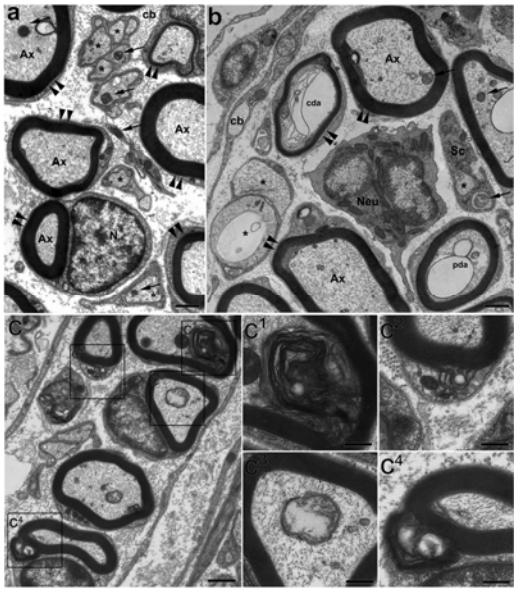


Figure 1: Morphometric analysis of sciatic nerves after injury. Representative semi-thin transversal sections of sciatic nerves from (a) uninjured animals, (e) vehicle-treated injured animals, (i) DNP2-treated injured animals, and (m) DNP4-treated injured animals. Axon and fiber areas from different experimental groups are shown as follows: (b and c) uninjured animals, (f and g) vehicle-treated injured animals, (j and k) DNP2-treated injured animals, and (n and o) DNP4-treated injured animals. Panels d, h, l and p show, respectively, the distributions of G ratios for the four experimental groups listed above. Panel q shows mean G ratio values for the four experimental groups. (*) and (**) indicate p = 0.05 and p = 0.01, respectively. Scale bar = 40 µm.

Transmission electron microscopy analysis of uninjured nerves showed well preserved myelinated fibers with axons completely filled with cytoskeleton components (microtubules and neurofilaments) and surrounded by thick, electron dense ring-shaped myelin sheaths (Fig. 2a). At 48 h post-injury, partially and completely degenerated axons, presenting partial or complete degradation of cytoskeleton components, respectively, were abundantly found in nerve sections (Fig. 2b). Degraded or abnormal myelin sheaths were frequently observed in injured nerves (Fig. 2c). Unmyelinated fibers were also observed and presented prominent alterations in cytoskeleton content (Fig. 2b).

Figure 2: Ultrastructural analysis of sciatic nerve. (a) Representative electron micrograph of a transversal section of an uninjured sciatic nerve presenting myelinated (Ax) and unmyelinated (*) axons

completely filled with cytoskeleton and mitochondria (arrows). Schwann cells myelinating axons (arrow heads) and extracellular space was filled with collagen bundles (cb). (b) Injured nerve showing normal axons (Ax), completely degenerated axons (cda) and partially degenerated axons (pda). Collagen bundles (cb), myelinating (arrow heads) and unmyelinating Schwann cells (Sc), mitochondria (arrows) and neutrophils (Neu) are also visible. (Panel C and insets) Injured nerves presenting structural alterations in myelin, such as internal (c^1) and external (c^2 and c^4) unwrapping, and mitochondrial swelling (c^3). Scale bars: (a-c) = 1 µm; (c^1-c^4) = 500 nm.



DNP treatment also attenuated post-injury decline in limb function (Fig. 3). Measurements of toe spread (specifically the distance between the first and fifth toes) were used as a parameter for evaluation of limb function. Following sciatic injury, animals were submitted to a walking track test and, as expected, uninjured animals maintained a constant toe spread (averaging 1 cm). By contrast, vehicle-treated injured animals presented a progressive decrease in toe spread over the course of six weeks following injury. Interestingly, DNP-treated injured mice exhibited significantly less reduction in toe spread, indicating that DNP treatment attenuated limb functional loss in the weeks following injury.

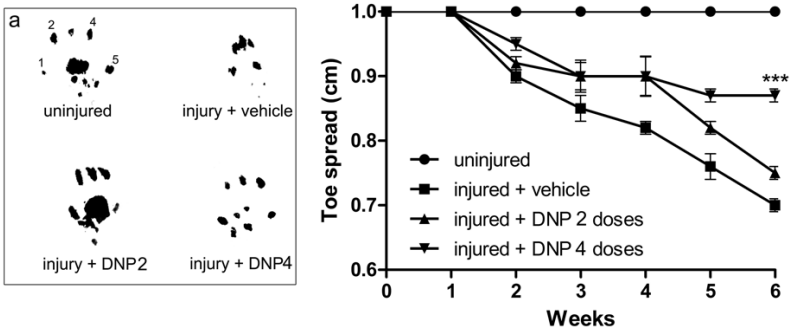


Figure 3: Paw finger-printing and sciatic function assay. Panel a: Left uppermost image is a representative print from an uninjured left hind paw with numbered toes (number 5 is the thumb). Other images are representative prints from the left hind paws of vehicle-treated, DNP2-treated or DNP4-treated injured animals (six weeks after injury), as indicated. Panel b: Limb loss of function was measured through a six week period. Curves correspond to toe spreads measured in different experimental groups, as indicated. (***) indicates statistically significant difference (p = 0.001).

Taken together, these results showed that DNP treatment preserved tissue architecture, reduced edema and prevented fibers from entering Wallerian degeneration, resulting in morphological and functional preservation of the injured nerves. These results argue that DNP can be used as a tool in the development of novel therapeutic approaches for treatment of nerve trauma and neurodegenerative diseases.

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Protection of synapses against Alzheimer's-linked toxins: Insulin signaling prevents the pathogenic binding of Aβ oligomers

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Alzheimer's disease (AD) is a neurological disorder characterized by profound memory loss and progressively catastrophic dementia. A large body of evidence indicates that memory loss in AD is a consequence of synapse failure in specific brain areas triggered by soluble Aβ oligomers that build up and accumulate in disease affected brains. Aβ oligomers, also known as ADDLs, have been shown to induce alterations in composition and function of synaptic receptors, neuronal ionic imbalance, oxidative stress and impairment of synaptic plasticity. Ultimately, exposing neurons to ADDLs leads to alterations in dendritic spine morphology and spine loss. The mechanisms by which ADDLs instigate such a multitude of detrimental consequences to neuronal and synaptic physiology are not completely understood (Ferreira et al., 2007). Nevertheless, preventing ADDL binding to synapses would predictably prevent ADDLs synaptic toxicity.

Although the brain once was considered insulin insensitive, it is now known that CNS insulin signaling is important for many aspects of neuronal function, including memory formation (Zhao and Alkon, 2001). Insulin plays a key role in plasticity mechanisms in the CNS and it recently has been shown that insulin and the insulin-sensitizing drug rosiglitazone improve cognitive performance in mouse models of AD and in patients with early AD.

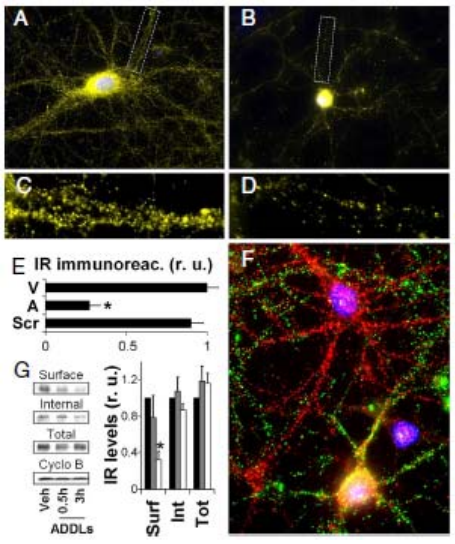

Fig. 1. ADDLs induce the removal of IRs from dendritic plasma membranes. Cultured hippocampal neurons were exposed to 100 nM ADDLs at 37 °C for 3 h followed by immunolabeling with anti-IR (yellow). Nuclear staining (DAPI) is shown in blue. A and B show representative images from vehicle- and ADDLtreated cultures, respectively. C and D show high-magnification images of dendrites contained in the dotted rectangles indicated in A and B, respectively. (E) Quantification of IR immunofluorescence levels for cultures treated with vehicle (V), ADDLs (A), or scrambled Abeta peptide (Scr). (F) A representative image showing double labeling for ADDL binding (NU4 oligomer antibody; green) and IR (red). (G) Surface abundance of IRs inhippocampal neurons exposed to vehicle or 100 nM ADDLs for 0.5 or 3 h, assessed by surface biotinylation. Asterisk indicates statistically significant (*, $P < 0.0002$) decrease compared to vehicle-treated cultures.

Conversely, insulin-resistant type 2 diabetes patients show significantly increased risk for developing AD. Moreover, experimental induction of diabetes in mouse models of AD results in premature cognitive failure and degeneration of synapse structure.

To test the hypothesis that insulin signaling provides a physiological defense mechanism against ADDLs' synaptotoxicity, we have used highly differentiated hippocampal nerve cell cultures, a preferred model for studies of synapse cell biology and mechanisms of ADDL pathogenicity (De Felice et al, 2009).

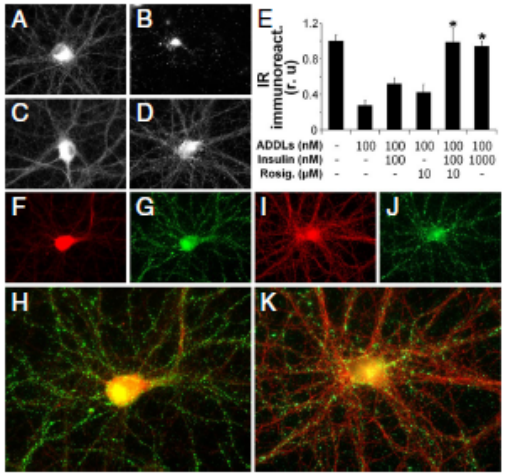

Fig. 2. Insulin prevents ADDL-induced pathological trafficking of IRs. (A–D) Representative IR immunofluorescence images from hippocampal neurons treated with vehicle (A), 100 nM ADDLs (B), 100 nM ADDLs 100 nM insulin (C), and 100 nM ADDLs 1 μM insulin (D). (E) Integrated IR immunofluorescence from 6 experiments using independent cultures (30 images analyzed per experimental condition per culture). Asterisk indicates statistically significant (*, P<0.005) increase compared to ADDL-treated cultures. (F–K) Neurons treated for 3 h with 100 nM ADDLs alone (F–H) or with 100 nM ADDLs 100 nM insulin 10 μM rosiglitazone (I–K) followed by double labeling for IR (red) and ADDLs (green). (H and K) Merged images of IR and ADDL immunolabeling. Note the inverse correlation between ADDL binding and dendritic IRα immunoreactivities on dendritic process.

Exposure of hippocampal neurons to ADDLs induced a robust redistribution of IRs from dendrites to neuronal cell body (Fig. 1 A-E). This effect was specific to neurons attacked by ADDLs. (Fig. 1F). Surface biotinylation experiments showed that IRs redistribution is accompanied by downregulation of the receptor in the neuronal surface (Fig. 1G).

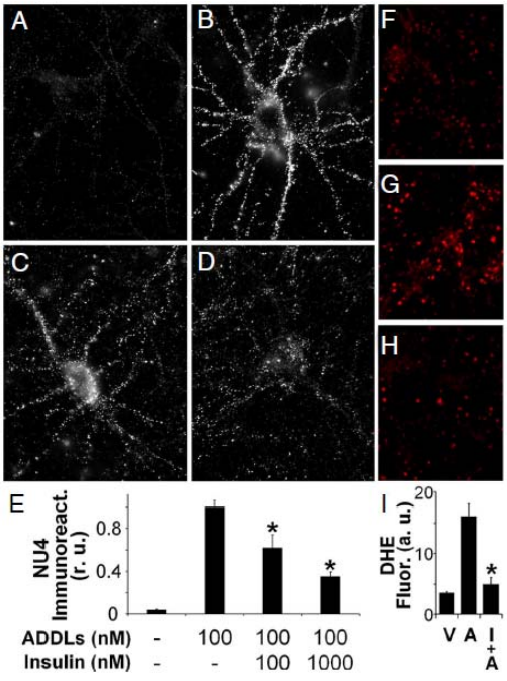

Fig. 3. Insulin blocks neuronal ADDL binding and ADDL-induced oxidative stress. (A–D) Representative images from hippocampal neurons treated with vehicle (A), 100nM ADDLs (B), 100nM ADDLs100nM insulin (C), and 100nM ADDLs1 μM insulin (D). ADDL binding was detected using NU4 antibody. (E) Integrated ADDL immunofluorescence intensities from 6 experiments using independent cultures (25 images analyzed per experimental condition per culture). Asterisk indicates statistically significant (*, P<0.01) decrease compared to ADDL-treated cultures. (F–H) Representative DHE fluorescence images in hippocampal cultures treated with vehicle (F), 1 μM ADDLs (G), or 1μM insulin 1 μM ADDLs (H). (I) Integrated DHE fluorescence. Asterisk indicates statistically significant (*, P<0.007) differences relative to ADDL-treated cultures.

Downregulation of IRs and the previously reported downregulation of NMDA-type glutamate receptor induced by ADDLs were dependent on the activities of calcium/calmodulin-dependent kinase II (CaMKII) and casein-kinase II (CKII) (data not shown).

Interestingly, pre-treatment of neuronal cultures with insulin prevented ADDL-induced redistribution of IRs (Fig. 2A-E), and the protective effect of insulin was potentiated by the insulin-sensitizing drug rosiglitazone (Fig. 2E-K). Moreover, insulin also blocked oxidative stress (Fig 3F-I) and spine loss (Fig. 4) induced by ADDLs, suggesting that insulin exerts its neuroprotective effects by acting in early stages of ADDLs toxicity.

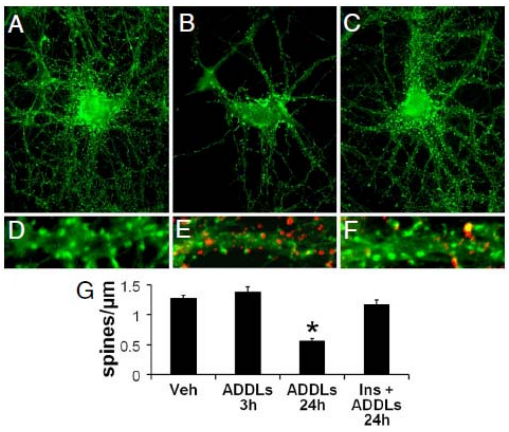

Fig. 4. Insulin blocks ADDL-induced synapse loss. (A–C) Representative images from hippocampal neurons treated with vehicle (A), 100nM ADDLs (B), or 100 nM ADDLs + 1 µM insulin (C) for 24 h. Spines were labeled using phalloidin (green). (D–F) Double-labeling high-magnification images of dendrites from neurons treated with vehicle (D), ADDLs (E), or ADDLs + insulin (F). Spines were labeled by phalloidin (green) and ADDLs were detected using the NU4 antibody (red). (G) Quantification of spine number per unit dendrite length. Asterisk indicates statistically significant difference (*, $P<0.001$) relative to vehicle-treated neurons.

To further investigate the mechanisms of insulin neuroprotection, we analyzed the effects of insulin pre-treatment on ADDLs binding to neurons. Remarkably, we found that insulin pre-treatment strongly reduces ADDLs binding to synapses in a concentration-dependent manner (Fig. 4A-E). Interestingly, the marked decrease in ADDL binding caused by insulin was prevented by a specific inhibitor of IR tyrosine kinase activity (data not shown), indicating that protection derives from activation of insulin neuronal signaling pathways rather than by simple ligand competition between ADDLs and insulin for a binding site at the synapse.

In summary, our results show that insulin blocks ADDL binding to synapses, thereby preventing the ensuing neurotoxicity. Decreased binding is the result of downregulation of ADDL binding sites through a mechanism requiring insulin receptor (IR) tyrosine kinase activity. This downregulation is the converse of ADDL-induced IR downregulation, which we have recently described as a mechanism underlying CNS insulin resistance in AD. Thus, physiological insulin and pathological ADDLs negatively regulate the abundance of each other's binding sites, creating a competitive balance between synapse survival and degeneration. Because insulin signaling in the brain is known to decline with age, the outcome of this balance represents a unique risk factor for AD well suited for therapeutic intervention. By restoring the balance to favor synapse survival, new drugs designed to specifically enhance CNS insulin signaling would provide a new and potentially significant class of AD therapeutics.

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Galectin-3: an old protein with new functions

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The success of peripheral nerve regeneration depends on intrinsic properties of neurons and a favorable environment, although the mechanisms underlying the molecular events during degeneration and regeneration are still not elucidated. Schwann cells are considered one of the best candidates to be closely involved in the success of peripheral nerve regeneration. These cells and invading macrophages are responsible for clearing myelin and axon debris, creating an appropriate route for a successful regeneration. After injury, Schwann cells express galectin-3, and this has been correlated with phagocytosis; also, in the presence of galectin-3, there is inhibition of Schwann-cell proliferation *in vitro*. In the present study we explored, *in vivo*, the effects of the absence of galectin-3 on Wallerian degeneration and nerve-fiber regeneration. Galectin-3 knockout mice showed earlier functional recovery and faster regeneration than the wild-type animals. We concluded that the absence of galectin-3 allowed faster regeneration, which may be associated with increased growth of Schwann cells and expression of β-catenin. This would favor neuron survival, followed by faster myelination, culminating in a better morphological and functional outcome.

Galectins are proteins that have a specific affinity for β-galactosides, with at least one carbohydrate recognition domain (CRD) at the C-terminal, and can be subdivided into three distinct groups (Ri-Yao Yang et al., 2008). Galectin function has been extensively studied with respect to its variable locations in cells (cytosol, nucleus, and other cell compartments). Because galectins can be secreted in the extracellular matrix, they have extracellular functions, including inflammatory processes, mainly related to cell recruitment and phagocyte activation. To date, fifteen galectins members have been identified in mammals, although galectin-3 is the most studied member.In the context of the nervous system, galectins-3 can be found in different cell types, such as macrophages, microglia, Schwann cells, some sensory neurons, and fibroblasts (Krześlak and Lipińsk, 2004).

The real biological properties of galectin-3 are controversial, whether in extra- or intracellular sites, or even inside the nucleus. After a peripheral nerve lesion myelin debris are paghocyted by Schwann cells and macrophages preparing the environment for nerve regeneration, since myelin debris contain proteins that are inhibitory to axon growth. Interestingly, while the group of Rotshenker has demonstrated that this molecule is important for myelin debris clearance by Schwann cells and macrophages after sciatic nerve damage (Reichert et al., 1994), we showed that the ablation of this protein enhanced sciatic nerve regeneration after injury (Figure 1) (Narciso et al., 2009).

The main result of our study was that the galectin-3 knockout mice exhibited a much faster regeneration of sciatic nerve fibers after surgical compression, compared to wild-type animals subjected to the same trauma.

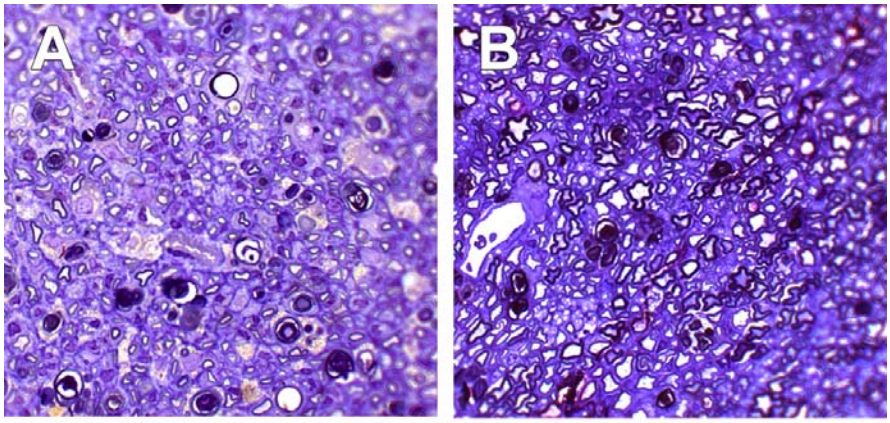

Figure 2. Semi-thin transverse sections (1 µm thick) of crushed right sciatic nerve of wild-type (A) and galectin-3−/− (B) mice 3 weeks after sciatic nerve crush. The differences between galectin-3−/− and wild-type mice after crush was pronounced at 3 weeks after lesion, when a much higher amount of myelinated nerve fibers is present in knockout animals. (Modified from Narciso et al., 2009).

These results were observed and confirmed by light microscopy, and quantification of axon area, fiber area, myelin sheath area, and number of myelinated fibers (figure 2). Calculation of the G ratio indicated that the majority of nerve fibers from galectin-3−/− animals showed a better axon X myelin relationship, which indicates a better functional outcome.

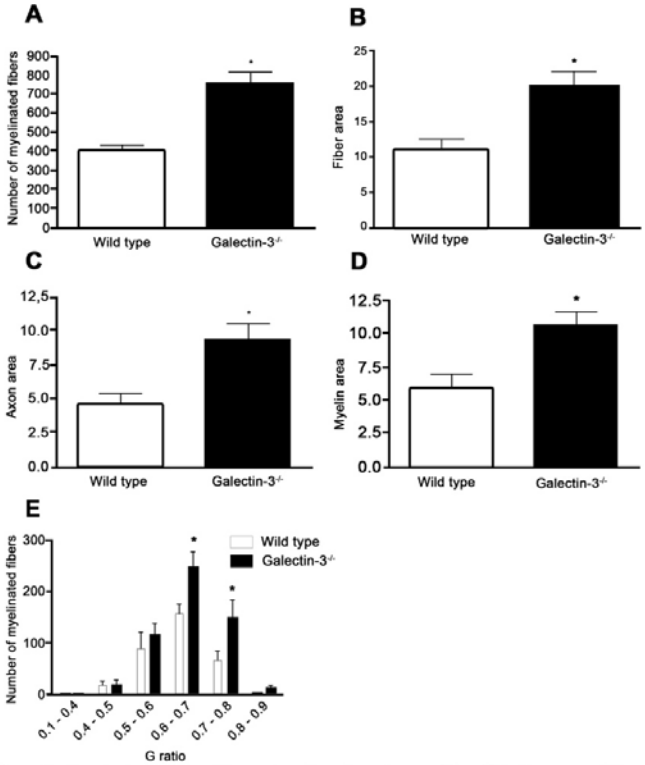

Figure 3 - Quantitative analysis of the number of myelinated nerve fibers (A), fiber area (B), axon area (C), myelin area (D) and G-ratio stratified by ranges (E) in galectin-3$^{-/-}$ and wild-type mice. Values represent mean ± SD. *P < 0.05.

However, the reasons which leaded to a rapid axonal regeneration in knockout animals are not still totally elucidated. Because galectin-3 interacts with multiple signaling pathways and influences many physiological and pathological cell behaviors, it is feasible to suppose that the absence of this molecule would lead to functional cellular compensations in knockout animals. In addition, since it is known that accelerated axon regeneration could be a consequence of faster axon degeneration, we also aimed to clarify the role of galectin-3 in the earlier steps of nerve degeneration in vivo. Recent results of our group showed that sciatic nerve WD in galectin-3-/- mice occurred in a more accelerated and efficient pattern, since myelin ovoid clearance from axonal microenvironment is faster in these knockout animals (figure 3).

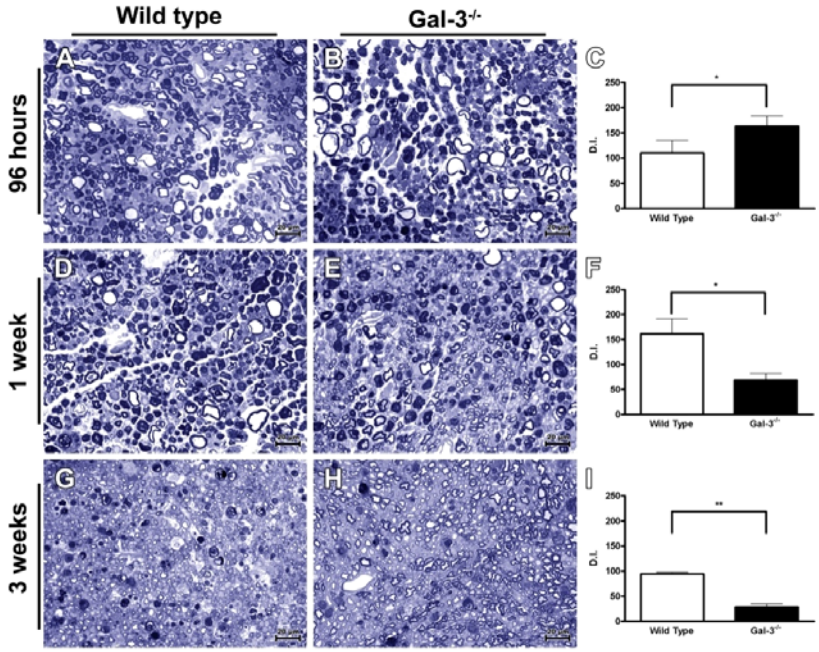

Figure 4. Semi-thin transverse sections (1 µm thick) of crushed right sciatic nerve of wild-type (A, D and G) and galectin-3-/- (B, E and H) mice at different times post-lesion (96 hours [A-B], 1 week [D-E], and 3 weeks [G-H]). We counted the fibers undergoing degeneration and the presence of myelin ovoids (indicated by index of degeneration [I.D.]) in all times (C, F and I) and found that myelin phagocytosis and Wallerian degeneration were faster in galectin-3-/- animals.

Our objectives now include the search for the molecular signaling pathway responsible for the faster axon degeneration in the absence of galectin-3.

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Prion Protein: Orchestrating Neurotrophic Activities

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The Transmissible spongiform encephalopathies (TSE) or prion diseases are a group of fatal neurodegenerative disorders that affects both animals and humans and can exhibit sporadic, inherited or infectious presentations. The propagation of the disease requires the expression of a GPI (glycosyl-phosphatidylinositol)-anchored cell surface protein, the prion protein (PrP^C) *(Prusiner et al., 1998)*. This protein is converted into an abnormal form, called $PrPS^c$, through a major conformational change. Most research in the prion field is directed at understanding the nature of the infectious agent, and the mechanistic and structural aspects of the PrP^c conversion to PrP^{sc} in either infectious or mutation-related pathologies. Nonetheless, the diagnostic procedures available for prion diseases are less sensitive than required and therapeutic interventions for these devastating diseases are still elusive *(Aguzzi et al., 2004)*.

The prion protein (PrP^C) is highly expressed in both the central and peripheral nervous systems from early stages of development and in adulthood. The massive neurodegeneration presented by individuals suffering from these diseases has been associated with the gain of neurotoxic activity of PrP^{Sc} *(Prusiner et al., 1998)*. On the other hand, major neurodegeneration is also observed in transgenic mice expressing PrP^C molecules deleted of specific domains, which points to important functional domains within this molecule, and supports the hypothesis that loss-of PrP^C function may contribute to the pathogenesis of TSEs *(Linden et al., 2008)*.

In a collaborative project between the groups described above we have demonstrated direct or indirect interaction of PrP^C with the extracellular matrix proteins laminin and vitronectin although it does not interacts with fibronectin (Fig.1A). PrP^C also interacts with, secreted factors as the co-chaperone Stress Inducible protein 1 (Fig. 1B) *(Martins et al., 2009)*. The interaction of these proteins with PrP^C modulates neuronal survival, neuronal plasticity and memory formation and consolidation. Other extracellular matrix receptors as the 37 kDa Laminin Receptor Precursor/ 67 kDa Laminin Receptor and integrins as well as glycosaminoglycans such as heparin and heparan sulfate also associate with PrP^C *(Linden et al., 2008)*.

The current review underscores the importance of PrP^C in the coordination of signaling related to neuronal survival and differentiation. The ability of PrP^C to mediate the assembly of multi-component complexes at the cell surface *(Linden et al., 2008)* is likely the basis for its neurotrophic properties *(Martins et al., 2009)*, and may be relevant for loss-of-function components of prion diseases, in particular those associated with neuronal death.

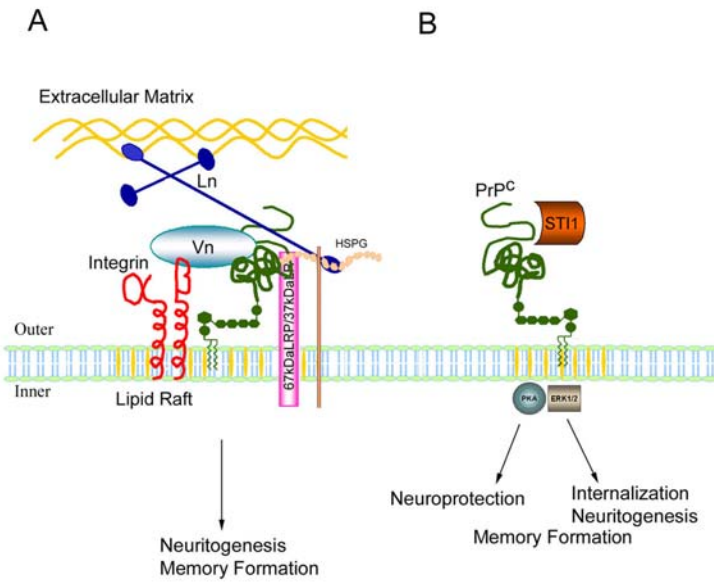

Figure 1: The PrP^C ligands mediating neurotrophic activity.
(A) PrP^C directly interacts with the ECM proteins: Laminin (Ln) and Vitronectin (Vn), and with the laminin receptor (37LRP/67LR), possibly forming a large complex involved in neuritogenesis and memory formation. Interaction of the laminin receptor with PrP^C is also mediated by Heparan Sulfate Proteoglycans (HSPG). Integrins are ECM receptors, and may also take part in this complex. **(B)** PrP^C interacts with the secreted Stress Inducible Protein 1 (STI1), which mediates neuroprotection and neuritogenesis through independent signaling pathways (PKA and ERK1/2).

In addition, the wide range of molecular interactions described for PrP^C suggests that this protein may be relevant for not only the spongiform encephalophaties, but also as an ancillary component of the pathogenesis of other neurodegenerative diseases, and therefore amenable to therapeutic targeting.

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Prion diseases are under compulsory notification in Brazil: surveillance of cases evaluated by biochemical and/or genetic markers in 2009

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Transmissible Spongiform Encephalophaties (TSE) are caused by an infectious agent, nominated as prions. They affect animals and humans with the latter presenting sporadic, familial or acquired forms of the disease. The transmission of bovine spongiform encephalopathy (BSE) to humans caused the new variant of Creutzfeldt-Jakob disease (vCJD). Many concerns have been addressed about iatrogenic transmission of CJD, since any screening test for prion contaminated material, as well as treatment are available. In 2005 the Brazilian Ministry of Heath asserted that all cases of possible human prion diseases should be notified and established collaborations with academic and scientific institutions to diagnose these disorders. In 2009 37 reported cases were evaluated by clinical, imaging, biochemical and/or genetic tests. The distribution of these notifications according to the Brazilian State is represented at Figure 1.

Figure 1- Notified cases with possible sCJD in Brazil in 2009.

The presence of protein 14.3.3 in cerebrospinal fluid was evaluated in 27 cases while 23 cases were analyzed by EEG (electroencephalogram) and 27 for MRI (magnetic resonance imaging) (Table 1).

According to the World Heath Organization criteria http://www.who.int/mediacentre/factsheets/fs180/en/), 68% (25 cases) of the cases were classified as possible sporatic CJD (sCJD) and from these 60% (15 cases) fulfill the parameters for probable sCJD (sCJD).

Table 1 Parameters evaluated in notified patients

14.3.3 (n=27)		EEG (n=23)			MRI (n=27)			
Negative	Positive	Normal	Typical	Atypical	Normal w/o dif	Normal w/ dif	Typical	Other abnor
n= 17	**n=10**	**n=1**	**n=14**	**n=8**	**n=1**	**n=4**	**n=13**	**n=9**
(63%)	**(37%)**	**(4%)**	**(61%)**	**(35%)**	**(4%)**	**(15%)**	**(48%)**	**(33%)**

w/o dif.= without diffusion; w/ dif.= with diffusion; abnor= abnormality

One case was confirmed as a genetic form of the disease with the mutation identified at codon 200 (E200K) of the prion protein gene (PRNP). The other 11 cases (21.7%) presented insufficient clinical signals to be classified as possible prion disease and 3 cases had an incomplete notification form (8.1%).

Table 2 – Classification of notified patients

Possible sCJD	Genetic CJD	Unclassified	
		Insufficient clinical signals	Incomplete form
n= 25 (67.5%)	n= 1 (2.7%)	n= 8 (21.7%)	n= 3 (8.1%)

From all analyzed cases, one presented a rare polymorphism at codon 171 (N171S) which is not associated with prion diseases. Polymorphisms at codon 117 (A117A$_{sil}$) were present in 5 cases (13%) while 60% (22 cases) of the cases presented M129M, 24% (9 cases) M129V and 16% (6 cases) V129V (Table 3). Brain tissues from 4 of notified cases were also evaluated by immunohistochemistry and all of them were positive for PrP^{Sc} and confirm the diagnosis of prion disease (Figure 2).

Residue Position	Genotype	Notified Cases n= 37 (%)
Octarepeat	R12234	37 (100)
		0 (0)
	R1234	0 (0)
117	Ala/Ala	32 (87)
	Ala/Ala$_{silent}$	5 (13)
129	Met/Met	22 (60)
	Met/Val	9 (24)
	Val/Val	6 (16)

Table 3 – Polymorphisms in PRNP gene in patients notified with possible prion diseases

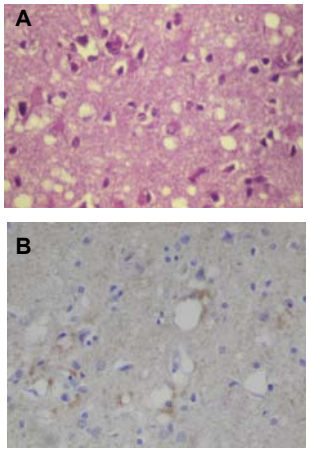

Figure 2- **(A)** HE from brain tissue with an intense vacuolization **(B)** Immnohistochemistry for PrP^{Sc} in brain tissue from a positive (B) case of CJD.

Role of astrocytes in the secretion of PrP^C ligands that mediate PrP^C-dependent functions in neurons and astrocytes

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Astrocytes and neurons interact in various neurophysiological events and represent an integral unit in brain function. Astrocytes are key elements in both the production of neurotrophic molecules and controlling the levels of neurotoxic substances. They can also modulate the production of extracellular matrix that supports neuronal development and differentiation.

The role of cellular prion protein (PrP^C) in neurotrophic interactions between astrocytes and neurons has been evaluated *(Lima et al., 2007)*. Astrocytes produce and secrete laminin, among other ECM proteins. Laminin secreted and deposited at the ECM by wild-type astrocytes showed a fibrilar organization **(Figure 1A)**, while that secreted from PrP^C-null astrocytes has a punctate pattern[2] **(Figure 1B)**. The pattern of laminin matrix influences neurite outgrowth either through modulation of its binding to PrP^C or to other cell surface receptors *(Lima et al., 2007)*.

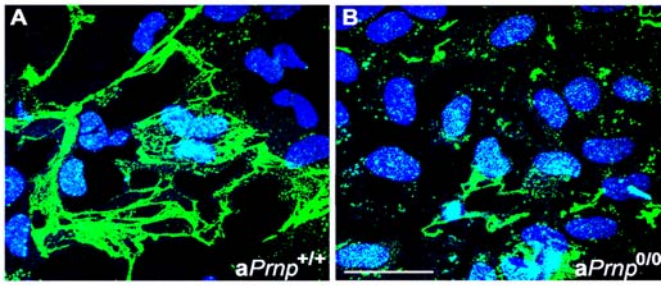

Figure 1- Immuno-fluorescence showing the laminin secreted and deposited by wild-type (A) and PrP^C-null (B) astrocytes.

Astrocytes also release soluble neurotrophic factors and proteins such as STI1 (Stress inducible protein 1) and PrP^C and these proteins can bind to neuronal PrP^C and NCAM respectively, mediating neuronal survival and differentiation *(Lima et al., 2007)*. Indeed, PrP^C expression in astrocytes is critical to sustain the organization of extracellular matrix and secretion of soluble factors, thus helping to maintain a healthy environment for neuronal survival and differentiation (**Figures 2A and 2B**). Therefore, PrP^C not only works as a neurotrophic factor for neuronal cells, but also modulates astrocyte performance on neuronal survival and differentiation.

In addition to the specific role of PrP^C-STI1 engagement in neurons the role of this interaction in astrocytes was also evaluated *(Arantes et al., 2009)*.

STI1 prevents cell death in wild-type astrocytes in a protein kinase A-dependent manner while PrP^C-null astrocytes were not affected by STI1 treatment *(Arantes et al., 2009)*.

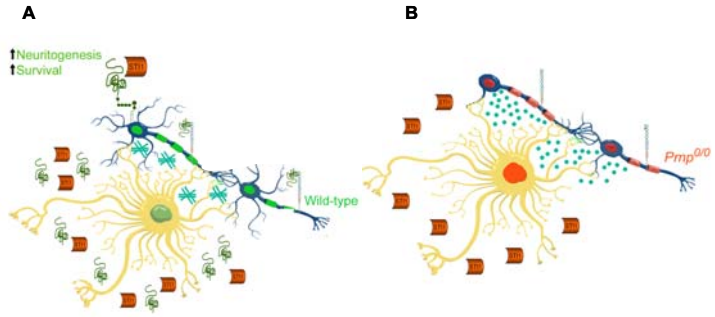

Figure 2- PrP^C and STI1 as neurotrophic factors in neuron-glia interaction. A prominent neuritogenesis is observed when wild-type neurons are co-cultured with wild-type astrocytes. Neurite outgrowth is impaired when both cell types are derived from PrP^C-null mice. Laminin secreted and deposited at the ECM by wild-type astrocytes has a fibrillar organization and promotes more intense neurite outgrowth than the puctated laminin secreted from PrP^C-null astrocytes. Astrocytes also release STI1 and PrP^C, which induce neuronal survival and differentiation. STI1 is also an autocrine factor mediating astrocyte survival, differentiation and proliferation.

STI1-PrP^C interaction also induces ERK1/2 activation which promotes differentiation of a flat to a process-bearing morphology while any alteration was observed in PrP^C-null astrocytes. Remarkably, STI1 inhibited proliferation of both wild-type and PrP^C-null astrocytes in a protein kinase C-dependent manner. Indeed, PrP^C and STI1 are essential to astrocyte development, and act through distinct signaling pathways *(Arantes et al., 2009)*.

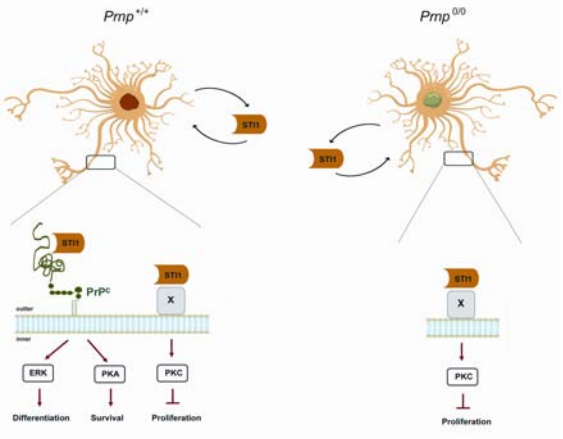

Figure 3 - Astrocytes secrete STI1 into the extracellular medium to act as an autocrine factor. At the cell surface STI1 interacts with PrP^C ($Prnp^{+/+}$) leading to differentiation through ERK1/2 activation and cell survival via the PKA pathway. In the absence of PrP^C ($Prnp^{0/0}$), astrocytes fail to undergo morphological changes and are more sensitive to staurosporine-induced cell death. STI1 is also capable of interacting with another putative receptor (X) to impair cell growth through PKC activation. The functional interplay of PrP^C and STI1 tightly regulates survival and differentiation in astrocytes, which are essential processes modulating neural activity.

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Purified retinal Müller cells differentiate dopaminergic properties and recover locomotor deficits in mouse model of Parkinson`s disease.

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Parkinson's disease (PD) is characterized by a progressive loss of dopaminergic neurons from *Substantia Nigra pars compacta* (SNpc). The most used symptomatic treatment of PD is levodopa (L-DOPA). This precursor of catecholamines alleviates PD symptoms but also causes adverse side effects after long periods of treatment (for review see Dauer and Przedborski 2003).

Alternative strategies involving the use of pluripotent stem cells have been proposed to restore dopaminergic function of deficient nigro-striatal circuit. Despite the great potential of pluripotent cell therapy, teratoma formation is a remaining challenge to be solved before clinical trials start (Lindvall and Kokaia, 2009). An alternative to this approach would be the use of non-pluripotent cells that naturally produce dopamine and that could be expanded *in vitro* to amounts that could be used in transplantations. Recently we demonstrated that Müller glial cells isolated from the avian retina express the enzymes tyrosine hydroxylase (TH) and dopa decarboxylase (DDC) and synthesize dopamine from L-DOPA. (Kubrusly et al 2008). Most, if not all dopamine synthesized by these cells is released to the extracellular space. Therefore, once purified, these cells spontaneously synthesize and release dopamine constitutively.

As for the avian Müller cells, murine glia also develop dopaminergic properties as illustrated in figure 1.

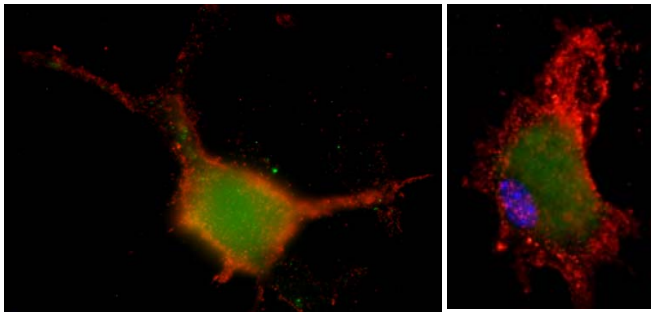

Figure1: Purified Müller from mice stained for **TH** (green); **S100β** , a specific marker for mammalian Müller (red); and **DAPI** (blue) for cell nuclei.

These cells not only synthesize dopamine but also release it to the extracellular space, a required property if one is to use these cells to promote dopaminergic activity (Figure 2)

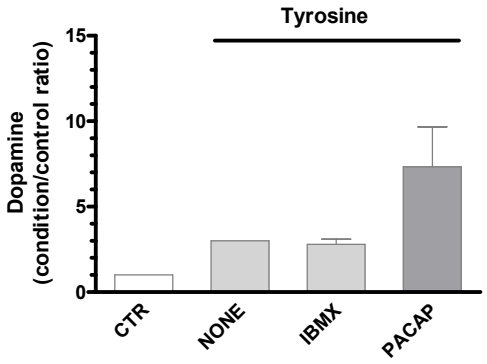

Figure 2: Müller glia purified cultures do not retain dopamine in its cytoplasm. Most of the dopamine produced is promptly released and found in the extracellular medium. **CTR** (empty bar) amount of dopamine release under control condition. The other bars indicate dopamine production and release after exposing cells for a few hours to the amino acid tyrosine, precursor of L-DOPA and dopamine. Note that this treatment increases by a factor of 3-fold the amount of dopamine in the medium (**NONE** bar). If cells are stimulated with PACAP, an agent that increases cAMP in these cells and activate tyrosine hydroxylase, the level of dopamine released increases by a factor of 8-9-fold (**PACAP** bar).

We propose that "dopaminergic" Müller cells could serve as a dopamine producing system, having as a default the release of this amine to the extracellular space, therefore, constituting a natural biological pump of dopamine. This proposal is schematized in figure 3.

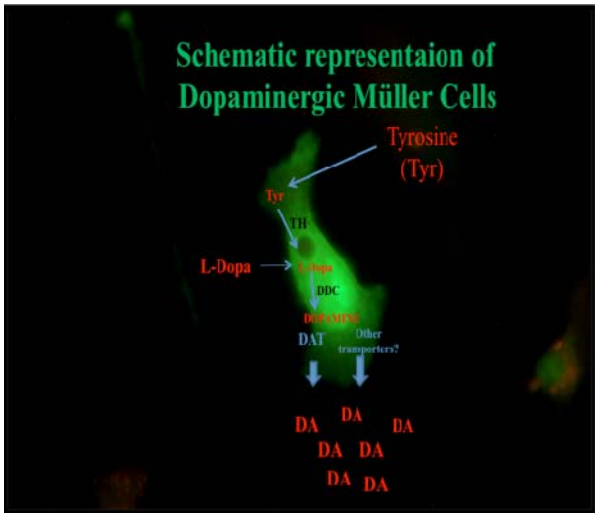

Figure 3: In green is a Müller cell as revealed by **GFAP** immunostaining. These cells take up **Tyrosine** from the extracellular enviroment that, once in the cytoplasm, is converted into **L-DOPA** by the action of **TH** (tyrosine hydroxylase), that is further transformed by **DDC** (dopa decarboxylase) into **DOPAMINE (DA)**. Dopamine is then released to the extracellular space via its transporter **DA**T.

We tested, then, the hypothesis that Müller glial cells isolated from mouse retina, after transplantation into the striatum, could recover locomotor deficits of hemiparkinsonian mice. These mice are produced by having one side of the brain injected with the neurotoxin 6-Hydroxydopamine (at the SN level). This agent destroys most of the dopaminergic neurons projecting to the corresponding

striatum, leaving intact the other side of the brain. When these animals are stimulated with agents (apomorfine or amphetamine) that activate locomotor activity, they rotate to the contra lateral side of the lesion. Therefore, locomotor activity can be quantified by measuring the number of rotations displayed by the animals. Non-lesioned animals or animals that have been transplanted with dopamine producing cells or treated with agents that compensate for the lack of dopamine display a reduced rate of rotation.
Figure 4 shows that transplantation of “dopaminergic” Müller cells into the striatum of the murine model of Parkinson`s disease restores the altered locomotor behavior of these animals.

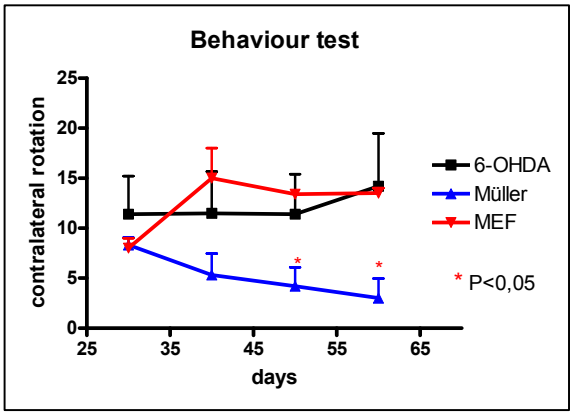

Figure 4: Contralateral rotations of mice stimulated with apomorfine 30 days after lesion of the nigro-striatal pathway. **Black line** shows the rotation of lesioned mice measured at intervals of 10 days up to one month of observation. **Blue line** depicts the rotation rate of animals with lesions and that were transplanted with Müller cells (on day 25) in the striatum lacking dopaminergic projections. Note that 30 days after transplantation the animals had their rotation rate reduced by more than 5-fold; close to the rotation rate of control, sham operated animals (not shown). **Red line** is the motor behavior of mice with lesions, transplanted with murine embryonic fibroblasts (MEF) that do not produce dopamine, do not compensate for the motor anomalies displayed by these animals.

These findings indicate that retinal Müller cells could be considered as an alternative source of dopamine releasing cells, free of genetic modifications and with reduced risk of teratoma formation. It also indicates that homologous transplantation of these cells can eventually be used to treat patients with PD. Work is in progress to see whether Müller cells from primates could reproduce what has been characterized for avian and mouse Müller cells.

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The repercussion of malnutrition on the Central Nervous System.

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We examined the effects of a multi-deficient diet after birth on the proliferation and differentiation of neurons in the hippocampus (BrdU, as a proliferation marker and doublecortin, as a neuronal migrating marker) as well as behavioral aspects (spatial memory, learning and exploratory activity were investigated using Morris water maze, passive avoidance and open field tests, respectively) linked to hippocampal functionality. This diet, referred to as regional basic diet (RBD), is qualitatively and quantitatively similar to the basic meal that is consumed by some rural communities in the northeast part of Brazil, as screened by a nutritional survey (*Teodósio et al. 1990*). It is composed of about 8% protein, being reduced in lipids and increased in carbohydrates. The majority of its content is of vegetal origin, revealing a low biological value. We applied this multi-deficient diet from birth to adulthood.

RDB malnutrition caused a significant decrease of 25% in the total cell number in the dentate gyrus (not shown), a consistent decrease of 45% in BrdU positive cells in both the granular and subgranular cell layer of the hippocampus and decrease of 85% in the number of DCX labeled cells (figure 1).

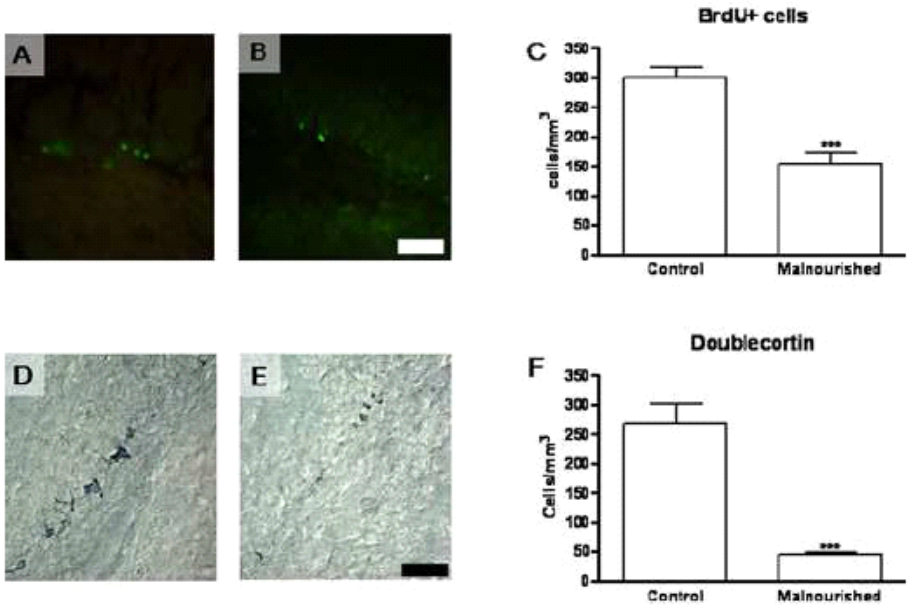

Figure 1 BrdU expression on dentate gyrus: (A) control group and (B) malnourished group. (C) density of proliferating cells (BrdU+) was decreased by malnutrition. ***p < 0.001 relative to control rats. DCX immunoreactivity in the dentate gyrus in the control group (D) and the malnourished group (E) – note that malnourished rats showed less DCX neurons in the dentate gyrus when compared with the control animals. ***P < 0.001 (F). Scale bar, 50 □m. **p < 0.01, ***p < 0.001.

Our data also show that the animals submitted to this multi-deficient diet (RDB) present deficits in learning and in spatial memory and less locomotor activity.

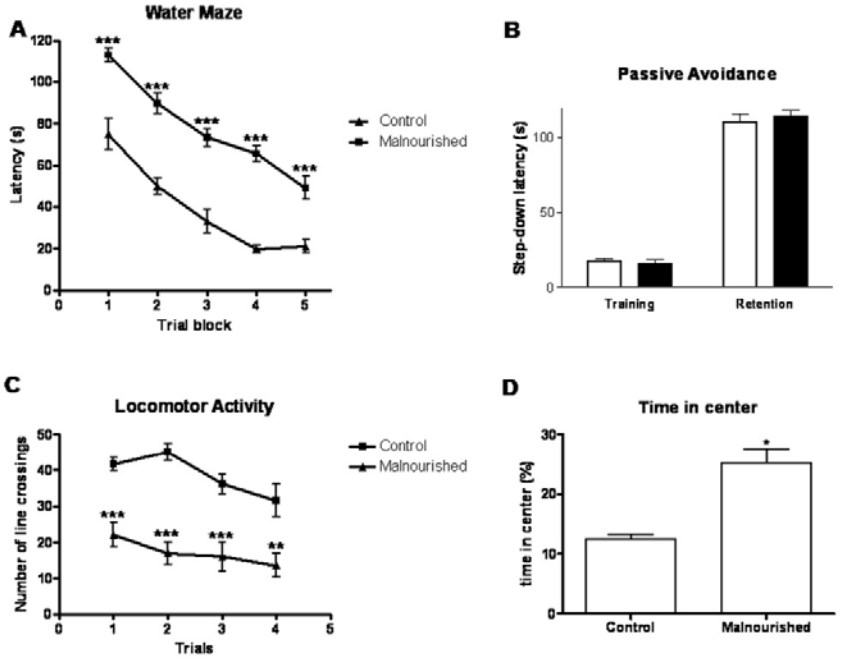

Figure 2: BEHAVIORAL TESTS (A) Bad performance of malnourished rats in the Water Maze. (B) No differences in the retention test of the passive avoidance task. Malnutrition affects the rat locomotor behavior. (C) Ambulatory activity of control and malnourished rats over four days, measured by number of line crossings. (D) Time spent in the center of the arena is significantly increased in the malnourished group. *P<0.05; **P < 0.01, ***P < 0.001.

Taken together, these results suggest that malnutrition can delay the maturation of newly generated cells in adult hippocampus, affecting behavioral features. Work is in progress to check if rich environment is capable to prevent hippocampus deficits under malnutrition conditions.

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Müller Cells: phenotype adaptation and survival of retinal neurons

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***Colaborative group*:** Instituto de Biofísica Carlos Chagas Filho-UFRJ **(INCT-Instituto Nacional de Neurociência Translacional)**.

Degeneration of neural retina is a major problem associated with visual dysfunction, leading to blindness. Cell replacement strategies have the potential to improve vision in patients who were previously considered to be untreatable. Two of the key target cells involved in most of the retinal disorders are photoreceptors (transduce light into electrical signals, identified by antibodies against opsin and rodopsin, green, Figure 1) and retinal ganglion cells (whose axons convey action potentials through the optic nerve, identified by Tuj1, red, Figure 1). Müller cells, on the other hand, are the main non-neuronal cell in the vertebrate retina and they interact with most, if not all neurons in this tissue, (top right, green, Figure 1). Recent data have given hope to hundreds of patients worldwide as progenitor cells in the retina, as well as the Müller glia, display regenerative properties that could be used to directed neuroprotection and regenerative medicine of the retina.

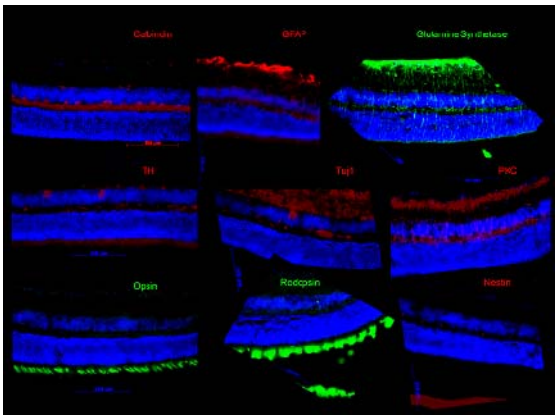

Figure 1
Adult mice (10 weeks) retina sections labeled with selective antibodies that recognize all the major cells of the retina. Note that Gluthamine synthetase (GS) label Müller glia (top right, green) that different from other cells, transverse the entire length of the retina.

Müller cells represent the main type of glia present in the retina interacting with most, if not all neurons in this tissue. Müller glia is an active player in the retina, responsible for the release of trophic factors, in the maintenance of the extracellular milieu homeostasis, and release of mediators that keep the bidirectional flow of neuronal-glia interactions. Müller glia trophic factors may regulate many different aspects of neuronal circuitry during synaptogenesis, differentiation, neuroprotection and survival of photoreceptors, Retinal Ganglion Cells (RGCs) and other targets in the retina (de Melo Reis et al 2008a).

Upon damage, Müller glia re-enter the cell cycle, dedifferentiate, acquire progenitor-like phenotypes, and produce new neurons and glia (Fischer and Reh, 2003). It is not known how this happens in the absence of neurons, but as Müller cells express functional receptors and transmitter enzymes *in vitro*, this could be of potential interest in directing the correct transformation of Müller cells into specific neurons. Different transmitter phenotypes have been associated with these cells in culture (Kubrusly et al., 2005; de Sampaio Schitine, 2007; Ramirez and Lamas, 2009). Recently, Müller cells have been shown to generate clonal neurospheres and upon retinal lesion are capable of differentiating into functional neurons and to generate site-specific neurons upon transplantation.

Neuro-glial interactions are based on a bidirectional communication mediated by trophic factors and modulators. Trophic activity secreted from Müller cells has been reported to support RGC survival and neuritogenesis (Figure 2). Moreover, Müller glia within the retina or in culture express mRNA and protein for a vast amount of tropic factors from several distinct families (de Melo Reis et al., 2008b)

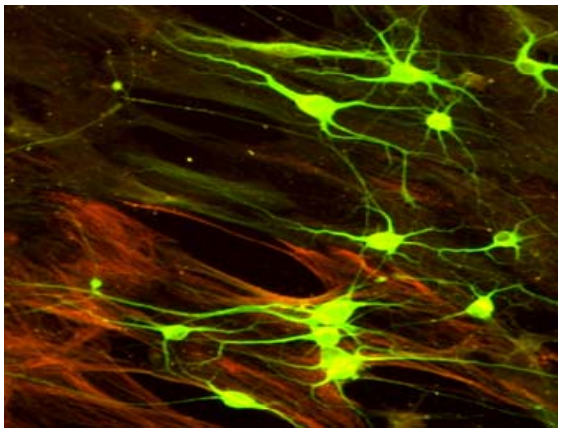

Figure 2.
Postnatal RGCs plated on top of purified Müller glial cells for 72h. The cells were then fixed, washed with PBS and prepared for immunocyto-chemistry. The primary antibodies used for staining for neurons were anti-HNK-NCAM, while for glial cells were anti-vimentin (de Melo Reis et al., 2008b).

Retinal cell neurospheres proliferate in the presence of growth factors and are easily prepared from single cell suspensions derived from early postnatal mice retina. In the proliferative state, several cells express molecular markers of neural stem/progenitor cells (Figure 3). We are developing a functional single cell imaging platform allowing the simultaneous identification of a diversity of cell phenotypes differentiating from retinal neurospheres, prepared from single cell suspensions derived from early postnatal mice retina and allowed to proliferate in the presence of growth factors.

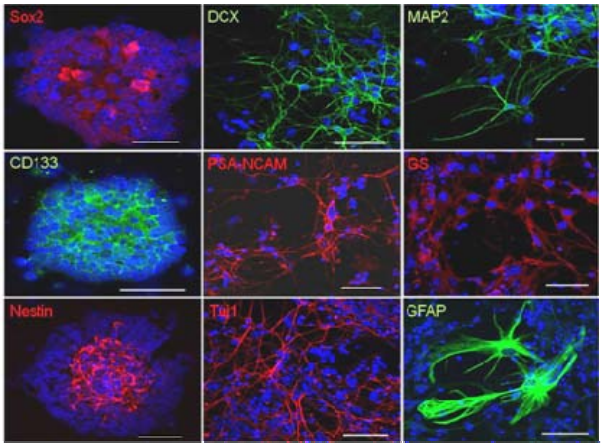

Figure 3 Expression of stem cell, progenitor, neuronal and glial markers on the three-dimensional structure of the retinal neurospheres

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The Glial Network

The Network Glial (RG, in portuguese) headed by Vivaldo Moura Neto put together laboratories from Instituto de Ciências Biomédicas leaded by Dr. Flavia Gomes and Dr. Jose Garcia Abreu, from Medical School of the Universidade Federal do Rio de Janeiro by Professor Leila Chimelli, from the Department of Cell Biology, Universidade Federal de Santa Catarina by Dr. Andrea Trentin and two other scientists; one from Universidade Federal da Bahia leaded by Dr. M Fatima Costa and from Universidade Federal de Pernambuco by Dr. Rubem Guedes. The central objective of the RG group is the study of the neuronal-glial cell interactions that supports the harmonious development of the brain as well as by looking elements involved in these interactions to understand glial tumors during tumoral progression. The expansion of this Network Glial has grown in the last year with the creation of Brazilian Glial Club, coordinated by RG team involving around 34 groups interested on Glial cells. These Glial Club was also associated to the latin American laboratories, Spanish and Portuguese to establish “la Red Glial Iberoamericana (RGIA)” looking for the enhacement and development of international colaboration between different glial groups and then permit the qualification of young researchers in this domain. Two other international activities of Brazilian RG was the plenary lecture presented in 2009 September by Vivaldo Moura Neto at two meetings: 9th European Meeting on Glial Cells and Spanish Neuroscience Society meeting. In the same occasion, Vivaldo also presented a lecture in National Institute of Biology of Slovenia and started a cooperation between RG and laboratories from this Institute. The next meeting of SBBC, 2010 July, will hold a great reunion of RG and RGIA with several speakers from different laboratories from Brazil and Europe. This meeting will consolidate the glial cooperation between Brazilian and foreign partners at Glial Studies and aim to apply for financial support in common research projects. The member José Abreu established a cooperation with Professor Xi He (Wu et al.,2009) at the Childrens Hospital Boston, Harvard Medical School to search for new genes involved in the formation of the vertebrate brain. From that cooperation a new mechanisms were uncovered about how the future brain is constructed involving Wnt/ß-catenin inhibition. This cooperation involved one PhD thesis and 3 papers had been published in international journals. Rubem Guedes, another member of this NTIN and UFPE develops a cooperation (Fregni et al, 2005; Liebetanz, D et al. 2006, Fregni, F et al. 2007) with, Felipe Fregni from Havard University to establish analysis of brain cells using transcranial stimulation (tDCS), transcranial magnetic stimulation (TMS) which permit evaluate cerebral excitability during injures as concussions, for instance. Flávia Gomes colaborates with Dr. Eva Anton from North Caroline University, USA. The aim of their project is to study the mechanisms and molecules that control neural stem cell commitment. They recently characterized a molecular pathway involved in the generation of astrocytes from radial glia cells (neural stem cell of the cerebral cortex): the TGF-b1/smad pathway (unpublished results). Their colaboration involved a PhD student who developed part of her thesis in the USA. The scientific contribution of RG to Glial understanding has

been focused on Glial cells in health and diseases and can be addressed by the following subtopics: a- cell-cell interactions; b-New insights with old drugs against glioblastoma proliferation; c-glial differentiation, looking at stem cells and mechanisms underlying neurological disorders associated with thyroid hormone deficiency.

Cell-cell interactions in neurological disorders and in normal brain

Flavia RS Lima[1] , Michel Mallat[2] , Vilma Martins[3] , Rafael Linden[4], Rubem Guedes[5], Jorge Marcondes [6] , Leila Chimelli [6] ,Vivaldo Moura Neto[1]

***Colaborative group*:** 1-Instituto Ciências Biomédicas-UFRJ, 2-INSERM-Hôpital de la Salpétrière, France, 3-Instituto Ludwig SP, 4-Instituto de Biofísica Carlos Chagas Filho, 5-Departamento de Nutrição- UFPE, 6-Faculdade de Medicina – UFRJ **(1-3,5,6 INCT- Instituto Nacional de Neurociência Translacional)**. Rio de Janeiro,RJ

Interactions between neuronal and glial cells are pivotal to harmonious development of the brain. We are interested to analyze the interactions with cerebral microenvironment and normal and tumoral glial cells to understand the progression of glioma. We previously demonstrated that normal astrocytes are the preferential substrate to development of neurons and recently (2006) that human glioblastoma cells (GBM) could support the neuritogenesis of neurons submitted to co-culture. Further, in collaboration with Vilma Martins (NTIT) and Rafael Linden (IBCCF-UFRJ) we also demonstrated that the co-chaperone STI1, a PrPc ligand, is secreted by glial cells and promotes glioblastoma (GBM) proliferation. Now, our microglia-GBM interaction studies focus at the influence of STI1 and PrPc in the tumoral growth and invasion. Highly pure cultures of microglial cells from neonatal mice and tumor cells from GBM95 human cell line express and secrete STI1 which seems induces GBM proliferation, 100% increased. MAPK/ERK, PI3K and PKA pathways might be involved in signaling pathways in the tumoral proliferation and cellular migration.

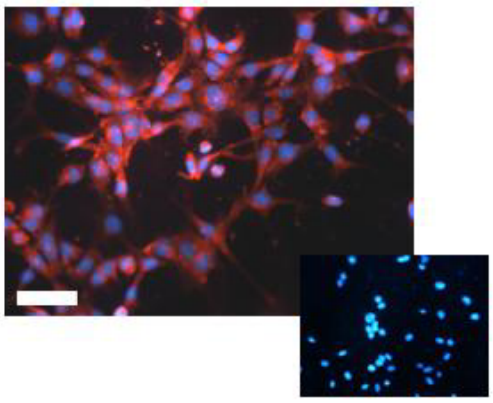

Figure 1- A172 cells express PrP^{C}.- Immunostaining.
Right panel is a negative control

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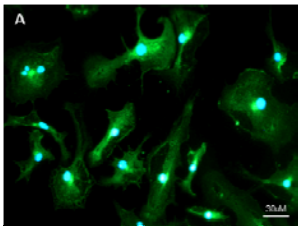

Figure 2- Microglia express and secrete STI1. Microglial cells were immunolabeled with anti-STI1 antibody and nuclei were stained with DAPI.

Xenotransplanted human glioblastoma into rodent brain shows this microglia-glioblastoma interaction supporting our in vitro data.

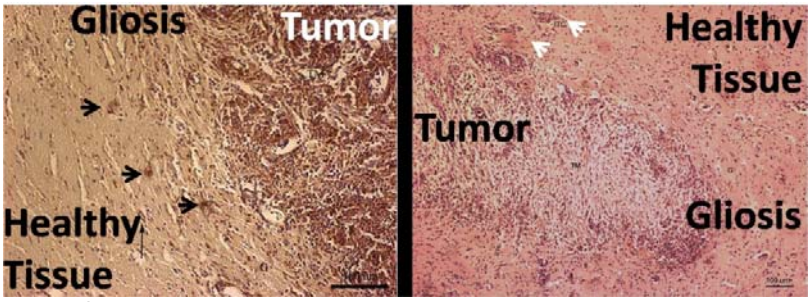

Figure 3- Xenotransplanted human glioblastoma – GBM 95. Isolectin B4 (left panel) peroxidase staining showing the microglial cells into the tumoral mass and around of it. Black arrows show IB4 positive cells around the tumor. H&E staining (right panel) for the tumor mass and the healthy tissue. Note gliosis and the white arrows show some tumor cells invading the tissue around.

Another line under investigation is the effects of spreading depression (CSD), a slowly propagating wave of suppressed electrical activity, It is characterized by a wave of depolarization of neurons and glia that propagates in the gray matter of the central nervous system (CNS). CSD can be recorded in various experimental pathological conditions that affect the cerebral cortex including brain trauma, subarachnoid hemorrhage, and stroke. However, the exact cause and the pathophysiological correlate of CSD remain elusive, and only a few studies have identified CSD in human brains. Cortical myelin can be severely affected in patients with demyelinating disorders of the central nervous system. However, the functional implication of cortical demyelination remains elusive. We have investigated whether cortical myelin influences cortical spreading depression. Rubem Guedes and collaborators published in Annals of Neurology (2009) 66:355–365, that " Propagation of Spreading Depression Inversely Correlates with Cortical Myelin Content". They demonstrated that Cortical demyelination, but not astrogliosis or inflammation per se, was associated with accelerated CSD. In contrast, hypermyelinated neuregulin-1 type I transgenic mice displayed a decelerated CSD propagation. These studies provide several lines of experimental evidence that the extent of cortical myelination inversely correlates with CSD propagation velocity. Thus, cortical demyelination, as can be noted in chronic MS and in other demyelinating diseases of the CNS, may represent a pathological substrate for altered CSD. Cortical myelin may be crucially involved

in the stabilization and buffering of extracellular ion content that is decisive for CSD propagation velocity and cortical excitability, respectively. Our data thus indicate that cortical involvement in human demyelinating diseases may lead to relevant alterations of cortical function.

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New insights with old drugs against glioblastoma proliferation

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Glioblastomas are the most common subtype of gliomas and are considered one of the deadliest human cancers. There is evidence that the incidence of brain tumors, such as glioblastomas has been rising for as much as fifty years. New efforts are focused on finding novel effective compounds and therapies against brain tumors, such as flavonoids, targeted toxins and pore-forming proteins. As a association of Flavia Gomes's group and Silvia Costa (UFBA) and her students we began to study the effects of the flavonoid casticin from Brazilian Croton betulaster in cerebral cortical progenitors in vitro: direct and indirect action through astrocytes. More recently, with José Garcia Abreu's team we have identified the flavonoid isoquercetrin as a potent negative regulator of glioblastoma proliferation. It inhibits cell cycle of glioblastoma cells by decreasing levels of cyclin D1 and increasing levels of p27. Different from other flavonoids such as quercetin and rutin, isoquercetrin did not induce apoptosis. In adition, we found two evidences that indicate isoquercetrin as a modulator of Wnt/□ß-catenin signaling. In glioblastoma cells treated with isoquercetrin ß-catenin changed from nuclear to cytoplasmic.

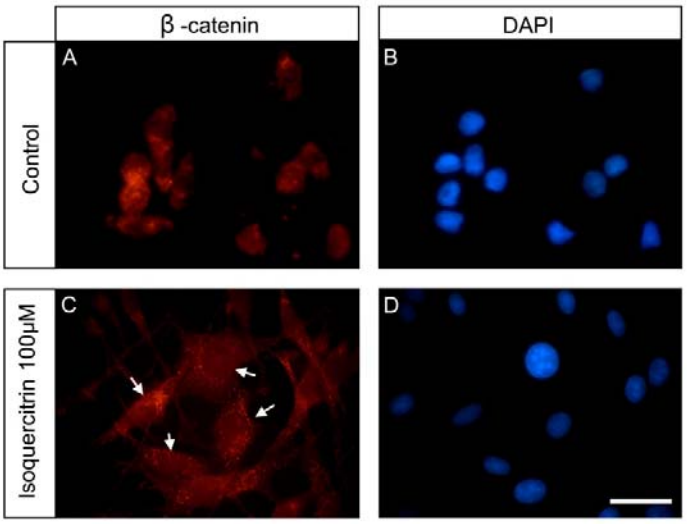

Figure 1-Isoquercitrin affects β-catenin localization. These figure showed β-catenin immunostaining of human glioblastoma (Gbm95) cells. After cells treated with isoquercitrin, β-catenin staining is primarily localized in the cytoplasm

Likewise, protein level of GSK3, a kinase involved in □β-catenin phosphorilation is increased after isoquercetrin treatment. Further, to control glioblastoma proliferation, we use natural pore-forming toxins to generate a novel class of anti-cancer drugs has been explored recently. The common mechanism of action of these toxins, also referred as cytolysins, is the formation

of pores on the targeted cell membrane that can be cytotoxic and cytolytic. Cytolysins either alone, or as hybrid proteins, fused to molecules, have been used to target and kill cancer cells selectively as previously demonstrated one of our collaborator from Slovenia, Gregor Anderluh (2002) We showed, with Anderluh and Javier Vernal (Spain), Hernán Terenzi and Nelson H. Gabilan from UFSC-Brazil, that equinatoxin-II (EqTx-II, isolated from the sea anemone *Actinia equina*) and toxin Bc2 (isolated from the sea anemone *Bunodosoma caissarum*), could be promising tools to selectively kill glioblastomas. Moreover, when given at non-toxic concentrations, EqTx-II enhanced up to 300-fold the cytotoxicity of the antimicrotubule agent vincristine in glioblastomas (Table I), a very great demonstration that these cytolysins can also target cancer cells when used in combination with conventional chemotherapeutic drugs.

Table 1. Cytotoxicity of chemotherapeutic drugs alone or combined with toxin Bc2 or EqTx-II against U87 cells

Compound	IC_{30} (µM)	$IC_{30\ Bc2}$	Ratio	$IC_{30\text{-}EqTx\text{-}II}$	Ratio
Vincristine	0.3	2.10^{-3} µM	150	10^{-3} µM	3.10^{2}

The mechanism of inducing cell death mediated by cytolysins in gliomas and some of the possible cell signaling pathways involved in their action, particularly the MAPK/ERK, PKC, PKA, CaMKII and PI3K pathways. Swollen, dead or dying cells U87 and A127 were negative for TUNEL-staining.The pre-treatment with inhibitors of mitogen-activated/extracellular regulated kinase (MEK1), protein kinase C (PKC) or Ca^{2+}/calmodulin-dependent kinase II (CaMKII) blocked the toxic effects of toxin Bc2 and EqTx-II, suggesting that calcium entry, activation of MEK1, PKC and CaMKII pathways are involved in the cytotoxicity induced by these cytolysins.

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Peripheral and Central Nervous System differentiation

Giordano W. Calloni[1], Ricardo C.Garcez[1], Marcio Alvarez-Silva[1], Andréa G. Trentin[1,] Elizabeth Dupin [2] ,Nicole Le Douarin[2], Rubem C Guedes[3] , Flavia R S Lima[4] Flavia Carvalho Alcantara Gomes[4] ,Vivaldo Moura Neto[4]

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Two actions are in course to approach this line of cellular differentiation:1-analyze the contribution of neural crest stem cells; 2-hormonal effects on neural differentiation. NC is a model system to investigate multipotency during vertebrate development. Environmental factors control NC cell fate decisions. By analyzing mass and clonal cultures of cephalic mouse and trunk quail NC cells; we show here for the first time that fibronectin (FN) promotes expression of the smooth muscle cell phenotype, while not affecting differentiation into glia, neurons and melanocytes.
Time course analysis indicated that the FN-induced effect is not related to massive cell death during the differentiation period of culture, or proliferation of smooth muscle cells.

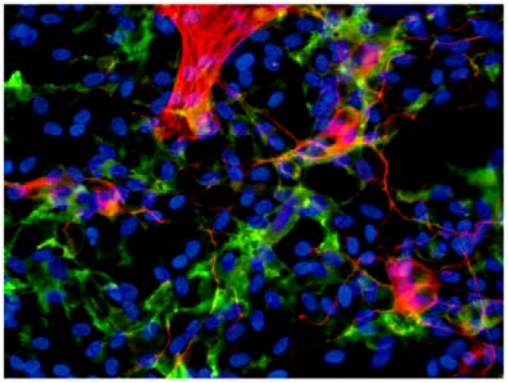

Figure1- Imunofluorescence picture of a tripotent progenitor from quail NC, endowed with glial, neuronal and smooth muscle potential. The progenitor was identified according to the presence of SMP^+ glial cells (green), $aSMA^+$ smooth muscle cells (red) and betaIII tubulin neurons (red ↙), under EGF+FGF.

We show for the first time that EGF induces differentiation of NC to the neuronal and melanocytic phenotypes, while fibroblast growth factor 2 (FGF2) promotes NC differentiation to Schwann cells. In the presence of both EGF and FGF2, the neuronal differentiation predominates. Our results suggest that FGF2 stimulates gliogenesis, while EGF promotes melanogenesis and neurogenesis. These results show that FN promotes NC cell differentiation along the smooth muscle lineage and, therefore, plays an important role in fate decisions of NC progenitor cells. The results also suggest that EGF and FGF2 may play an important role in the fate decision of NC progenitors and in the development of the peripheral nervous system.

Vivaldo Moura Neto, Flavia Gomes, Flavia Lima,all from UFRJ, Andrea Trentin from UFSC and Rubem Guedes from UFPE have a good experience studing thyroid hormone (T3) effects on neuronal and glial cells. Years ago, we

described a new way to neuronal action of T3 via glial cells. This T3 induces glial cells to secret growth factors like EGF, FGF, TGFbeta to autocrinally act on differentiation of undifferentiated glial cells with increased expression of GFAP (a protein marker of differentiated glial cells) and paracrinally induce differentiation of cortical neuron and proliferation of cerebellar neuroblast. Recently, we showed that T3 induces increased expression of Myosin Va and its RNA in tumoral and normal glial cells during differentiation (1B). This observation reinforce knowed role of T3 in actin-myosin cargo transport system and open a new via to understand the communication between cells during histogenesis. The development of newborn is dramatically affected by Thyroid Hormones (THs) and it is impaired under hypothiroidism. Then to understand the cellular and molecular, mechanism of THs action during nervous system development, we have been studying the cerebellum, one of the best known targets of these hormones and theirs nuclear receptors (TRα and TRβ, looking to the efficience of Bergmann glia (BG) (Fig.2) with its radial morphology, an essential characteristic to proper neuronal migration in cerebellum.

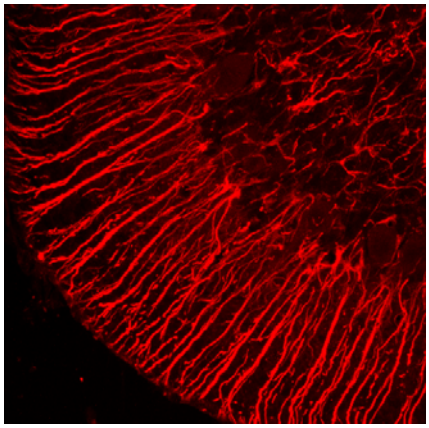

Figure 2 : GFAP immunostainning (glial marker) of 9-days of mice cerebellum. Note the elongated morphology of BG cells.

Using a natural human mutation (337T) in the TRβ locus, which impairs T3 binding to its receptor, on the mouse cerebellum ontogenesis and *in vivo* /*in vitro* experiments we suggest that TRβ might be a mediator of TH action in BG maturation

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How do we recognize familiarity?

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There are numerous evidences indicating the existence of multiple forms of long-term memory (LTM). In this context, declarative -or explicit- memory can be is defined as the memory for facts and events and is often sub-divided into episodic (memory for events within one's own life) and semantic memory (general knowledge about aspects of the world). Information about the sensorial, spatial and contextual attributes of previously encountered objects is an important element of most declarative memories. Indeed, difficulty to recognize familiar items or to discriminate them from novel ones is one of the early symptoms of cognitive decline in Alzheimer's disease (AD; Dudas et al., 2005; Budson et al., 2005). For these and other reasons, learning paradigms based upon the recognition and discrimination of novel and familiar objects are essential to understand the neurobiology of declarative memory (Winters et al., 2008).
Functional integrity of the medial temporal lobe, including the hippocampus, the amygdala and the entorhinal, perirhinal, and parahippocampal cortices, is essential for recognition memory processing (Ennaceur and Delacour, 1988; Logothetis and Sheinberg, 1996; Riesenhuber and Poggio, 2002). In particular, lesion and pharmacological studies suggest that the hippocampal formation is required for acquisition and storage of information regarding contextual details and the temporal order of previous experiences (Balderas et al., 2008). However, very little is known at the molecular and electrophysiological levels about the mechanisms contributing to recognition memory consolidation in the hippocampus. Thus, research about this topic in our laboratories concentrates on unraveling the differential involvement of the hippocampus and associated cortexes, including the entorhinal, perirhinal and prefrontal cortex participation, in recognition processing.

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How are memories stored?

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A prevailing view of the modern science of memory is that memories are initially fragile but become more stable with time. In other words, memory takes time to stabilize or consolidate (McGaugh et al., 1966). Ribot, the great French psychologist, was the first to envisage that memories might be processed over time (the so-called Ribot gradient; Ribot, 1981) and Muller and Pilzecker were the first to use the term consolidation to describe the post-acquisition processes that stabilize memories thus explaining retroactive interference (Muller & Pilzecker, 1900). Memory consolidation was thereafter defined on the basis of a great body of evidence showing that a newly formed memory undergoes a lingering process, becoming stronger and resilient over time until it is insensitive to disruption. Consequently, new learned material consolidates progressively with time being the memory trace vulnerable to disruption only for a limited period after training. This idea has dominated thought in the field ever since (McGaugh, 2000).

Work in our laboratories has tried to answer several questions concerning memory consolidation: (1) which is the time course of memory consolidation? (2) Is consolidation a unitary process? (3) What are the brain regions engaged in consolidation? (4) How can the brain form persistent memories? (5) How is information in structures of the medial temporal lobe, i.e. the hippocampus, transferred for ultimate consolidation in the neocortex? And why this sequence of events occurs in some but not all memories?

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When remembering can make us to forget

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Without retrieval memories would be unusable. However, retrieval can weaken memories. It is known that, under certain conditions memory reactivation destabilizes the consolidated trace, which to persist must undergo a protein synthesis-dependent process called reconsolidation. In addition, when retrieval in the absence of appropriate reinforcement is repeated regularly enough it induces memory extinction, a phenomenon characterized by a decrease in the amplitude and/or frequency of the learned response that also requires protein synthesis in definite areas of the brain.
Reconsolidation and extinction are functionally related; both require acquisition of retrieval-related information connected to previous learning. However, they are mechanistically different; extinction involves new learning and replaces the expression of the original memory with a newly formed one (Quirk and Mueller, 2008; Pape and Pare, 2010) whereas reconsolidation restabilizes the old memory opened to modification or strengthening by retrieval (Hupbach et al., 2007; Lee, 2008; Nader and Hardt, 2009; Forcato et al., 2010).
The clinical relevance that reconsolidation blockade and extinction enhancement could have (Monfils et al., 2009; Schiller et al., 2010) is obvious. Thus, research in our laboratories concentrates on the molecular, neuroanatomical and electrophysiological requirements of reconsolidation and extinction, as well as on the elucidation of the behavioral and neurochemical conditions that constraint or facilitates either process. In addition, we study the potential interaction between extinction and reconsolidation at different levels to answer the question of whether these processes are or are not independent.

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Neuron/Astrocyte chat during the inflammatory cascade: a clue for understanding neurodegeneration and neuroprotection

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Over the last years, increasing evidence has shown that immune response activation could be related to upstream mechanism involved in epileptogenesis. Experimental studies in rodents show that inflammatory reaction can enhance neuronal excitability, impair cell survival and increase the permeability of blood-brain barrier to blood-borne molecules and cells. In addition, the alteration in the microenvironment during inflammation and cytokines release may affect progenitor cells activity causing ectopic neurogenesis and astrogliosis.
Among cytokines already described as being up-regulated in TLE such as IL1β, IL6 and TNFα, molecules from complement system C1q, C3c, C3d as well as the membrane attack protein complex (C5b-C9) were detected in glial cells from sclerotic hippocampus. Cyclooxygenase-2 (COX2), another proinflammatory mediator, acts on epileptogenesis through the progressive loss of hippocampal neurons, ectopic neurogenesis and astrogliosis in the epileptic focus. In addition, the prolonged tissular retention of immune response markers sustains an inflammatory process that could destabilize neuronal networks. Other important molecules that are also involved in inflammatory process are kinins, which have been shown to participate in neuronal loss mediated by excitotoxicity in TLE (Arganãraz et al., 2004 a, b; Perosa et al., 2007).
Kinins like bradykinin (BK) and Lys-BK are short-living peptides that are released after cleavage of their precursor kininogen. These active polypeptides exert their biological activity through different heterotrimeric G-protein coupled receptors named B1 or B2. Growing evidence indicates that like other peptides and their receptors, kinin B1 receptors could be involved in glutamate mobilization and excitotoxicity. The peptide des-Arg^9-BK, a potent kinin B1 receptor agonist is able to increase the release of glutamate in rat brain slices or submitted to the kainic and kindling models of epilepsy, supporting the idea that kallikrein-kinin system could contribute to the modulation of epileptic neuronal excitability.
Previous works from our group have shown that kinin B1 as well as B2 receptors are up regulated in the hippocampus of patients with TLE (Perosa et al., 2007; Figure 1).

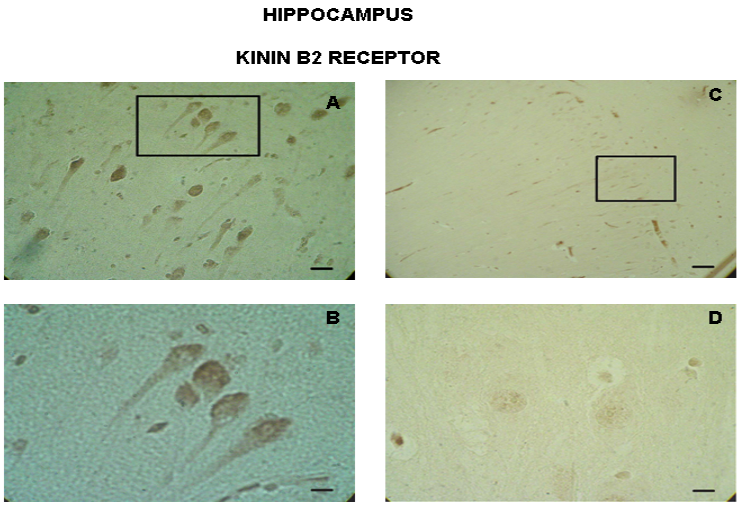

Figure 1: Immuno-histochemistry of hippocampal slices against kinin B2 receptor (CA3 area). Fig3A: Sclerotic hippocampus at 200X magnification showing several stained neuronal cells; Fig3B: Amplification (1000X) of the area showed in Fig3A; Fig3C: Control hippocampus at 200X magnification showing absence of neuronal staining; Fig3D: Amplification (1000X) of the area showed in Fig3C. (Scale Bars: A- C =50 μm; B- D= 4 μm).

Argañaraz (2004a) also showed increased expression of these receptors in neurons from hippocampal formation, mainly in those found in vulnerable areas such as CA1, CA3, hilus and dentate gyrus of rats. In addition, studying kinin B1 knockout mice in experimental model of epilepsy, Argañaraz (2004b) showed that this receptor has epileptogenic action, while kinin B2 receptor has neuroprotector function. These receptors were co-localized with NeuN, a nuclear and neuronal protein marker, in the hippocampus of rats (Argañaraz et al., 2004a).

In the brain, the enzyme responsible for kinin liberation is denominated kallikrein 1 (hK1), described as a serine-protease with diverse additional physiological function. The hK1 is also related to cancer growth and malignancy activity since it may degrade extracellular matrix components. In this context, we studied the distribution of the enzyme hK1 in the hippocampal formation of patients with TLE (Figure 2) as well as the levels of mRNA KLK1 in the same tissues. We also performed double staining employing anti-NeuN and anti-kinin B1 receptor. Co-localization studies of kinin B1 and B2 receptors in the same neuron were also carried out, in order to understand the localization and the function of each

component of kallikrein-kinin system in the hippocampus.

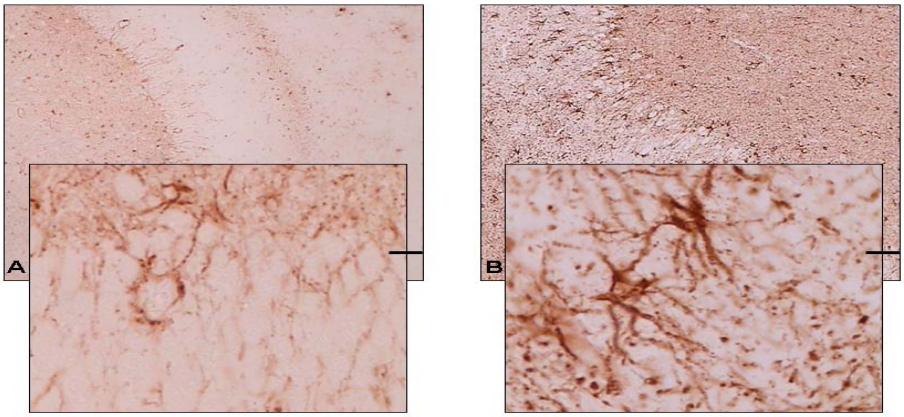

Figure 2- Immuno-histochemistry against hK1 (CA3)
Figure 2 shows the immunoreactivity against hK1 presented by cells from CA3 regions from control (Fig. 2A) and sclerotic hippocampus (Fig 2B). Note the intense immunostaining observed in the hippocampus of patients with TLE, when compared with control tissue. Scale bar: 100µm

The results showed that kinin B1 and B2 receptors were co-localized with NeuN protein and that the enzyme hK1 was up-regulated and co-localized with GFAP in the hippocampus of patients with TLE (Figure 3), using confocal microscopy.

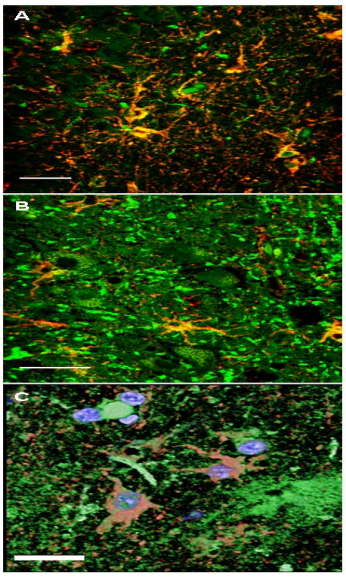

Figure 3- Immuno-Fluorescence – Double staining (GFAP-hK1)
Figure 3 shows the double staining of control and sclerotic hippocampus using GFAP (red) and hK1 (green). Panel 3A shows the co-localization of GFAP and hK1 in the sclerotic hippocampus. Panel 3B shows the co-localization of GFAP and hK1 in control tissue. Note the intense immunoreactivity found in the hippocampus of epileptic patients, when compared with control tissue. Scale bars: 20 µm. Panel 3C shows a Voxx (http://www.nephrology.iupui.edu/imaging/voxx/download) renderized projection of serial optical sections obtained by confocal microscopy. Scale bar: 30 µm

Kinin B1 and B2 receptors were up-regulated in neurons suggesting a cross-talk between neuronal and glial cells in kallikrein-kinin system this condition. As shown (Figure 4) hK1 may act on their main substrate, the low molecular weight kininogen, releasing Lys-BK that could be hydrolyzed to BK, Des-Arg^{9}BK or des-Arg^{10}-Lys-BK by kininases. These short living peptides will act on neuronal surface and**:** **A**) binding to kinin B1 receptors they will induce increase in glutamate release, thus augmenting neuronal excitability. **B**) Acting on kinin B2 receptors these peptides will produce neuroprotection (Argañaraz, 2004). Curiously, kinin B1 and kinin B2 receptors could be found in the same neuron, showing that a unique cell could express both receptors.

This data allow us to suggest that the relationship or the rate between the kinin B1 and B2 receptors agonists, present in the hippocampus, could modulate the neural excitability in normal and pathological conditions. Furthermore, the up-regulation of hK1 expression by astrocytes could be the reflex of the reactive gliosis that perpetuates the inflammatory process.

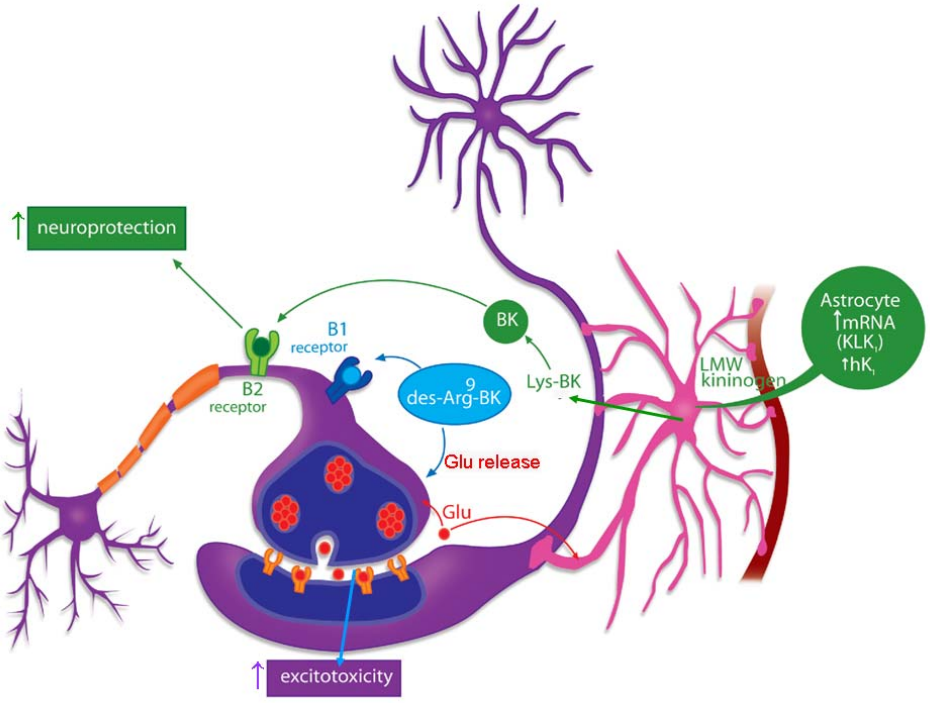

Figure 4- Cross-talk between glial and neural cells related to kallikrein-kinin system
Figure 4 was an adaptation based on the image found at the Site:
http://learn.genetics.utah.edu/units/addiction/reward/images/neuronsAstrocyte.jpg

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The potential role of physical exercise in the treatment of epilepsy

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Considering that physical activity has been found to be beneficial for treating animal models of Parkinson's, Alzheimer's and Huntington's diseases, there is considerable interest in determining the efficacy of this strategy for preventing or treating chronic temporal lobe epilepsy. Temporal lobe epilepsy is the most common form of partial epilepsy, characterized by atrophy of mesial temporal structures, mossy fiber sprouting, spontaneous recurrent seizures and cognitive deficits. In this regard, seizure frequency has significant impact on quality of life with a particularly strong effect on physical role functioning. Unfortunately, persons with epilepsy have previously been discouraged from participation in physical activity and sports for fear of inducing seizures or increasing seizure frequency. For this purpose, clinical and experimental studies have analysed the effect of physical exercise on epilepsy and promising data are presented.

The effects of exercise in animal models of epilepsy have been investigated before and after brain insults induced by status epilepticus (Arida et al., 2009). In this regard, various investigations have been performed attempting to prevent epilepsy or answer the question whether the physical activity influences the brain susceptibility to seizures. The results have demonstrated that an aerobic exercise program before brain insult can reduce seizure susceptibility induced by kindling or pilocarpine model of epilepsy (figure 1).

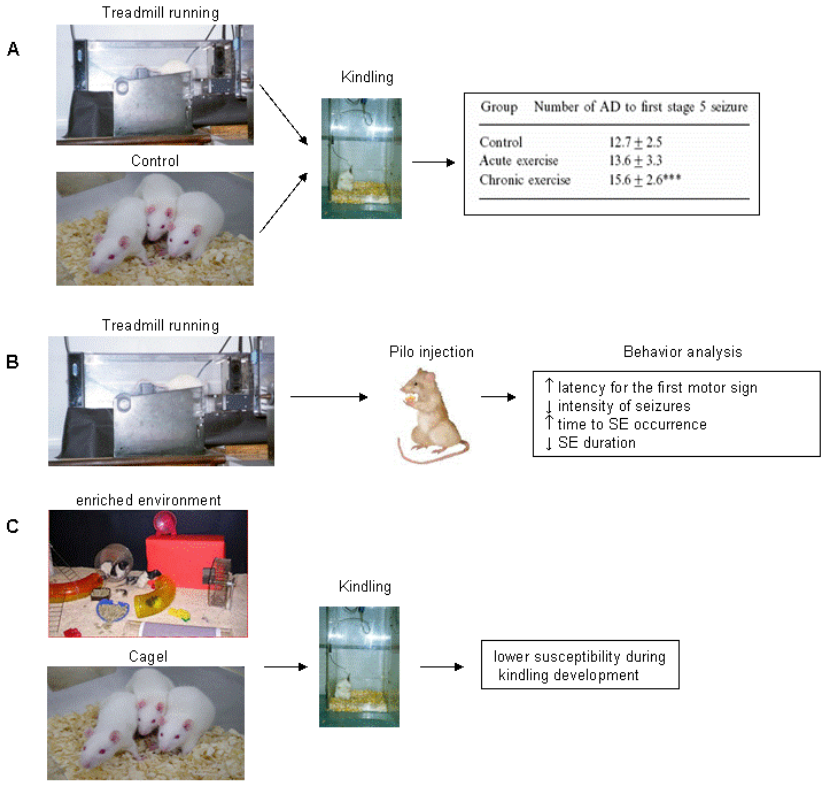

Figure 1. Effect of aerobic exercise program before brain insults induced by SE. A- In the kindling model, the number of stimulations required to reach stage 5 is higher for the chronic exercise animals. B- Trained animals present longer latency for the first motor sign, reduced seizure intensity, prolonged time to SE occurrence and shorter SE duration. C- animals housed in enriched environment and submitted to kindling model of epilepsy present lower susceptibility during kindling development than those housed in isolated conditions.

In view of the beneficial effect of exercise during the epileptogenesis process, we found necessary evaluate whether this effect could occur after the development of chronic epilepsy. Physical exercise was able to reduce seizure frequency, reverse the low metabolic rates in several structures of animals with epilepsy required or not to prevent seizures, induce marked reduction in epilepsy-induced hippocampal electrophysiological abnormalities and increase parvalbumin-immunoreactivity in number and in fibers staining (hilus) (figure 2).

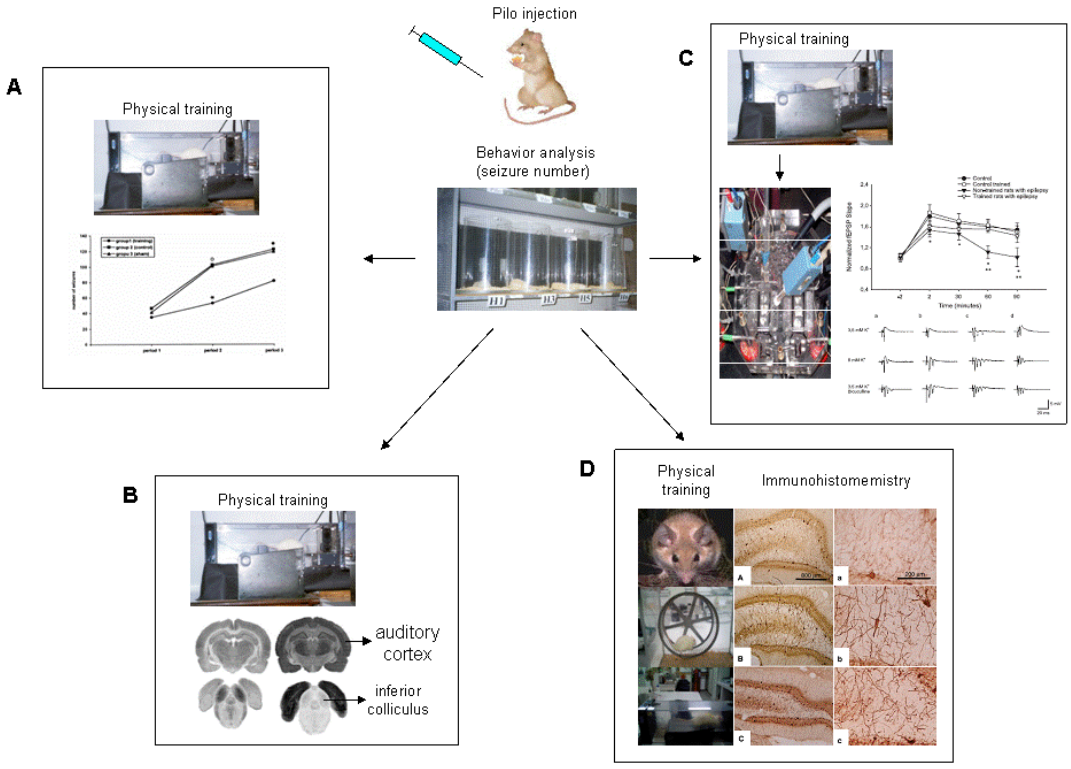

Figure 2. Effect of aerobic exercise programs after brain insults induced by SE. A- Trained animals with epilepsy present reduced seizure frequency during the physical exercise program. B- Increase in interictal local cerebral metabolic rates for glucose in inferior colliculus and auditory cortex in the trained rats with epilepsy when compared to non-trained rats with epilepsy. C- rats with epilepsy exposed to regular physical training exhibit marked reduction in epilepsy-induced hippocampal electrophysiological abnormalities when compared to non-trained rats with epilepsy. D- Immunohistochemical analyses of animals with epilepsy show an increased parvalbumin-immunoreactivity in number and in fibers staining (hilus) of animals with epilepsy submitted to acute treadmill running or voluntary wheel running.

People with epilepsy frequently experience psychiatric comorbidities, most commonly depressive and anxiety disorders. Furthermore, stress is among the most frequently self-reported precipitants of seizures. Therefore, the effects of exercise on mood disorders and stress are of great interest. In the general population, regular exercise has been shown to provide mood benefits, aid in the treatment of depression, and attenuate the impact of stressful life events. The literature documents that physical stress, that is, physical exercise, can provide beneficial effects in people with epilepsy. In view of evidence indicating that sensitivity to stress is reduced after a physical exercise program, physical activity could be a potential candidate for stress reduction in people with epilepsy. For that reason, we hypothesized the possible mechanisms by which exercise may help persons with epilepsy cope positively with stress (Arida et al., 2009) (figure 3).

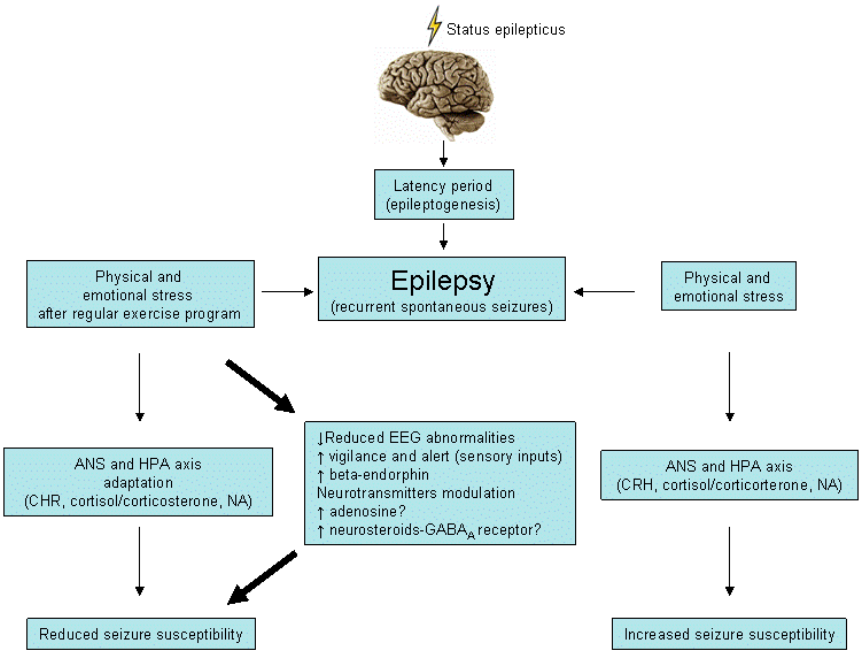

Figure 3. Summary of possible mechanisms by which exercise cope positively with stress. Exposure to a stressful event is processed in limbic areas, which project to the hypothalamus (activates the hypothalamic-pituitary-adrenal axis (HPA). From there the autonomic nervous system (ANS) is activated, resulting in the release of adrenaline; this in turn, can indirectly elevate the release of noradrenaline (NA) in brain, from projections originating in the *locus coeruleus*. Stress also leads to the release of corticotropin-releasing hormone (CRH), which results in pituitary secretion of adrenocorticotropic hormone (ACTH). As a result, corticosterone is released from the adrenal cortex. Corticosterone acts on many peripheral organs but also feeds back on the pituitary and hypothalamus, thereby turning down the activity of the axis. Furthermore, corticosterone reaches extra-hypothalamic brain regions (prefrontal cortex, amygdala and hippocampus). Within the hippocampus, several stress-related hormones affect cellular function. The adapted brain stress system induced by regular exercise induces the activation of several seizures inhibitory components (NA, adenonise, neurosteroids GABAa receptor, sensory inputs) reducing seizure susceptibility.

Considering the positive findings of exercise on epilepsy described above, we might also consider the regular physical exercise as a nonpharmacological therapy. Nonpharmacological therapies, including complementary and alternative medicine (CAM), are often used by people with epilepsy. The most commonly cited CAMs include acupuncture, botanical/herbals, chiropractic care, magnet therapy, prayers, stress management, and yoga. However, individuals with epilepsy and health care professionals do not usually include physical exercise as complementary therapy. In this concern, controlled trials, and epidemiological surveys suggest a role for exercise as an adjunctive form of therapy for epilepsy and as a tool for health promotion and wellness in this population. Here, we present data from human and animal studies supporting the role of exercise as a therapy for epilepsy complementary to standard treatments (Arida et al., 2010) (figure 4).

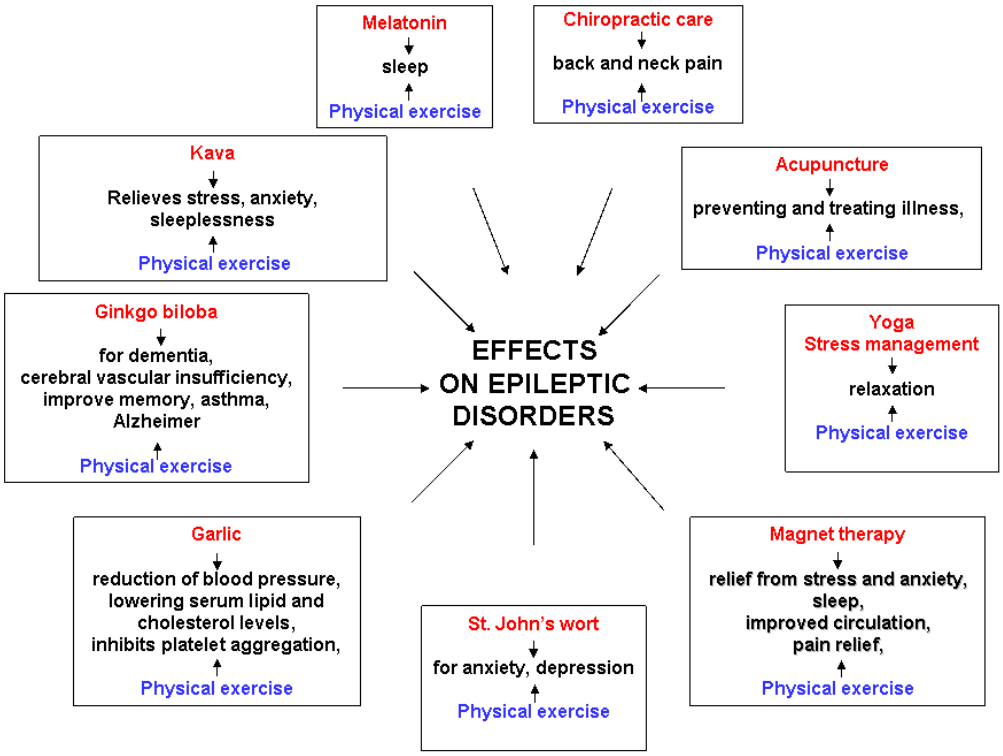

Figure 4. Comparison among the effects of complementary and alternative medicine (CAM) and physical exercise for both seizure and non-seizure conditions. Adequate physical exercise can exert most of the positive benefits as observed in CAM without some adverse effects of CAM.

In conclusion, we need to answer many questions to know how best to use exercise strategy for preventing (to maintain the brain prepared to confront brain injury) or treating epilepsy (recovery from brain injury after SE). A better understanding of how something as simple as a program of physical exercise influences the brain is unquestionably important because there is growing evidence that this intervention may improve function before and after the epileptogenic event (figure 5). Taking into account that the beneficial effects of exercise have been increasingly reported for persons with epilepsy, both for seizure control and for improvement of quality of life, more attention should be taken to understand and reinforce the role of exercise as complementary treatment of epilepsy.

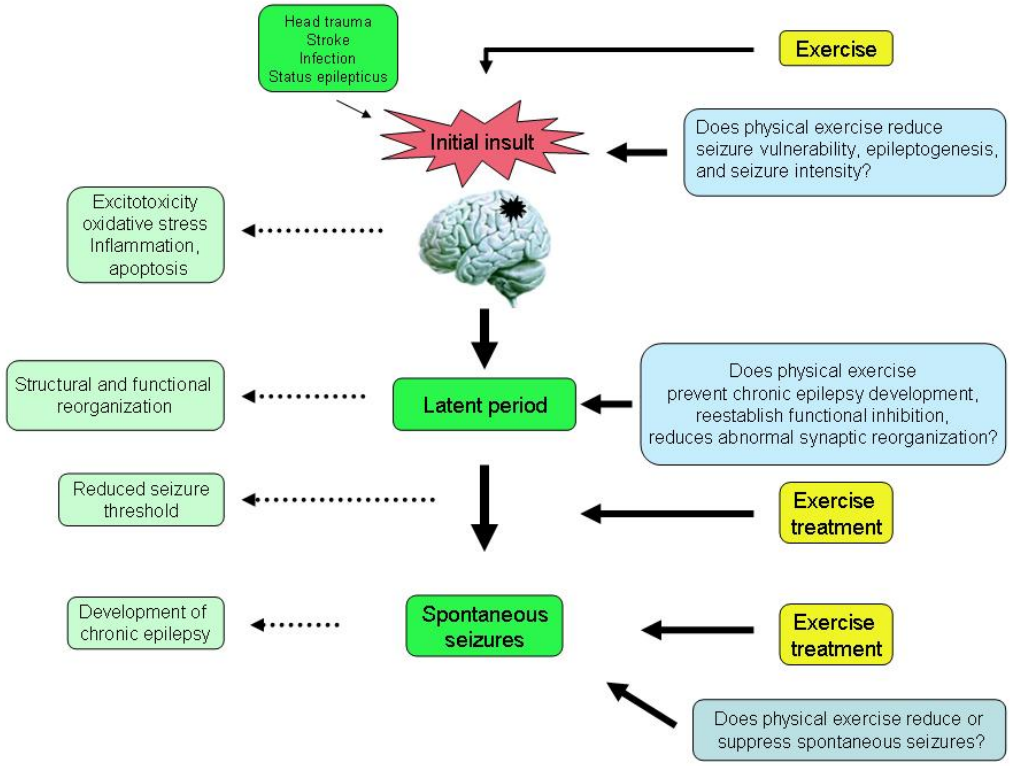

Figure 5. Exercise as neuroprotective and anti-epileptogenic strategy. Potential neuroprotection and treatment strategies for preventing epilepsy development before and after an initial precipitating injury (*status epilepticus* – SE). SE leads to abnormal structural and functional reorganization of the brain circuitry, which gradually develops into epilepsy characterized by recurrent spontaneous seizures (SRS). The neuroprotective interventions such as exercise may be beneficial before or after SE for preventing subsequent epileptogenesis. The potential exercise treatment during the latent period may also help in controlling the SRS. Adapted with permission (Munjal M. Acharya, Bharathi Hattiangady and Ashok K. Shetty, *Prog Neurobiol*. 2008; 84(4): 363–404).

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Differential neuroanatomic characteristics in Amazonian rodents that could account for the resistance to epileptogenesis

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Lorente de Nó (1934) used the term cornu Ammonis (CA) for the hippocampus proprius and defined the CA2 subfield as a narrow zone of cells interposed between CA3 and CA1 that had large cell bodies like CA3 but did not receive mossy fiber innervations. However, CA2 has remained somewhat of an anomaly, sometimes considered a separate region and sometimes simply as an intermingling of cells from CA1 and CA3 (Grove and Tole, 1999). In the Nissl stained sections, most authors rarely identify the CA2 (El Falougy et al., 2008). Thus, possibly because of its small size and difficult encountered in defining its borders, CA2 has been little studied (Mercer at al., 2007). Recently, there has been evidence in favour of distinguishing features of the CA2 field relative to rest of the hippocampus. For example, a relative resistance to CA2 pyramidal cell loss has been noted in head trauma, refractory epilepsy, ischemia and early isolation. Moreover, CA2 possesses a unique pathology in schizophrenic patients. Thus, CA2 appears to be dissimilar from other CA regions with respect to death and survival from injury or age (Zhao et al., 2007). The CA2 region is unique in receiving inputs from the hypothalamic supramammillary nucleus and the interruption of supramammillohipocampal afferents prevents the genesis and spread of limbic seizures in the hippocampus (Saji et al., 2000). Functionally, the CA2 area has been proposed as a pacemaker region for synchrony (Wong and Traub, 1983). Taken together, the evidence points to an active participation of CA2 neurons in the hippocampal network. In this line of evidence, a great deal of the knowledge that has improved our understanding of the brain disorders has derived from appropriate animal models. This is certainly the case in mesial temporal lobe epilepsy (MTLE), the most common type of partial complex seizure in adulthood. Thus, understanding the pathophysiogenesis of temporal lobe epilepsy largely rests on the use of models of *status epilepticus* (SE). Surprisingly, data from our laboratory indicated that similar methodologies to induce MTLE failed to produce chronic epilepsy conditions in a group of rodents of the genus *Proechimys*, suggesting natural endogenous inhibitory mechanisms in these animals (Carvalho et al., 2003; Arida et al., 2005 a,b). In this line of evidence, *Proechimys* was designated as an animal model of resistance to

epilepsy (Fabene et al., 2001; Carvalho et al., 2003; Arida et al., 2005 a,b). For this reason, these rodents from Amazonian Neotropical rainforests may represent a useful tool for investigation. Quite interesting, recent evidence suggested that the CA2 subfield could be involved in seizure generation. In brief, Wittner et al. (2009) showed that the CA2 region of the sclerotic human hippocampus can generate an independent epileptiform activity. Furthermore, numbers of parvalbumin-positive inhibitory cells and axons were shown to be decreased in the epileptic tissue.

In this work, we present the distinctive CA2 subfield of the epilepsy-resistant Amazonian rodent *Proechimys,* a unique cytoarchitectonically subfield exhibiting dispersed and disorganized cell presentation, strongly contrasting to the densely packed CA2 cells in the Wistar rats. The NeuN immunostaining (Fig.1) revealed a clear distinguished CA2 pyramidal cell layer and atypical dispersion of the pyramidal-like cells to the *stratum oriens* in these rodents. Wistar´s CA2 subfield has the narrowest and most densely packed pyramidal cell layer of the hippocampal sectors, strongly contrasting to the dispersed and disorganized cell presentation found in *Proechimys*. Furthermore, *Proechimys* presents large limbic structures (Fig. 2) and at this point, we asked whether such dissimilarities could reflect evolutionary or adaptive advantages of an animal that lives in the Amazon rainforest. We can brainstorm and dream about the functional roles and why these CA2 cells are arranged in such peculiar cytoarchitecture. Has the hippocampus of these rodents evolved and changed in response to their wild and complex habitat? Or even, structures and functions of the Proechimys´s brain are adaptations to this rodent´s physical and social environment?

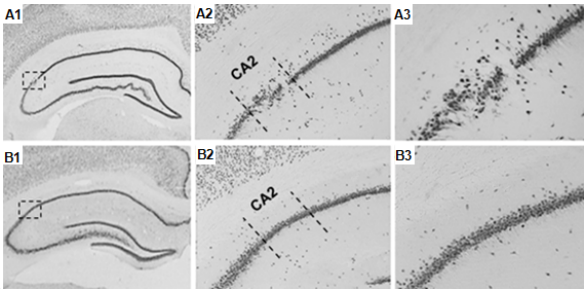

Fig.1. Photomicrographs of NeuN stained coronal sections showing CA2 section. A1-A3 *Proechimys*, B1-B3 Wistar. A1, B1 Hippocampal formation (20x). *Proechimys* shows a very distinctive neuronal distribution in the CA2 subfield of the hippocampal formation (A2-A3), strongly contrasting to the densely packed CA2 cells and unclear boundaries seen in the Wistar rats (B2-B3). A2, B2: 100x; A3, B3: 200x.

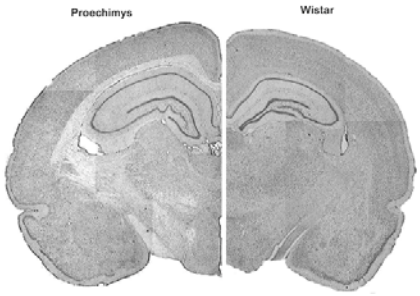

Fig.2.Nissl-stained coronal sections illustrating larger hippocampal formation of *Proechimys* (left) versus Wistar (right). 20x

Several hypotheses have pointed to a key role of hippocampal interneurons in epileptogenesis and it has been shown that in *Proechimys* the resistance to pro-convulsant insults involves a persistent activation of hippocampal interneurons, at least of those that contain PV (Fabene et al.,

2004). Following this reasoning, we investigated the calcium-binding proteins of the *Proechimys* CA2 subfield. The evidence points to an active participation of CA2 neurons in the hippocampal network. Moreover, precise calcium transient control is required to maintain the normal spontaneous arrhythmic and asynchronous firing pattern. In our study, the parvalbumin-immunostaining (Fig.5) was intense in both subject groups and no significant difference in the optical density was found (Fig.6). However, *Proechimys* presented significant higher optical density of calbindin (Fig.3,6) and calretinin-immunoreactivity (Fig.4,6) than found in Wistar rats. Since intracellular Ca^{2+} signaling is highly regulated by those buffers that modulate the amplitude, duration and extent of an influx of free Ca^{2+} in the cytoplasm, a role for the elevated Ca^{2+} buffering proteins in *Proechimys* could be suggested.

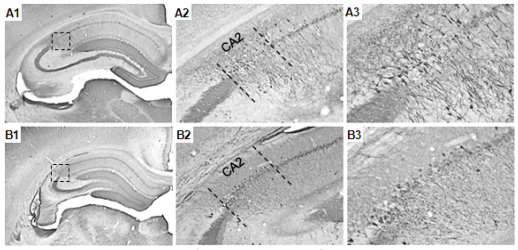

Fig3. Distribution of calbindin (CB) immunoreactive (IR) elements. A1-A3 *Proechimys*, B1-B3 Wistar.

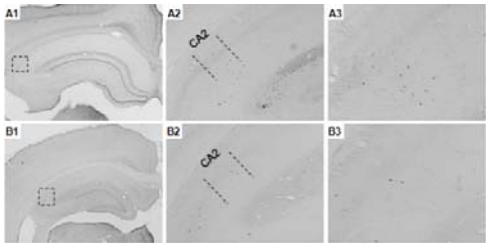

Fig4. Distribution of calretinin (CR) immunostaining. A1-A3 *Proechimys*, B1-B3 Wistar.

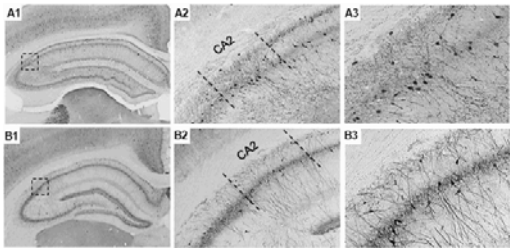

Fig5. Distribution of parvalbumin (PV) immunoreactive (IR) elements. A1-A3 *Proechimys*, B1-B3 Wistar.

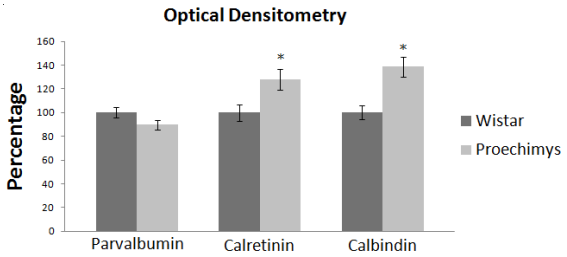

Fig6. Optical Densitometry. PV Wistar (WT) 100% ± 4.506% x *Proechimys(PC)*: 89.617% ± 3.841% (p>0.05); CR WT: 100% ± 7.041% x PC: 127.756% ± 8.836% (*p<0.05) and CB WT: 100% ± 5.946% x PC 138.768% ± 8.311% (*p<0.05).

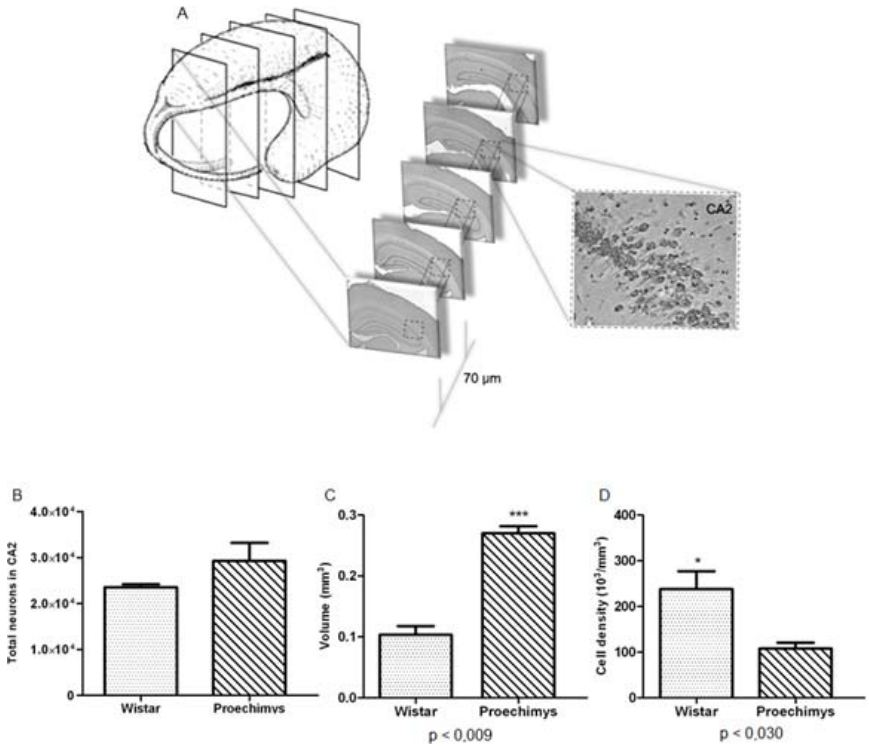

Fig.7

We evaluated number of neurons, volume and neuronal density of the CA2 sector (Fig.7). We did not notice statistically significant differences in the total number of neurons of the CA2 region between *Proechimys* and Wistar. The total numbers of pyramidal neurons of Wistar rats estimated in our study is consistent with those obtained by other authors (Sadowski et al., 1999). Furthermore, we found that both the volume and the neuronal density of the CA2 region show statistically significant differences between the two species studied. *Proechimys* rodents presented higher CA2 volume than Wistar rats, however, CA2 neuronal density was higher in Wistar animals. The neuronal density reflects number of neurons per unit of area. Despite the larger CA2 volume found in *Proechimys*, these rodents did not present a statistically significant number of neurons in comparison to Wistar rats, culminating in lower neuronal density, which is consistent with the dispersed neurons along the CA2 sector observed in the *Proechimys* rodents.

In summary, *Proechimys* presents distinctive features of CA2 neurons that may play a unique role in hippocampal circuitry. In addition, the high level of calcium buffer proteins found in these rodents could be a distinct Ca^{2+} signaling toolkit in order to exactly adjust their spatiotemporal aspects of calcium signalling to their physiological function. In this context, this unique CA2 subfield seen in *Proechimys* opens up a new set of possibilities to explore the contribution of CA2 neurons in normal and pathological brain circuits.

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4- HUMAN RESOURCES

4.1 – Students and Postdoctorals

GROUP	UNDER-GRAD.	MS	PhD	POST DOCTORAL
Roberto Lent	15	03	06	02
Vilma Martins	02	01	03	04
Sergio Ferreira	16	09	13	05
Fernando Mello	17	05	09	01
Esper Cavalheiro	04	14	17	04
Ivan Izquierdo	-	05	08	04
Vivaldo Moura Neto	39	18	12	04

4.2 – Completed Thesis

GROUP	MS	PhD
Roberto Lent	01	-
Vilma Martins	-	02
Sergio Ferreira	07	03
Fernando Mello	05	01
Esper Cavalheiro	05	05
Ivan Izquierdo	-	-
Vivaldo Moura Neto	12	07

5- NEW FACILITIES (with INNT grant)

Confocal Facility

A confocal microscope Leica TCS SP5 II model (Figure 1) was aquired with financial support from INCT-MCT in 2009.

The Leica TCS SP5 II Confocal covers a broad range of requirements in confocal and multiphoton imaging - with the full array of scan speeds at highest resolution. With its high-efficiency SP detection (five channels simultaneously) and the optional AOBS (Acousto-Optical Beam Splitter), the system delivers bright, noise-free images with minimal photo damage at high speed.

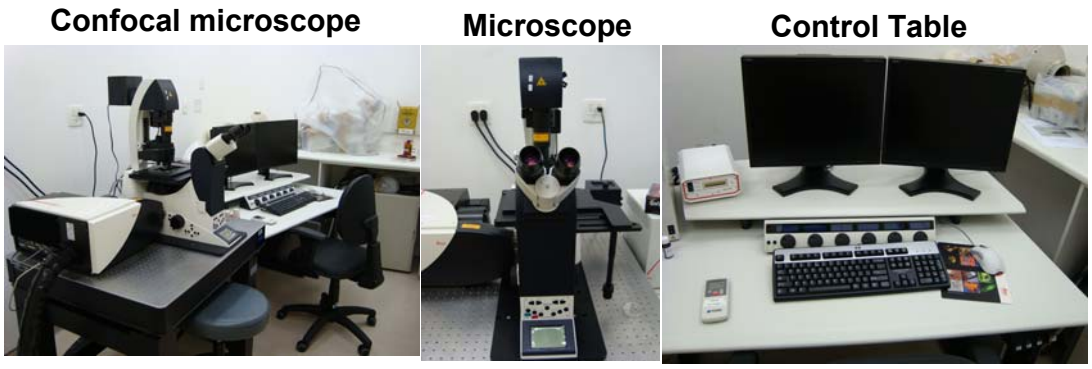

Figure 1: Pictures of Confocal Microscope Leica TCS SP5 II installed in the facility.

Confocal microscopy or Laser Scanning Microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph by using a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images.

This technique has gained popularity in the scientific and industrial communities and typical applications in biomedical research. There has been a tremendous explosion in the popularity of confocal microscopy in recent years, due in part to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional optical microscopy, and in its great number of applications in many areas of current research interest

In the work developed by the INNT group several strategies can be approached using confocal microscopy offering unprecedented precision in three-dimensional imaging and exact examination of subcellular structures and dynamic processes in distinct biological processes.

The technology can be used to evaluate cell signaling, *in situ* cell markers distribution, protein-protein interaction through FRET (Fluorescence resonance

energy transfer), live-cell imaging, including protein trafficking and intracellular calcium dynamics.

To approach those events various antibodies, proteins and peptides can be conjugated/fused with fluorescent markers or fluorescent indicators. For example, the effect of specific proteins in neural progenitor/stem cells biology can be approached by evaluation of the distribution of neural markers (Figure 2).

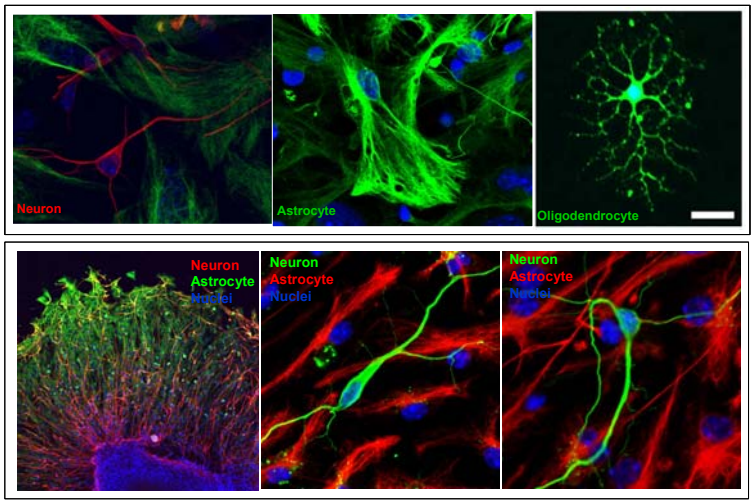

Figure 2: Expression of neural markers in neural stem/ progenitors cells derived from mice forebrain and cultured as neuro-sphere.
Neural stem cells are able to differentiate in neurons, astrocyte and oligoden-drocytes (upper panels). Lower panels, neurospheres cultured onto laminin, an extracellular protein able to induce differentiation, labeled with neural markers (□III-tubulin for neurons and Glial fibrillary acidic protein for astrocyte). Nuclei were stained with DAPI.

The equipment can be also used to evaluate intracellular protein trafficking in live cell lines. In the case presented here, PrP^C was fused to green, cyan or yellow fluorescent protein (GFP, CFP or YFP, respectively) (Figure 3 - CFP-PrP^C). The internalization of PrP^C mediated by copper can be observed in Figure 4.

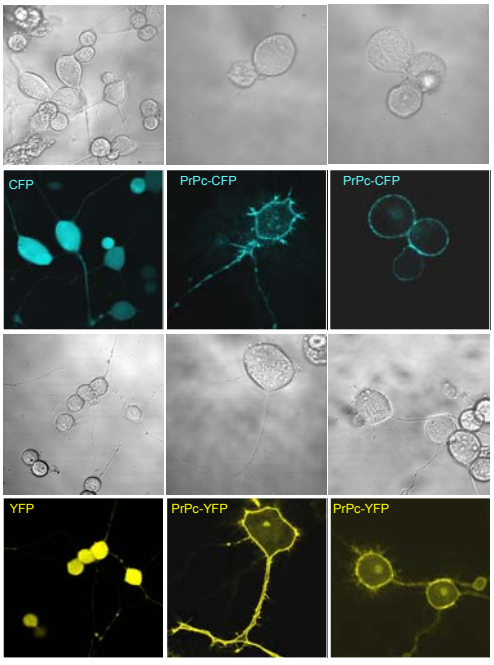

Figure 3: Transfection of SN-56 neuronal cell line with constructions PrP^C-YFP e PrP^C-YFP. The panels represents images of transmitted light (upper panels) and fluorescence (lower panels) of SN-56 cell line transfected with empty vector (CFP or YFP) or with CFP or YFP fused with PrP^C. Note cell surface and perinuclear distribution

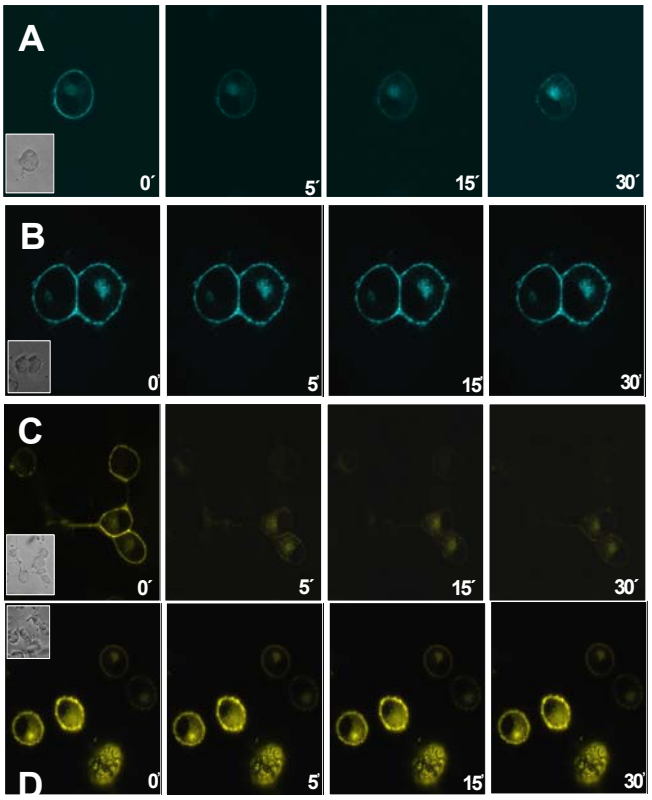

Figure 4: PrP^C fused to YFP e CFP is internalized by copper in Sn-56. The panels repre-sent confocal images of SN-56 transfected with PrP^C-CFP (panels A and B) e PrP^C-YFP (panels C and D) treated with CuCl2 (500μM) during different periods. The insets represent transmitted light images. Controls without $CuCl_2$ treat-ment (panels B and D). Note an increasing of PrP^C from membrane surface to peri-nuclear region.

It is also possible to evaluate the traffic of proteins to specific cellular compartment. We have approach that to demonstrate a high co-localization between STI1 and endosomal vesicles (Figure 5).

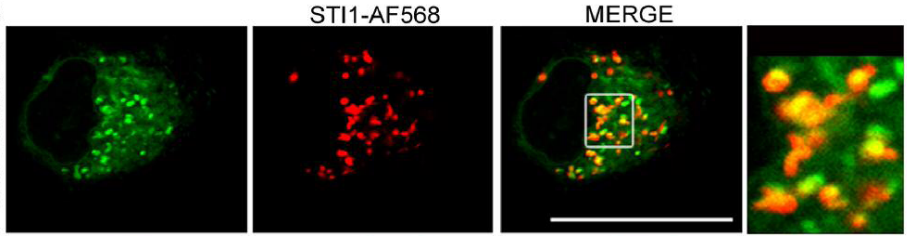

Figure 5: Subcellular localization of STI1 in SN-56 cell line. Acid vesicles were labeled with green fluorescent marker and STI1 was stained with red fluorophore. Note a high colocalization (yellow) in merge picture.

Confocal microscopy is also a powerful toll to measure Ca^{2+} signaling. We assessed intracellular Ca^{2+} levels in live cells using the fluorescent indicator fluo-3. Fluorescence upon the addition of the prion protein ligand (i.e., STI1) to the medium, intracellular calcium increasing is measured in function of fluorescence emission (Figure 6).

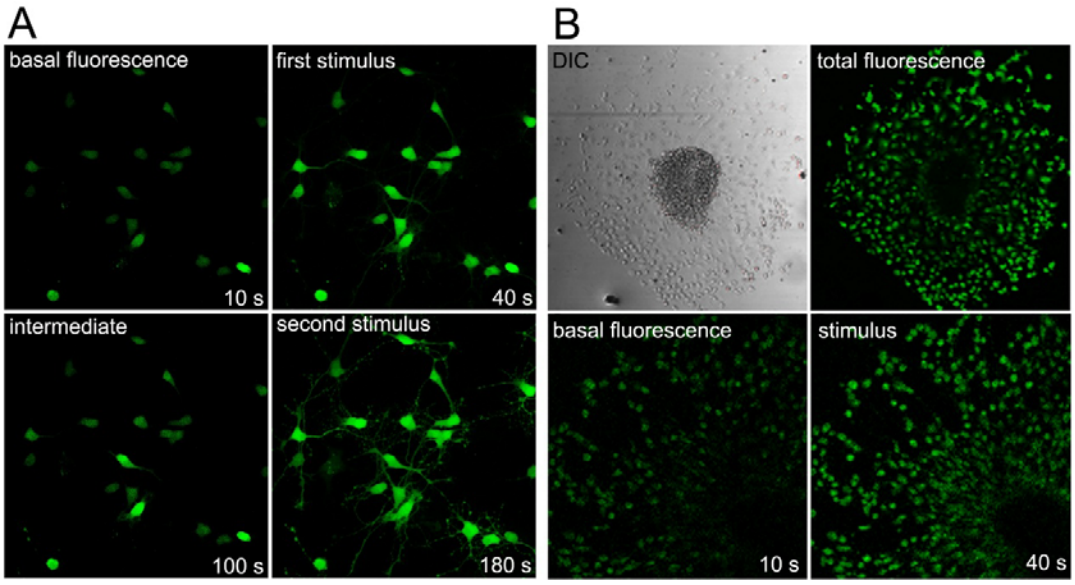

Figure 6: Intracellular calcium measurement in neural cells: Time-lapse of cell-live fluorescence imaging with calcium indicator Fluo-3. **A**, primary hippocampal neurons were stimulated with recombinant STI1, leading to calcium channel opening on plasma membrane. After calcium buffering, a second stimulus with thapsgargin promotes another round of intracellular calcium increasing. **B**, neural progenitor/stem cells cultured as neurospheres were treated with STI1 and followed by calcium fluorescence monitoring.