Role of reactive oxygen species in the regulation of signaling pathways during neuronal death

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Reactive oxygen species (ROS) modulate apoptosis of cerebellar granule neurons (CGN), but the mechanisms implicated have not been clarified. It has been demonstrated an involvement of Akt and TXNIP (thioredoxin-interacting protein) in the excitotoxic death induced by NMDA receptor (NMDAR) stimulation. According to a previous study, it has been suggested that synaptic activity turns off TXNIP transcription (ubiquitously expressed redox protein that promotes apoptosis) by inducing the PI3K-Akt pathway and triggers the inactivation of transcription factors that modulate transcription of pro-death genes (including TXNIP). In this regard, in non-neuronal cells it has recently been reported that Akt is redox sensitive. Based on these evidences, one possible scenario in the CGN is that ROS generated by apoptotic conditions could regulate the activation of Akt, which would modulate the activation of transcription factors and as a result the regulation of TXNIP, leading to the apoptotic death of CGN. In this study, we evaluated this possibility by using a model of apoptotic death of cultured CGN induced by potassium deprivation (K5) and staurosporine (Sts). Under these conditions, we found an early increase in the generation of ROS induced by K5 and Sts treatment. On the other hand, using western blot assays we found that hydrogen peroxide inhibits the activation of Akt (indicating that this kinase is redox sensitive in CGN), and that K5 and Sts induce the inactivation of Akt at short times. In addition we found that hydrogen peroxide induces the expression of TXNIP in these cells (indicating that this protein is redox sensitive), and that the apoptotic conditions K5 and Sts are also able to induce the expression of TXNIP at short times. To confirm the above, we use antioxidant conditions under the stimuli of K5 and Sts and we found that in all cases the expression of TXNIP is inhibited. Taken together, these data suggest that the ROS generated by K5 and Sts induce the expression of TXNIP through regulation by Akt, modulating apoptosis of CGN.

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Pioglitazone protects human differentiated SH-SY5Y cells against MPP^+-induced damage

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The pathogenic mechanisms of Parkinson's disease (PD) remain unknown, PD has been associated with impaired mitochondrial complex I activity and complex I inhibitors cause pathological and neurochemical changes with a remarkable similarity to PD. Pioglitazone is a member of thiazolidinediones (TZDs), used clinically as insulin-sensitizing drugs. These compounds selectively bind to peroxisome proliferator-activated receptor gamma (PPARγ) a nuclear receptor and ligand-dependent transcription factor that regulates the expression of several genes involved in lipid and carbohydrate metabolism, upregulates antioxidant defences and promotes mitochondrial biogenesis. We previously found that rotenone decreased mitochondrial mass, membrane potential and oxygen consumption, while increasing free radical generation and autophagy in differentiated SHSY5Y cells. Similar changes were seen in PINK1 knockdown cells, in which the membrane potential, oxygen consumption and mitochondrial mass were all decreased. In both models, all these changes were reversed by treatment with the PPARγ agonist rosiglitazone (Exp Neurol 253:16, 2014). We now have studied whether PPARγ activation with pioglitazone, prevents damage of human differentiated SH-SY5Y cells exposed to 1-Methyl-4-phenylpyridinium ion (MPP^+). Our data show that inhibition of complex I with MPP^+ in human differentiated SH-SY5Y cells, decreases mitochondrial mass, membrane potential, while increases ROS generation. All these changes were reversed by pre-treatment of the cells with the PPARγ agonist pioglitazone. Thus, pioglitazone is neuroprotective in a model of mitochondrial dysfunction associated with Parkinson's disease through a direct impact on mitochondrial function.

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Induction of autophagy as a neuroprotective mechanism in an animal model of Parkinson´s disease.

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Parkinson´s disease (PD) is the second most common chronic and progressive neurodegenerative disorder after Alzheimer´s disease. Most of the cases of PD affect people over the age of 60 and above. This disorder is characterized by the selective loss of dopaminergic neurons in the *substantia nigra pars compacta*, which leads to a deficiency of the neurotransmitter dopamine and subsequent motor and cognitive dysfunction. There are two types of PD, the familial form, which is related to specific mutations in genes such as LRRK2, PARK2, PARK7, PINK1 or SNCA; and the sporadic form, which involves the interaction between environmental exposition, genetic background and ageing. PD etiology is still unknown. However, three main events have been related: mitochondrial dysfunction, oxidative stress and impairment of the protein degradation pathways mediated by the proteasome and autophagy. Autophagy is a self-regulatory mechanism of cells and involves degradation of damaged organelles, misfolded proteins and other cytoplasmic components through lysosomes, and the degradation products can be re-used for cell survival. Impairment of autophagy is associated with cell death and neurodegeneration. Therefore, we wanted to determine whether the induction of autophagy can protect the loss of dopaminergic neurons in an animal model of PD. We established the experimental model of PD using the herbicide paraquat (PQ), which induces oxidative stress, autophagy disruption and cell death. PQ induced a decreased of the dopaminergic neuronal population, which was demonstrated by detecting the dopaminergic marker tyrosine hydroxylase (TH) through immunohistochemistry and western blot. Autophagy was stimulated with rapamycin one week before intoxication with PQ. After 6 weeks of treatment we observed that both motor and cognitive functions were slightly affected by PQ, with a mild improvement when autophagy was stimulated. An increase of LC3-II, the hallmark of autophagy, was confirmed by western blot in rapamycin-treated mice. Interestingly, autophagy induction increased TH marker in PQ-treated mice. Our results suggest that autophagy stimulation might have a protective effect on dopaminergic cells in PD.

C/EBPβ regulates the expression of progesterone receptor in human glioblastoma cells

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Many progesterone (P4) effects are mediated by its intracellular receptor (PR), which has two isoforms (PR-A and PR-B). Differential isoform expression ratios have been associated to several types of cancer. In human astrocytomas, the most frequent and aggressive brain tumors, P4 induces proliferation and infiltration of astrocytoma cells, and PR isoforms expression is directly correlated to the tumor evolution grade, being PR-B the predominant isoform. Each PR isoform is transcribed from a separate promoter and their differential expression is regulated by specific transcription factors such as the CCAAT/Enhancer Binding Protein β (CEBP/β). An *in silico* study showed that only PR-B promoter has three putative C/EBP binding sites near a conserved CCAAT box and two Specific protein 1 (Sp1) binding sites. In this study, we evaluated the expression of CEBP/β and its role in the regulation of PR-B expression in U251, D54 and U87 cells derived from human astrocytomas grade IV (glioblastomas). Cells were cultured, and Western blot, ChIP, and qPCR were performed to study the role of CEBP/β in PR expression. Immunofluorescence assays against CEBP/β were performed in high-grade human astrocytoma biopsies. U251, D54 and U87 astrocytoma derived cell lines expressed both PR isoforms (PR-B:PR-A ratio of 3:1, 1:1 and 2:1, respectively) as well as all three CEBP/β isoforms (LAP1, LAP2 and LIP) with a predominance of LAP2. CEBP/β was expressed in the cytoplasm and nuclei of high-grade human astrocytoma biopsies. ChIP assays in U251 cells showed a significant basal binding of CEBP/β, Sp1, and estrogen receptor alpha to PR-B promoter. When silencing CEBP/β in U251 cells with a lentivirus carrying a shRNA, PR-B expression was increased both at mRNA and protein levels. P4 induced genes were differently regulated when CEBP/β was silenced. Treatment with estradiol (10 nM) reduced CEBP/β binding to the promoter from 3 to 48 hours and up-regulated the expression PR in CEBP/β knockdown cells. These data show that CEBP/β participates in the regulation of PR-B expression in human glioblastoma cell lines.

The role of thrombospondin-1 (TSP-1), in neuronal changes induced by enriched environments

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Abstract

Enriched Environment (EE) are models used to study brain plasticity experience-dependent. EE modify the morphology of the neuron and stimulate the formation of dendritic spines. However, the cellular mechanisms involved in this process are not fully understood. Thrombospondin-1 (TSP-1), is an extracellular matrix protein synthesized and released by astrocytes, involved in the development and maintenance of neurites, dendritic spines and synapses. The aim of this study was to determine whether TSP-1 is involved in neuronal morphology changes induced by EE. Briefly WT and KO TSP-1 mice were exposed for five weeks to EE, subsequently were sacrificed and their brains processed for Golgi staining. Sholl analysis was performed to know changes in neuronal morphology and density of dendritic spines in hippocampal neurons. TSP-1 levels were determined by immunoassay. Results: Wt mice in EE show changes in number of nerve arborizations, decrease in the length of secondary and tertiary dendrites; associated with an increased neural complexity, enhanced dendritic spine density and an increase of TSP-1 levels. TSP-1 KO mice exposed to EE show reduction on number of dendrites, increased length of the dendrites and decreased neural complexity. EE increase dendritic spine density in distal dendrite but not in

proximal dendrite. Our results suggest that TSP-1 could have an important role in the mechanisms that control changes in neuronal morphology, and brain plasticity experience-dependent.

Activation of adult-born neurons after a focal hippocampal lesion and in response to a context-fear memory task

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Adult hippocampal neurogenesis is a plastic process that takes place in the dentate gyrus (DG) of the hippocampus, a structure involved in processing contextual fear memory. A fraction of the adult-born neurons (ABNs) become functional and integrate into the hippocampal circuit in physiological conditions. Brain damage leads to neurogenesis in the adult DG and a fraction of the newly-ABNs achieve maturation, so neurogenesis has been proposed to play a role in neural reorganization after damage. However it remains unclear if neurons born after damage even activate in response to hippocampal dependent tasks. In this work we aimed at evaluating if new neurons born after a DG lesion express c-fos as a reporter of neuronal activation. For this purpose, we induced a focal lesion in the DG of adult male rats, and after 9 days we conditioned animals in a contextual fear memory task. 24 hrs later, we induced and evaluated contextual fear retrieval and analyzed through immunohistochemistry cellular proliferation through BrdU incorporation, fate specification and maturation by the expression of DCX and neuronal activation with c-fos as a reporter. Lesion animals show a significant deficit in recalling contextual fear memory ($p<0.001$) and a significant increase in activated-immature neurons DCX+/c-fos+ in the granular layer ($p=0.0436$), while the number of activated-new immature neurons BrdU+/DCX+/c-fos+ increase both in the granular layer and in the hilus as compared to the control group ($p=0.0015$). As the activation in ABNs has been reported only as they get 3 weeks-old in physiological conditions, our results suggest that DG damage may contribute to the premature activation of young neuroblasts born pre- and post-lesion and that although still immature, they are prone to activation after a cognitive process.

Naloxone blocks neurogenesis in the olfactory bulb induced by paced mating in the female rat

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Neurogenesis is a process that occurs continuously in the adult mammalian brain. But environmental conditions or internal states can modify different stages of this process.

Our group has demonstrated that when a female rat is allowed to pace the sexual interaction, increases the number of newborn cells in the accessory olfactory bulb (AOB). Moreover, this paced mating develops a positive affective (reward) state on them, evaluated by the conditioned place preference paradigm. This reward state is mediated by opioids because we also demonstrate that the intraperitoneal injection of naloxone (opioid receptor antagonist) blocks the conditional place preference.

In the present study we evaluated if blocking the opioid receptors with naloxone could reduce the number of new cells that incorporate to the AOB generated during the first sexual experience in paced mating. Sexually-naïve female rats, bilaterally ovariectomized and hormonally supplemented were randomly assigned to one of five groups: 1) without sexual contact injected with saline, 2) without sexual contact injected with naloxone, 3) females that mated without pacing the sexual interaction, 4) females injected with saline before paced mating and 5) females injected with naloxone before paced mating.

Females treated with saline that paced the sexual interaction, showed an increase in newborn cells in the AOB compared to the other groups. In contrast, females treated with naloxone that paced the sexual interaction before paced mating showed no increase.

These data support that opioid peptides have a fundamental role in the production of new cells in the olfactory bulb induced by paced mating in the female rat. Further research will demonstrate if these newborn cells are involved in the physiology of reproduction.

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To see is to believe

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In 1993 I published the book “To believe is to see” as the outcome of my investigations into the properties of human vision. It was based on the assertion that the traditional saying originating in folk wisdom, “To See is to Believe” is fundamentally incorrect. “To Believe is To See”, or perhaps more to the point, “Believing Leads to Vision”. Which is perhaps that was we see with our brain is not some pixel generated binary formed image of what our eyes take in and transmit to our cerebrum but what our brain constructs from that image as a result of its own internally processed interpretation of that image. Some twenty years later, after revisiting the progress made in vision science since 1993, there has been a reevaluation. The magnetic resonance imaging and other advances in the neurophysiology, the visual system neuroscience have brought about a new understanding: what we “see” is what the brain tell us about that, not what the raw images from the eyes tell us.

In this short presentation I will explain how these new findings acquired from new data, have confirmed my original interpretations of the relationship of what is going on between the eyes “seeing” and the brain telling.

MicroRNAs and response to nicotine in *Drosophila melanogater*

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Nicotine is an alkaloid that is synthesized in the roots of the plant of the *Nicotiana tabacum* species. It is a very toxic compound that acts on the Central Nervous System (CNS) through nicotinic Acetylcholine Receptors (nAChR). The effect and the mechanisms involved in the assimilation and metabolism of nicotine are conserved in different organisms. *Drosophila melanogaster* is a model organism that has been used for the study of the effects of alkaloids, drugs and other substances of abuse that act in the CNS. A screening for nicotine hypersensitive flies was performed in a collection of P{GawB} insertion lines. Two lines, L70 and L4, showed greater sensitivity to volatilized nicotine when compared to control lines *white^{1118}* (*w^{1118}*) and *Oregon-R* (*Ore-R*). We demonstrated that the mutation in the line L70 deregulates the expression of microRNAs *mir-310, mir-311, mir-312 and mir-313* (*miR-310/313 cluster*) in the larval, pupal and adult stages. Adult expression of the *mir-310/313 cluster*, caused altered behavior when exposed to nicotine. We also found that the mutation in the line L4 causes a loss of function of the *escargot* (*esg*) gene. This gene encodes a transcriptional factor, interestingly,

esg has target sites for the *310-313 microRNAs*. In order to demonstrate that these microRNAs regulate *esg* gene directly, we used the bipartite system UAS/GAL4 and UAS/GAL4/GAL80 tripartite system to control the time of expression of these microRNAs. We demonstrate that overexpression of *mir-3010/313 cluster* using as an *driver esg* increases the phenotype caused by loss of function of the protein Esg (malformation of the tergites and sternites in the abdomen) and also showed that these flies phenocopy both L70 and L4 mutant sensitivity to nicotine. Constitutive expression of the *mir-310/313 cluster* using as a *driver* an actin-GAL4 line, also causes a nicotine sensitivity phenotype. When the *mir-310-313/313 cluster* expression is repressed, nicotine sensitivity becomes normal. This indicates that this phenotype is dependent on the expression of the *miR-310/313 cluster*. When over-expression was induced, also in actin-GAL4 in embryos, protein Esg levels were decreased completely.

Based on these results, we hypothesized that the trancripcional factor *esg* that regulates genes may be involved in the correct formation and development of the organs involved the perception and metabolism of nicotine. These organs could be chemical receptor sencilla, neurons or sensory organs that receive and integrate a response to this stimulus. Adult expressión of the *miR-310/313 cluster*, when they are not normally expressed, may also have other targets genes that participate in a hypersensitivity to nicotine. These genes may encode for signaling molecules, neurotransmitters or metabolic enzymes are activated or repressed depending on the stimulus. Thus it is probable that in the flies exist a regulatory mechanism where the hypersensitivity to nicotine is induced by a decreased into dosage of the Esg transcriptional factor (L4) and/or overexpression of *mir-310/313 cluster* (line L70).

A functional metabotropic-like NMDAR in rat cultured astrocytes.*

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The ionotropic glutamate N-methyl D-aspartate (NMDA) receptor (NMDAR) is critical for CNS functions, but its expression and function in astrocytes is still a matter of research and debate. Here, we demonstrate immunofluorescence (IF) labeling in rat cultured cortical astrocytes (rCCA) of all NMDAR subunits, with phenotypes suggesting their intracellular transport, and their mRNA were detected by qRT-PCR. IF and Western Blot revealed GluN1 full length synthesis, subunit critical for NMDAR assembly and transport, and its plasma membrane localization. Functionally, we found an $_i Ca^{2+}$ rise after NMDA treatment in Fluo-4-AM labeled rCCA, an effect blocked by the NMDAR competitive inhibitors D(-)-2-amino-5-phosphonopentanoic acid (APV) and Kynurenic acid (KYNA), and dependent upon GluN1 expression as evidenced by siRNA knock down. Surprisingly, the $_i Ca^{2+}$ rise was not blocked by MK-801, an NMDAR channel blocker, or by extracellular Ca^{2+} depletion, indicating flux-independent NMDAR function. In contrast, the IP_3 receptor (IP_3R) inhibitor XestosponginC did block this response, whereas a Ryanodine Receptor inhibitor did so only partially. Furthermore, tyrosine kinase inhibition with genistein enhanced the NMDA elicited $_i Ca^{2+}$ rise to levels comparable to those reached by the gliotransmitter ATP, but with different population dynamics. Finally, NMDA depleted the rCCA mitochondrial membrane potential ($m\Delta\psi$) measured with JC-1. Our results demonstrate that rCCA express NMDAR subunits which assemble into functional receptors that mediate a metabotropic-like, non-canonical, flux-independent $_i Ca^{2+}$ increase.

*These results have been published in PLOS One.

Bidirectional regulation of neuronal excitability of L-lactate via activation of a Gi-protein coupled receptor

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Lactate has an important role as a metabolic and energetic substrate for the brain, yet lactate can modulate neuronal excitability through the activation of the receptor HCA1. Therefore, we investigated the effect of L-lactate on the passive and firing properties of hippocampal cells on acute slice. Extracellular perfusion of L-lactate (5 mM) was effective to decrease the neuronal input resistance and increase the rectification phase of the I-V relationship. Additionally, L-lactate increased the rheobase current to elicit action potentials. The direct activation of the L-lactate receptor HCA1, with the agonist 3,5-DHBA, yielded similar results on the input resistance and I-V rectification. Interestingly, lower concentrations of L-lactate or 3,5-DHBA reduced the fire frequency, but higher concentrations within a relevant physiological range, increased the firing of pyramidal neurons. Bath perfusion of the enantiomer D-lactate (5 mM) did not modulate any electrophysiological response; however, the perfusion of the L-lactate precursor, pyruvate, mimicked the reduction in excitability (5 mM). This effect was prevented when slices were preincubated in the lactate dehydrogenase inhibitor oxamate (20 mM), suggesting that L-lactate controls the neuronal excitability through a mechanism independent of metabolic pathways. To discard that the effects of L-lactate are a consequence of the active transport of L-lactate via the monocarboxilate transporter (MCTs), we incubated slices in the presence of the MCTs inhibitor, 4-CIN (0.5 mM). Under these conditions, L-lactate still controls the passive and firing properties of pyramidal cells, suggesting that in the hippocampus, L-lactate acts via a membrane receptor, plausibly the HCA1. As the HCA1 is a G_i protein-coupled receptor, in another series of experiments we treated the cells with intracellular pertussis toxin, a $G_{\alpha i}$ subunit inhibitor. Under these experimental conditions, the modulatory actions of L-lactate at the lower and higher concentrations were abolished. Our experiments also revealed that the excitatory effects of L-lactate do not involve the signaling cascades related to G_s or G_q protein since incubation or intracellular treatment with the correspondent antagonists did not revert the effects of L-lactate. Lastly, an additional analysis of the action potential spikes indicates that L-lactate changes the kinetic parameters of sodium and potassium conductances in a concentration dependent manner. However, more experiments are required to clarify the specific conductances modulated by L-lactate and their influence in excitability.

Peripheral oxytocin receptors as modulators of nociceptive transmission

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Oxytocin from the paraventricular hypothalamic nuclei has emerged as a mediator of endogenous analgesia. Recently, some studies suggest that oxytocin could be synthetized in the epidermal cells but the function remain unknown. The aim of the present study was to investigate in electrophysiological recordings, behavioral nociceptive tests, protein expression and neuronal tracing, the potential role of peripheral oxytocin receptors (OTR) in the nociceptive transmission.

In anesthetized (2-3 % sevoflurane) male Sprague-Dawley rats the nociceptive responses evoked on the wide dynamic range (WDR) cells at the lumbar segments (L3-L6) of the spinal cord was tested. Extracellular unitary recordings were made (4-8 MΩ). The neuronal responses were evoked by 20 electrical stimuli (0.5 Hz, 1-ms pulse duration, 0.1-3.3 mA) delivered on the receptive field located in the ipsilateral (to recording site) hindpaw. Furthermore, using the 1% formalin test (50 μl formalin injected s.c. into the dorsal surface of the hindpaw) we evaluated the effects of peripheral oxytocin on nociception. In both cases (electrophysiological and formalin test) dose-response curves to oxytocin (0.1-100 μg/paw; 50 μl) were performed. Additionally, we labeled the peripheral neuronal terminals using the neuronal tracers Fluoro-Gold® and Fluoro-Rubi® injected in the sciatic nerve; this tissue was also collected in order to perform an immunofluorescence to OTR, SP, CGRP and IB4. Finally, the OTR protein was identified using the western blot technique. The oxytocin was administered subcutaneously (s.c.) 5 min before the test whereas in the experiments with the OTR antagonist (L-368,899; 10 or 100 μg/50 μl), the drug was given 5 min (s.c.) before the oxytocin.

We found that oxytocin (10-100 μg/paw) inhibits selectively the neuronal activity related to Aδ- and C-fibers but not Aβ-fibers, an effect probably mediated by OTR activation, since pre-treatment with the antagonist (L-368,899) blocked the oxytocin-induced antinociception. Similar results were obtained in the formalin behavioral nociceptive test. Consistent with this, OTR is expressed in the sciatic nerve. Moreover, fluorescent labeling of primary afferent fibers show that OTR is mainly found in the skin nociceptive terminals.

Taken together our results show that the OTR are expressed preferentially in the nociceptive terminal nerve endings and upon activation are able to inhibit selectively the nociceptive input.