Committee for Aid and Education in Neurochemistry (CAEN)
CATEGORY 1B: Research supplies for use in the applicant's home laboratory
April 2015 Round

Dr Juan Carlos Corona
Children´s Hospital of Mexico Federico Gómez
Dr. Márquez 162, Col. Doctores, Delegación Cuauhtémoc, Mexico City, C.P. 06720.
Tel: +52 (55) 52289917 Ext: 2352
Email address: jccc93@gmail.com
Current Position: *Chief, Laboratory of Neurosciences*

Final Report

Background

The attention deficit hyperactivity disorder (ADHD) is characterized by inattention, impulsivity and hyperactivity. The ADHD is highly prevalent, affecting around 5-10 % children and 4% of adults (Faraone et al., 2003). The long-term consequences include lower educational and occupational achievement and increased risk for developing other psychiatric disorders (Mannuzza et al., 1997; Mannuzza et al., 1998). Methylphenidate (MPH) is currently used in the treatment of children with ADHD (Biederman et al., 2000; Volkow et al., 2002). The application of MPH has been standard for more than 50 years as an effective treatment of ADHD (Biederman, 2003) due of its ability to reduce the symptoms in up to 70% of children and adolescents (Swanson et al., 1998). Another approved drug is atomoxetine (ATX), a non-psychostimulant and highly selective norepinephrine re-uptake inhibitor, which effectively reduces the symptoms of ADHD (Buitelaar et al., 2004; Michelson et al., 2001). There are still few works regarding the effects of MPH and ATX on mitochondrial function. It has been shown that MPH produces short-term changes in neurotransmission in brain regions of young rats involved in motivation, behaviour, cognition, appetite and stress (Gray et al., 2007). A significant dose-dependent alterations in metabolic activity were found in the components of extra-pyramidal system, nucleus accumbens and olfactory tubercle, evaluated by rates of local cerebral glucose utilization following acute administration of MPH (Porrino and Lucignani, 1987). The chronic administration of MPH increased the activities of mitochondrial respiratory chain complexes II and IV in the brain of young rats (Fagundes et al., 2007). So far, little is known about the molecular basis of the therapeutic effects of ATX. However, it has been shown that long-term treatment with ATX induced the regulation of several genes in the prefrontal cortex of young rats (Lempp et al., 2013), suggesting that ATX has additional active therapeutic mechanisms. Oxidative stress has been postulated as a possible hypothesis on the development of ADHD (Ceylan et al., 2010). In addition, in specific brain regions of young rats, chronic application of MPH resulted in a dose dependent increase of lipid peroxidation products and protein carbonyls, which are substances formed by oxidative damage of proteins (Martins et al., 2006). The acute or chronic MPH treatment led to altered activities of catalase and superoxide dismutase in brain areas of young rats (Gomes et al., 2008). The acute and chronic administration of MPH increased creatine kinase activity in brain of young and adult rats (Scaini et al., 2008).

Specific Aims

Study the mechanisms and consequences of altered mitochondrial function following treatment with psychostimulant and non-psychostimulant drugs commonly used in the ADHD in vitro.

1. To explore the mechanisms and consequences on mitochondrial bioenergetics following the treatment with MPH and ATX.

2. To determine the effect of the treatment with MPH and ATX on mitochondrial mass, autophagy and mitochondrial biogenesis.

Outcomes

All the experiments in this study are carried out using human neuroblastoma SH-SY5Y cells, grown in DMEM/F12 medium supplemented with 10% FBS, containing penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively), in a humidified incubator at 37 °C and 5% CO2. The cells are differentiated into human neuron like cells, as follows. 6 × 103 cells/cm2 are seeded on Matrigel basement membrane matrix-coated culture dishes and allowed to attach overnight. The FBS content of the culture medium is then reduced to 2% and cells exposed to 10 µM retinoic acid. The cells are kept under these conditions for 7 days, changing the culture medium every 2 days. The cells are pre-treated for 7 days with different concentrations of ATX (ATX are added every 2 days at the same time that retinoic acid).

To study the consequences of treatment with ATX on the mitochondrial function, we started the measurement in real-time through confocal microscopy of parameters such as mitochondrial membrane potential (ΔΨm) for which we used (TMRM), (Fig. 1) and for the measurement of mitochondrial mass we used (TMRM and Calcein-AM), (Fig. 2) as follow: Differentiated cells are grown on 22 mm cover slips, cells are loaded with recording medium, containing 25 nM TMRM and 1 µM Calcein-AM for 30 min at room temperature (RT). After 30 min cells are washed with the same recording medium containing 25 nM TMRM. Images are acquired using a Zeiss 510 CLSM confocal microscope at RT. Images are analysed using the software program Image J.

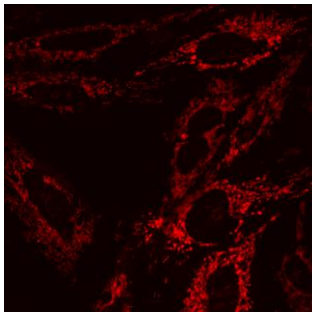

Fig. 1. Representative confocal image of mitochondrial membrane potential (ΔΨm) was measured by the retention of TMRM (red), in differentiated SH-SY5Y cells.

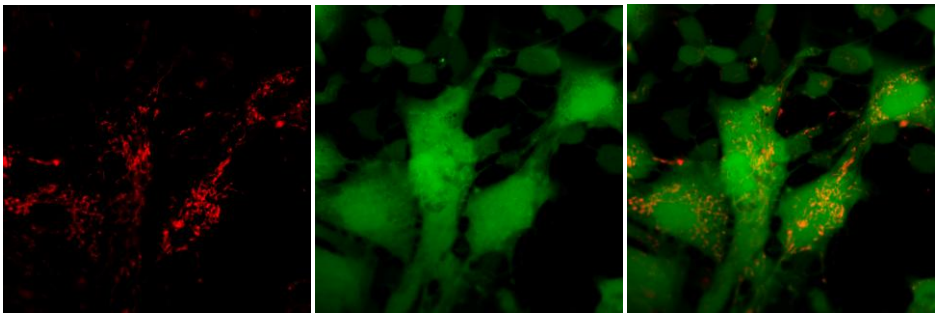

Fig. 2. Representative confocal images of ΔΨm were measured by the retention of TMRM (red), and mitochondrial mass which was calculated from the images using calcein-AM (green) to define the cytosol.

To study the effect of treatment with different concentrations of ATX increase or decrease in the expression of proteins involved in mitochondrial biogenesis, the expression of oxidant or antioxidant proteins and finally the involvement of autophagy, all these parameters are determined using Western Blot (MitoBiogenesis cocktail, Beta-Actin, NQO1 and LC3-B antibodies), as follow: Cells are processed for the determination of protein content using standard protocols. Proteins are transferred onto activated polyvinylidene difluoride (PVDF) membrane. The membranes are blocked with 10% non-fat dried milk in PBS, 0.2% Tween-20. Blocked

membranes are incubated overnight with primary antibodies diluted in albumin solution at 4 °C. The membranes are then rinsed three times in PBST and incubated with the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature (RT). Peroxidase conjugated secondary antibodies are enhanced by chemiluminescence and detected by Odyssey CLx Infrared Imagine system.

At the moment, we have found that treatment with different concentrations of ATX with a range of 1 to 50 uM in differentiated SH-SY5Y cells, produced changes on mitochondrial function, as demonstrated in the changes observed in both the mitochondrial membrane potential and as well as in mitochondrial mass (Fig.3) . We conclude that depending on the concentration of ATX, there are effects on mitochondrial function, indicating that the ATX produces additional effects independent of the inhibition in the norepinephrine reuptake.
We have begun to make the standardization of Western blot in the laboratory, which includes the measuring the amount of protein and the optimal conditions for the acrylamide gels, in order to study the characteristics of ATX treatment on the expression or decrease of mitochondrial proteins and the characterization in the expression of antioxidant proteins. Also we have begun to study the impact of treatment with ATX on mitochondrial quality control.

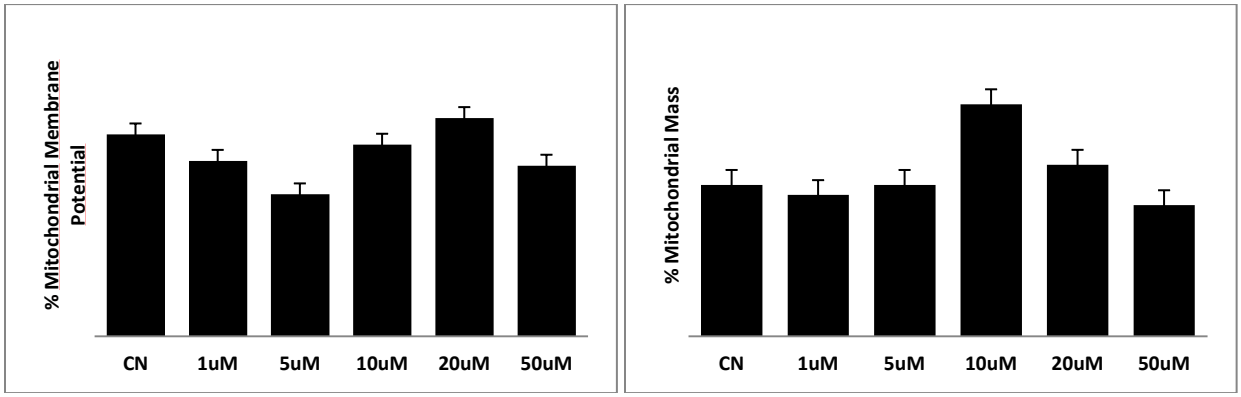

Fig. 3. Preliminary data of the quantification of mitochondrial membrane potential and mitochondrial mass of cells treated with different concentrations of ATX.

The research project that we are carrying out in the laboratory, has a major impact on the research of the ADHD. Therefore, this project will determine whether the use of drugs psychostimulant and non-psychostimulant commonly used in the ADHD, can produce changes on the mitochondrial function (mitochondrial membrane potential, mitochondrial mass, mitochondrial biogenesis, and whether have influence in the process of autophagy, all parameters are not determined yet, despite there are some *in vivo* data that provide evidence of such alterations in the energy metabolism with MPH and ATX) to generate an integrated picture of the mechanisms involved. Therefore, this project will help us to establish new therapeutic approaches for the management of this disorder in children, and the use and refinement of such compounds. This is quite important because it has been shown that MPH has a clear potential for drug abuse in children and adolescents as a result of long-term treatment. The ADHD is the most frequently diagnosed disorder in children and is becoming a major economic burden for the modern society.

Acknowledgements
I would like to deeply thank to the Committee for Aid and Education in Neurochemistry (CAEN) and Dr Roberto Cappai (Chair) for the opportunity given to me. This grant support the research: the use of drugs psychostimulant and non-psychostimulant commonly used in the ADHD and the changes produced on the mitochondrial function. The data will be presented in an International Meeting and when we published our results, the support will be properly acknowledged.

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Below I describe the prices of each of the reagents that I bought as a part of the support:

TMRM, 25mg	USD$ 220.44
Retinoic Acid 50 mg X 3	USD$ 135.31
FBS USDA 500 ml X 2	USD$ 654
DHE 10x1mg	USD$ 331
Anti-beta Actin antibody, 100ug	USD$ 508.5
Calcein-AM, 20x50 mg	USD$ 347.61
LC3-B antibody, 100ul	USD$ 444
NQO1 antibody, 100ul	USD$ 444
Mitobiogenesis WB cocktail 100 ug	USD$ 638
DMEM/F12 media GlutaMAX	USD$ 198
Propidium iodide solution (1.0 mg/ml in water), 10ml	USD$ 94.5
NuPAGE precast gels X2	USD$ 492.5
Cover slips 22 mm	USD$ 275
Culture Dish 35 mm box/500 p X2	USD$ 231.28
	Total USD$ 5014.14