ACETYL-L-CARNITINE NEUROPROTECTION IN MITOCHONDRIAL DYSFUNCTION

Tese de Candidatura ao Grau de Doutor em Patologia e Genética Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto

Orientadora: Doutora Maria Teresa Burnay Summavielle
Categoria: Investigador Auxiliar
Afiliação: Instituto de Biologia Molecular e Celular
Nesta Tese foram apresentados os resultados contidos nos artigos publicados ou em vias de publicação seguidamente mencionados:


**Preclinical imaging: an essential ally in biosciences and drug development**

(Submitted for publication)


**Acetyl-L-Carnitine preconditioning in methamphetamine exposure leads to excessive glucose uptake impairing reference memory and deregulated mitochondrial membrane function**

(Submitted for publication)


**Comunicações orais apresentadas em congressos internacionais:**


**The role of mitochondria in acetyl-L-carnitine neuroprotective action**

European Association of Nuclear Medicine Annual Congress. October 2012. Birmingham, UK.

Apresentações sob a forma de poster em congressos internacionais:

**Acetyl-L-carnitine improves cell bioenergetics**  
European Association of Nuclear Medicine Annual Congress. October 2012. Milan, Italy.  
**Acetyl-L-carnitine neuroprotection and mitochondria bioenergetics**  
Society for Neurosciences Annual Congress. October 2012. New Orleans, USA

**Cunha L**, Damiani D, Alves C, Metello LF, Summavielle T  
**Study of mechanisms for acetyl-L-carnitine neuroprotective action**  
European Association of Nuclear Medicine Annual Congress. October 2010. Vienna, Austria.  

Apresentações sob a forma de poster em congressos nacionais:

**Cunha L**, Bravo J, Gonçalves R, Metello LF, Rodrigues A, Summavielle T  
**Mitochondrial function and acetyl-L-carnitine neuroprotection**  

**Bravo J**, **Cunha L**, Fernandes S, Binienda Z, Summavielle T  
**Acetyl-L-carnitine neuroprotection through autophagy and UPS activity**  
L-Carnitine (LC) is a naturally occurring compound responsible for the transport of long chain fatty acids across the mitochondrial membranes into the matrix, where they undergo β-oxidation. LC, absorbed from diet or endogenously synthesized, can be acetylated, originating esterified compounds such as acetyl-L-carnitine (ALC). ALC plays a variety of vital functions in the body, mainly related to the mitochondria. For instance, ALC is involved in the transmembrane trafficking of acetyl groups and, consequently, in the control of acetyl-CoA/CoA intramitochondrial ratio, and therefore ALC is also involved in the regulation of glucose oxidation. The close interaction between ALC and cell bioenergetics, determines its involvement in many diseases, especially those related to the mitochondria. ALC has been proposed to have beneficial effects in chronic neurodegenerative disorders in which the main outcome from ALC supplementation has been the improvement of mitochondrial function. Recently, our group has demonstrated that pre-treatment with ALC confers effective neuroprotection against 3,4-methylenedioximethamphetamine (MDMA)-induced neurotoxicity, preventing mitochondrial oxidative damage and, most importantly, preventing the typical MDMA-induced serotonin loss. Although the role of ALC at the mitochondrial level has been widely investigated, the molecular mechanisms underlying the action of ALC remain elusive. This dissertation aims to contribute for a better understanding of the mitochondria-related molecular mechanisms underlying the action of ALC in brain bioenergetics.

We used C57BL/6J mice to evaluate the role of ALC in brain glucose uptake. In addition, methamphetamine (METH), known to cause hypoglycemia in the human brain, was used to decrease glucose uptake and to clarify the role of ALC under glucose-deprived conditions. Animals were divided into four groups and treated with: 1) saline (control), 2) ALC (100 mg/kg), 3) METH (10 mg/kg) or 4) ALC + METH (30 min later). A radioactive analogue of glucose molecule, 2-deoxy-2-[18F]fluoro-D-glucose (18F-FDG), was then injected in the tail vein. In vivo images of the mice brain were acquired in a PET-MRI scan for 50 minutes. Here, we observed a generalized increase in glucose uptake in several brain regions immediately after the administration of METH when mice were preconditioned with ALC, which was particularly evident in regions receiving a strong dopaminergic input and therefore more susceptible to METH action, the striatum and the prefrontal cortex.
Since carnitine supplementation activates the insulin growth factor 1 (IGF1) signaling cascade and consequently promotes glucose utilization, we investigated if transcript levels of Igf1 and Igf1 receptor were affected by the different experimental conditions. We found that exposure to METH induced a significant decrease in Igf1 and Igf1R levels in the striatum. The combined administration of ALC and METH prevented the decrease in Igf1 levels. Of relevance, in this group, there was an augment in Igf1R in levels that paralleled the increased glucose uptake. ALC by itself increased Igf1 expression, which may be relevant for the putative neuroprotective features of the compound.

To further understand the mechanisms underlying ALC action, we used a dopaminergic cell line (PC12 cells), exposed to METH in deprived glucose conditions (LG) to explore the role of ALC in glucose uptake and mitochondrial function. Similarly to what was observed in vivo, preconditioning with an ALC dose of 0.01 mM increased glucose uptake in PC12 cells exposed to METH in a LG medium. This was associated with decreased cell viability, lower ATP synthesis, reduced mitochondrial function, higher levels of intracellular ROS, decreased mitochondrial membrane potential and translocation of activated Bax to the nucleus.

To understand the effect of ALC over the dopaminergic functionality, we exposed PC12 cells to METH and evaluated the intra- and extracellular levels of DA. Moreover, using a radioactive compound, antagonist of dopamine D2 receptors (D2R), we acquired in vivo images to evaluate the striatal binding ratio of the radiotracer. Our results revealed that, in vitro, ALC can interfere with DA release, decreasing the amount of extracellular DA in the presence of METH. These data corroborated those obtained in vivo, in which ALC was able to counteract the effects of METH on D2R occupancy by increasing the receptor displacement of METH.

Overall, our results show that ALC when combined with METH exerted its action by increasing glucose uptake and Igf1R levels in the striatum. Concerning dopaminergic functionality, ALC showed not only to increase the intracellular levels of DA, but also to improve the availability of D2R by promoting METH displacement, relevant for its putative neuroprotective features. In vitro data showed that the combination of ALC 0.01 mM and METH increased glucose uptake at 24h. However this was associated to decreased viability and ATP synthesis, increased ROS and loss of mitochondrial membrane potential.
A L-carnitina (LC) é um composto natural, responsável pelo transporte dos ácidos gordos de cadeia longa através das membranas mitocondriais até à matriz, onde decorre o processo de β-oxidação. A LC, quer seja absorvida a partir da dieta, quer seja produzida endogenamente, pode ser acetilada, originando compostos esterificados, como por exemplo, a acetil-L-carnitina (ALC). A ALC desempenha diversas funções no organismo, especialmente relacionadas com a mitocôndria. Por exemplo, a ALC está envolvida no tráfego transmembranar de grupos acetil ao nível mitocondrial e, consequentemente, no controlo da razão intramitochondrial de acetil-CoA/CoA, estando assim igualmente envolvida na regulação da oxidação da glucose. Esta interacção entre a ALC e a bioenergética celular é determinante no seu envolvimento em muitos processos patológicos, particularmente os que estão relacionados com a mitocôndria. Inclusivamente, a administração de ALC tem demonstrado benefícios ao nível das patologias neurodegenerativas, cujo efeito principal se reflecte na melhoria da função mitocondrial. Recentemente, o nosso grupo demonstrou que o pré-tratamento com ALC confere neuroprotecção efectiva contra a toxicidade induzida pela 3,4-metilenodioximetanfetamina (MDMA), prevenindo não só o dano mitocondrial oxidativo, mas também a perda de neurónios serotoninérgicos, típica da acção da MDMA. Apesar de o papel da ALC ao nível mitocondrial estar bem estudado, os mecanismos moleculares subjacentes à sua acção não são totalmente compreendidos. Esta dissertação pretende contribuir para uma melhor compreensão dos mecanismos moleculares, relacionados com a mitocôndria, subjacentes à acção da ALC na bioenergética cerebral.

Usamos ratinhos C57BL/6J para avaliar o papel da ALC na captação cerebral de glucose. A metanfetamina (METH), conhecida por provocar hipoglicemia no cérebro humano, foi utilizada com o objectivo de diminuir a captação da glicose e esclarecer o papel da ALC em condições de carência de glucose. Os animais foram divididos em quatro grupos e tratados com: 1) solução salina (controlo), 2) ALC (100 mg/kg), 3) METH (10 mg/kg), 4) ALC + METH (30 minutos depois). Um análogo radioativo da molécula de glucose, 2-desoxi-2-[^{18}F]fluor-D-glucose (^{18}F-FDG), foi injectado na veia da cauda. Foram adquiridas imagens in vivo dos cérebros dos ratinhos num tomógrafo PET-MRI durante 50 minutos. Observávamo um aumento generalizado na captação da glucose em várias regiões cerebrais imediatamente após a administração de METH, quando os animais foram precondicionados com ALC. Este aumento foi mais evidente em regiões como o estriado.
e o córtex pré-frontal, que possuem um considerável input dopaminérgico e, portanto, mais susceptíveis à acção da METH.

Dado que a suplementação com carnitina activa a cascata do factor de crescimento da insulina 1 (IGF1) e, consequentemente promove a utilização de glucose, investigámos se os níveis do gene Igf1 e do respectivo receptor (Igf1R) estariam afectados nas diferentes condições experimentais. Verificámos que a exposição à METH induziu um decréscimo significativo nos níveis de Igf1 e Igf1R no estriado. A administração conjunta de ALC e METH preveniu o decréscimo nos níveis de Igf1. De salientar que neste grupo, se verificou um aumento nos níveis de Igf1R, concomitante com um aumento na captação da glucose. A ALC per si induziu um aumento dos níveis de Igf1, aspecto que poderá ser relevante para o alegado efeito neuroprotector do composto.

No sentido de melhor compreender os mecanismos subjacentes à acção da ALC, usámos uma linha celular dopaminérgica (células PC12), e expusémo-la à acção da METH em condições de carência de glucose, explorando assim, o papel da ALC na captação da glucose e na função mitocondrial. Semelhantemente ao que observámos in vivo, o pré-tratamento com ALC aumentou a captação da glucose nas células PC12 expostas à METH, na presença de meio de cultura com baixa concentração de glucose. Associado a este aumento na captação da glucose, verificámos uma redução da viabilidade celular, da síntese de ATP e da função mitocondrial, bem como um aumento dos níveis intracelulares de espécies reactivas de oxigénio (ROS), diminuição do potencial de membrana mitocondrial, bem como a translocação da Bax activa para o núcleo.

De modo a explorar o efeito da ALC na função dopaminérgica, expusemos as células PC12 à METH e avaliamos os níveis intra- e extracelulares de dopamina (DA). Além disso, utilizando um composto radioactivo, antagonista dos receptores D2 (D2R), adquirimos imagens in vivo para avaliar a razão de ligação estriatal do radiotraçador. Os nossos resultados revelaram que, in vitro, a ALC pode interferir com a libertação de DA, diminuindo a quantidade de DA extracelular, na presença de METH. Estes dados estão de acordo com os obtidos in vivo, nos quais a ALC foi capaz de contrariar os efeitos da METH na ocupação dos D2R, promovendo a saída da METH dos mesmos.

Globalmente, os nossos resultados mostram que a ALC, quando administrada em conjunto com a METH, exerce a sua acção promovendo um aumento da captação da glucose e dos níveis de Igf1R no estriado. Relativamente à função dopaminérgica, a ALC não só aumentou os níveis intracelulares de DA, como também melhorou a
disponibilidade dos D2R, promovendo a saída da METH, aspectos relevantes para as características neuroprotectoras da ALC. Os dados adquiridos in vitro mostram que a administração conjunta de ALC 0.01 mM e METH, aumentou a captação da glucose pelas células PC12 às 24h. Contudo, este aumento foi acompanhado por uma diminuição da viabilidade celular, da síntese de ATP e da perda do potencial de membrana mitocondrial.
AGRADECIMENTOS

Uma importante meta da jornada que tem sido a frequência do Programa Doutoral em Patologia e Genética Molecular, está prestes a ser atingida: a entrega do manuscrito. Outra meta importante será a defesa pública do mesmo. Evidentemente, um trabalho desta natureza nunca é o esforço de uma só pessoa. Reconhecendo esse facto, gostaria de deixar uma palavra de gratidão a todos quantos me acompanharam no percurso e que, com o seu contributo, me permitiram ultrapassar dificuldades e enriquecer o presente trabalho, de tal modo que o alcance desta primeira meta se tornasse uma realidade. O percorrer desta jornada foi muito importante não só para o meu desenvolvimento pessoal, profissional e científico, como também me permitiu adquirir novas competências em áreas distintas daquelas que estão relacionadas com a minha formação de base. Sem falsas modéstias, tenho consciência que hoje sou uma profissional diferente e com um leque de competências mais amplo. Acredito que isso já se refleita hoje e se tornará mais evidente no futuro do meu desempenho profissional, pois de outra forma, todo o esforço (meu e daqueles que acreditaram em mim) terá sido em vão…

Passo então a expressar a minha gratidão a todas as pessoas que, das mais diversas formas, foram importantes para o desenvolvimento do presente trabalho:

A. Apoio profissional e/ou científico

Começo por endereçar o meu agradecimento à Professora Doutora Teresa Summavielle, orientadora científica deste trabalho, que me acolheu, desde o primeiro momento, com entusiasmo e boa vontade no Laboratório de Neuroprotecção do Instituto de Biologia Molecular e Celular e que, com a sua experiência e os seus ensinamentos, me permitiu desenvolver um espírito crítico face aos resultados obtidos. Agradeço igualmente a dedicação, a disponibilidade e, acima de tudo, a confiança demonstradas.

À Dra. Joana Bravo, colega de laboratório e “camarada de luta”! Obrigada Joana pelo teu auxílio ao longo destes anos, pela tua paciência e persistência e por, em cada dia e em cada tarefa procurares fazer sempre melhor.

À Dra. Sílvia Fernandes, que se encontra a fazer um percurso semelhante ao meu, obrigada pelo auxílio nas experiências. Espero que possas atingir a meta por que lutas o mais brevemente possível!
À Mestre Danira Damiani, cujo percurso do Mestrado se cruzou com o início da minha jornada. Um agradecimento pelas longas horas de trabalho em conjunto, sempre com boa disposição e competência.

Aos restantes investigadores do Laboratório de Neuroproteção, agradeço a simpatia com que me acolheram na equipa.

Aos meus superiores e colegas de trabalho, pelo seu incentivo sempre presente.

Neste apartado, um agradecimento muito especial ao Professor Luís F. Metello, Coordenador do Curso de Medicina Nuclear da Escola Superior de Tecnologia da Saúde, Instituto Politécnico do Porto (ESTSP.IPP), por sempre ter acompanhado de perto a evolução deste trabalho, por toda a colaboração prestada na realização da parte prática e pelo seu incentivo sempre presente. “Boss é boss!” … e este é daqueles não nos deixa acomodar nem por um segundo, com a sua inquietude contagiantes!

Ao Dr. Pedro Costa, colega de trabalho, agradeço o auxílio na realização dos testes de captação da glucose (in vitro), bem como no processamento das imagens PET e SPECT. Obrigada, Pedro pelo teu profissionalismo, pela tua dedicação e preocupação constantes.

Ao Dr. Domingos Vieira, igualmente colega de trabalho, agradeço a paciência, a imaginação e a disponibilidade patentes no auxílio prestado na procura das melhores soluções para o processamento das imagens de PET e SPECT.

A todos os colegas da Área Técnico-Científica de Medicina Nuclear, expresso a minha gratidão pelo vosso incentivo permanente, por perdoarem algumas fases de menor disponibilidade e por partilharem responsabilidades profissionais, mesmo quando isso implicou um acréscimo de trabalho. Graças à vossa dedicação e empenho pessoal, em conjunto fizemos importantes conquistas durante os últimos anos!

À Doutora Paula Sampaio, responsável do serviço “Advanced Light Microscopy” por me ajudar a dar os primeiros passos na microscopia de fluorescência: “um admirável mundo novo!” e pelas preciosas dicas.

À Doutora Raquel Gonçalves, investigadora do grupo “NEWTherapies” do Instituto de Engenharia Biomédica (INEB), pela iniciación na citometria de fluxo.

À Doutora Margarida Duarte, investigadora de Parasitologia Molecular, pelos seus conselhos úteis em termos de função mitocondrial.
I also would like to thank to Drs. Jan Breteling and Markus Diemling, respectively CEO and Research Director from HERMES Medical Solutions for their permanent availability and for the technical support on using advanced tools in imaging processing and for always answering “present!” when we needed.

B. Apoio pessoal

Um agradecimento muito especial à minha família, pelo carinho, pelo suporte e preocupação que desde sempre demonstraram. Aos meus pais, pelo seu exemplo de vida, bem como pela educação que me proporcionaram e sem os quais, não seria quem sou. Às minhas irmãs, com quem posso sempre contar, apesar da distância que por vezes nos separa.

Ao Luís, meu companheiro de vida, sempre disposto a aliviar o meu fardo. Pelo privilégio que tem sido partilhar a vida contigo, tu que és dono de uma boa disposição e criatividade ímpares!

À minha amiga Ana Teles (quase Doutora!), cujo destino se cruzou com o meu logo no início desta jornada. Muito obrigada pelo teu carinho e amizade!

C. Apoio institucional

Ao Instituto de Ciências Nucleares Aplicadas à Saúde (ICNAS), o meu agradecimento pela cedência do $^{18}$F-FDG, que tornou possível a realização dos testes de captação da glucose nas linhas celulares.

I also thank to the Semmelweis University for receiving me in the Nanobiotechnology & In Vivo Imaging Center, allowing the performance of great experiences with the wonderful equipment that allowed me to obtain those valuable PET, SPECT, CT and MRI images. To Dr. Domokos Máthé, Director of CROmed Ltd, I thank for the opportunity and the pleasure that was to work in the Department and to learn with his staff. Ildiko, thank you for your sympathy and professionalism!

O meu agradecimento ao Instituto de Biologia Molecular e Celular, por ter permitido a realização do presente trabalho no Laboratório de Neuroprotecção.
À Fundação para a Ciência e Tecnologia (FCT) pelo financiamento concedido do Programa de Apoio à Formação Avançada de Docentes do Ensino Superior Politécnico (Bolsa SFRH/PROTEC/49698/2009).

À Escola Superior de Tecnologia da Saúde do Porto e ao Instituto Politécnico do Porto pelo apoio concedido através do Programa de Formação Avançada, materializado através de apoio financeiro e redução de serviço docente. Este apoio foi de facto um factor decisivo para o sucesso no atingimento desta meta. Por tudo isto e também por ser, com toda a probabilidade, a instituição que mais directamente virá a beneficiar da aquisição de conhecimentos e competências acrescidas que este trabalho permitiu desenvolver, parece-me particularmente relevante deixar “duas palavras” a testemunhar o meu apreço e mais sincero reconhecimento para todos os que estiveram envolvidos nas tomadas de decisão inerentes a todas as etapas do processo de apoio ao Programa de Formação Avançada de que beneficiei. Um bem-haja pela vossa Visão e sentido de Missão para o Politécnico do Porto e para a ESTSP.IPP! Podem contar comigo para demonstrar que este investimento e esta aposta foram bem aplicados!
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Chemical structure of L-carnitine</td>
<td>24</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic representation of LC biosynthesis</td>
<td>27</td>
</tr>
<tr>
<td>1.3</td>
<td>Chemical structure of acetyl-L-carnitine</td>
<td>32</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic representation of carnitine shuttle</td>
<td>35</td>
</tr>
<tr>
<td>1.5</td>
<td>The mitochondrial spiral</td>
<td>46</td>
</tr>
<tr>
<td>1.6</td>
<td>Free radical production from catecholamines breakdown and autoxidation and glutamate receptors</td>
<td>51</td>
</tr>
<tr>
<td>1.7</td>
<td>Molecular structure of the most common amphetamines and its similarities with dopamine</td>
<td>57</td>
</tr>
<tr>
<td>1.8</td>
<td>Mechanisms through which METH increases synaptic levels of catecholamines</td>
<td>58</td>
</tr>
<tr>
<td>1.9</td>
<td>Temporal sequence of events associated to METH in vitro toxicity</td>
<td>60</td>
</tr>
<tr>
<td>1.10</td>
<td>Positron emission tomography images of dopamine D2 receptors</td>
<td>63</td>
</tr>
<tr>
<td>1.11</td>
<td>18F-FDG PET images depicting brain glucose uptake under control condition or after METH administration</td>
<td>64</td>
</tr>
<tr>
<td>1.12</td>
<td>Schematic representation of PC12 cells in comparison to dopaminergic nerve terminal</td>
<td>67</td>
</tr>
<tr>
<td>2.1</td>
<td>Increased glucose uptake in the brain after the administration of METH to mice preconditioned with ALC</td>
<td>81</td>
</tr>
<tr>
<td>2.2</td>
<td>Igf1 (A) and Igf1R (B) transcript levels in the striatum of mice from different groups</td>
<td>82</td>
</tr>
<tr>
<td>2.3</td>
<td>Altered glucose uptake in long time starvation in PC12 cells exposed ALC</td>
<td>84</td>
</tr>
<tr>
<td>2.4</td>
<td>Cell viability of PC12 cells at 24 and 72h</td>
<td>85</td>
</tr>
<tr>
<td>2.5</td>
<td>Decreased mitochondrial function in conditions of long time starvation, induced by METH</td>
<td>86</td>
</tr>
<tr>
<td>2.6</td>
<td>ATP synthesis of PC12 cells under different conditions</td>
<td>88</td>
</tr>
<tr>
<td>2.7</td>
<td>Decreased membrane potential in cells preconditioned with ALC and exposed to METH</td>
<td>89</td>
</tr>
<tr>
<td>2.8</td>
<td>Translocation of active Bax to the nucleus</td>
<td>90</td>
</tr>
<tr>
<td>2.9</td>
<td>The combined use of ALC and METH decreases mitochondrial mass</td>
<td>91</td>
</tr>
<tr>
<td>3.1</td>
<td>Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM on DA release</td>
<td>101</td>
</tr>
<tr>
<td>3.2</td>
<td>Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM and METH 1.0 µM on DA release</td>
<td>103</td>
</tr>
<tr>
<td>3.3</td>
<td>Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM and METH 100 µM on DA release</td>
<td>104</td>
</tr>
<tr>
<td>3.4</td>
<td>ALC reverses the METH-induced increased D2 receptors occupancy</td>
<td>106</td>
</tr>
<tr>
<td>3.5</td>
<td>Representative 123I-IBZM SPECT/MR images from 12-week old male mice</td>
<td>107</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1 – Carnitine content of some foods ........................................................................26
Table 1.2 - Carnitine concentration in several organs .........................................................30
Table 1.3 - Biological putative functions and effects of carnitine ......................................40
Table 1.4 – Main neurodegenerative diseases and mitochondria involvement .................54
Table 1.5 – Effects of METH ...............................................................................................65
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{123}$IBZM</td>
<td>Iodine-123-labeled(S)-2-hydroxy-6-methoxy-N-((1-ethyl-2-pyrrolidinyl)methyl) benzamide</td>
</tr>
<tr>
<td>$^{18}$F-FDG</td>
<td>Fluorine-18-labeled 2-deoxy-2-fluoro-D-glucose</td>
</tr>
<tr>
<td>3-NT</td>
<td>3-nitrotyrosine</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ALC</td>
<td>Acetyl-L-carnitine</td>
</tr>
<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>beta-amyloid</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>CAT</td>
<td>Carnitine translocase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome-c oxidase</td>
</tr>
<tr>
<td>CPT I</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CPT II</td>
<td>Carnitine palmitoyltransferase II</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>D1R</td>
<td>Dopamine D1 receptor</td>
</tr>
<tr>
<td>D2R</td>
<td>Dopamine D2 receptor</td>
</tr>
<tr>
<td>D3R</td>
<td>Dopamine D3 receptor</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>EHC</td>
<td>Euglycemic hyperinsulinemic clamp</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide 2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HG</td>
<td>High glucose</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KGDHC</td>
<td>α-ketoglutarate dehydrogenase complex</td>
</tr>
<tr>
<td>LC</td>
<td>L-carnitine</td>
</tr>
<tr>
<td>LG</td>
<td>Low glucose</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-methylenedioximethamphetamine</td>
</tr>
<tr>
<td>METH</td>
<td>Methamphetamine</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>O2−</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDHC</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SNPC</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>SUV</td>
<td>Standardized uptake value</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial-transcription factor A</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
1.1. Brief History of Carnitine Discovery

Carnitine (\(\beta\)-hydroxy-\(\gamma\)-N-trimethylaminobutyric acid) is a naturally occurring compound in all mammalian species having important roles in the transport of fatty acids across the inner mitochondrial membrane into the matrix where they undergo \(\beta\)-oxidation, a key source of energy production (Evans and Fornasini, 2003; Sharma and Black, 2009). Carnitine was first described in the beginning of the last century, in 1905, as a constituent of muscle tissue, owing its name to the high concentration present in meat (from the Latin word *Carnis*) (Gulewitsch and Krimberg, 1905). The chemical structure was only established 22 years later (Tomita and Sendju, 1927). Despite intense research during the 1930s, stimulated by the similarities between carnitine and choline, it was not before the 1950s that its biological functions started to be unveiled, when researchers found an essential growth factor for the mealworm *Tenebrio molitor* (common as fish food), called vitamin B\(_T\) (T from *Tenebrio*), that later on was identified as being L-carnitine (LC). Subsequent studies showed that mealworm larvae growing in a state of carnitine deficiency accumulated high amounts of fat, but still apparently died from starvation, as they were unable to use their fat stores to survive. This suggested that LC might play a role in fat oxidation. In the middle 1950s, two important papers renewed the interest on carnitines and revealed some of the biological functions of carnitine in mammals. It was shown that carnitine stimulates fatty acid oxidation in liver homogenates as well as that it can be acetylated by acetyl coenzyme A (acetyl-CoA) in reversible way (Friedman and Fraenkel, 1955; Fritz, 1955), leading to the discovery that carnitine functions as a carrier of active acetyl groups through the mitochondrial membrane (Bremer, 1962). However, these observations only acquired true clinical significance when the first cases of human inborn errors in carnitine transport and metabolism were described, in 1973 (DiMauro and DiMauro, 1973; Engel and Angelini, 1973). Since then, carnitine has become an interesting topic of research over a variety of fields, namely, clinical medicine (neurology, cardiology and oncology), sports medicine, nutrition and aging. A demonstration of this is the number of papers on the subject listed in the “Pubmed” over the last ten years (above 4.300).

1.2. Carnitine Chemistry and Biochemistry

L-Carnitine (molecular formula: \(C_7H_{15}NO_3\); molecular weight: 161.2 g/mol) is a white, crystalline and hygroscopic powder. As it is a zwitterion (dipolar salt) (figure 1.1), in aqueous solution, LC is freely soluble in water since more than 90% of its ionizable
groups (COO⁻ and N⁺(CH₃)₃) dissociate at a physiological pH (~7.4) (Virmani and Binienda, 2004).

The hydroxyl group at the C2 position (figure 1.1) is virtually undissociated in solution, being of great physiological significance because it determines some of LC functions. LC participates in reversible transesterification reactions, in which an acyl group is transferred from coenzyme A to the hydroxyl group of LC, originating carnitine esters such as acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC). Other carnitine esters are also biosynthesized in this manner (Rebouche, 1992; Sharma and Black, 2009). Due to its four different ligands, the C-atom of the hydroxyl group is optically active and occurs in two stereoisomeric forms, the D- and the L-form. Despite only the L-isomer is biological active, the D-isomer is capable of interfering with the membrane transport of LC (Gross et al., 1986; Wu et al., 1999).

LC is present in all animal species as well as in many microorganisms and plants (although in the latter, the availability is limited). Carnitine homeostasis is maintained through a relatively low rate of several mechanisms such as endogenous synthesis (25%), absorption from diet (75%) through enterocyte membranes, renal reabsorption and other mechanisms present in tissues that are able to maintain adequate concentration gradients between intracellular and extracellular carnitine pools¹ (Rebouche and Seim, 1998; Evans and Fornasini, 2003; Virmani and Binienda, 2004; Flanagan et al., 2010). The status of carnitine in humans varies with body composition, gender and diet. A brief description of both exogenous and endogenous ways of carnitine provision will follow.

---

¹ The term “carnitine pool” comprises free carnitine and short-, medium- and long chain carnitine esters, generally designated as acylcarnitines.
1.2.1. Exogenous Carnitine

Exogenous carnitine may be absorbed from food or food supplements through active or passive mechanisms and its bioavailability varies with diet composition (Rebouche and Chenard, 1991; Evans and Fornasini, 2003). In vegetarians, who are used to low carnitine diets, the bioavailability is superior (66% to 86% of available carnitine) comparatively to regular red meat eaters, used to high carnitine diets (54% to 72% of available carnitine). Omnivorous ingest 2-12 µmol/kg of body weight/day (23-135 mg) which represents 75% of body carnitine sources. Bioavailability of oral carnitine dietary supplements is very low comparatively to those obtained from food (14 to 18% of dose) and unabsorbed LC is mostly degraded by microorganisms in the large intestine (Rebouche and Chenard, 1991; Evans and Fornasini, 2003; Brass, 2004; Flanagan et al., 2010). On the other hand, orally administered ALC is deacetylated soon after its uptake by the intestinal cells and part of the recently formed free carnitine is reacetylated (Gross et al., 1986). However, although high doses of LC or ALC are generally well tolerated, the absorption of oral dosage forms is very low (Rosca et al., 2009), as described by Parnetti et al. who treated patients with senile dementia with 2.0 g/day for 50 consecutive days of oral ALC and observed only a modest increase in plasma concentration (Parnetti et al., 1992). They also observed that intravenous administration of 500 mg led to a rapid increase in plasma concentration, with gradual clearance until reaching the baseline 12h after administration. The most important metabolites are trimethylamine oxide in urine and γ-butyrobetaine in feces (Rebouche and Chenard, 1991). Diet effects on plasma or serum free and total carnitine concentrations among individuals of different gender and age has been extensively studied and was summarized by Rebouche and Chenard (Rebouche and Chenard, 1991). There is evidence that meat is the main source of LC. Dairy products, seafood and fish are relatively low in carnitine whereas vegetables are generally very low in carnitine (Demarquoy et al., 2004; Steiber et al., 2004). There is no recommended dietary allowance or dietary reference intake for LC. Table 1.1 summarizes the carnitine content of some selected foods.
Table 1.1 – Carnitine content of some foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Carnitine (µmol/100 g)</th>
<th>Carnitine (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>401.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Chicken</td>
<td>64.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Turkey</td>
<td>131.0</td>
<td>21.2</td>
</tr>
<tr>
<td>Shrimp</td>
<td>4.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Salmon</td>
<td>36.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Milk dry whole</td>
<td>62.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Milk 4% fat</td>
<td>14.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Yogurt (regular)</td>
<td>75.0</td>
<td>12.2</td>
</tr>
<tr>
<td>Egg (white)</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Egg (yolk)</td>
<td>5.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Apple / Banana</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Avocado</td>
<td>50.0</td>
<td>8.1</td>
</tr>
<tr>
<td>Asparagus / Spinach / Tomato</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Potato</td>
<td>15.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Garlic</td>
<td>8.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

[Adapted from (Demarquoy et al., 2004)]

Unabsorbed LC in the gastrointestinal tract is metabolized by bacteria of the intestinal flora, originating trimethylamine and γ-butyrobetaine. Bacteria metabolism increases as the oral intake of LC also increases, because in this case, the amount of LC that reaches the gastrointestinal tract, available for breaking down by bacteria is higher (Evans and Fornasini, 2003).

1.2.2. Endogenous Carnitine

Carnitine not obtained from diet, is endogenously synthesized from two essential amino acids, lysine and methionine, primarily in the liver and kidneys. L-lysine provides the carbon chain and nitrogen atom and L-methionine provides the methyl groups of LC (Rebouche, 1992; Rebouche and Seim, 1998; Vaz et al., 1999; Virmani and Binienda, 2004). Synthesis of carnitine begins with methylation of lysine residues (in proteins like myosin, actin and histones) by S-adenosylmethionine, the donor of methyl groups. 6-N-trimethyl lysine is the first product of the reaction, being initially bound in proteins, is then released through proteolysis in lysosomes. The next step occurs in the cytosol, where the hydroxylation of 6-N-trimethyl lysine (C3) originates 3-hydroxy-6-N-trimethyl lysine. Then, the cleavage of hydroxytrimethyl lysine originates glycine and γ-trimethylaminobutyraldehyde. Oxidation of the carboxyl group results in the production of γ-
butyrobetaine (deoxy L-carnitine), which is finally oxidized, leading to the formation of L-carnitine (β-hydroxy-γ-N-trimethylaminobutyric acid) (Bremer, 1983; Rebouche, 1992; Rebouche and Seim, 1998; Vaz et al., 1999). In humans, the major sites of carnitine synthesis are the liver, the kidneys and the brain because these are the only tissues with a high activity of γ-butyrobetaine dioxygenase (BBD), the enzyme that hydroxylates γ-butyrobetaine to form carnitine (Ringseis et al., 2012). Other substances such as magnesium, vitamins C, B3 and B6, iron and α-ketoglutarate, as well as the cofactors required to originate S-adenosylmethionine (methionine, folic acid, vitamin B12, and betaine) are vital participants in the endogenous synthesis of carnitine. Under normal circumstances, the rate of LC synthesis is approximately 1-2 µmol/kg body weight/day, being regulated by the availability of 6-N-trimethyl lysine. Thus, conditions that increase protein methylation and/or protein turnover may increase the rate of LC biosynthesis (Rebouche, 1992; Vaz et al., 1999; Steiber et al., 2004; Sharma and Black, 2009; Flanagan et al., 2010). A scheme of the described process is shown in figure 1.2.

Figure 1.2 – Schematic representation of LC biosynthesis

In humans, the enzyme that catalyzes the final step of LC synthesis, is present in liver, kidneys and brain, meaning that inter-organ transport must occur in order to widely distribute LC, making it available for all body tissues (Bremer, 1983; Flanagan et al., 2010).

1.2.3. The Transport of Carnitine

L-carnitine and acylcarnitines are present in all tissues. In most of them, because the concentration of carnitine is about 10 fold higher in tissues than in plasma, an active
uptake of carnitine occurs. Plasma concentration of free carnitine is the result of a
dynamic balance with acylcarnitines (the ratio acylcarnitines/free carnitine ≤ 0.4 is
considered normal) (Bellinghieri et al., 2003). Circulating LC concentrations are
maintained at a fairly constant level of around 50 µM, predominantly through
efficient reabsorption by the kidneys (in humans, 90–98% of filtered carnitine is
reabsorbed under normal homeostatic conditions) (Rebouche and Seim, 1998; Evans and
Fornasini, 2003). The uptake rate is highly variable among different organs.
Concentrations of carnitine and acylcarnitines change as a function of dietary conditions.
During starvation and after a high-fat meal intake, the fraction of acetylated carnitine in the
liver and kidneys rises significantly, whereas a carbohydrate-rich diet lowers the levels of
acetylated carnitine in the liver (Jones et al., 2010). In humans, the decrease in plasma
LC does not occur immediately, but the increase in long- and short-chain acylcarnitines
during fasting or diabetic ketosis seem to be fast (Frohlich et al., 1978; Genuth and
Hoppel, 1979; Rebouche and Seim, 1998). Although plasma levels have no direct
correlation with brain levels, a remarkable increase in brain acylcarnitine concentration
was observed by Murakami et al. in neonatal starved rats comparing to control group, with
almost all of the increase attributed to short-chain acylcarnitines. They suggested that
during starvation, carnitine and its esters may be redistributed to the brain, which may use
them for energy supply through fatty acid oxidation or for the delivery of acetyl groups
(Murakami et al., 1997).

Carnitine reabsorption is also sensitive to the amount in the diet. For instance, if filtration
of LC increases as a result of higher consumption or after intravenous infusion, the
efficiency of reabsorption drops promptly (Rebouche and Seim, 1998). It has been shown
that LC as well as ALC and γ-butyrobetaine are synthesized in the kidneys, which are
then secreted into the tubular lumen. Once the kinetics of these metabolites by the
transport systems (mentioned below) are the same, the fraction excreted in the urine is
the result of both the amount present in the glomerular filtrate and the amount secreted in
the tubular lumen by the epithelial cells. A rapid intracellular synthesis of acylcarnitine
(from LC) or direct accumulation from the circulation, will lead to a higher secretion and,
consequently, to a higher proportion of acetylated species in the urine comparatively to
that in the circulation. In fact, kidneys play an important role in the regulation and
homeostasis of acylcarnitines (Rebouche and Seim, 1998). Only in case plasma carnitine
concentration exceeds the normal range (supraphysiologic levels), the excess is rapidly
eliminated as a consequence of saturation of the reabsorption mechanism (Ringseis et al.,
2012).
The carnitine system consists of carrier proteins that transport carnitine across the membranes and enzymes, carnitine acyltransferases that catalyze the reversible equilibrium: acyl-CoA + L-carnitine ⇌ CoA + acyl-L-carnitine (Ramsay et al., 2001; Evans and Fornasini, 2003; Sharma and Black, 2009). Carnitine transport is mediated by a family of organic cation transporters (OCT), playing a pivotal role in the homeostasis of intracellular carnitine. The most important members of this family are OCTN1, OCTN2 and OCTN3. OCTN1 has a very low affinity for carnitine transport (Nalecz et al., 2004). OCTN2 is the most important controlling factor for carnitine pools in the plasma membrane (and needs sodium for the co-transport of carnitine, but not for other organic cations) (Indiveri et al., 2010). OCTN3 is present in the peroxisomal membrane of mammals, having the highest specificity for carnitine (Ramsay et al., 2001; Nalecz et al., 2004; Sharma and Black, 2009). Carnitine and acylcarnitine accumulate in most tissues through the high affinity, sodium-dependent organic cation transporter OCTN2, being highly expressed in the heart, placenta, skeletal muscle, pancreas, kidneys, testis and epididymis and poorly expressed in the brain, lungs and liver (Nalecz et al., 2004; Rytting and Audus, 2005; Sharma and Black, 2009). Mutations in the OCTN2 gene can cause primary systemic carnitine deficiency, markedly reducing serum carnitine levels (0–5 µmol/L) because most of the filtered carnitine will be eliminated through urine (Scaglia et al., 1999). The discovery of OCTN2 as the responsible protein for carnitine transport was presented by Nezu et al., who were the first to demonstrate that patients with systemic carnitine deficiency have a loss of OCTN2 carnitine transporter function (Nezu et al., 1999).

It has been reported that carnitine transport may also occur through a Na⁺-, Cl⁻-dependent way by an amino acid transporter ATB⁰⁺ (Nakanishi et al., 2001), characterized as a low-affinity transporter of carnitine, being expressed in the hippocampus, trachea, lungs, mammary glands and intestinal tract (Sloan and Mager, 1999; Nakanishi et al., 2001; Nalecz et al., 2004). In vitro studies demonstrated that both OCTN2 and ATB⁰⁺ may be involved in the transport of carnitine into the brain as they are expressed in the apical as well as in the basolateral membrane of the blood brain barrier (BBB) (Kido et al., 2001; Berezowski et al., 2004; Nalecz et al., 2004).

The Michaelis-Menten (K_m) values mentioned in the literature for the tissue uptake of LC are highly variable. In part, this variability is due to the method chosen to perform the assay (Evans and Fornasini, 2003). Yet, it seems that brain and liver have K_m values considerably higher (1–5 mmol/L) than kidney (0.1–0.5 mmol/L) and cardiac and skeletal muscle (20–100 µmol/L). However, direct comparisons between tissues and experimental
conditions are difficult to establish, not only due to the presence of multiple transporters with different $K_m$ and maximum rates ($V_{max}$) values, but also to the presence of a passive transport component (Bremer, 1983; Evans and Fornasini, 2003; Stephens et al., 2007).

1.2.4. The Carnitine Pool

Not only the $K_m$ values, but also the concentration of LC varies widely among different tissues. Tissue distribution and uptake are partially controlled by hormones, especially in the liver and epididymis (Bremer, 1983). The latter exhibits extremely high concentrations of carnitine, which, in rats can reach 2000 times the plasma concentration. Skeletal muscle is the second in the ranking, concentrating more than almost 4 mmol/kg, representing 100 fold of plasma concentration. Brain, liver and kidneys contain intermediate levels of 300–1000 µmol/kg. Plasma and extracellular fluid show very low amounts (40-60 µmol/kg) of LC (Bremer, 1983; Harper et al., 1993; Evans and Fornasini, 2003). Table 1.2 summarizes the carnitine concentrations in some human tissues.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Carnitine Concentration (µmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>1140 – 3940</td>
</tr>
<tr>
<td>Heart</td>
<td>610 – 1300</td>
</tr>
<tr>
<td>Liver</td>
<td>500 – 1000</td>
</tr>
<tr>
<td>Brain</td>
<td>500 – 1000</td>
</tr>
<tr>
<td>Kidney</td>
<td>330 – 600</td>
</tr>
<tr>
<td>Plasma</td>
<td>40 – 60</td>
</tr>
</tbody>
</table>

Table 1.2 - Carnitine concentration in several organs

[Adapted from (Angelini et al., 1992; Harper et al., 1993; Evans and Fornasini, 2003)]

As can be seen, the ratios of tissue-to-plasma levels are remarkably high, emphasizing the importance of maintaining adequate levels of the compound within the primary sites of fatty acid oxidation (Evans and Fornasini, 2003). It has been estimated that the total LC pool in a healthy standard (70 kg) adult is about 128 mmol (21 grams). Note that measurements of LC plasma levels may not give reliable information about the real carnitine status in the body, because this compartment accounts with less than 1% of the total carnitine pool, making quantification difficult. Plasma levels of the most abundant carnitine ester, ALC, are around 3-6 µmol/kg, meaning that the concentration of total carnitine (including LC and ALC) is about 50–60 µmol/kg. LC and ALC do not bind to
plasma proteins and although it is known that blood cells contain LC, binding to erythrocytes seems to be minor or negligible (Evans and Fornasini, 2003). Rebouche and Engel have conducted a pioneer study on the pharmacokinetics of an exogenous dose of radiolabeled of L-[methyl-\(^3\)H]-carnitine (Rebouche and Engel, 1984). The compound was administered by intravenous injection to healthy volunteers as well as to patients with carnitine deficiency pathologies. Blood samples were collected for up to 28 days. Results identified three distinct compartments for carnitine in the body: the extracellular fluid (representing the initial distribution volume), fast equilibrating tissues (most probable to be the liver and the kidneys) and slow equilibrating tissues (cardiac and skeletal muscle). In healthy volunteers, the mean residence times (turnover) of LC in these compartments were approximately 1h, 12h and 191h, respectively, being the whole body turnover time about 66 days. In fact, LC exhibits both a slow uptake and a prolonged residence time in muscle tissue, reason why muscle LC content changes very little after acute or short-term administration to healthy individuals (Rebouche and Engel, 1984). However, it has been documented that chronic administration of either intravenous or oral forms of LC for a sufficient length of time, increases muscle LC levels (Sharma and Black, 2009).

1.2.5. Carnitine and Carnitine Esters

L-carnitine is present in the body in both non-esterified and esterified forms. Short-chain (C2 – C5) organic acids and medium- (C6 – C12) or long-chain (C14-C24) fatty acids are transferred to and from CoA and the hydroxyl group of carnitine. These reversible reactions are catalyzed by a group of enzymes designated as carnitine acyltransferases. Acylcarnitine esters are produced intracellularly during the normal cell metabolic activity, in conjunction with the above mentioned carnitine functions, namely the generation of long-chain acylcarnitine esters in order to transport the fatty acyl moieties into mitochondria that then undergo \(\beta\)-oxidation. In fact, long-chain fatty acids can only enter mitochondria as acylcarnitine esters. Short- and medium-chain acylcarnitine esters are generated either in mitochondria and peroxisomes, partially as a way to remove organic acids from these organelles as high-energy compounds (Rebouche and Seim, 1998). In turn, acylcarnitine esters may also have diverse functions, either at cellular or organ level. For example, benefits of PLC in the contractile function of heart and in the protection of the ischemic heart from reperfusion injury, are widely studied (Broderick et al., 2000; Felix et al., 2001; Lango et al., 2005; Vargiu et al., 2008; Mingorance et al., 2011). ALC (figure 1.3) is the most abundant carnitine ester and has a putative antioxidant effect in mitochondria (Shigenaga et al., 1994) by increasing the endogenous pool of antioxidant

---

31
glutathione (Aureli et al., 1999) and promoting the activity of antioxidant enzymes (El-Awady et al., 2010; Hao et al., 2011; Haorah et al., 2011). In fact, antioxidant properties of ALC are still a matter of controversy, mainly because in vivo studies not always match the findings of in vitro studies. Schinetti et al. explained antioxidant properties of ALC based on its iron-chelating ability in vitro (Schinetti et al., 1987). This was partially corroborated by Reznick et al., who administered carnitine derivatives to ischemia-reperfused rat hearts and observed that none of the carnitine derivatives were able to scavenge peroxyl or superoxide radicals. However, propionyl-L-carnitine and propionyl-D-carnitine were the only derivatives able to suppress hydroxyl radical production in the Fenton system, probably by chelating the iron required for the generation of hydroxyl radicals (Reznick et al., 1992). In vitro, ALC has demonstrated to protect the electron transport chain (ETC) from electron leakage and superoxide production (Shen et al., 2008).

ALC also shown to improve impaired mitochondrial DNA expression in the brain and heart of aged rats (Gadaleta et al., 1990), to improve cognitive function after ischemic injury and ameliorate damage induced by traumatic spinal cord injury through improvement of mitochondrial function (Zanelli et al., 2005; Kobayashi et al., 2010; Goo et al., 2012; Patel et al., 2012; Zhang et al., 2012). ALC will be further discussed in this work.

**Figure 1.3 - Chemical structure of acetyl-L-carnitine**

As previously mentioned, starvation induces an increase in acylcarnitine in plasma and liver (Yamaguti et al., 1996). After feeding, a quick decrease in plasma is observed and is concomitant with the increase in liver concentration. It was shown that during fasting, short-chain acylcarnitine esters (particularly ALC) uptake by muscles parallels the release of non-esterified carnitine. These findings suggest that, carnitine esters in plasma might be contributing for the exchange of metabolic fuels from the liver to the muscle (Rebouche and Seim, 1998). Likewise, during high-intensity exercise, there is a marked increase in
short-chain acylcarnitine esters concentration in skeletal muscle paralleled with a decrease in free carnitine levels. Low-intensity exercise does not change carnitine metabolism (Hiatt et al., 1989; Rebouche and Seim, 1998; Stephens et al., 2007). The study of carnitine metabolism during exercise has given evidence to support the concept that carnitine and acetyltransferase enzyme have an important contribution for the modulation of the acyl-CoA to free (non-esterified) CoA ratio during metabolic stress (Rebouche and Seim, 1998).

1.6. BIOLOGICAL FUNCTIONS OF CARNITINE AND MITOCHONDRIAL BIOENERGETICS

A variety of important biological functions in the body for carnitine, emphasized by carnitine deficiency diseases, has been described. Two of these functions, and perhaps the most important, are the catalytic function in the mitochondrial combustion of fatty acids and the metabolic function as a buffer for the excess of acyl residues. For the catalytic function, only tiny amounts of LC are needed. In the metabolic function, free LC is esterified (mainly to ALC), involving larger amounts of LC in the process (Jones et al., 2010). ALC exerts many of its biological actions through the effects of LC itself (described above) as well as acetyl moieties. The latter, may acetylate –NH₂ and –OH functional groups in amino acids as lysine, serine, threonine, tyrosine and N-terminal amino acids in peptides and proteins, eventually modifying their structure, function and turnover. It has been described that ALC has a higher potential for transacetylation reactions, transferring acetyl groups more easily than acetylcholine or the terminal phosphate of ATP molecule (Pettegrew et al., 2000).

Carnitine is essential for the shuttle of long-chain fatty acids across the inner mitochondrial membrane, whose rate of β-oxidation is also controlled by LC. Thus, LC plays an important role in energy metabolism (Rebouche and Seim, 1998). If alterations in carnitine levels occur, this will have repercussions on energy production by the mitochondria. The entry of long-chain fatty acids, released by lipolysis, into mitochondria involves several steps. The first of them consisting in the activation of the fatty acid to CoA occurs in the cytosol and originates fatty acetyl-CoA, which, however, is not able to cross the mitochondrial membrane unless it is transported by carnitine (Kerner and Hoppel, 2000). This transport system consists of three distinct proteins: carnitine palmitoyltransferase I (CPT I), carnitine translocase (CAT) and carnitine palmitoyltransferase II (CPT II). CPT I, present in the outer mitochondrial membrane
(OMM), catalyzes the transfer of the fatty acid moiety from long-chain fatty acyl-CoA to carnitine (esterification of carnitine with long-chain acyl-CoA to form long-chain acylcarnitine) and this is the rate-limiting step for β-oxidation of fatty acids, because CPT I is inhibited by malonyl-CoA. In fact, there is an inverse relationship between the rate of fatty acid oxidation and tissue malonyl-CoA concentration (Kerner and Hoppel, 2000; Stephens et al., 2007). In the second step, the reaction products, long-chain acylcarnitines, are transported from the outer to the inner mitochondrial membrane. This is catalyzed by CAT that allows the transfer of one long-chain acylcarnitine molecule into the mitochondria and the release of one molecule of free LC or acylcarnitine out of the mitochondria (Kerner and Hoppel, 2000; Stephens et al., 2007). CAT also participates in other functions such as buffering the mitochondrial acetyl-CoA/free CoA ratio and shuttling chain-shortened fatty acid oxidation products from the peroxisomes into the mitochondria (Ramsay and Arduini, 1993; Kerner and Hoppel, 2000). Once inside the mitochondria, long-chain acylcarnitine molecules are transferred to CoA (transesterification of acylcarnitine back to free carnitine and long-chain acyl-CoA) through CPT II (an enzyme located on the matrix side of the inner mitochondrial membrane), originating long-chain acyl-CoA that is finally ready to undergo β-oxidation in which successive oxidative removal of two carbon units in the form of acetyl-CoA occurs, starting at the carbonyl end of the fatty acid chain (for example, palmitoyl-CoA yields eight acetyl-CoA molecules) (Pettegrew et al., 2000). Carnitine is then transferred back to the intermembrane space via CAT in an exchange with an incoming acylcarnitine. Each molecule of acetyl-CoA produced from β-oxidation of fatty acids, originates one molecule of flavin adenine dinucleotide (FADH<sub>2</sub>) and one molecule of nicotinamide adenine dinucleotide (NADH). The acetyl-CoA is then oxidized to CO<sub>2</sub> in the tricarboxylic acid (TCA) cycle, producing one guanosine triphosphate (GTP) (or one adenosine triphosphate, ATP) molecule, three NADH molecules and one FADH<sub>2</sub> molecule. FADH<sub>2</sub> and NADH undergo oxidation in the ETC with each NADH molecule generating three ATP molecules and each FADH<sub>2</sub> molecule generating two ATP molecules. In summary, each acetyl-CoA molecule generated by β-oxidation of fatty acids can produce a total of 17 ATP molecules (Pettegrew et al., 2000).

The link between β-oxidation and the electron pathway within the mitochondria originates chemical energy. On the other hand, carnitine released in the mitochondrial matrix can return to the cytosol via CAT. Therefore, carnitine has a pivotal role in the mitochondrial utilization of long-chain fatty acids for energy production (Angelini et al., 1992; Carter et al., 1995; Kerner and Hoppel, 2000; Stephens et al., 2007), which, in many organs,
represent a crucial energy source (for example, liver and muscle). Figure 1.4 depicts the carnitine shuttle and the above mentioned interactions.

Lipid storage in most organs occurs only in small amounts, making energy production dependent on continuous supply of fatty acids mainly from adipose tissue. Once within the cell, free fatty acids bind to fatty acids binding proteins, which are abundant in the cytosol. Then, the fate of fatty acids depends on the tissue and its metabolic needs. It can be converted into triglycerides or membrane phospholipids or oxidized in the mitochondria for energy production. However, whatever the fate, fatty acids have always to be activated first (Kerner and Hoppel, 2000). The acetyl-CoA produced is oxidized to CO\textsubscript{2} and energy (plus free CoA) in the TCA cycle of all mitochondria. It may be temporarily converted into acetyl-L-carnitine and in ketone bodies in the fasting liver. These molecules are exported to the blood and are oxidized via acetyl-CoA in other cells. In other organs than the liver, the mitochondrial oxidation of fatty acids is deeply related with the TCA cycle.

![Figure 1.4 – Schematic representation of carnitine shuttle](From (Finn and Dice, 2006), with permission).

On the other hand, the control of fatty acid oxidation and ketogenesis takes place in the carnitine dependent transfer of activated fatty acids into the mitochondria, at CPT I level. A high rate of fatty acid oxidation is correlated with a high acylcarnitine/free carnitine ratio. The complete oxidation of acylcarnitines to CO\textsubscript{2} and water is the result of two interacting cycles, the β-oxidation cycle and the TCA cycle: one turn of the β-oxidation cycle
originates one acetyl-CoA molecule and one turn of the TCA cycle converts one acetyl-CoA into one \( \text{CO}_2 \) molecule. In muscle, the two cycles work like gearwheels as there is practically no use of acetyl-CoA outside the TCA cycle. Consequently, if the TCA cycle is blocked, fatty acid oxidation will be inhibited in muscle tissues. In liver things are different because the ketogenic enzymes are able to convert acetyl-CoA in to ketone bodies (Bremer, 1983).

Due to the reversible transesterification of the acyl-CoAs with carnitine and the ability of acylcarnitine to cross the mitochondrial membrane, the intramitochondrial relationship between acyl-CoA and free CoA has an impact on the extramitochondrial acylcarnitine to free carnitine ratio. This ratio, very sensitive to changes in mitochondrial metabolism, is considered normal around 0.25 and abnormal above 0.4, representing a state of carnitine insufficiency indicating that more carnitine is needed to handle any increased need for the production of acylcarnitines (Angelini et al., 1992; Carter et al., 1995).

It was found by several groups that following a few minutes of intense exercise, there is a remarkable reduction on free carnitine content in skeletal muscle (from approximately 75% of the total muscle carnitine pool to 20%), being this reduction correlated to the formation of ALC, which in turn is associated to the increase of muscle acetyl-CoA. Stephens et al. suggested that rate the of acetyl-CoA formation from pyruvate oxidation, and catalyzed by the pyruvate dehydrogenase complex (PDHC), is in excess comparatively to its rate of utilization by the TCA cycle, meaning that the rate of condensation with oxaloacetate is less than its rate of production, leading to accumulation (Stephens et al., 2007). The PDHC is a mitochondrial multi-enzyme complex that regulates the oxidation of glycolytic pyruvate, generating acetyl-CoA for metabolism by the TCA cycle or fatty acid synthesis, according to the following reaction (Randle, 1998):

\[
\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{NADH}.
\]

In the presence of increased PDHC flux (as during intense exercise), carnitine buffers the excess of acetyl groups, ensuring that a viable pool of free CoA is kept for the continuation of the PDHC and TCA cycle reactions. Moreover, the accumulation of ALC itself provides a pool of acetyl groups that are available for transacylation back to acetyl-CoA, to be used in the TCA cycle when needed (Stephens et al., 2007).

Carnitine is responsible for maintaining adequate concentrations of free and esterified CoA, as it provides a way for the removal of poorly metabolized and potentially toxic acyl-CoA out of mitochondria (Carter et al., 1995; Ringsseis et al., 2012). In fact, it has been demonstrated that intracellular acyl-CoA derivatives accumulation contributes to the
development of insulin resistance in skeletal and heart muscle. In this context, oral carnitine supplementation has demonstrated to be a useful tool for the treatment or prevention of insulin resistance and type 2 diabetes mellitus (Mingorance et al., 2011). In addition, removal of acetyl-CoA disinhbits pyruvate dehydrogenase, leading to a decrease in lactate levels and an increase in glucose oxidation (Stephens et al., 2007). The role of carnitine (free carnitine and ALC) in reducing intra-mitochondrial acetyl-CoA levels results in a 10- to 20-fold decrease in the acetyl-CoA/CoA ratio. This mechanism is thought to be the responsible for the enhancement of glucose utilization by healthy and type 2 diabetic individuals (De Gaetano et al., 1999; Mingrone et al., 1999). In a study conducted by Mingrone et al. this stimulating effect on glucose oxidation was not observed in healthy control subjects, but only in type 2 diabetic patients, whose PDHC impaired activity was restored to normal as a result of carnitine infusion. In healthy subjects, after carnitine infusion, an increase in glucose uptake was observed, but instead of being used as a fuel, was stored in the form of glycogen (Mingrone et al., 1999). Similarly, in a study with healthy subjects, under euglycemic hyperinsulinemic Clamp (EHC) conditions, Stephens et al. observed an increase in glucose uptake, but also an inhibition of glucose oxidation at the level of PDHC (increased muscle glycogen), probably due to an increase in fatty acid oxidation mediated by carnitine (Stephens et al., 2006b). Similar results were obtained by other groups, demonstrating an augmentation of non-oxidative glucose disposal as a result of carnitine administration in EHC conditions (Ferrannini et al., 1988; Wyss et al., 1990). These findings indicate that carnitine may have an important role in the modulation of PDHC activity and thus, exert an effect on whole body glucose homeostasis (Ringseis et al., 2012).

Carnitine has another important role in the mitochondria as it is a reservoir for excess acyl residues, generated by high rates of β-oxidation, in which the acyl residue is transesterified from CoA to carnitine, liberating CoA for other cellular reactions (Rebouche, 2005; Ringseis et al., 2012). The acylcarnitine thus formed may have several destinations: it may remain in the site of production and used when needed or may leave from the cell and be used by other cells or tissues or it may be finally excreted from the body. This represents an advantage of carnitine and its esters that unlike CoA and its esters are able to easily cross most membranes, facilitated by specific carriers and transporters. Clearly, this function has important implications for cellular energy metabolism (Rebouche, 2005). For example, carnitine facilitates the oxidation of glucose in working hearts by relieving inhibition of PDHC by fatty acids (Broderick et al., 1992). The mechanism involves removal of acetyl groups generated from fatty acid β-oxidation by transesterification from acetyl-CoA to carnitine, liberating CoA to participate in the PDH
reaction. Carnitine may also increase the rate of glucose production and oxidation secondarily by facilitating utilization of long-chain acyl-CoA in the hypothalamus. In addition, it has been shown that inhibition of CPT I and, consequently, the increase in long-chain acyl-CoA concentration in the hypothalamus, induces anorexia and decreases hepatic glucose production (Obici et al., 2003).

Furthermore, it is thought that ALC may be a form of energy storage for the cell, at least in sperm and macrophages. Peroxisomes also contain carnitine acyltransferases and a β-oxidation enzyme system, particularly active in the shortening of very long chain fatty acids (Bremer, 1990). Carnitine acyltransferases from peroxisomes seem to be active in the transfer of the shortened acyl-CoAs and the acetyl-CoA to the mitochondria for complete oxidation. The carnitine acyltransferases of the mitochondria can catalyze the formation of PLC and branched chain acylcarnitines from branched chain amino acids and methylthiopropionylcarnitine from methionine. According to Bermer, their formation apparently represents a security system to prevent acyl-CoA accumulation in the mitochondria (Bremer, 1990). In addition, another function attributed to carnitine is the stabilization of cell membranes and enhancement of calcium transport (Hülsmann et al., 1985).

Besides the modulation of the PDHC by carnitine, some authors have come up with alternative explanations for the improvement of glucose tolerance associated to carnitine supplementation, involving the modulation of expression of glycolytic and gluconeogenic enzymes. In type 2 diabetic patients, oral ALC has been shown to correct a common abnormality in these patients consisting in an inappropriate shift in the substrate use from carbohydrates to lipids (Ruggenenti et al., 2009). Similar effect were observed after intravenous infusion of L-carnitine in insulin resistant subjects with type 2 diabetes mellitus, which increased whole body glucose utilization and decreased plasma free fatty acids (Capaldo et al., 1991). Taken together, these results suggest that carnitine acts as an essential cofactor for β-oxidation of fatty acids, facilitating the transport of long-chain fatty acids across the mitochondrial membrane in the form of acylcarnitine esters. Other mechanisms involve the modulation of key enzymes implicated in glycolysis and gluconeogenesis (Stephens et al., 2007; Ruggenenti et al., 2009).

In another study, using a mouse model of primary carnitine deficiency (juvenile visceral steatosis, JVS mouse model) it was demonstrated that hepatic transcript levels of glycolytic enzymes (glucokinase and pyruvate kinase) are reduced, while hepatic transcript levels of the gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, PCK1) are increased. Carnitine administration reversed these alterations in gene
expression to normal levels, demonstrating the contribution of carnitine in the modulation of genes involved in glucose metabolism (Hotta et al., 1996). More recently, carnitine has also been shown to be involved in several other aspects related to glucose metabolism, namely the increase in the expression of genes involved in glucose transport (e.g., GLUT8), the conversion of glucose into glucose 6-phosphate and glycolysis and the down-regulation of genes involved in gluconeogenesis in pig liver. This shows that suppression of hepatic gluconeogenesis also contributes for the positive effect of carnitine on glucose utilization (Keller et al., 2011) and that the cause of this inhibitory effect on gluconeogenic enzymes resides in the improvement of insulin action (Mingrone et al., 1999). DNA microarray analysis in the liver of pigs whose diet was supplemented with 500 mg/kg of oral carnitine for 21 days, exhibited alterations in the expression of several genes involved in insulin signaling cascade (insulin receptor substrate-2, phosphatidylinositol 3-kinase regulatory alpha subunit and receptor protein-tyrosine kinase erbB-3 precursor) (Keller et al., 2011).

The observation that carnitine improves glucose disposal and oxidation in healthy volunteers is indicative that carnitine influences glucose metabolism acting at insulin receptor level, by increasing transmembrane glucose transport or at post-receptor level, influencing the insulin signaling cascade (Ferrannini et al., 1988; De Gaetano et al., 1999).

More recently, it was found that carnitine also has beneficial effects through its action on the regeneration of endocrine pancreas, namely through the modulation of the insulin-like growth factors (IGFs) and IGF binding proteins (Heo et al., 2001; Molfino et al., 2010). Molfino et al. found that caloric restriction increases intestinal uptake of carnitine, which, associated to oral carnitine supplementation, improves insulin resistance and may be an adjunctive treatment in patients with impaired fasting glucose and type 2 diabetes mellitus (Molfino et al., 2010). It has been shown that carnitine administration to diabetic rats increases plasma concentrations of IGF-1 and IGF-2, possibly activating the IGF-1 signaling pathway, improving glucose tolerance (Heo et al., 2001).

In cardiac disease, the administration of carnitine to patients has also shown beneficial effects by improving heart function. Surprisingly, the improvement of heart function in diabetic or carnitine-deficient rat hearts was not due to an improvement in fatty acid oxidation rates but to an increase in the overall glucose utilization (Broderick et al., 1992; Broderick et al., 1995b; Broderick et al., 1995a). The reason for this is that carnitine increases cytosolic acetyl-CoA, which in turn increases malonyl-CoA. As stated before, the latter is a CPT I potent inhibitor. Indirectly, carnitine is decreasing fatty acid oxidation, by decreasing fatty acid entry into the mitochondria (Broderick et al., 1995b; Broderick et
al., 1995a). On the other hand, the transfer of intramitochondrial acetyl groups from acetyl-CoA to carnitine (forming ALC) relieves PDHC inhibition, thus increasing glucose oxidation (Broderick et al., 1992). The carnitine derivative PLC is able to improve both fatty acid oxidation and glucose utilization in diabetic hearts, because the propionyl group is able to enter the TCA cycle as succinyl-CoA stimulating the TCA cycle flux and ultimately, stimulating glucose utilization. On the other hand, the carnitine moiety released from this reaction can also enhance fatty acid oxidation through the stimulation of the carnitine shuttle system, therefore, preventing the accumulation of lipid intermediates and improving insulin sensitivity (Schonekess et al., 1995). Table 1.3 summarizes the main known biological functions and effects of carnitine.

### Table 1.3 - Biological putative functions and effects of carnitine

<table>
<thead>
<tr>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promotion of mitochondrial oxidation of long-chain fatty acids</td>
<td>(Pande and Blanchaer, 1970; Kerner and Hoppel, 2000; Jones et al., 2010; Ringseis et al., 2012)</td>
</tr>
<tr>
<td>Stimulation of mitochondrial metabolism by decreasing acyl-CoA and increasing free CoA</td>
<td>(Bremer, 1990; Broderick et al., 1992; Sharma and Black, 2009)</td>
</tr>
<tr>
<td>Membranes stabilization, abolition of Ca(^{2+})-overload and stimulation of microcirculation in ischemia</td>
<td>(Hülsmann et al., 1985; Aureli et al., 2000)</td>
</tr>
<tr>
<td>Involvement in membrane repair by reacylation</td>
<td>(Arduini et al., 1994)</td>
</tr>
<tr>
<td>Stimulation of immune response and decreasing of inflammatory cytokines</td>
<td>(Famularo et al., 1994; Winter et al., 1995)</td>
</tr>
<tr>
<td>Promotion of the maturation process of the fetus, lungs and sperm</td>
<td>(Lohninger et al., 1996; Palmero et al., 2000; Kang et al., 2011)</td>
</tr>
<tr>
<td>Decreasing of oxidative stress</td>
<td>(Aureli et al., 1999; El-Awady et al., 2010; Hao et al., 2011)</td>
</tr>
<tr>
<td>Inhibition of apoptosis and opening of the permeability transition pore. Inhibition of caspases</td>
<td>(Pastorino et al., 1993; Citone et al., 1997; Mutomba et al., 2000)</td>
</tr>
<tr>
<td>Protection against (neuro)toxins and correction of mitochondrial, lipid and neurotransmitter receptor abnormalities in aging brain and heart</td>
<td>(Gadaleta et al., 1990; Aureli et al., 1994; Aureli et al., 2000; Mazzio et al., 2003a; Virmani et al., 2003; Kobayashi et al., 2010)</td>
</tr>
<tr>
<td>Prevention of mitochondrial damage and ATP depletion</td>
<td>(Dhitavat et al., 2005)</td>
</tr>
<tr>
<td>Enhancement of glucose utilization by stimulating the PDHC activity</td>
<td>(Ferrannini et al., 1988; Uziel et al., 1988; Wyss et al., 1990; Stephens et al., 2006a)</td>
</tr>
<tr>
<td>Improvement of mitochondrial bioenergetics following spinal cord injury</td>
<td>(Patel et al., 2012)</td>
</tr>
<tr>
<td>Amelioration of arterial hypertension, insulin resistance and impaired glucose tolerance in subjects at increased cardiovascular risk</td>
<td>(Ruggenenti et al., 2009)</td>
</tr>
<tr>
<td>Improvement of learning capacity in rats</td>
<td>(Kobayashi et al., 2010)</td>
</tr>
<tr>
<td>Modulation of glucose metabolism and stimulation of glycogen synthesis</td>
<td>(Aureli et al., 1998)</td>
</tr>
</tbody>
</table>
Stephens et al. proposed that an increase in carnitine-mediated long-chain fatty acid oxidation, might inhibit glycolytic flux and carbohydrate oxidation (decreasing the PDHC activity and lactate content and increasing glycogen storage), even in the presence of high carbohydrate content (Stephens et al., 2007). This is a process known since the 1960s from studies conducted by Randle et al. on rat hearts and diaphragm muscle (Randle et al., 1963; Randle et al., 1964). According to Randle’s glucose – fatty acid cycle, an increase in β-oxidation will enhance acetyl-CoA concentration and, consequently, citrate and glucose-6-phosphate content will increase. This, in turn, results in the down-regulation of carbohydrate flux, due to product inhibition of PDHC, phosphofructokinase and hexokinase, respectively (Randle, 1998). In fact, increases in the mitochondrial ratio of acetyl-CoA/CoA will result in the activation of PDH kinase, resulting in an increased phosphorylation and consequently in the inhibition of PDHC. This will result in a decrease in glucose oxidation rates. An important determinant of the ratio of acetyl-CoA/CoA is both the rate of removal of intramitochondrial acetyl-CoA by the TCA cycle and the rate of fatty acid β-oxidation, which is an alternate source of acetyl-CoA (Randle cycle).

The above mentioned effects of free LC can also be applied to ALC, which after ingestion, is in large part hydrolyzed to L-carnitine (half-life: 10 to 45 hours) (Rebouche, 2004; Ruggenenti et al., 2009). The acetyl moiety is either rapidly used or stored as acyl-esters of varying chain length, in particular, in skeletal and cardiac muscles (Rebouche, 2004). ALC is the main acylcarnitine ester, both intracellularly and in the plasma. Like LC, ALC participates in both anabolic and catabolic pathways in cellular metabolism. 14C from [acetyl-1-14C] acetyl-L-carnitine injected into mice appeared both as 14CO2 and in the phospholipid and triacylglycerol fractions predominantly in liver (Farrell et al., 1986; Rebouche and Seim, 1998).

In the adult brain, about 80% of total carnitine remains in the free from, 10-15% is present as ALC and about 10% as long-chain acylcarnitines. In the developing brain (as observed in suckling rats), the amount of ALC is higher, while free from diminishes by 40% (Nalecz et al., 2004). Due to the similarities in the chemical structure between ALC and acetylcholine, it has been proposed that ALC might play a role in neurotransmission (cholinomimetic activity) (Falchetta et al., 1971; Pettegrew et al., 2000). ALC, like free carnitine, is both transported across the BBB through OCTN2 and synthesized in the brain (Wawrzenczyk et al., 1995; Nalecz et al., 2004). It has been reported that ALC crosses more easily cell membranes and the BBB compared to free carnitine (Rebouche, 1992). Two substrates are needed for acetylcholine synthesis: acetyl-CoA and choline. Choline is able to cross the BBB and may originate from the intracellular pool, while acetyl-CoA can
be synthesized in peroxisomes as well as in mitochondria. Acetyl-CoA generated inside the mitochondria can cross the inner mitochondrial membrane in the direction of cytosol, as citrate or as ALC. However, ALC is the main source of acetyl moieties for acetylcholine synthesis in the brain (Tucek, 1990; Rosca et al., 2009). In addition, it was demonstrated that in case of acetylcholine content depletion, its synthesis could be stimulated by the administration of glucose, carnitine or ALC (Tucek, 1983). Studies performed with cortical neurons and neuroblastoma cell line, showed that the addition of carnitine increased the production of acetylcholine in the presence of glucose and choline (Wawrzenczyk et al., 1995).

ALC has also been shown to improve membrane cytoskeletal protein-protein interactions, increasing membrane stability and possibly altering cell membrane dynamics and modulating its function (Arduini et al., 1990). Other evidences that ALC induces changes in membranes was found by Paradies et al., who observed that after ALC administration, there was a recovery on mitochondrial membrane phospholipid composition (cardiolipin) and a restoration of phosphate carrier activity, which were altered as a result of aging (Paradies et al., 1992).

A putative role of ALC in nerve growth factor (NGF) levels has been reported. NGF regulates neuronal development and maintenance of the differentiated state in certain neurons of the peripheral and central nervous system of mammals. ALC shown not only to improve NGF-binding capacity in the hippocampus and in the basal forebrain regions of aged rats, improving cognitive performance (Angelucci et al., 1988; Tagliatala et al., 1996), but also to increase NGF receptor expression in the striatum of developing rats (De Simone et al., 1991).

As will be presented in the next section, mitochondria-related enzymes are involved in the process of neurodegeneration. A considerable number of both in vivo and in vitro studies demonstrated that ALC is able to modulate a variety of mitochondrial and other intracellular enzymes. Examples are the increase in the activity of key enzymes such as cytochrome oxidase and α-ketoglutarate dehydrogenase induced by ALC, particularly in synaptic mitochondria and acetylcholinesterase in plasma membrane from rat frontal cortex (Gorini et al., 1996; Gorini et al., 1998). According to the literature, ALC exerts its actions possibly through direct physicochemical interaction or through protein acetylation (Pettegrew et al., 2000).

Furthermore, ALC has been described as having neuromodulatory effects on synaptic transmission, namely by restoring the number of synaptic vesicles of giant synapses in the
hippocampus of aged rats (Laschi et al., 1990). ALC also increases choline uptake into nerve terminals, increasing acetylcholine synthesis, and enhances acetylcholine release in the striatum and hippocampus of freely moving rats (Imperato et al., 1989). ALC is able to attenuate the age-related reduction of a number of central nervous system receptors, including N-methyl-D-aspartate (NMDA), NGF and glucocorticoid receptors and prevents NMDA receptor loss in the hippocampus, striatum and frontal cortex of aged rats (Castorina et al., 1994).

Fariello et al. found that ALC administration to mice for 5 consecutive days raised nigral GABA, but striatal levels of dopamine and metabolites were not significantly affected. The authors suggested that ALC would be useful in treating symptoms of neuronal dysfunction related to the accumulation of metabolic waste (Fariello et al., 1988). Intracerebral ALC administration by microdialysis in different brain regions, increases the release of neurotransmitters as dopamine and acetylcholine as well as amino acids, particularly aspartate, glutamate and taurine in a concentration-dependent manner (Toth et al., 1993). The repeated administration of ALC led to consistent increases in DA output in the nucleus accumbens, subsequent to acute stress conditions exposure (Tolu et al., 2002). More recently, Zaitone et al. observed increased striatal DA levels in rats treated with rotenone (an inhibitor of mitochondrial complex I) and ALC. This increase was not observed in the control group, suggesting that ALC increases DA synthesis and release as a response to injury (Zaitone et al., 2011). Using $^{13}$C-ALC, it was demonstrated that the acetyl moiety of ALC is used as an energy source by both astrocytes and neurons and rapidly incorporated into glutamate and GABA synthesis (Scafidi et al., 2010b). This clarified the direct involvement of ALC in increased glutamine, glutamate and GABA levels, through α-ketoglutarate production, via the tricarboxilic acid (TCA) cycle. Importantly, biosynthesis of both tyrosine and L-tryptophan, dopamine and 5-HT precursors, involves either a transamination reaction with glutamate or the participation of glutamine as an amine donor (Yudkoff, 1997), which may account to explain the action of ALC over monoamine production.

Moreover, ALC has a putative neuroprotective effect, both in vitro and in vivo, against a variety of drugs such as ethanol (Rump et al., 2010; Abdul Muneer et al., 2011a; Haorah et al., 2011), 1-methyl-4-phenylpyridinium (MPP$^+$) (Mazzio et al., 2003b; Virmani et al., 2004), methamphetamine (METH) (Coccurello et al., 2007; Abdul Muneer et al., 2011b), 3,4-methylenedioximethamphetamine (MDMA) (Alves et al., 2009) and rotenone (Zaitone et al., 2011). These ALC features, as well as its neuromodulatory ability will be further discussed.
Since the human body is able to endogenously synthetize carnitine in sufficient amounts, normally it is not required in the human diet. Until now, no clinical pathological conditions have been associated to carnitine nutritional deficiencies. Vegans, who normally absorbed lower carnitine amounts from diet, have lower plasmatic amounts, but no relevant clinical deficiency (Rebouche, 1992). However, in some conditions, carnitine deficiencies may develop, meaning that carnitine concentration in plasma or tissues is under the required to assure the normal function of the body. Clinically, carnitine deficiencies are diagnosed based on plasma levels of carnitine (below 2 µmol/L) and may have genetic or acquired origin (medical conditions or iatrogenic states) (Pons and De Vivo, 1995).

Primary systemic carnitine deficiency of genetic origin results in mutations in the gene for carnitine transporter OCTN2, which causes poor intestinal absorption of dietary LC and impaired LC reabsorption by the kidneys, increasing urinary loss of the compound. Clinical manifestation of this deficiency occurs in early childhood and includes cardiomyopathy and often skeletal myopathy and/or hepatic encephalopathy. There are criteria to establish this condition, namely: decreased plasma or tissue carnitine levels, impaired fatty acid oxidation, the metabolic abnormalities are corrected when carnitine concentration is restored to normal levels, absence of other primary defects in fatty acid oxidation (Treem et al., 1988; Pons and De Vivo, 1995). Without treatment, primary systemic carnitine deficiency is fatal. Treatment consists of pharmacological doses of carnitine.

Secondary carnitine deficiencies are more common and include both genetic and acquired conditions, characterized by decreased carnitine levels in plasma or tissues or both. Hereditary causes include genetic defects in amino acid degradation and lipid metabolism leading to a build-up of organic acids, which are subsequently removed from the body via urinary excretion of acylcarnitine esters. Kidney malfunction also results in increased urinary losses of carnitine, by decreasing renal reabsorption of carnitine. Moreover, the administration of some drugs also induces carnitine deficiencies (e.g. anthracyclines, valproate, pivaloyl-antibiotics, cephalosporins, salicylic acid and aspirin, etc.) (Pons and De Vivo, 1995).

1.7. MITOCHONDRIA AND NEURODEGENERATIVE DISEASES

Mitochondria are double membrane organelles enclosing a matrix playing a central role in cell bioenergetics and cell death pathways. These two membranes invaginate, forming mitochondrial cristae. Mitochondria have their own DNA (mtDNA), composed by 16.500
base pairs and 37 genes, being rather complex and dynamic organelles, as they are able to move, fuse and split. Besides mtDNA, the mitochondrial matrix also contains RNA, detoxification systems and enzymes involved in β-oxidation and TCA cycle (Filosto et al., 2011). The outer membrane is permeable to ions and solutes up to 50 kDa (Harris and Thompson, 2000). The intermembrane space contains proteins encoded by nuclear DNA, such as apoptosis promoting proteins (Harris and Thompson, 2000) and metallic ions and enzymes responsible for several metabolic processes (Terziyska et al., 2005).

The inner membrane is impregnated with protein complexes (I–IV) constituting the ETC through which the reducing equivalents coming from TCA cycle and β-oxidation pass along, as well as two electron carriers (coenzyme Q and cytochrome c). Energy conservation is maintained through charge separation at the level of the inner mitochondrial membrane. The oxidation of substrates gives rise to electrons that are transported to oxygen by the redox carriers of the respiratory chain simultaneously with H⁺ ejection on the redox pumps at complexes I, III and IV. This creates an electrochemical proton gradient, which at normal physiological conditions is equivalent to -220 mV (Bernardi et al., 1999) and is used to generate ATP. As mitochondria are the main power plants of cells, any condition that interferes with energy homeostasis may be translated into pathological conditions. The degree of dependence of a certain organ from oxidative phosphorylation will determine the number of mitochondria. Because they are huge energy consumers, the brain, heart and skeletal muscle are quite abundant in mitochondria. Beyond their well-known role in ATP synthesis, mitochondria are a source of reactive oxygen species (ROS), being involved in cellular responses to oxidative stress and apoptosis (Filosto et al., 2011).

In the recent years, increasing interest has arisen on the possible role of mitochondria in the onset and development of neurodegenerative diseases as it participates in a diversity of biochemical processes such as energy production and reactive oxygen species generation, regulation of intracellular calcium (Ca²⁺), excitotoxicity and apoptotic cascade (Filosto et al., 2011). The question of whether are the oxidative stress and mitochondria impairment the responsible for the onset and development of neurodegenerative diseases or are the former a consequent of the latter is still to be answered (Petrozzi et al., 2007; Filosto et al., 2011). In fact, energy impairment in the brain is tightly correlated to intracellular Ca²⁺ regulation as well as ROS generation (Blass, 2000). It has been reported that impairments in glucose metabolism seems to impair the mechanisms that quench free radicals, contributing to free radical damage. This idea has been formulated as “the mitochondrial spiral”, described by Blass (figure 1.5) (Blass, 2000). Moreover, abnormalities in glucose oxidation are associated to neurodegenerative diseases as Alzheimer’s and Parkinson’s Diseases (Blass, 2000; Hu et al., 2000). The reduction in
cerebral metabolic rate occur prior to functional impairments are evident on neuropsychological testing or brain atrophy being detected by neuromorphological imaging, but they can induce cognitive impairments in AD patients (Blass, 2000). Beneficial effects as a result of glucose or insulin administration indicate that the defects in glucose metabolism in AD brain are functionally significant (Boyt et al., 2000; Craft et al., 2000).

A variety of factors may contribute to trigger the mitochondrial spiral in the brain, such as vascular disease, glutamate excitotoxicity, mitochondrial or nuclear DNA mutations, toxins, etc. Initiation of mitochondrial spiral has an early impact on cellular function as energy charge decreases, biosynthetic and other anabolic processes are spared in order to maintain cell structure and osmotic regulation. Mitochondrial spiral can also result in cell death due to apoptotic (cytochrome c release) or necrotic mechanisms (loss of ion homeostasis), or the combination of both (Blass, 2000).

Figure 1.5 – The mitochondrial spiral. Energy impairments, changes in calcium homeostasis and excessive ROS production interact with in mitochondria. Inducing any one of them leads to abnormalities in the other two. The arrows pointing to initiators of the mitochondrial spiral are grouped only for graphic convenience. Initiators can start the cycle at several points (e.g. glutamate excitotoxicity may work directly on dysregulating Ca\(^{2+}\) and reperfusion injury after ischemia damage, directly increases ROS. [From (Blass, 2000), with permission]

The brain cortex is mainly constituted by neurons and astrocytes; other cellular types, which account for only a small proportion of the brain, are oligodendrocytes, microglia and nonparenchymal cells like brain endothelial cells. In humans, astrocytes represent about
30% of brain cortical volume, exhibiting the correspondent fraction of brain glucose oxidation (one-third). About two-thirds of glucose metabolism in cerebral cortex occurs in neurons. Oligodendrocytes, prominent in the white matter, exhibit low levels of glycolytic enzymes and, consequently, low rates of glucose utilization (Hertz and Dienel, 2002).

Glucose oxidative metabolism is the main source of energy to the brain, being tightly regulated to generate ATP and provide carbon atoms for biosynthetic reactions in conjunction with local functional brain activities. Pyruvate oxidation through the TCA cycle originates a high yield of ATP via the ECT and establishes a link between bioenergetics and amino acid pools. At steady state, more than 90% of the available glucose undergoes oxidative degradation in the whole brain. In fact, brain activation is associated to increased glucose utilization (that can sometimes be higher than the concomitant increase in oxygen consumption. The exact mechanisms and type of cells involved in this “metabolic mismatch” are still not fully understood, but some authors have suggested that it may include the accumulation and/or the release of lactate and eventually the generation of other incompletely oxidized glucose metabolites (Hertz and Dienel, 2002). Regions functionally more active and consequently, with increased metabolic rate of glucose, exhibit greater capacity for fuel delivery and recruitment of the related components (as transporters and enzymes) in order to match the local metabolic needs with adequate fuel delivery (Hertz and Dienel, 2002). In the last years, in vivo imaging studies such as positron emission tomography (PET) using an analog of the glucose molecule labeled with a radioactive isotope (2-deoxy-2-[\(^{18}\)F]fluoro-D-glucose, \(^{18}\)F-FDG) have provided valuable information concerning glucose metabolism in the brain in resting as well as activated conditions and cellular participation in the bioenergetics of brain activation.

Besides being a source of energy, glucose oxidation also results in the production of important neurotransmitters, such as glutamate, an amino acid neurotransmitter that is produced by the transamination of \(\alpha\)-ketoglutarate (an intermediate of the TCA cycle). Another important neurotransmitter, derived from glutamate (and therefore from glucose) is \(\gamma\)-aminobutyric acid (GABA). The first step of the glucose oxidation occurs in the cytosol (glycolysis) and the subsequent steps take place into the mitochondria (PDHC, TCA cycle and ETC). The TCA cycle is important for amino acids metabolism as well as for the conversion of sugar metabolites into fragments that are then converted into amino acids by the transamination. In some conditions, brain can also obtain energy from its modest glycogen storage (located mainly in astrocytes), which may supply the needed fuel when metabolic demands exceed the supply of glucose from the blood (Blass, 2002).
The passage of glucose from the blood to the brain cells requires the action of several isoforms of the glucose transporters. Endothelial cells forming the blood brain barrier (BBB) express the glucose transporter GLUT1 and neurons express the isoform GLUT3. There is no evidence that GLUT3 is expressed in other brain cells than neurons (astrocytes, oligodendrocytes or endothelial cells) (Simpson et al., 2008). GLUT5 is expressed in the microglia (Maher et al., 1994; Vannucci et al., 1997). Although glucose is the main energy source, brain cells are able to use other sources to produce energy, as fatty acid oxidation by the astrocytes. Additionally, ketone bodies can be used as a fuel by neurons, astrocytes and oligodendrocytes (Edmond, 1992). Whereas stimulation of brain activity increases glucose utilization in specific brain areas, activity inhibition or neuronal loss reduces it. Glycolysis and glycogenolysis are stimulated in astrocytes by glutamate, released in the course of neuronal activity (Magistretti et al., 1994; Pellerin and Magistretti, 1994). Glucose is metabolized to lactate and together with glucose itself, is released into the extracellular space and taken up by neurons, being used as a substrate for oxidative phosphorylation (Dringen et al., 1993). If glucose becomes scarce, astrocytes are able to supply ketone bodies, because in these conditions, glucose is too precious to serve as a fuel (Randle et al., 1978). However, the brain is the last organ to use substantial ketone bodies metabolism after long periods of starvation (Heininger, 2002a). Although neurons cannot use fatty acids, astroglial cells can degrade them by β-oxidation, providing ketone bodies to the neurons (Edmond, 1992). By one side, oxidative metabolism yields much higher amounts of ATP, but by the other side, it represents an increased risk of oxidative stress, contributing to the enhanced vulnerability of neurons (Almeida et al., 2001).

It has been shown that the levels of activity of the enzymes involved in glucose metabolism not only vary significantly, but also do not distribute uniformly in the brain, indicating that glucose metabolism may present cellular compartmentalization in the brain, with high rates of glycolysis in astroglia and high rates of PDHC, TCA cycle and electron transport in neurons (Blass, 2002). Moreover, it has been reported that the activity of key enzymes for energy metabolism is reduced in neurodegenerative diseases. They include the PDHC, the α-ketoglutarate dehydrogenase complex (KGDHC) and the cytochrome oxidase (COX) (Blass, 1999; Gibson et al., 2005). Abnormalities in mtDNA can occur, at least in part, as a result of oxidative stress. Additionally, ECT complexes III, IV and V are also impaired in neurodegenerative diseases (table 1.4).

Heininger defined aging as a genetically programmed deprivation syndrome in which the soma, stressed by the degeneration of the trophic milieu, develops mechanisms to find or maintain survival pathways, characterized by hypometabolism, adjustments of the
glucose-fatty acids cycle, oxidative stress and modulation of DNA repair (Heininger, 2002b). The adaptive response of the glucose-fatty acids cycle occurs during the normal process of brain aging and results in the reduction of both cerebral blood flow and glucose utilization (Heininger, 1999), the mismatch of oxygen and glucose consumption, the increase use of ketone bodies as a fuel (Heininger, 2002a) and the decrease of brain insulin receptor densities and signal transduction (Frolich et al., 1999). Moreover, during aging, degeneration of proglycolytic neurotransmitters (acetylcholine, noradrenaline and serotonin) is observed (Heininger, 1999).

The brain is highly susceptible to oxidative damage because it presents a high oxygen consumption rate, it is rich in easily peroxidizable unsaturated fatty acids and relatively low content in antioxidant enzymes (Nunomura et al., 2006; Gandhi and Abramov, 2012). Moreover, three main reasons contribute for mtDNA increased susceptibility to oxidative damage: a) it is not protected by histones, making their mutation rate ten times higher than nuclear DNA (Filosto et al., 2011), b) it is too close to the source of ROS, the ETC, c) less efficient repair machinery (Petrozzi et al., 2007). On the other hand, calcium homeostasis deregulation can lead to intracellular calcium overload, disrupting proton-motive force and consequently, inducing mitochondrial permeability transition (MPT), which, once activated, results in the release of cytochrome c and caspase-mediated apoptosis (Lin and Beal, 2006).

Oxidative stress has been considered one of the most important factors associated to neurodegeneration, in which there is an imbalance between the production of ROS or reactive nitrogen species (RNS) and its inactivation by cellular antioxidant machinery, resulting in cell damage. ROS and RNS attack key molecules, causing DNA, RNA and protein oxidation and lipid peroxidation (Gandhi and Abramov, 2012). More recent data suggest that ROS and RNS are involved in signaling cascades, regulating normal cell function (Droge, 2002; Valko et al., 2007). It has been shown that many of the ROS-mediated responses can protect the cells against oxidative stress and reestablish the so-called redox homeostasis (Droge, 2002; Valko et al., 2007). The term ROS encloses both (free) radicals (substances that have unpaired electrons) and chemicals that participate in radical type reactions (substances that gain or lose electrons, but are not considered true radicals as they do not have unpaired electrons) (Magder, 2006). Examples of free radicals are atomic hydrogen (with only one electron), molecular oxygen (O₂, with two unpaired electrons in the outer orbitals). Partially reduced forms of oxygen are highly unstable because they must react either by accepting or donating electrons, originating
free radicals as superoxide (O$_2^-$) and hydroxyl radical (OH$^-$) (Magder, 2006; Gandhi and Abramov, 2012).

Examples of non-radical ROS are hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCI), ozone (O$_3$) and singlet oxygen (1^O$_2$). In addition to the oxygen radicals, there are also nitrogen-based radicals such as nitric oxide (NO), nitric dioxide (NO$_2$), peroxynitrite (ONOO$^-$), sulfur-based radicals such as thinyl (RS) and perthinyl (RSS), carbon-based radicals as trichloromethyl (CCl$_3$*) (Magder, 2006).

Due to their instability, ROS or RNS establish chemical reactions with biological molecules, inducing changes in cell functions that can, ultimately, cause cell death. Because of its unique features, brain cells and neurons in particular, need a robust and efficient antioxidant system. In fact, their increased vulnerability to oxidative stress is essentially due to three main factors (Gandhi and Abramov, 2012): a) greater oxygen consumption when compared to other tissues (around 10-fold); b) neurons are non-dividing, and long living cells, presenting cumulative exposure to all kind of insults; c) presence of nitric oxide, having physiological role in the brain, which can originate RNS such as ONOO$^-$, in the presence of O$_2^-$ (Gandhi and Abramov, 2012).

ROS produce deleterious effects in living organisms through its actions at cellular and molecular level. The origin of these species can be exogenous (produced from xenobiotics, chemical toxins or radiation exposure) or endogenously produced (from ETC, cytochrome P-450) (Cadet and Brannock, 1998). They attack lipid molecules from cell membranes, causing lipid peroxidation, changing membrane fluidity and shape. This damage disrupts calcium homeostasis, fundamental for intracellular signaling cascades. Moreover, peroxides can also cause damage to other key molecules such as proteins and DNA (Cadet and Brannock, 1998; Magder, 2006). Oxidative stress conditions favor Fenton reaction, in which iron reduction by O$_2^-$ originates the highly toxic radical OH$^-$ (Thomas et al., 2009). Activation of Poly(ADP) polymerase (PARP) is the result of the indirect damage resulting from ROS, leading to changes in gene expression, ATP depletion and, ultimately, to apoptosis (Zingarelli et al., 1996). Other important targets of ROS are proteins that undergo oxidation, disturbing receptors, enzymes, cell signaling pathways and the production of carbonyl groups.

Under normal physiological conditions, electron leakage from the ETC is the major source of O$_2^-$*. About 1-3% of mitochondrial reduced O$_2$ is in the form of O$_2^-$* originated at complexes I and III. Additionally, some enzymes are also able to produce O$_2^-$ in the course of their normal functions, such as amino acid oxidase, cytochrome oxidases,
monoamine oxidases, xanthine oxidase and aldehyde oxidase (Cadet and Brannock, 1998; Magder, 2006). Other known sources of $\text{O}_2^{-}$ are: the autoxidation of catecholamines, reperfusion after ischemia episode and the stimulation of glutamate receptors (Cadet and Brannock, 1998). In the brain, catecholamines are indeed an important source of free radicals as its autoxidation induces the production of quinones and, subsequently, the generation of $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ radicals and damage to proteins. Moreover, the breakdown of dopamine, serotonin and norepinephrine catalyzed by monoamine oxidase, originates $\text{H}_2\text{O}_2$, as shown in figure 1.6.

![Figure 1.6 – Free radical production from catecholamines breakdown and autoxidation and glutamate receptors.](image)

Although the idea that mitochondria are the major source of ROS is generally accepted, in fact, under pathological circumstances cytosolic enzymes as nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase produce much higher amounts of free radicals than mitochondria (Gandhi and Abramov, 2012). Furthermore, more recently, Brown and Borutaite have demonstrated that other organelles such as the endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS than mitochondria (Brown and Borutaite, 2011). Because they are continuously exposed to free radicals, mitochondria have a very good antioxidant system. Overproduction of ROS by one side and suboptimal
response by the antioxidant systems by the other, induces oxidative stress and neurodegeneration (Gandhi and Abramov, 2012). Neurodegenerative diseases are characterized by a progressive and selective loss of neurons, anatomically or physiologically correlated (Lin and Beal, 2006). Aging is the major risk factor for the onset of the most common neurodegenerative diseases (such as Alzheimer’s, Parkinson’s and Huntington’s diseases) in which mitochondria have been implicated (Lin and Beal, 2006; Gandhi and Abramov, 2012). A very brief discussion about the involvement of mitochondria in these pathologies will be presented (table 1.4).

Since we live in oxygen rich environments and, as mentioned above, ROS are generated in the course of normal metabolic functions, cells were forced to develop defense mechanisms in order to guarantee homeostasis and health status. The existence of effective scavenging systems makes that oxygen radicals have very short lives (Cadet and Brannock, 1998; Valko et al., 2007). There are different types of defense mechanisms against oxidative stress that may act at different phases concerning oxidative injury, namely preventive and repair mechanisms, physical barriers and antioxidant defenses (Valko et al., 2007). The enzymes involved in antioxidant defenses are: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). Examples of non-enzymatic antioxidants are: vitamins C and E, glutathione (GSH), carotenoids and flavonoids (Magder, 2006; Valko et al., 2007).

GSH is a tripeptide thiol particularly abundant in the cytosol, nuclei and mitochondria, exerting its antioxidant action by working as a cofactor of several detoxifying enzymes, by scavenging OH\(^{−}\) and \(\cdot O_2\), by regenerating other antioxidants to their active form and by working in conjunction with other antioxidants as GSH-Px (Magder, 2006; Gandhi and Abramov, 2012). SOD exerts its action particularly against \(\cdot O_2^{−}\), existing in three different forms: the cytosolic and releasable copper-zinc SOD (CuZn-SOD) and the mitochondrial manganese SOD (MnSOD). CAT exists in the peroxisomes, lysosomes and mitochondria, neutralizing hydrogen peroxide to water and molecular oxygen, in conjunction with GSH-Px. The latter also constitutes another line of defense against organic hydroperoxides and prevents lipid peroxidation, membrane destabilization and membrane dysfunction (Cadet and Brannock, 1998). Vitamins E and C and \(\beta\)-carotene are absorbed from diet, mostly from fruits and vegetables, constituting important lines of defense in the brain. Vitamin E traps peroxyl radicals, originating tocopheroxyl radicals which are then reduced back to the original tocopherol by vitamin C. Moreover, vitamin C reacts with ROS, originating dehydroascorbate, which is reduced by GSH (Cadet and Brannock, 1998).
In fact, ROS and RNS are physiological products from normal cellular metabolism, able to induce cell damage or work as secondary messengers in several cell signaling cascades. Ironically, these reactive species may also participate in a number of redox-regulatory mechanisms and protect cells against oxidative stress and maintenance of the so called redox homeostasis. However, an imbalance between ROS or RNS production and its inactivation by the cellular antioxidant machinery, results in cell damage and ultimately in cell dysfunction and death (Cadet and Brannock, 1998; Valko et al., 2007).

Carnitines and ALC in particular have been reported to have antioxidant properties, namely by increasing the endogenous pool of GSH (Aureli et al., 1999) and promoting the activity of antioxidant enzymes (El-Awady et al., 2010; Hao et al., 2011; Haorah et al., 2011). Lower doses of ALC seem to be more efficient in preventing oxidative stress (Liu et al., 2002). As an example, ALC improved age-related decline in mitochondrial cristae in the hippocampus, while higher doses not only did not decreased lipid peroxidation in the brain, but also increased protein carbonyl content. Based on these results, the authors suggest that increased oxidative stress may be a side effect of high doses of ALC (Liu et al., 2002).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Characterization</th>
<th>Clinical Symptoms</th>
<th>Evidence of Mitochondrial Involvement</th>
<th>References</th>
</tr>
</thead>
</table>
| **Alzheimer’s Disease** | - Late onset and progressive disease, characterized by neuronal loss, particularly in the cortex and hippocampus. <br> - Presence of extracellular senile plaques (containing the peptide β-amyloid) and neurofibrillary tangles (containing hyperphosphorylated of the microtubular protein tau). | Cognitive functions deterioration with behavioral and personality alterations. | - Disruption of several mitochondrial enzymes such as PDHC, KGHC, COX, results in decreased brain metabolism and function.  
- Aβ accumulates in damaged mitochondria, impairing ETC and inhibiting mitochondrial enzymes (e.g. COX, KGDHC). These events lead to MPT opening and trigger the apoptotic cascade.  
- Complex I and COX inhibition promotes tau phosphorylation and thus facilitates microtubules aggregation. This leads to the formation of tangles, which consist in microtubules aggregates with hyperphosphorylated tau protein.  
- mtDNA mutations, alterations in mitochondrial fission and fusion processes as well as morphological changes in mitochondria have also been reported. | (Pereira et al., 1998; Blass, 2002; Coskun et al., 2004; Virmani and Binienda, 2004; Lin and Beal, 2006; Petrozzi et al., 2007; Valko et al., 2007; Wang et al., 2008; Filosto et al., 2011) |
| **Parkinson’s Disease** | - Late onset and progressive disease, characterized by loss of dopaminergic neurons in SNPC and the accumulation of inclusions positive for α-synuclein and ubiquitin (Lewy bodies). | Rigidity, bradykinesia and tremor | - Accumulation of point mutations and deletions in mtDNA in PD brains.  
- Inhibition of mitochondrial respiratory complex I in the SNPC.  
- Six out of nine nuclear genes that have been associated to PD are directly or indirectly linked to mitochondrial function, marked in blue: α-synuclein, DJ-1, leucine-rich-repeat kinase 2 (LRRK2), parkin, phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1), nuclear receptor NURR1, HTRA2, tau and ubiquitin carboxy-terminal hydrolase L1.  
- α-synuclein overexpression impairs mitochondrial function by reducing | (Blass, 2002; Lin and Beal, 2006; Petrozzi et al., 2007; Valko et al., 2007; Chinta et al., 2010; Filosto et al., 2011) |
| **Huntington’s Disease** | Complex I activity and increasing free radical production.  
- DJ-1 translocates to the mitochondrial matrix, downregulating PTEN-tumour suppressor, protecting cells from oxidative stress.  
- Parkin located at OMM inhibits cytochrome c release; it may also interact with TFAM, regulating mitochondrial biogenesis and mitophagy.  
- PINK1, a mitochondrial kinase protects against apoptosis, suppressing cytochrome c release. PINK1 deficiency leads to mitochondrial calcium overload and, in combination with ROS, initiates opening of MTP.  
- Mutations in the above mentioned proteins have been associated to PD. | - Genetic autosomic disease characterized by loss of GABAergic neurons in the striatum, resulting in the progressive atrophy of the caudate nucleus, putamen and globus pallidus.  
- Caused by the expansion of CAG trinucleotide repeat in the huntingtin (HTT) gene, expanding the polyglutamine stretch in the corresponding protein, with subsequent induced toxicity. | - Reduced activity of ETC complexes II, III and IV.  
- High levels of lactate in the cortex and basal ganglia.  
- Huntingtin associates with OMM, increasing sensitivity to calcium-induced mitochondrial permeabilization and cytochrome c release.  
- Mutant huntingtin binds to p53 (tumour suppressor that regulates genes involved in mitochondrial function and oxidative stress), translocating it to mitochondria, causing subsequently Bax activation. | - Legend: Aβ – β-amyloid; COX – cytochrome-c oxidase; ETC – electron transport chain; KGHC- ketoglutarate dehydrogenase complex; mtDNA – mitochondrial DNA; MPT – mitochondrial pore transition; OMM – outer mitochondrial membrane; PD – Parkinson’s disease; PDHC – pyruvate dehydrogenase complex; SNPC – substantia nigra pars compacta; TFAM – mitochondrial-transcription factor A.  
(Blass, 2002; Bae et al., 2005; Lin and Beal, 2006; Petrozzi et al., 2007; Filosto et al., 2011) |
1.8. Methamphetamine as an Oxidative Stress Inducer

Methamphetamine (N-methyl-O-phenylisopropylamine, METH) is a psychostimulant and a highly addictive drug, abused worldwide, particularly popular among the youngest due to its acute effects, namely the onset of euphoria, the improvement of alertness and concentration, the sensation of well-being and self-confidence, the increased libido and decreased appetite (Barr et al., 2006; Fleckenstein et al., 2007). However, a long list of acute side effects causing serious health problems may also occur, partially due to the effects of METH on epinephrine and norepinephrine release by the adrenal glands: tachycardia, increased blood pressure, hyperthermia, muscle rigidity and ataxia, organ failure (liver and kidneys), nausea, blurred vision, anxiety, psychosis and seizures (Barr et al., 2006; Cadet et al., 2007). Human and animal studies have shown that withdrawing the administration of high doses of amphetamines originates the opposite effects seen on acute administration, leading to the onset of fatigue, anxiety, anhedonia, depression and lack of concentration (Barr et al., 2006). Chronic administration of amphetamines is associated with attention and memory deficits, impaired learning capacity, as well as compromised decision making (Cadet et al., 2007). Moreover, schizophrenia has been associated with METH abuse (McKetin et al., 2010).

Under physiologic conditions neuronal activation induces vesicular dopamine release in the synaptic cleft, that will be then removed by dopamine transporters (DAT) and stored again in the vesicles by the vesicular monoamine transporter 2 (VMAT-2), thus protecting DA from oxidation. However, in the presence of METH, DA homeostasis will be affected in a variety of ways which will be described below.

Due to its relatively high lipophilicity, METH crosses the blood brain barrier (BBB) very easily (Barr et al., 2006; Fleckenstein et al., 2007), and after entering monoaminergic terminals by DA or serotonin transporters, it causes the release of monoaminergic neurotransmitters as dopamine, norepinephrine and serotonin, which will be oxidized to produce ROS, resulting in neuronal damage and death (Kuczenski et al., 1995; Davidson et al., 2001; Sulzer et al., 2005). METH belongs to the group of amphetamine-type stimulants, which are synthetic compounds including amphetamine (1-methyl-2-phenylethylamine), METH itself and MDMA (Fleckenstein et al., 2007). All these compounds share similarities in their chemical structure with the monoaminergic neurotransmitter dopamine (DA), as shown in figure 1.7 (Fleckenstein et al., 2007). This is the reason why METH is able to compete so efficiently with DA for access to DAT. The latter, expressed in the dopaminergic neurons, are responsible for DA reuptake after each
round of neurotransmission, in order to store it again into new DA vesicles for future events of excitation.

Concerning the effects on catecholamine-containing neurons, psychostimulants can be categorized into two classes: amphetamines, that inhibit the reuptake of catecholamines and are potent releasers and non-amphetamines (e.g. cocaine), that inhibit reuptake but are more limited in their releasing capacities (Ritz et al., 1987). Amphetamines cause DA release from the synapses because they are weak bases (the “weak base hypothesis”), highly permeable, able to enter into vesicles and bind to free protons. This induces alkalization of the existing acidic pH gradient and decreases the energy that provides accumulation of neurotransmitters in the cytosol and in the synaptic cleft (Sulzer et al., 2005). Additionally to this redistribution of catecholamines from the vesicles to the cytosol, it was demonstrated that amphetamines not only reverse the activity of monoamine transporters, but also decrease the expression of DAT at the cell surface (Saunders et al., 2000; Barr et al., 2006). Other mechanisms involved in the increase of the cytosolic levels of monoamines include the inhibition of the monoamine oxidase (MAO) activity and the increase in the expression and activity of tyrosine hydroxylase (TH), the DA-synthesizing enzyme (Mandell and Morgan, 1970; Mantle et al., 1976; Sulzer et al., 2005). Altogether, these mechanisms (represented in figure 1.8) contribute to the powerful action of amphetamines on monoamines release, presenting prolonged behavioral effects comparatively to other psychostimulants due to its longer biological half-life (8-13h for METH and 1-3h for cocaine) (Barr et al., 2006).
Figure 1.8 – Mechanisms through which METH increases synaptic levels of catecholamines. The represented mechanisms involve: (1) the redistribution of catecholamines from the vesicles into the cytosol and the reverse transport through plasma membrane transporters; (2) the impairment of monoamine transporters activity; (3) the decrease in the expression of DA transporters at the cell surface; (4) the inhibition of MAO activity; (5) the increase in the expression and activity of tyrosine. The cytosolic dopamine is then oxidized originating reactive oxygen species. DA – Dopamine; DAT – Dopamine transporter; MAO – Monoamine oxidase; ROS – Reactive oxygen species; VMAT - vesicular monoamine transporter. [Adapted from (Barr et al., 2006)]

Abnormalities in the dopaminergic system are a common characteristic of METH-induced toxicity as well as Parkinson’s disease (Brotchie and Fitzer-Attas, 2009; Yagi et al., 2010) and schizophrenia (Akiyama et al., 1994; Kurachi, 2003). The nigrostriatal DA pathway is the most affected by METH-induced neurotoxicity comparing to the mesocorticolimbic dopaminergic projections from the ventral tegmental area to the forebrain regions, such as the nucleus accumbens (Eisch et al., 1992; Cass, 1997; Barr et al., 2006). Multiple high-doses of METH administration cause a greater impact on the DA depletion or on the reduction in DAT density in the striatum rather than in the nucleus accumbens, making the former more sensitive than the nucleus accumbens to neurotoxic effects of METH (Eisch et al., 1992; Cass, 1997). Additionally, a region-specific study has shown that the dorsal caudate putamen is less vulnerable to dopaminergic deficits than the ventral caudate putamen region (Eisch et al., 1992; Cass, 1997). There is extensive evidence about the involvement of DAT in METH neurotoxicity as the administration of DAT inhibitors protects
against METH toxicity (Marek et al., 1990b, a; Pu et al., 1994). Moreover, heterozygotic DAT knock-out mice show resistance to dopaminergic deficits induced by METH (Cubells et al., 1994; Fumagalli et al., 1998). Under normal physiologic conditions, DAT are responsible for the transport of extracellular DA back into the nerve terminals. After METH administration, however, a reduction in DAT activity and/or cell surface localization may occur, decreasing DA efflux, promoting its oxidation (Riddle et al., 2006). It has been reported that a single high dose of METH reversibly decreases DAT function, as a result of phosphorylation and internalization of the transporter (Saunders et al., 2000). Although the exact mechanisms involved in the increased vulnerability of the striatum are not known, some experiments have shown that the striatum has a significantly higher density and activity of DAT (Eisch et al., 1992; Povlock and Schenk, 1997).

Other experiments based on the administration of TH inhibitors (α-methyl-p-tyrosine) (Gibb and Kogan, 1979; Uehara et al., 2004; Fleckenstein et al., 2007; Thomas et al., 2008) or DA receptor antagonists (chlorpromazine or haloperidol) (Sano et al., 1982; Nagai et al., 2007; Oshibuchi et al., 2009) shown to prevent METH-induced deficits, indicating that DA itself is also involved in METH-induced toxicity. As mentioned before, METH induces an abnormal accumulation of DA in the cytosol, which, in the presence of METH is rapidly oxidized into ROS (figure 1.8) as quinones and semiquinones, in such amount that exceeds the capacity of the antioxidant systems, thus damaging DA-containing neurons (De Vito and Wagner, 1989; Cubells et al., 1994; Cadet and Brannock, 1998; Sulzer and Zecca, 2000). Striatal extracellular levels of DA are four to five fold of those in nucleus accumbens, reinforcing the increased vulnerability of the striatum (Hernandez et al., 1987; Cass, 1997). Furthermore, accumbal antioxidant levels (particularly superoxide dismutase) are more than twice those of striatum, contributing to the differential vulnerability to METH of these brain regions (Perumal et al., 1992; Kunikowska and Jenner, 2002). The VMAT-2 is the responsible for the sequestration of cytosolic DA into the vesicles until posterior release and by doing its function is also contributing for the protection of dopaminergic neurons from DA oxidation and thus, from oxidative damage (Riddle et al., 2006; Fleckenstein et al., 2007; Sulzer, 2011). However, the presence of METH seems to induce a subcellular redistribution of VMAT-2 such that fewer transporter associated to storage vesicles will be available to capture DA, preventing its subsequent oxidation (Riddle et al., 2006).

It is reported that RNS are formed concomitantly to ROS as a result of DA oxidation. The major cellular consequences from these oxidative species are lipid peroxidation and the production of protein carbonyls (Yamamoto and Zhu, 1998; Wu et al., 2007; Alves et al.,
In vivo studies show that a single administration of METH to Wistar rats, induced mitochondrial oxidative damage both in the striatum and cortex (Shiba et al., 2011). Repeated doses of METH (4 x 10mg/kg/injection 2h apart) resulted in increased oxidative stress markers in the striatum and hippocampus in mice (Gluck et al., 2001). Despite this observation, the cellular antioxidant systems exhibited increased activity as the same dose scheduling induced an early (2h) increased in GSH and oxidized GSH in Sprague–Dawley rat striatum (Harold et al., 2000). Furthermore, RNS play a major role in METH-induced dopaminergic neurotoxicity. Both in vitro experiments using PC12 cells and in vivo studies in mice, demonstrated a significant increase in the production of 3-nitrotyrosine (3-NT) induced by METH, paralleled by DA depletion, respectively in PC12 cells and in mice striatum (Imam et al., 2001b).

Human postmortem studies of METH abusers displayed increased activity of antioxidants: CuZn-SOD and oxidized GSH levels increased 14% and 58%, respectively. Additionally, in METH abusers with severe DA loss, a tendency for decreased GSH levels was observed. Authors suggest that antioxidant systems may be preserved in METH administration and that GSH depletion occurs only as a result of severe dopamine loss (Mirecki et al., 2004).

In vitro studies using dopaminergic cell lines shown that METH induces a temporal sequence of several cellular events (figure 1.9), from decreased mitochondrial membrane potential to apoptosis (Wu et al., 2007).

**Figure 1.9 – Temporal sequence of events associated to METH in vitro toxicity.**

Legend: ↓ - decreased; ↓↓ - strongly decreased; ↑ - increased; ROS – reactive oxygen species; mtDNA - mitochondrial deoxyribonucleic acid
As shown in figure 1.9, mitochondria are heavily involved in METH toxicity, being a known source of ROS. Antioxidants and free radical scavengers have beneficial effects in the reduction of METH-induced oxidative damage (Perumal et al., 1992; Dhitavat et al., 2005; Alves et al., 2009; Koo et al., 2011). METH causes impairment of mitochondrial function by inhibiting the complexes I (Virmani et al., 2004), II, III (Brown et al., 2005) and IV (Burrows et al., 2000) of the ETC and this is likely to increase the production of free radicals, resulting in oxidative damage (Brown and Yamamoto, 2003) and energy depletion due to decreased ATP generation. Other mechanisms for METH-induced neurotoxicity may comprise the activation of apoptotic cascades, involving caspases, pro-apoptotic genes from Bcl-2 family and the tumor suppressor gene p53 (Imam et al., 2001a; Koo et al., 2011). A single high dose of METH (40 mg/kg) induced apoptosis in monoaminergic cells from the frontal cortex by overexpressing pro-apoptotic proteins (Bax, Bad and Bid) and decreasing the expression of anti-apoptotic Bcl-2-related proteins (Bcl-2 and Bcl-X\textsubscript{L}) (Jayanthi et al., 2004). At mitochondrial level, a cascade of events occurs, namely the early release (30 minutes post METH injection) of apoptosis-inducing factor (AIF), smac/DIABLO (a pro-apoptotic molecule working as an indirect activator of caspases through the inhibition of the inhibitor of apoptosis proteins, IAPs) and cytochrome c, with subsequent activation of caspases (Du et al., 2000; Imam et al., 2003; Jayanthi et al., 2004). Other proteases such as calpain (a calcium-dependent enzyme) are also involved in METH-mediated neuronal cell degeneration, as shown in human neuroblastoma SH-SY5Y cultured cells (Suwanjang et al., 2010). Calpain exists as an inactive pro-enzyme in the cytosol, being activated in response to excessive calcium influx and thus able to cleave several proteins from the cytoskeleton, other proteases and signaling molecules (Suwanjang et al., 2012).

More recently, the involvement of both pro- and anti-inflammatory immune mediators has been demonstrated subsequently to METH administration (Itzhak and Achat-Mendes, 2004; Gonçalves et al., 2008; Tocharus et al., 2010; Pendyala et al., 2012).

The role of environmental and body temperature in METH toxicity is still a topic of discussion due to controversial findings. Ali et al. reported that multiple doses of METH (4 x 10 or 20 mg/kg) administered in a cold room (4\textdegree C) decreased significantly striatal dopamine and serotonin depletion, observed when the same doses were given in a 23\textdegree C room (Ali et al., 1994; Baucum et al., 2004). The underlying mechanism might somehow interact with glutamate neurotransmission. The administration of a glutamate receptor antagonist (NMDA), which prevents body temperature to rise, induced a protective mechanism over striatal DA and serotonin depletion (Bowyer, 1995). Conversely, more
recently, studies have shown that mechanisms underlying METH neurotoxicity are temperature-independent (Thomas et al., 2008).

Although research has been focused primarily on the neurotoxicity of METH in dopaminergic neurons, lately there has been an increasing interest on the effects of METH upon serotonergic terminals. Similar findings were obtained, in which high doses of METH caused significant loss of serotonergic terminals, with changes in the levels of serotonin and serotonin transporters (Gross et al., 2011; Weng et al., 2012). However, effects on serotonin neurons are more diffuse, including regions as the hippocampus and perirhinal cortex, anterior cingulate cortex, caudate nucleus, nucleus accumbens and septum (Armstrong and Noguchi, 2004; Kish et al., 2009).

Increased levels of glutamate (the main excitatory neurotransmitter in mammals) in several brain areas as the striatum, the hippocampus and the ventral tegmental area (VTA) subsequently to METH administration, as well as changes in glutamate transporters, suggest the involvement of the neurotransmitter in METH toxicity (Mark et al., 2007; Kerdsan et al., 2012). Neuronal excitotoxic damage occurs due to excessive glutamate release and activation of glutamate receptors (Cadet et al., 2003). Moreover, a single dose of 30 mg/kg induced significant changes in the striatal glutamatergic/GABAergic homeostasis, paralleled by impaired mice motor activity (Pereira et al., 2012). Striatal dopaminergic cells present the receptors α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA), which are activated by glutamate, promoting Ca\(^{2+}\) influx into the neuron, leading to calcium overload that is damaging to the mitochondria, and ultimately to the cell (Schinder et al., 1996).

Finally, more recently, it has been demonstrated that the BBB plays a key role in METH-induced neurotoxicity. METH acts either by inducing a severe disruption of BBB integrity in several brain regions (as the cortex, thalamus, hypothalamus, hippocampus, amygdala and cerebellum) or by increasing BBB permeability (Kiyatkin and Sharma, 2009; Ramirez et al., 2009). Possible mechanisms involve decreasing the levels of tight-junction proteins in the hippocampus and increasing activity and expression of matrix metalloproteinases (Martins et al., 2011), increasing glial activation (Snider et al., 2012), increasing enzyme activation related to BBB cytoskeleton remodeling (Kousik et al., 2012) and the induction of neuroinflammatory pathways (Gonçalves et al., 2008; Kousik et al., 2012).

Animal experiments have elucidated that METH effects are largely dependent on dose schedule (the number of doses, the amount per dose and the time interval between doses). Extrapolating the results obtained in animals to humans may reveal a difficult task
due to the fact that animal studies are mainly based on acute exposure to multiple high
doses of METH during one or two days (which may cause 30-60% loss of dopaminergic
terminals), but human studies are based on months or years of exposure (Barr et al.,
2006). This question was well addressed by Krasnova et al. (Krasnova et al., 2010).
Additionally, the degree of purity of administered drugs might be rather different. Human
neuroimaging studies have been invaluable in the characterization of cerebral alterations
induced by METH abuse both at functional (positron emission tomography, PET and
functional magnetic resonance imaging, fMRI) and morphological level (magnetic
resonance imaging, MRI). Major morphological imaging findings include loss of gray
matter in the cingulate, limbic and paralimbic cortices, hippocampus atrophy and white
matter hypertrophy (Thompson et al., 2004; Daumann et al., 2011; Nakama et al., 2011;
Orikabe et al., 2011). Concerning function, PET studies revealed significant decreases in
dopamine-relevant proteins in the orbitofrontal cortex, dorsolateral prefrontal cortex,
striatum, nucleus accumbens and amygdala, which presented a good correlation with
cognitive impairments and the severity of psychiatric symptoms (Volkow et al., 2001b;
Volkow et al., 2001c). The density of dopamine D2 receptors in the striatum is significantly
reduced in METH abusers (Volkow et al., 2001d). More recently this loss of dopamine D2
receptors (figure 1.10) has been associated with decreased glucose metabolism by the
brain (figure 1.11) (Gouzoulis-Mayfrank et al., 1999), which over time, tends to substitute
glucose as the main source of energy by acetate in some brain areas. These findings
about changes on brain metabolism are also applicable to other addictive substances
such as alcohol and cocaine (Volkow et al., 2006; Volkow et al., 2013).

Figure 1.10 – Positron emission tomography images of dopamine D2 receptors. Images show
the uptake of 11C-raclopride by dopamine D2 receptors in the striatum of a healthy volunteer and a
METH abuser. Note the significant loss of D2 receptors in the METH abuser. [From (Volkow et al.,
2001d)]
The rewarding effects of METH and drugs of abuse in general are due to their ability in increasing DA, particularly in the nucleus accumbens. Thus, the mesolimbic DA pathway, which involves cells in the VTA that projects to the nucleus accumbens, plays an important role in drug reward. The mesostriatal (DA cells in substantia nigra that project to the dorsal striatum) and mesocortical (DA cells in VTA that project to the frontal cortex) are also involved in mechanisms of drug reward and addiction (Wise, 2009; Volkow et al., 2011). METH is able to increase DA-evoked long-term adaptive alterations in dopaminergic transmission by either disturbing or desensitizing the reward system, whose activation by METH induces addiction, characterized by an obsessive drug seeking behavior (Volkow et al., 2011). Table 1.5 summarizes the main biochemical alterations induced by METH.
Table 1.5 – Effects of METH

<table>
<thead>
<tr>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impairment of mitochondrial function</td>
<td>(Schinder et al., 1996; Brown and Yamamoto, 2003; Wu et al., 2007)</td>
</tr>
<tr>
<td>Decrease in DAT activity and expression</td>
<td>(Saunders et al., 2000; Volkow et al., 2001c; Barr et al., 2006; Riddle et al., 2006)</td>
</tr>
<tr>
<td>Decrease in dopamine D2 receptors</td>
<td>(Volkow et al., 2001d)</td>
</tr>
<tr>
<td>Decrease in brain glucose metabolism under high doses</td>
<td>(Huang et al., 1999)</td>
</tr>
<tr>
<td>Changes in VMAT-2 activity and subcellular localization</td>
<td>(Riddle et al., 2006)</td>
</tr>
<tr>
<td>Ca^{2+} influx</td>
<td>(Schinder et al., 1996)</td>
</tr>
<tr>
<td>Dopamine oxidation and ROS generation</td>
<td>(Cadet and Brannock, 1998; Sulzer et al., 2005; Barr et al., 2006)</td>
</tr>
<tr>
<td>Release of monoaminergic neurotransmitters</td>
<td>(Kuczenski et al., 1995; Sulzer et al., 2005)</td>
</tr>
<tr>
<td>Neuronal cell death</td>
<td>(Davidson et al., 2001; Cadet et al., 2003)</td>
</tr>
<tr>
<td>Inhibition of MAO activity</td>
<td>(Mantle et al., 1976)</td>
</tr>
<tr>
<td>Increase the expression of TH</td>
<td>(Sulzer et al., 2005)</td>
</tr>
<tr>
<td>Lipid peroxidation and protein carbonyls generation</td>
<td>(Yamamoto and Zhu, 1998; Wu et al., 2007; Alves et al., 2009)</td>
</tr>
<tr>
<td>Increase activity of GSH</td>
<td>(Harold et al., 2000)</td>
</tr>
<tr>
<td>RNS generation</td>
<td>(Imam et al., 2001b)</td>
</tr>
<tr>
<td>Inhibition of the ETC</td>
<td>(Burrows et al., 2000; Virmani et al., 2004; Brown et al., 2005)</td>
</tr>
<tr>
<td>Activation of pro-apoptotic cascades</td>
<td>(Imam et al., 2001a; Jayanthi et al., 2004; Koo et al., 2011)</td>
</tr>
<tr>
<td>Decrease activity serotonin and serotonin transporters</td>
<td>(Armstrong and Noguchi, 2004; Kish et al., 2009)</td>
</tr>
<tr>
<td>Increase of glutamate levels in several brain regions</td>
<td>(Mark et al., 2007; Kerdsan et al., 2012)</td>
</tr>
<tr>
<td>BBB disruption and increase in the permeability</td>
<td>(Kiyatkin and Sharma, 2009; Ramirez et al., 2009)</td>
</tr>
<tr>
<td>Induction of pro- or anti-inflammatory mediators</td>
<td>(Gonçalves et al., 2008; Pendyala et al., 2012)</td>
</tr>
</tbody>
</table>

Legend: BBB – Blood brain barrier; DAT – Dopamine transporter; ETC – Electron transport chain; GSH – Glutathione; MAO – Monoamine oxidase; RNS – Reactive nitrogen species; ROS – Reactive oxygen species; TH – Tyrosine hydroxylase; VMAT-2 – Vesicular monoamine transporter 2;

Exposure to METH was previously shown to provide an excellent model to mimic these neurodegenerative processes, both in vivo and in vitro (Ferrucci et al., 2008a). In the present study, METH is used in order to clarify the molecular mechanisms underlying the
putative neuroprotective role of ALC. METH is known to affect both the mitochondrial (Brown and Yamamoto, 2003) and the dopaminergic function (Barr et al., 2006) by promoting the production of ROS. In addition, METH-induced activation of mitochondria-mediated intrinsic cell death signaling events is known to be associated with the loss of mitochondrial membrane potential, the release of apoptogenic factors and subsequent activation of apoptotic cascades, creating a suitable model for exploring the mechanisms of action of ALC. METH doses were established based on previous studies from Fornai and collaborators (Fornai et al., 2004) for the in vitro assays and from groups of Bowyer and Fukumura for the in vivo assays (Bowyer et al., 1994; Fukumura et al., 1998).

The present work has urged subsequently to previous observations from our group demonstrating that pre-treatment with ALC confers effective neuroprotection against MDMA-induced neurotoxicity, preventing mitochondrial oxidative damage, reducing carbonyl formation, decreasing mtDNA deletion and improving the expression of respiratory chain components (Alves et al., 2009). These results emphasize the benefits from ALC administration in neurodegenerative disorders. However, as the underlying molecular mechanisms of ALC action remain unclear, here we propose to study those mechanisms that are associated to mitochondria bioenergetics and to dopamine neurotransmission.

1.9. CELLULAR AND ANIMAL MODELS

In the present study, the experimental component was divided into two phases: before using animal models, experiments were performed using a suitable dopaminergic cell line, aiming to determine the role of ALC in mitochondrial bioenergetics, morphology and functionality. The rat pheochromocytoma (PC12) cell line is well known for its catecholamine production. PC12 cell lines are often used as an in vitro model to mimic dopaminergic function and predict the neurotoxicity of a variety of compounds acting on central DA neurons (Schlegel et al., 2004; Piga et al., 2005). This cell line is widely used due to the similarities with central dopaminergic neurons, easy handling and replication and low cost maintenance. PC12 cells are able to produce DA (Greene and Tischler, 1976) and contain DA receptors in their external membranes (Sampath et al., 1994) (figure 1.12). Comparing PC12 cells to dopaminergic central neurons, PC12 cells also express TH, but have less DAT and no expression of VMAT-2 (Fornai et al., 2007). Dopamine content in PC12 cells is 10-fold less than that of DA terminals, L-DOPA amount is very
similar, but norepinephrine levels are very low, while epinephrine is not produced by these cells. Moreover, storage mechanisms in PC12 cells are less efficient (Fornai et al., 2007).

Figure 1.12 – Schematic representation of PC12 cells in comparison to dopaminergic nerve terminal. Main metabolic steps are also depicted in the figure. [From (Fornai et al., 2007), with permission].

In the present study, aiming to evaluate the putative role of ALC over the function of neurotransmitter systems, we have exposed PC12 cells pre-treated with ALC to different concentrations of METH, for 24h or 72h. Despite the differences between PC12 cells and central dopaminergic neurons, PC12 cells possess unique dopaminergic features that make them particularly sensitive to METH and thus, suitable for our purposes (Greene and Tischler, 1976; Fornai et al., 2007).

In the second phase of the experimental procedures, young adult C57BL/6J male mice were used. The option for this strain is related to the well characterized brain anatomy (MacKenzie-Graham et al., 2004) as well as neurotoxicity profile of METH (O’Callaghan and Miller, 1994).
1.10. Objectives

Aging and neurodegenerative diseases have been associated to significant changes in the brain bioenergetics, involving not only impairments in glucose metabolism and neurotransmitter systems, but also increased oxidative damage due to less efficient antioxidant systems. On one hand, there is extensive evidence in the literature regarding the mitochondrial involvement in these processes. On the other hand, it is also reported that ALC might improve deleterious effects induced by aging and neurodegeneration by acting at the mitochondrial level and neurotransmitters modulation. This dissertation aims to contribute for a better understanding of the mitochondria-related molecular mechanisms underlying the action of ALC in the brain.

In order to fulfil this general goal, more specific objectives were outlined:

A. ALC action on glucose uptake and mitochondrial function
   - Evaluate if the administration of ALC has an effect on brain glucose metabolism through the *in vivo* evaluation of glucose brain uptake, using $^{18}$F-FDG.
   - Explore the role of ALC in mitochondrial bioenergetics, using a dopaminergic cell line (PC12 cells), exposed to METH, known to induce reductions on glucose metabolism.
   - Determine if ALC is able to protect cells from loss of mitochondrial membrane potential and prevent the activation of the apoptotic cascade.

B. ALC action on the dopaminergic function
   - Evaluate the role of ALC over the dopaminergic function *in vitro*, through the quantification of intra and extracellular DA levels.
   - Verify the presumed role of dopamine D2 receptor expression as a mechanism of action of ALC’s ability to inhibit dopaminergic toxicity through DA receptor imaging with the radiotracer $^{123}$I-Iodobenzamide.
CHAPTER 2

ACETYL-L-CARNITINE IN MITOCHONDRIAL BIOENERGETICS AND FUNCTIONALITY
2.1. INTRODUCTION

Acetyl-L-Carnitine (ALC) is the most widely distributed ester of L-carnitine in the body, playing a variety of vital functions. Due to its close interaction with cell bioenergetics, ALC plays a role in metabolic compromising diseases, especially those related to the mitochondria (Rosca et al., 2009). Although the role of ALC at the mitochondrial level has been widely investigated, the molecular underlying mechanisms remain elusive.

It is currently thought that improving mitochondrial function and raising ATP levels may be relevant for improving neuronal dysfunction (Owen and Sunram-Lea, 2011). In patients with compromised cognitive function, ALC promoted an effective recovery of attention capacity, mental flexibility, short-term memory, visual scanning and tracking, motor control and orientation ability [revised by (Flanagan et al., 2010)]. We have demonstrated that ALC confers effective neuroprotection against 3,4-methylenedioximethamphetamine (MDMA)-induced neurotoxicity, preventing mitochondrial oxidative damage and the typical MDMA-induced loss of serotonin (Alves et al., 2009). However, until now, the majority of such studies were conducted in aged or demented populations with depleted energy resources. Therefore, the benefits of ALC to healthy and young populations are unknown.

Besides the well-known role of ALC in fatty acid oxidation, it has been shown to exert beneficial effects in patients with impaired fasting glucose and type 2 diabetes (Owen and Sunram-Lea, 2011). Carnitine participates in the glucose metabolism by reducing the acyl-CoA/CoA ratio in mitochondria, which in turn increases pyruvate dehydrogenase activity, improving glucose disposal (Stephens et al., 2007). In addition, carnitine seems to have beneficial effects through the modulation of insulin-like growth factors (IGFs) and IGF binding proteins (Heo et al., 2001; Molfino et al., 2010) and by affecting the expression of glycolytic and gluconeogenic enzymes (Hotta et al., 1996; Ruggenenti et al., 2009). Recently, ALC was used in vitro to prevent METH-induced decrease of glucose uptake and to stabilize the glucose transporters GLUT1 and GLUT3 (Abdul Muneer et al., 2011b).

In order to clarify the role of ALC in the neuronal metabolism of both healthy and compromised subjects, we have exposed C57BL/6J mice to the action of a well known dose of METH. We used positron emission tomography (PET) to evaluate changes in the regional glucose uptake, which were paired to cognitive performances in a Water Maze (WM). To further understand the mechanisms involved at the mitochondrial level, an in vitro model of exposure to METH and ALC, was also used.
2.2. MATERIALS AND METHODS

2.2.1. In Vivo Assessment of Glucose Uptake

Twelve week-old male C57BL/6J mice (21.6 – 26.0 g) were purchased from Charles River (Innovo, Gödöllő, Hungary) and maintained on a 12-h light/dark cycle. All experimental procedures were approved by the Local Animal Experimental Ethical Committee of Semmelweis University, Budapest, Hungary, in compliance with the European Community Council Directive of September 22, 2010 (2010/63/UE). All efforts were made to ensure minimal animal stress and discomfort. Room temperature was kept at 22°C and animals were allowed water and food ad libitum except in the day of the study when they were deprived of food 4h before 2-deoxy-2-[\(^{18}\)F]fluoro-D-glucose (\(^{18}\)F-FDG) administration (free access to water was maintained). FDG consists in a modified glucose molecule in which the C-2 hydroxyl group was substituted by fluorine (\(^{18}\)F). This modification allows FDG to continue to be a substrate for the glucose transporter and for hexokinase conversion to FDG-6-phosphate, but it cannot be metabolized beyond this first step. Consequently, \(^{18}\)F-FDG is trapped in the cell as FDG-6-phosphate and its uptake can be used to determine glucose uptake. Twelve animals were divided into four groups, according to different treatments: group 1 (control, saline), group 2 (ALC, 100 mg/kg), group 3 (METH, 10 mg/kg) and group 4 (ALC + METH 30 min later). Drugs were administered by intraperitoneal (ip) injection. Thirty minutes after the last dose, 10.5 ± 1.1 MBq of \(^{18}\)F-FDG was injected in the tail vein, under anesthesia (isoflurane 2%). After injection, animals recovered very quickly from isoflurane anesthesia and were returned to their home cage for the \(^{18}\)F-FDG uptake period (45 minutes). Immediately before image acquisition, animals were anaesthetized with urethane ip injection (1g/kg), the head was fixed to a specific head holder bed (MultiCell™, Mediso, Hungary) and the body immobilized with tapes to prevent movement. Images were acquired in a PET-MRI scanner (nanoScan PM, Mediso, Hungary), equipped with full ring LYSO crystals and one Tesla magnet. Images were acquired for 50 minutes (25 minutes for each imaging modality).

List mode PET files were reconstructed into images of 3D volume of 0.3x0.3x0.3 mm voxels by proprietary 3D ordered subset expectation maximization (OSEM) algorithm (Tera-Tomo™, Mediso, Hungary) correcting for scatter. Decay-, random and dead time correction was performed. A gamma photon energy window of 400 to 600 keV was used, with 5 ns coincidence timing and 1:5 coincidence mode of the detector modules in the ring. Regions of interest (ROIs) were drawn on several brain regions using the transverse and coronal planes. For \(^{18}\)F-FDG brain uptake, the standardized uptake value (SUV) was
calculated at each time point by dividing the mean radioactivity concentration (MBq/mL) by the injected dose per body weight (MBq/g). For the MRI scans, a T1-weighted three-dimensional Gradient Echo (GRE) sequence of the nanoScan PET-MRI scanner was applied at the brain region, in 50 slices and 0.4 mm slice thickness. The frequency direction being horizontal, the frequency/phase distribution was 192/192, with 5 excitations, 25 ns repetition time, 2.3 ns echo time and 25 degrees flip angle. All images were co-registered and visualized using Interview Fusion™ (Mediso, Hungary) and VivoQuant (inviCRO, Boston, USA) dedicated image processing software. We also used advanced post processing Hybrid Viewer™, courtesy from HERMES Medical Solutions (Stockholm, Sweden). Volume of interest delineation in 3D mode was done by fusing the Brookhaven Laboratory Mouse MRI atlas developed at the University of Florida (http://brainatlas.mbi.ufl.edu/) to the isotopic modality and matching the regional volumes with the animal’s own MRI scans.

2.2.2. Assessment of Brain Igf1 and Igf1R Transcript Levels by Real Time Polymerase Chain Reaction (RT-PCR)

Another set of 12 C57BL/6J mice were treated as described above and killed under ketamine (75mg/kg)/medetomidine (1mg/kg) anesthesia. Brains were immediately dissected on ice and brain regions frozen on dry ice cooled isopentane and stored at -80°C until use. For each brain region, total RNA was extracted using RNeasy® Mini Kit (Qiagen, Hilden, Germany). RNA quality was confirmed by Experion automated electrophoresis system (Bio-Rad Hercules, CA, USA). Synthesis of cDNA was performed by Qiagen RT² HT First Strand cDNA kit. A total amount of 2.0 µg of RNA was used to study gene expression through quantitative RT-PCR.

2.2.3. Cell Culture and Drug Regimen

Rat pheochromocytoma PC12 cells (American Type Cell Culture Collection, Manassas, VA) were used as an in vitro model to study neuroprotective effects of ALC. The cells were used in their undifferentiated form between passage 12 and 16. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM + GlutaMAX™-I, Gibco®) supplemented with 10% of fetal bovine serum (FBS, Gibco®) and 1% of penicillin (50 IU/mL) streptomycin (50 mg/mL) (PS, Gibco®) in a CO₂ incubator at 37°C, with 5% CO₂ and 95% filtered air. Cells
in log phase were treated with increasing doses of ALC (0.01, 0.1 and 1.0 mM) alone or in combination with METH 1.0 or 100 µM, directly dissolved in low glucose (LG, 5.56 mM, MEM-Alpha, Gibco®) or high glucose (HG, 25.0 mM, DMEM + GlutaMAX™-I, Gibco®) concentration culture medium for 24h or 72h. When ALC and METH were combined, pre-treatment with ALC preceded METH exposure in 30 min. The selected doses of METH were previously described and well characterized (Fornai et al., 2004; Fornai et al., 2007). ALC was kindly provided by Sigma-Tau, S.p.A. (Pomezia, Italy). METH hydrochloride was obtained from Sigma-Aldrich® (St. Louis, MO). All subsequent experiments were performed using culture medium without FBS in order to counteract the protective effect conferred by the serum.

2.2.4. In Vitro Assessment of Glucose Uptake

Glucose uptake was measured through the quantification of the cellular uptake of \(^{18}\)F-FDG. 8x10\(^5\) cells/mL were seeded in 12-well plates 24h prior to treatment with ALC and METH. Cells were then incubated with 37 kBq/mL of \(^{18}\)F-FDG in a low glucose serum-free medium for 60 min. Cells were lysed by adding NaOH (1.0 M), assayed for radioactivity by gamma-counting (Berthold LB 2111 gamma counter) and the total protein content was determined (Alj et al., 1999; Smith, 2001).

2.2.5. Cell Viability Assay

To assess cell viability the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. This is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving a purple color. This reaction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells (Mosmann, 1983; Ahmadian et al., 2009). Cells were seeded in 24-well plates at a density of 8x10\(^4\) cells/mL. At cell confluence, drugs were added and cultures incubated for 24 or 72h either in LG and HG culture medium. Then, medium was discarded and replaced by 100 µL of fresh medium containing 1.0 mM of MTT (Sigma Chemical Co., St. Louis, MO) and incubated for 3h at 37°C in the CO\(_2\) incubator. Medium was discarded and 100 µL of dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO) was added to dissolve the produced formazan crystals. Absorbance at wavelength 540 nm was measured in a microplate reader.
2.2.6. Determination of Mitochondrial Membrane Integrity

MitoTracker<sup>®</sup>Red CMXros (Molecular Probes) was used to assess mitochondrial membrane integrity; this mitochondria-selective probe, passively diffuses across the plasma membrane and accumulates in active mitochondria. Procedures were carried out according to manufacturer’s instructions. Briefly, cells were seeded in 24-well plates at a density of 3x10<sup>5</sup> cells/mL. 24 hours later, cells were treated with the above mentioned concentrations of ALC and METH and incubated for additional 24h or 72h. Cells were then stained with 1.0 µM of MitoTracker<sup>®</sup> Red CMXros and incubated at 37°C for 30 min in the dark. Cells were washed twice, harvested and centrifuged. Cells were then transferred to a tube on ice for analysis of the fluorescence intensity by flow cytometry (FACS Calibur<sup>™</sup>, BD Biosciences). Fluorescence images were also acquired in confocal microscope Leica TCS SP5 II.

2.2.7. Assessment of ATP Synthesis

ATP synthesis was evaluated by luminescence using the kit CellTiter-Glo® (Promega), which signals metabolically active cells through the quantitation of the ATP present. Cells were seeded in 24-well plates at a density of 4x10<sup>4</sup> cells/mL. Drugs were added 24 hours later and incubated for additional 24h or 72h in LG or HG concentration culture medium. CellTiter-Glo® reagent was added to each well in an equal volume to the cell culture medium. Cells were transferred to 96-well opaque-walled plates and readings were performed in the plate reader (BioTek Synergy 2) with an integration time of 1 second/well. Control wells (medium without cells) were prepared to determine background luminescence. A standard curve per assay was also generated. In order to study the origin of the synthesized ATP (glycolysis or oxidative phosphorilation), an inhibitor of the complex III of the electron transport chain (antimycin A) was also added in some experiments. Antimycin A (4.0 µM) was added to culture medium 30 minutes before ATP assessment.

2.2.8. Assessment of Mitochondrial Mass

MitoTracker® Green FM (Molecular Probes) was used to quantify mitochondrial mass; this mitochondria-selective probe, passively diffuses across the plasma membrane and binds to lipids from the inner mitochondrial membrane (Lee et al., 2000). Procedures were
carried out according to manufacturer's instructions. Briefly, cells were seeded in 24-well plates at a density of 3x10^5 cells/mL. 24 hours later, cells were treated with the above mentioned concentrations of ALC and METH and incubated for additional 24h or 72h. Cells were then stained with 800 nM of MitoTracker® Green FM and incubated at 37°C for 30 min in the dark. Cells were washed twice, harvested and centrifuged. Cells were then transferred to a tube on ice for analysis of the fluorescence intensity by flow cytometry (FACS Calibur™, BD Biosciences).

2.2.9. Determination of Mitochondrial Membrane Potential (Δψ_m)

Mitochondrial membrane potential was measured using the radioactive lipophilic and cationic agent, hexakis (2-methoxyisobutylisonitrile)-technetium-99m (⁹⁹mTc-sestamibi), initially developed for myocardial perfusion imaging. The uptake of this agent depends on mitochondrial metabolism as well as negative potential of the inner membrane (Piwnica-Worms et al., 1990; Moretti et al., 2005). Briefly, 25 x10⁴ cells were seeded in 12-well plates 24h prior to treatment with ALC and METH. Cells were then incubated with 74 kBq/mL of ⁹⁹mTc-sestamibi for 45 min. Cells were lysed by adding NaOH (1.0 M), assayed for radioactivity by gamma-counting (Berthold LB 2111 gamma counter) and total protein content was determined.

2.2.10. Determination of Bax Activation and Location

The expression of Bax protein was monitored by western blot, using two different antibodies, one for active Bax (6A7, abcam) and another one for inactive form (Cell Signaling Technology). Cells were seeded in 100 mm tissue culture dishes (Orange Scientific) at a density of 4x10⁴ cells/mL. At 80% of confluence, ALC 0.01 mM alone or in combination with METH 1.0 µM and 100 µM was added and cells were incubated for 24h. Cells were then lysed in TEN buffer (50 mM Tris-HCl, 0.2 M EDTA, 150 mM NaCl, 1% NP-40, 4.5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.0 mM sodium fluoride, 1.0 mM sodium orthovanadate and proteases inhibitors 1:500, Sigma, Missouri, USA) and proteins were extracted. The supernatant was taken for western blot analysis, 40 µg of protein was separated on 10% SDS-polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride membrane for blotting (0.2 µm, Bio-Rad) by electroblotting (Trans-Blot Turbo Transfer System for Protein, Bio-Rad). The membranes were blocked in 3% BSA (Nzytech) in 0.1% TBS Tween 20 (50 mM Tris, 150 mM NaCl,
pH 7.6), 1h at room temperature. Then, were incubated with primary antibodies, active anti-Bax (0.5 µg/mL) and anti-GAPDH (1:200000) (5G4, HyTest) diluted in 3% BSA in 0.1% TBS-T, overnight at 4°C. Membranes were washed three times for 10 min in the same buffer and incubated for 1h at room temperature with Immunopure Goat anti-mouse IgG peroxidase conjugated (1:1000) (Thermo Scientific) and anti-rabbit IgG peroxidase conjugate (1:1000) (Sigma).

Immunoreactive proteins were revealed using enhanced chemiluminescence method (Immun-Star HRP Chemiluminescent kit, Bio-Rad). The membranes were washed and incubated in a Restore Western Blot Stripping Buffer (Thermo Scientific), for 15 min at room temperature. Then the membranes were blocked in 3% BSA in 0.1% TBS-T, 1h at room temperature and were incubated with primary antibodies inactive anti-Bax (1:1000, Cell Signaling) and anti-GAPDH, overnight at 4°C. The blots were probed with horseradish peroxidase-labeled secondary antibodies. Blots were analyzed with Quantity One 1-D Analysis Software, version 4.6 (Bio-Rad). Additionally, Bax location was monitored by confocal microscopy. Briefly, cells were seeded in 24-well plates over glass coverslips treated with poly-L-lysine at a density of 3x105 cells/mL. 24 hours later, cells were treated with ALC 0.01 mM and METH and incubated for additional 24h. Cells were then washed and 500 nM of MitoTracker CMXRos was added for 30 min at 37°C in the dark. 4% formaldehyde was used for cell fixation during 20 minutes after which cells were permeabilized with 0.2% Triton for 2 minutes and blocked for 1h with 5% BSA. Cells then incubated with anti-Bax antibody (5ug/ml, diluted in 5% BSA) overnight at 4°C. Cells were washed and the secondary antibody was added (anti-mouse 1:1000, Alexa Fluor 488) followed by 1h of incubation. Cells were washed four times and coverslips were mounted directly in aqueous mounting medium with DAPI (1:10 000, Sigma). Immunofluorescence images were acquired in confocal microscope Leica TCS SP5 II.

2.2.11. Statistical Analysis

All data are representative of at least three independent experiments. Data are presented as mean+SEM. Statistical significance was determined by using one-way ANOVA with Bonferoni’s post hoc test. 18F-FDG-PET data were analyzed by two-way ANOVA with Bonferoni’s post hoc test. Differences were considered to be statistically significant at p<0.05 level. All analyses were performed using Prism 6 for Mac OS X (GraphPad Software, San Diego, California, USA).
2.3. RESULTS

2.3.1. ALC Preconditioning Increased Glucose Uptake in METH Exposed Animals

We have used a C57BL/6J mice model to evaluate the role of ALC in the cerebral glucose metabolism. We have used a well-described dose of METH to clarify the role of ALC under glucose-deprived conditions. Our analysis was focused in regions receiving relevant dopaminergic inputs (and thus preferentially affected by METH), such as the prefrontal cortex (PFC), the striatum (left and right) and the hippocampus. Due to its strong regulation by GABAergic striatal inputs, the thalamic region was also included. The dose of ALC administered (100 mg/kg) was previously shown to modulate glucose metabolism in the brain (Scafidi et al., 2010a). Furthermore, we have previously demonstrated that this ALC dose was effective in preventing the toxic effects of MDMA at the mitochondrial level (Alves et al., 2009). Interestingly, our present results show that neither ALC nor METH had a significant impact on glucose uptake in none of the assessed brain regions. However, when ALC is administered 30 min before METH, glucose uptake is highly augmented (two-way ANOVA (treatment x region), F(12,40)=0.5408, p<0.0001 for treatment, p=0.0016 for brain region, and no interaction between treatment and region). The PFC was one of the most affected regions, displaying an increased uptake in the ALC/METH group that was highly significant when compared to all other conditions (p<0.00001 to ALC and METH and p=0.0228 to control, Bonferroni’s post-hoc, figure 2.1-A and B). The right striatum was also highly affected, displaying a very similar increase in the ALC/METH group (p<0.00001 to ALC and p=0.0002 to METH and p=0.0097 to control, Bonferroni’s post-hoc, figure 2.1-A and B). In the left striatum, differences were significant when compared to ALC (p=0.0006, figure 2.1-A and B) and METH (p=0.0083, figure 2.1-A and B), but not to control. Likewise, the thalamic region displayed an increased uptake for the ALC/METH group when compared to ALC (p=0.0008, figure 2.1-A and B) and METH (p=0.0353, figure 2.1-A and B). The uptake in the hippocampus was less affected by ALC/METH and presented only an augmented uptake when compared to ALC (p=0.0065, figure 2.1-A and B). Of note, the intense uptake characteristic of the tongue and salivary glands, particularly in the presence of an adrenergic agent such as METH, is no longer observed. Likewise, the uptake in the hardierian glands seems to be clearly reduced, however these extra cerebral regions were not quantified.
Figure 2.1 - Increased glucose uptake in the brain after the administration of METH to mice preconditioned with ALC. A: Representative $^{18}$F-FDG-PET-MRI images from 12-week old male mice from different groups: control, ALC, METH and ALC/METH. In the upper row, representation of the regions of interest (ROI) used for SUV quantification. Tomographic images were reconstructed by OSEM algorithm and the resultant planes are shown: sagittal, transverse and coronal. Note the generalized increase in glucose uptake on ALC/METH group. Extra cerebral intense $^{18}$F-FDG uptake corresponds to a characteristic pattern of hardierian glands (very well depicted in the control group) and to the tongue and salivary glands, particularly in the presence of METH. B: Bar graph representing SUV quantification of brain glucose uptake using $^{18}$F-FDG-PET. Glucose uptake was assessed in the prefrontal cortex (PFC), left and right striatum (left and right striat), hippocampus (hippoc) and in the thalamus. Data represent mean±SEM (n=3 mice/group). SUV is consistently higher in ALC/METH group comparing to ALC in all brain regions. Significant differences are marked as **p<0.01 as compared with the control condition; *p<0.05, **p<0.01 and ***p< 0.001 as compared with METH and ▽p<0.05, ▽▽p<0.01 and ▽▽▽p<0.001 as compared with ALC (as determined by two-way ANOVA, followed by a post-hoc Bonferroni’s test).

2.3.2. Igf1 and Igf1R Regulation

IGF1 promotes glucose utilization and is known to be strongly expressed in response to several types of brain insults (Gehrmann et al., 1994; Jin et al., 2001; Fernandez and Torres-Aleman, 2012). Additionally, carnitine supplementation was seen to activate the
IGF1 signaling cascade (Heo et al., 2001; Bondy and Cheng, 2004) and increase the plasmatic levels of IGF1 (Heo et al., 2001). On the other hand, METH seems to decrease IGF1 receptors levels (Bourque et al., 2011). Therefore, we investigated the transcript levels of Igf1 and the Igf1R in the different experimental conditions used in our in vivo model, measured 24h after the drug administration period. This evaluation was conducted in the striatum, which is known to be particularly susceptible to METH. Our results evidenced that exposure to METH induced a significant decrease in Igf1 levels in the striatum (one-way ANOVA, F(3,12)=28.700, p=0.0007, Bonferroni’s post-hoc, figure 2.2-A). Importantly, the combined administration of ALC and METH prevented the decrease in Igf1 induced by METH (p=0.0010). ALC administration by itself increased Igf1 expression (p=0.0206, figure 2.2-A), which is in accordance with previous reports (Ahmed, 2012) and seems to be relevant for the putative neuroprotective features of ALC. Therefore, Igf1 levels were affected within the experimental groups (ALC and METH) that did not show a significant decrease in glucose uptake, but unaltered in the ALC/METH that displayed highly increased glucose uptake. Of note, transcript levels of Igf1R were significantly increased in the ALC/METH group (one-way ANOVA, F(3,11)=17.23, p=0.0032 to control, p=0.0001 to METH and p=0.0104 to ALC Bonferroni’s post-hoc, figure 2.2-B).

![Figure 2.2](image)

**Figure 2.2 - Igf1 (A) and Igf1R (B) transcript levels in the striatum of mice from different groups** (n=3 mice/group). Bar graphs represent the relative fold change to the control condition. Note that ALC increases Igf1 levels, while METH decreases it. Animals preconditioned with ALC and exposed to METH showed increased levels of Igf1R when compared to all other groups. Significant differences are marked as *p<0.05, **p<0.01 and ***p<0.001, as compared with the control condition; ••••p< 0.001 as compared with METH and ▽p<0.05 as compared with ALC (as determined by one-way ANOVA, followed by a post-hoc Bonferroni’s test).
2.3.3. ALC Affects Glucose Uptake in a Dose-dependent Way

To clarify the action of ALC in the metabolism of neuronal cells, we have also assayed the uptake of glucose in the PC12 dopaminergic cell line. Despite the differences between PC12 cells and the central dopaminergic neurons, PC12 cells possess unique dopaminergic features that make them particularly sensitive to METH (Greene and Tischler, 1976; Fornai et al., 2007) and, therefore, suitable for our purposes. The assay is performed necessarily in cells kept in a LG medium. Consistently with what we observed in vivo, METH did not show an effect on glucose uptake. ALC however, seems to have a dose-dependent effect on the uptake of glucose, since at 24h ALC 0.01 mM, by itself, significantly increased the uptake of glucose (one-way ANOVA, F(3,22)=3.432, p=0.0352, Bonferroni’s post-hoc, figure 2.3-A). Likewise, the combined action of ALC 0.01 mM and METH 1.0 or 100 µM, induced an increased glucose uptake (one-way ANOVA, F(4,21)=3.256, p=0.0368 for ALC/METH1 and F(4,30)=9.232, p=0.0004 for ALC/METH100, Bonferroni’s post-hoc, figure 2.3-B and C) but at 72h this effect was no longer seen. For METH 1.0 µM, at 72h, the combined action of ALC 0.01 mM was even lower than in control conditions (one-way ANOVA, F(4,45)=24.430, p=0.0015 to control and p<0.0001 to METH1, Bonferroni’s post-hoc, figure 2.3-E). ALC 1.0 mM, however, lead to increased uptake at 72h, either by itself (one-way ANOVA, F(3,36)=26.510, p<0.0001, Bonferroni’s post-hoc, figure 2.3-D), or in the presence of METH1 (one-way ANOVA, p<0.0001 to control and p=0.0015 to METH1, Bonferroni’s post-hoc, figure 2.3-E) and METH100 (one-way ANOVA, F(4,47)=4.005, p=0.0117, Bonferroni’s post-hoc, figure 2.3-F). The selected doses of METH were based on previous dose-response studies (Fornai et al., 2007) demonstrating that an intraperitoneal dose of 5 mg/kg of this compound results in a striatal dose of 1.0 µM, while a 100 µM dose is much more toxic. The absolute glucose uptake by control cells was very similar at 24h and 72h (data not shown).

Since the ALC 0.01 mM dose seems to have more impact on the short term glucose uptake, we selected this dose to proceed with the in vitro evaluation.
Figure 2.3 - Altered glucose uptake in long time starvation in PC12 cells exposed to ALC. A to C: Cells treated with ALC at 0.01, 0.1, 1.0 mM alone or in combination with METH 1.0 µM or 100 µM for 24h. The lowest ALC dose increases glucose uptake at 24h, alone (p=0.0352) or in combination with METH 1.0 and 100 µM (p=0.0004). D to F: Cells treated with ALC at 0.01, 0.1, 1.0 mM alone or in combination with METH 1.0 µM or 100 µM for 72h. In conditions of long time starvation, ALC seems to have a dose-dependent effect, increasing significantly glucose uptake with the ALC dose (p<0.0001 for ALC 1.0 mM comparing to control). Graphic representation of glucose uptake per mg of total protein content, comparing to control cells. Columns represent mean+SEM (n=9). Significant differences are marked as *p<0.05, **p<0.01 and ***p<0.001, as compared with the control condition and /circle5 p<0.05, /circle5 /circle5 p<0.01 and /circle5 /circle5 /circle5 p<0.001 as compared with METH 1.0 or 100 µM (as determined by one-way ANOVA followed by a post-hoc Bonferroni’s test). Dashed lines represent the control condition.

2.3.4. ALC/METH Prevented the METH-induced Decrease in Mitochondrial Function

To better characterize the action of the selected ALC dose, we have performed an MTT assay, which measures the activity of dehydrogenase enzymes, in metabolically active mitochondria. This assay reflects both cell viability and the enzymatic functionality of mitochondria. We evaluated the action of ALC in undifferentiated PC12 cells cultured in a
low glucose environment. At 24h, the combination of ALC 0.01 mM and METH resulted in a significant decrease in MTT reduction (one-way ANOVA, F(5,145)=9.701, p<0.0001 for ALC0.01/METH1 and for ALC0.01/METH100, Bonferroni’s post-hoc, figure 2.4-A) reflecting decreased cellular viability. METH by itself was not different from the control. At 72h the decrease in MTT reduction was still present for ALC0.01/METH1 (one-way ANOVA, F(5,130)=7.096, p=0.0002, Bonferroni’s post-hoc, figure 2.4-B). At 72 h, METH 100 µM also led to lower levels of MTT reduction (p=0.0188) (figure 2.4-B), and therefore at this time point ALC may have contributed to balance the effect of METH, since the combined action of METH100 and ALC 0.01 mM is not different from the control.

Figure 2.4 – Cell viability of PC12 cells at 24 and 72h. A: Decrease in cell viability when ALC 0.01 mM was used in combination with METH1 or METH 100 (p<0.0001 for both). B: Cell viability in PC12 cells treated for 72h. Note the significant decrease in cell viability when cells were treated with ALC 0.01/METH1 (p=0.0002) and METH100 (p=0.0188). In the latter case, ALC 0.01 mM was able to prevent the decline in mitochondrial function induced by METH100. Significant differences are marked as *p<0.05 and ***p<0.001, as compared with the control condition and •••p<0.01 and ••••p<0.001 as compared with METH 1.0 or 100 µM (as determined by one-way ANOVA followed by a post-hoc Bonferroni’s test). Dashed lines represent the control condition.

Mitochondrial function was assessed with the MitoTracker®CMXRos, a dye retained by active mitochondria that are effectively contributing to cell respiration. At 24h, we did not observe any significant differences between conditions (data not shown). However, for prolonged exposures, METH significantly reduced mitochondrial function (both METH 1.0
and 100 µM: one-way ANOVA, F(5,67)=11.270, p<0.0001, Bonferroni’s post-hoc, figure 2.5-A, C and D). This reduction was also visible in confocal microscopy (figure 2.5-E and F). Importantly, in this case, the ALC dose 0.01 mM was effective in preventing the reduction induced by METH 1.0 µM (one-way ANOVA, F(5,67)=11.270, p=0.0002, Bonferroni’s post-hoc, figure 2.5-A, C and G).

Figure 2.5 - Decreased mitochondrial function in conditions of long time starvation, induced by METH. A: Mitochondrial function at 72h measured by flow cytometry using the MitoTracker® CMXRos. In long time starvation, METH induced a near 50% reduction in mitochondrial function, partially prevented by ALC 0.01 mM (for METH1). Graphic representation of the percent of cells contributing to cell respiration comparing to control. Columns represent mean+SEM (n=9). Significant differences are marked as *p<0.05 and **p<0.001, when compared with the control condition and ***p<0.01 and ****p<0.001 when compared with METH (as determined by one-way ANOVA followed by a post-hoc Bonferroni’s test). Dashed lines represent control conditions. B to D: representative fluorescence histograms of the flow cytometry quantification presented in A. Graphics represent the range of red fluorescence and the number of cells associated to that emission. E to G: Fluorescence images of PC12 cells stained with 150 nM of MitoTracker® CMXRos (red staining) depicting mitochondrial function, and 4 µM of DAPI depicting cell nucleus (blue staining). E, control; F, METH 1.0 µM; G, ALC 0.01 mM/METH 1.0 µM. Scale bar = 10 µm.

2.3.5. ALC/METH Decreased ATP Synthesis in Glucose Deprived Cells

Reduced levels of mitochondrial function should result in reduced ATP levels and therefore we have measured the amount of ATP being synthesized within the different
experimental conditions. In a LG medium, we have observed that the combined action of ALC and METH resulted in lower ATP levels (one-way ANOVA, F(5,86)=7.597, p=0.0040 for ALC0.01/METH1 and p=0.0088 for ALC0.01/METH100, Bonferroni’s post-hoc, figure 2.6-A). Of note, this early reduction of ATP synthesis may be protective in the long term.

To verify if the amount of glucose in the medium was affecting the synthesis of ATP, we have repeated the whole evaluation in a HG medium. Although in control conditions the absolute production of ATP had always been higher in LG than in HG medium (p=0.0411 at 24h and p=0.0017 at 72h, figure 2.6-C), in the presence of ALC, even when combined with METH, the ATP production is much higher in HG (one-way ANOVA, F(5,86)=6.011, p<0.0001 for ALC 0.01 mM and p=0.0062 for ALC0.01/METH1, Bonferroni’s post-hoc, figure 2.6-B). Interestingly, although mitochondrial function was more affected at 72h than at 24h, the decrease in ATP synthesis, under the action of ALC and METH, seems to precede it. Of note, the absolute amount of ATP produced by control cells at 72h was 25% higher than at 24h (one-way ANOVA, F(3,68)=10.980, p=0.0386, Bonferroni’s post-hoc figure 2.6-C). To verify if in HG conditions the extra amount of ATP is provided by the activity of the respiratory chain, we added antimycin A to the culture medium to block complex III activity. In the presence of antimycin A the amount of ATP produced was similar to the control cells in all tested conditions (data not shown). Although PC12 cells are known to be relatively resistant to antimycin A (Ohsawa et al., 2003; Han and Im, 2008), our results show that the absolute amount of ATP synthesized in the presence of ALC alone or in combination with METH was significantly reduced when antimycin was added, showing that the increase in ATP production in HG conditions is due to the ALC effect over the respiratory chain activity.
Figure 2.6 – ATP synthesis of PC12 cells under different conditions. A: Relative ATP synthesis of cells treated for 24h in LG medium expressed as a percentage of control. Note the decreased ATP synthesis induced by the combined use of ALC 0.01 mM and METH1 (p=0.0004) or METH100 (p=0.0088). B: Relative ATP synthesis of cells treated for 24h in HG medium, expressed as a percentage of control. Note the increased ATP synthesis promoted by ALC 0.01 mM in HG medium. C: Absolute ATP synthesis of control cells at 24h and 72h in low and high glucose culture medium. Control cells in LG medium exhibit a considerably higher ATP synthesis than those cultured in HG medium (p=0.0411 for LG-24h vs. HG-24h and p=0.0017 for LG-72h vs. HG-72h). Moreover, in LG medium, ATP synthesis was higher at 72h (p=0.0386). Columns represent mean+SEM (n=14-18). Significant differences are marked as *p<0.05, **p<0.01 and ***p<0.001 as compared with the control condition and /circle4/p<0.05 and /circle4/circle4/circle4p<0.001 when compared to the respective concentration of METH (as determined by one-way ANOVA followed by a post-hoc Bonferroni’s test). Dashed lines represent control conditions.

2.3.6. Increased ROS Formation Associated Translocation of Active Bax to the Nucleus

The production of intracellular ROS is a hallmark of METH action; therefore, we evaluated the formation of ROS in our experimental model. Although ALC is often referred as an antioxidant compound (Calabrese et al., 2006; Rump et al., 2010), we verified that in the presence of both METH 1.0 and 100 µM, ALC 0.01 mM augmented the formation of ROS when compared to control (one-way ANOVA, F(5,37)=2.591, p=0.0136 for ALC0.01/METH1 and p=0.0193 for ALC0.01/METH100, Bonferroni’s post-hoc, figure 2.7-A). Importantly, ROS production is in accordance with the results obtained for the ATP synthesis. As ROS production might also influence mitochondrial membrane potential, we questioned if the membrane potential would be compromised as well. To verify that, we used a radioactive lipophilic and cationic agent, 99mTc-sestamibi, whose uptake is dependent on the electropotential gradient across the mitochondrial membrane. We observed a significant reduction in the membrane potential at 24h in cells preconditioned with ALC and exposed to METH 1.0 µM (one-way ANOVA, F(5,45)=5.159; ALC0.01/METH1 p=0.0100 to control and p=0.0004 to METH1, Bonferroni’s post-hoc, figure 2.7-B). Importantly, reduced membrane potential is inversely related to ROS production, which is in accordance with previous reports (Kim et al., 2004).
Figure 2.7 - Decreased membrane potential in cells preconditioned with ALC and exposed to METH. A: Intracellular ROS produced by cells treated in LG medium for 24h, expressed as mean fluorescence intensity (MFI) per mg of protein (percent of control condition, n=9). Note the increase in intracellular ROS production in the presence of ALC0.01/METH1 (p=0.0136) and ALC0.01/METH100 (p=0.0193). B: Mitochondrial membrane potential of cells treated in LG medium for 24h, expressed as percent uptake of $^{99m}$Tc-sestamibi per unit of mitochondrial mass (n=9). The combined use of ALC and METH significantly reduced the mitochondrial membrane potential, particularly for ALC0.01/METH1 (p=0.0100 to the control p=0.0004 to METH1). Significant differences are marked as *p<0.05 as compared with the control condition and ***p<0.001 when compared to METH1 (as determined by one-way ANOVA followed by a post-hoc Bonferroni's test). Dashed lines represent control conditions.

As a consequence of increased ROS, Bax, a protein involved in the regulation of apoptotic cascade (Hsu and Youle, 1997), can be activated leading to increased mitochondrial transition pore formation (Lee et al., 2012), associated with loss of mitochondrial membrane potential. When evaluating the presence of activated Bax, we found no significant differences between groups (figure 2.8-B). However, immunocitochemistry of activated Bax, revealed interesting differences in its redistribution. Of note, while METH seems to reduce its translocation to the mitochondria (figure 2.8-A), ALC seems to promote its translocation to the nucleus. Moreover, when ALC was combined with METH, Bax is seen in the nucleus, but also in the mitochondria. These observations match previous reports, showing Bax translocation from the cytosol to the mitochondria, nucleus and endoplasmic reticulum after the exposure to cytotoxic agents (Gajkowska et al., 2001).
Figure 2.8 – Translocation of active Bax to the nucleus. A: Immunofluorescence images of cells treated in LG medium for 24h and stained with DAPI (blue), 6A7 antibody for active Bax (green) and MitoTracker® CMXRos (red). Note the translocation of Bax to the nucleus when cells were treated with ALC0.01/METH1. Scale bar = 10 µm. B: Ratio of active to total Bax, expressed as a percentage of the control condition. Columns represent mean±SEM (n=3).

Furthermore, mitochondrial mass was evaluated using the lipophilic and cationic properties of Mitotracker® Green FM that accumulates preferentially in the mitochondria, binding to the lipids from the inner mitochondrial membrane, independently of the mitochondrial membrane potential (Lee et al., 2000; Pendergrass et al., 2004). Data obtained for mitochondrial mass at 24h revealed decreased mass for ALC0.01/METH100 (one-way ANOVA, F(5,58)=7.475, p=0.0034, Bonferroni’s post-hoc), but increased for METH100 (p=0.0248) (figure 2.9-A). At 72h, both METH100 and ALC0.01/METH led to lower mitochondrial mass (one-way ANOVA, F(5,78)=5.307, p<0.0001 for METH100 and p=0.0170 ALC0.01/METH100, Bonferroni’s post-hoc, figure 2.9-B).
Figure 2.9 – The combined use of ALC and METH decreases mitochondrial mass. This effect is seen both at 24h (A) and 72h (B). Of note is the early transitory increase in mitochondrial mass after exposure to METH 100 µM, which drops significantly after a prolonged exposure. Significant differences are marked as *p<0.05, **p<0.01 and ***p<0.001 as compared with the control condition and •••p<0.001 when compared to METH100 (as determined by one-way ANOVA followed by a post-hoc Bonferroni’s test). Dashed lines represent control conditions.

2.4. DISCUSSION AND CONCLUSION

The present study unravels potential deleterious effects of ALC supplementation to healthy or metabolically compromised individuals. In mice preconditioned with ALC and exposed to METH, we verified a generalized increase in glucose uptake in several brain regions, which was particularly evident in regions receiving a strong dopaminergic input. Importantly, these animals performed poorly in a water maze test (performed in the lab, but not addressed in the present work), reflecting impaired reference memory. In vitro, this was associated with higher levels of intracellular ROS, decreased mitochondrial membrane potential and translocation of active Bax to the nucleus.

In human METH abusers glucose metabolism is persistently reduced in the striatum, the hippocampus or the thalamic region (Volkow et al., 2001a; Wang et al., 2004; London et al., 2005). However, when assessing the effect of a single low dose of METH in naïve mice, we verified that glucose uptake was not affected in any of the analyzed regions. Since the acute effect of a single low dose is not comparable to a chronic exposure, this is not unexpected. Importantly, in animals preconditioned with ALC, the administration of
METH led to a robust and general increase of glucose metabolism in the whole brain, which was particularly relevant in the PFC.

Recently, using $^{13}$C-ALC, it was demonstrated that the acetyl moiety of ALC both in astrocytes and neurons is rapidly incorporated into glutamate and GABA synthesis (Scafidi et al., 2010b). This establishes the direct involvement of ALC in increased glutamine, glutamate and GABA levels, via the tricarboxylic acid (TCA) cycle. Importantly, biosynthesis of both tyrosine and L-tryptophan, dopamine and 5-HT precursors, involves either a transamination reaction with glutamate or the participation of glutamine as an amine donor (Yudkoff, 1997), which may account to explain the action of ALC over monoamine production.

In astrocytes, the increased routing of glutamate into the TCA cycle seems to rise ATP levels, leading to phosphofructokinase and glycolysis inhibition, and promoting glycogen synthesis [reviewed by (DiNuzzo et al., 2011)]. On the other hand, stress events, such as METH exposure, would favor glycogen degradation (Cruz and Dienel, 2002). In vivo, although glutamatergic neurotransmission accounts for nearly 85% of intracortical signaling, it seems that in regions receiving stronger noradrenergic and serotonergic inputs, the activity of these neurotransmitters may be crucial in determining the amount of glycogen that will be mobilized (DiNuzzo et al., 2011). Therefore, the combined action of METH over dopaminergic and adrenergic receptors and transporters, allied to ALC-induced synthesis of neurotransmitters may justify the increase of glucose uptake that we have observed.

It has been suggested that glucose uptake is influenced by the expression of IGF1 through the indirect promotion of GLUTs (1, 3 and 4) translocation to the plasma membrane, thus augmenting glucose transport into the cell (Bondy and Cheng, 2004). Because the brain relies essentially on glucose oxidation for energy production, insulin and IGF receptors are highly expressed in this organ, particularly in neurons and, to a lesser extent, in glia and other areas (Gerozissis et al., 2001). Within the present work, we observed that METH exposure reduced Igf1 levels, while ALC enhanced it. Of note, ALC preconditioning in METH exposed animals led to Igf1 levels that were similar to the control. Increasing IGF1 expression may be an attempt to promote GLUTs translocation to the outer cell membrane, thus augmenting glucose uptake by the brain, which seems to be relevant for the action of ALC. In accordance, increased IGF1 expression has been reported in response to several types of injury (Genead et al., 2012; Schober et al., 2012). While it is possible that increased levels of IGF1 in the brain may constitute a compensatory mechanism to prevent metabolism disruption, it was also suggested that
IGF1 may not be directly involved in the uptake of glucose by neurons (Tafreshi et al., 2010). Interestingly, we verified that the levels of Igf1R were only upregulated for the ALC/METH group, where Igf1 was unaltered, but glucose uptake was highly increased. Augmented Igf1R levels were observed in the brain of rats kept under a low carbohydrate diet (Cheng et al., 2003). Of note, it was demonstrated that IGF1 uptake through the blood brain barrier is modulated by neural activity (Nishijima et al., 2010) and therefore, unaltered Igf1 levels do not necessarily correspond to unaltered IGF1 levels.

To better understand the role of ALC in neuronal glucose uptake under the action of METH, we have used a dopamine producing cell line that was exposed to METH and ALC in a glucose-deprived condition. In these cells, an ALC dose of 0.01 mM led to improved glucose uptake alone or in combination with METH, which was followed by a strong decrease at 72h. In a previous work, in a primary neuronal culture, reduced neuronal glucose uptake after METH (100µM) was paralleled by lower expression of GLUT3 (Abdul Muneer et al., 2011b). Interestingly, these authors demonstrated that ALC (0.01 mM) was able to significantly prevent GLUT3 decrease, probably by donating an acetyl group to glucosamine, promoting its stabilization. Importantly, in the present study, increased glucose uptake in PC12 cells exposed to ALC and METH, resulted in decreased cell viability, which was associated with early (24h) reduction of ATP synthesis, but seems to protect mitochondrial function later on (72h). Conversely, in a high-glucose medium, ATP synthesis in cells exposed to equal concentrations of ALC and METH was much higher. These results suggest that when glucose is available, ALC promotes glycolysis for energy production, but if glucose is scarce, ALC may promote alternative metabolic pathways, such as β-oxidation of fatty acids (Flanagan et al., 2010; Abdul Muneer et al., 2011b). In a glucose-deprived conditions, METH seems no to affect ATP production, that was seen in primary neuronal cells, where no clear evidence of METH action over ATP synthesis was observed (Abdul Muneer et al., 2011b).

The binding of IGF1 to its receptor can act via different pathways, including the PI3-K–Akt, which inhibits pro-apoptotic proteins of the Bcl-2 family and induces anti-apoptotic proteins of the same family, connecting IGF1 and cell survival (Bondy and Cheng, 2004; Fernandez and Torres-Aleman, 2012). Bax is a pro-apoptotic homologue of Bcl-2 protein present in the cytosol, which is activated in apoptosis, undergoing conformational modifications and translocation to the outer mitochondrial membrane or to the nucleus (Dewson et al., 2001). Here, although the ratio of activated Bax was unaltered, ALC alone or in combination with METH, led to the translocation of active Bax mostly to the nucleus. Although Bax may be associated with the nuclear matrix even in the absence of an
apoptotic stimulus (Wang et al., 1999; Motyl et al., 2000), Bax translocation is commonly seen as an early event of apoptosis (Gajkowska et al., 2001; Gill et al., 2008). Adding to this, we observed that Bax translocation was concomitant with decreased \( \Delta \psi_m \) and higher ROS formation. ROS are important mediators in mitochondrial apoptotic signaling (Cande et al., 2002). ALC is often seen as an energy enhancer, improving cellular oxygen consumption, which can increase oxidative stress. In a number of pathologic conditions, electron leakage augmenting ROS production is preceded by bioenergetic impairment due to loss of mitochondrial function (Adams et al., 2001; Virmani et al., 2004).

In summary, the present results show that ALC preconditioning in mice exposed to a low METH dose led to a robust increase in glucose uptake that was concomitant with impaired reference memory in the WM. Furthermore, in vitro, ALC/METH combination led to decreased viability, reduced ATP synthesis, increased ROS and loss of mitochondrial membrane potential. The translocation of active Bax to the nucleus can be associated with apoptotic events.
CHAPTER 3

NEUROPROTECTIVE ACTION OF ALC IN DOPAMINERGIC NEUROTTRANSMISSION
3.1. INTRODUCTION

As stated before, pre-treatment with ALC revealed a neuroprotective effect against MDMA-induced neurotoxicity, not only at the mitochondrial level, but it also prevented a MDMA-induced decrease of serotonin levels in several regions of the rat brain (Alves et al., 2009). However, the molecular mechanisms underlying the mechanism of action of ALC, particularly regarding the induction of changes in neurotransmitter systems are still not fully understood. This section aims to contribute to a better understanding of these mechanisms and explore the potential neuroprotective effect of ALC in the therapy of neurodegenerative disorders that result from mitochondrial dysfunction.

Exposure to METH provides an excellent model to mimic these neurodegenerative processes, both in vivo and in vitro (Ferrucci et al., 2008a). Using a cell line model of exposure to METH, we aim to contribute to clarify the mechanism by which administration of ALC alters neurotransmitter release. METH exposure is well known to induce altered function of the dopaminergic system, initially increasing striatal DA content, which increases oxidative stress, leading to intracellular alterations at the nigral DA cell bodies, followed by degeneration of dopaminergic terminals and decreased striatal release of DA (Ferrucci et al., 2008b). These features of METH action provide an excellent model to study the action of ALC over DA release.

As mentioned in the first chapter, the PC12 cell line derived from the rat pheochromocytoma is well known for its catecholamine production and is often used as an in vitro model to mimic dopaminergic function and may predict the neurotoxicity of a variety of compounds acting on central dopaminergic neurons (Schlegel et al., 2004; Piga et al., 2005). In the present study, aiming to evaluate the putative role of ALC over the function of neurotransmitter systems, we have exposed PC12 cells pre-treated with ALC, to different concentrations of METH, for either 24h or 72h. The intra- and extracellular levels of DA were assessed to evaluate the influence of ALC on dopaminergic function.

A differential effect of METH and DA itself on post-synaptic neurons was previously demonstrated (Fornai et al., 2007). DA receptors seem to have a pivotal role in DA-induced cell death. As D2 receptors (D2R) are well known to function as auto-receptors and are also possible modulators of D1 receptors, we aim to identify the putative protective role of ALC in D2R mediated METH neurotoxicity. Since the D2R play an important role in the release of DA, a potential effect of ALC on the function of these receptors may be relevant to improve our understanding of the mechanisms of action of ALC as a potential neuroprotective drug for dopaminergic neurons. In the present work we
also aim to verify the presumed role of D2R availability as a mechanism of action of ALC’s ability to protect dopaminergic toxicity through DA receptor imaging with the radiotracer iodine-123-labeled (S)-2-hydroxy-6-methoxy-N-((1-ethyl-2-pyrrolidinyl)methyl) benzamide (\textsuperscript{123}I-IBZM). This radiotracer was first proposed by Kung et al. for the assessment of D2R availability (Kung et al., 1988), exhibiting a high specificity to these receptors with no or minimal metabolism (Kung et al., 1989). \textsuperscript{123}I-IBZM is a DA D2/3R antagonist exhibiting high selectivity and moderate affinity to these receptors (K\textsubscript{D} D2R = 0.43 nM) (Kung et al., 1989), making it a suitable agent to image DA release in regions of high D2R density (Laruelle et al., 1995).

### 3.2. MATERIAL AND METHODS

#### 3.2.1. Cell Culture and Drug Regimen

Rat pheochromocytoma PC12 cells (American Type Cell Culture Collection, Manassas, VA) were used as an in vitro model to study neuroprotective effects of ALC. The cells were used in their undifferentiated form between passage 12 and 16. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM + GlutaMAX™-I, Gibco\textsuperscript{®}) supplemented with 10% of fetal bovine serum (FBS, Gibco\textsuperscript{®}) and 1% of penicillin (50 IU/mL) streptomycin (50 mg/mL) (PS, Gibco\textsuperscript{®}) in a CO\textsubscript{2} incubator at 37°C, with 5% CO\textsubscript{2} and 95% filtered air. Cells in log phase were treated with increasing doses of ALC (0.01, 0.1, 0.5 and 1.0 mM) alone or in combination with METH 1.0 or 100 µM. When ALC and METH were combined, pretreatment with ALC preceded METH exposure in 30 min. The selected doses of METH were previously described and well characterized (Fornai et al., 2004; Fornai et al., 2007). ALC was kindly provided by Sigma-Tau, SpA (Pomezia, Italy). METH hydrochloride was obtained from Sigma-Aldrich\textsuperscript{®} (St. Louis, MO). All subsequent experiments were performed using culture medium without FBS in order to counteract the protective effect conferred by the serum.

#### 3.2.2. Dopamine Quantification

Cells were grown in 24-well plates (100,000 cells/well) and incubated with increasing doses of ALC (0.01 mM to 1.0 mM), alone or in combination with METH 1.0 µM or 100 µM. Drugs were dissolved directly into the culture medium and incubated for 24h or 72h. Cell culture medium was collected and stored at -80° C and cells were trypsinized and
centrifuged. The pellets were re-suspended in perchloric acid (HClO₄) 0.1 N, disrupted by ultrasonication and centrifuged at 15,000 g for 5 min. Supernatant was collected and filtered through a 0.2 µm nylon filter (Costar micro-centrifuge filter, Corning, NY). When cell culture medium was defrosted, an equal volume of HClO₄ 0.2 N was added, disrupted by ultrasonication and centrifuged at 15,000 g for 5 min. The supernatant was collected and filtered through a 0.2 µm nylon filter (Costar micro-centrifuge filter, Corning, NY). DA content was determined by high performance liquid chromatography, combined with electrochemical detection (HPLC-EC) as previously described (Alves et al., 2009). Aliquots of 50 µl were used to assay DA levels by HPLC-EC using a Gilson instrument (Gilson, Inc., Middleton, WI, USA), fitted with an analytical column (Supelco Supelcosil LC-18 3 µM; 7.5 cm x 4.6 mm; flow rate: 0.8-1.0 mL/min; Supelco, Bellefonte, PA, USA). A mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/L) and Na-EDTA (40 mg/L) was used. Concentrations of DA were calculated using a standard curve. Dopamine standard was purchased from Sigma (St. Louis, MO, USA). Final results were expressed in terms of monoamine content per cell to allow direct comparison between intra- and extracellular levels.

3.2.3. In Vivo Quantification of Dopamine Striatal Binding

Twelve week-old male C57BL/6J mice (21.6 – 26.0 g) were purchased from Charles River (Innovo, Gödöllö, Hungary) and maintained on a 12-h light/dark cycle. All experimental procedures were approved by the Local Animal Experimental Ethical Committee of Semmelweis University, Budapest, Hungary, in compliance with the European Community Council Directive of September 22, 2010 (2010/63/UE). All efforts were made to ensure minimal animal stress and discomfort. Room temperature was kept at 22ºC and animals were allowed water and food ad libitum. Twelve animals were divided into four groups, according to the different treatments: group 1 (control, saline), group 2 (ALC, 100 mg/kg), group 3 (METH, 10 mg/kg) and group 4 (ALC + METH 30 min later). Drugs were administered by intraperitoneal injection. Thirty minutes after the last dose, 14.2 ± 0.9 MBq ¹²³I-IBZM (General Electric Healthcare, Eindhoven, the Netherlands) with a specific activity higher than 74 TBq/mmol (radiochemical purity >95% and radionuclide purity >99.9% at the reference time) was injected in the tail vein, under anesthesia (isoflurane 2%). After injection, animals recovered very quickly from isoflurane anesthesia and were returned to their home cage for the radiotracer uptake period (70 minutes). It was previously shown that specific striatal ¹²³I-IBZM binding in the human and rat brain, is
maximal at approximately 40 minutes after injection, remaining stable for up to 2 hours (Verhoeff et al., 1991b; Verhoeff et al., 1991a). In this study, image acquisition started 70 minutes after radiotracer intravenous injection and prolonged for 45 minutes. Three consecutive SPECT acquisitions were performed at 70, 85 and 100 minutes after radiopharmaceutical intravenous injection. Immediately before image acquisition, animals were anaesthetized with isoflurane 2%, the head was fixed to a specific head holder bed (MultiCell™, Mediso, Hungary) and the body immobilized with tapes to prevent movement. Images were acquired in a NanoSPECT/CT™ system (Mediso/Bioscan, Budapest, Hungary). Multiplexing multi-pinholes were used (9 in total; 0.7 mm diameter apertures) were used (SciVis, Göttingen, Germany). Thirty circular SPECT projections over a full circle were collected each for 84 seconds in a 159 keV ± 20% photopeak energy window. Projections were reconstructed into three-dimensional images using the HiSpect software (SciVis, Göttingen, Germany) with a proprietary 3D OSEM algorithm, in 0.2 x 0.2 x 0.2 mm voxels with decay correction. Magnetic resonance images (MRI) were acquired in a PET-MRI scanner (nanoScan PM, Mediso, Hungary), equipped with one Tesla magnet for 25 minutes. All images were co-registered and visualized using Interview Fusion™ (Mediso, Hungary) and VivoQuant (inviCRO, Boston, USA) dedicated image processing software. We also used advanced post processing Hybrid Viewer™ software, courtesy from HERMES Medical Solutions (Stockholm, Sweden). Regions of interest (ROI) were drawn over the left and right striatum as well as in the cerebellum in order to determine the striatal binding ratio (expressed as the quotient between striatal binding minus cerebellar binding divided by cerebellar binding) (Jongen et al., 2008). For $^{123}$I-IBZM uptake quantification, the secular equilibrium method was used, since there is no need of plasma sampling nor the application of a reference region based on dynamic image acquisition. Since the chosen radiotracer ($^{123}$I-IBZM) presents an adequate time period of binding equilibrium, it is possible to inject a single bolus instead of a continuous infusion (Scherfler et al., 2005).

### 3.2.4. Statistical Analysis

A one-way analysis of variance (ANOVA, treatment) was used to analyze differences between treatments. Significant differences were further tested using the post-hoc Tukey honest significant differences (HSD). Differences were considered to be statistically significant at $p<0.05$ level. All analyses were performed using Prism 6 for Mac OX (GraphPad Software, San Diego, California, USA).
3.3. RESULTS

3.3.1. ALC Action on Dopamine Release in PC12 Cells

After 24h of incubation, as described above, intracellular levels of DA were shown to be significantly increased in cells treated with ALC 0.5 mM (one-way ANOVA, F(4,12)=6.152, p=0.085, Tukey’s post-hoc). At this time point, no other differences compared to the control condition were observed, either in intra- or extracellular levels of DA (figure 3.1). In contrast, at 72h, the levels of intracellular DA were increased in PC12 cells treated with ALC 0.01 and 0.1 mM (one-way ANOVA, F(4,17)=43.950, p<0.0001, for both conditions, Tukey’s post-hoc) and still, no changes were observed in extracellular DA amounts (figure 3.1). This seems to indicate that ALC itself is able to increase the production of DA in PC12 cells, without interfering with DA release and uptake processes.

Figure 3.1 – Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM on DA release.
Graphic representation of intra- and extracellular levels of DA measured by HPLC-EC, in PC12 cells, when treated with ALC for 24h or 72h. Columns represent mean+SEM, expressed as pg of neurotransmitter/cell for each experimental condition (n=6-9). Significant differences are marked as **p<0.01 and ***p<0.001, as compared with the control condition (determined by one-way ANOVA followed by a post hoc Tukey’s HSD).
3.3.2. ALC Neuroprotective Action over METH 1.0 µM Exposure

Exposure to METH 1.0 µM is known to induce, within a few hours, the formation of intracellular multilamellar inclusions that are both α-synuclein and ubiquitin-positive, without a substantial impact on DA function (Ferrucci et al., 2008b; Fornai et al., 2008). Accordingly, in the present work, a 24h exposure to METH 1.0 µM did not induce any significant alteration in the intra- and extracellular levels of DA (figure 3.2).

However, when cells were exposed to METH 1.0 µM for a longer period (72h), a significant increase in the intracellular levels of DA was apparent (one-way ANOVA, F(5,257)=10.490, p<0.0001, Tukey’s post-hoc). Interestingly, when cells were pre-treated with ALC, before exposure to METH, this increase was reduced and only reached significance in cells pre-treated with ALC 0.5 mM (p<0.0001) (figure 3.2). At 72 hours, extracellular levels of DA in cells exposed to METH 1.0 µM were increased when compared to control conditions (one-way ANOVA, F(5,25)=4.751, p=0.0256, Tukey’s post-hoc). Importantly, ALC 0.01 mM was able to decrease DA extracellular levels induced by METH (p=0.0305). Furthermore, in cells pre-treated with other ALC concentrations, the extracellular levels of DA almost reached significance when cells were treated with the highest dose (ALC 1.0 mM, p=0.0771, figure 3.2). It is noteworthy that at this time point, the control levels of extracellular DA were markedly increased when compared with the intracellular DA levels (t test, p<0.0001). This reflects an increased release of DA through the incubation period.
Figure 3.2 – Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM and METH 1.0 µM on DA release. Graphic representation of intra- and extracellular levels of DA measured by HPLC-EC, in PC12 cells pre-treated with ALC and exposed to METH 1.0 µM for 24h or 72h. Columns represent mean+SEM, expressed as pg of neurotransmitter/cell for each experimental condition (n=6-9 in control and METH1 conditions, and n= 3 replicated experiments for combinations of METH 1 and ALC). Significant differences are marked as follows: *p<0.05 and ***p<0.001 as compared with the control condition; and •p<0.05 as compared with METH 1.0 µM (determined by one-way ANOVA followed by a post hoc Tukey’s HSD).

3.3.3. ALC Neuroprotective Action over METH 100 µM Exposure

Exposure to METH 100 µM is known to cause substantial cell loss, paralleled by massive DA release (Fornai et al., 2004). In the present work, when cells were exposed to METH 100 µM, the expected decrease in intracellular DA levels was clearly observed (one-way ANOVA, F(5,18)=182.4, p=0.0034, Tukey’s post-hoc). Notably, all tested doses of ALC were effective in preventing the METH-induced decrease of DA concentrations (p<0.0001). Moreover, in cells pre-treated with ALC 0.1 mM, 0.5 mM and 1.0 mM, intracellular levels of DA at 24h was markedly increased when compared to the control
However, at this time point, the levels of DA in the extracellular medium were unaltered for all tested conditions. Consequently, these results indicate that ALC not only effectively prevented the METH-induced decrease of DA concentrations, but also increased DA production.

At 72h, exposure to METH 100 µM led to increased intracellular levels of DA (one-way ANOVA, F(5,26)=5.046, p=0.0192, Tukey’s post-hoc), which were prevented by all tested doses of ALC except 0.1 mM (figure 3.3). As observed for METH 1.0 µM, a 72h exposure caused a massive release of DA into the extracellular medium (one-way ANOVA, F(5,24)=4.961, p=0.0113, Tukey’s post-hoc). In spite of this, in cells pre-treated with ALC, extracellular levels of DA were not increased when compared to the control, except for the lowest dose of ALC. Interestingly, this same ALC dose was the most effective in preventing DA loss after exposure to METH 1.0 µM.

**Figure 3.3 – Effects of pre-treatment with ALC at 0.01, 0.1, 0.5, 1.0 mM and METH 100 µM on DA release.** Graphic representation of intra- and extracellular levels of DA measured by HPLC-EC, in PC12 cells pre-treated with ALC and exposed to METH 100 µM for 24h or 72h. Columns represent mean±SEM, expressed as pg of neurotransmitter/cell for each experimental condition.
(n=6-9 in control and METH100, n= 3 replicated experiments for combinations of METH100 and ALC). Significant differences are marked as follows: *p<0.05; **p<0.01 and ***p<0.001 as compared with the control condition; and •••p<0.001 as compared with METH (determined by one-way ANOVA followed by a post hoc Tukey’s HSD).

3.3.4. ALC Neuroprotective Action over DA Receptors In Vivo

Drug-occupancy studies are often performed to evaluate the selectivity of drugs to specific brain regions, using radiopharmaceuticals with high specific activity in tracer amounts (such that radiotracer occupancy represents less than 5% of the total available receptor sites) (Hume et al., 1998; Henriksen and Drzezga, 2011). In the present study, $^{123}$I-IBZM was used to evaluate D2R occupancy in C57BL/6J male mice, after the administration of ALC alone or in combination with METH, a DA agonist. Images were acquired in three different time points with 15 minutes intervals, starting 70 minutes after $^{123}$I-IBZM injection.

A single dose of METH induced a significant decrease of striatal $^{123}$I-IBZM binding in both the left and right striatum that was evident since the first acquisition point (i.e., 70 min post-injection) [two-way ANOVA (treatment x acquisition time), F(6,24)=0.7784, p<0.0001 for both factors, but no interaction between them].

In the left striatum, there was a progressive displacement of the radiotracer binding over time (two-way ANOVA followed by a post-hoc Tukey’s test F(3,24)=21.07, p=0.0336 comparing to control at T70 and p=0.0012 at T100, figure 3.4-A). Interestingly, over time, ALC was able to reverse the decrease on the radiotracer binding induced by METH. At the beginning of image acquisition period, the ALC/METH group presented a significant decrease in radiotracer binding ratios (p=0.0024 to control and p=0.0116 to ALC, figure 3.4-A), but in the end, this condition was no longer different from control.

The right striatum was less affected than the left, but the progressive decreasing pattern induced by METH was similar to that observed in the left striatum. From the 85th minute after tracer injection and onwards, $^{123}$I-IBZM binding in the right striatum was significantly reduced comparing to the control condition (two-way ANOVA followed by a post-hoc Tukey’s test F(3,33)=13.26, p=0.0221 at T85 and p=0.0012 at T100, figure 3.4-B). In this case, although less evident, ALC was also able to reverse the decrease in $^{123}$I-IBZM binding induced by METH. However, conversely to the left striatum, at 85 minutes after radiotracer administration, in the right striatum ALC still did not reversed this decrease, which was only visible 100 minutes after tracer injection (figure 3.4-B). ALC per se did not
interfere with D2R binding, since the average binding ratio was always similar to the control group (figure 3.4-A and B).

**Figure 3.4 – ALC reverses the METH-induced increased D2 receptors occupancy.** A: Striatal binding ratio of $^{123}$I-IBZM in the left striatum. B: Striatal binding ratio of $^{123}$I-IBZM in the right striatum. It is evident the significant progressive radiotracer displacement in the D2R induced by METH in both striatal regions. Right striatum was less affected than the left. ALC was able to reverse the radiotracer displacement induced by METH. Significant differences are marked as *p<0.05 and **p<0.01, as compared with the control condition; ▽p<0.05 and ▽▽p<0.01 as compared with ALC (as determined by one-way ANOVA, followed by a post-hoc Tukey’s test).

In figure 3.5 representative tomographic coronal slices from SPECT image acquisition of mice brain are shown. Images clearly demonstrate a sharp decrease of striatal $^{123}$I-IBZM binding in the METH group comparing to all other groups.
Figure 3.5 – Representative $^{123}$I-IBZM SPECT-MRI images from 12-week old male mice. The displayed tomographic images were acquired 100 minutes after radiotracer injection and were reconstructed by OSEM algorithm. The coronal planes, representative of each group are shown. Note the decrease on radiotracer uptake on METH group, which is increased when animals were preconditioned with ALC.

3.4. DISCUSSION

The present study uses both a cell line and an animal model of neurotoxicity to evaluate the action of ALC on the dopaminergic neurotransmission system. It revealed that, in vitro, ALC can interfere with DA release, decreasing the amount of extracellular DA in the presence of METH. Extracellular DA may be neurotoxic to dopaminergic neurons. These data substantiate those obtained in vivo in C57BL/6J mice in which ALC was able to counteract the effects of METH on D2R occupancy by increasing the receptor displacement of METH. In fact, METH is well-known to increase the synaptic concentration of DA (Di Chiara and Imperato, 1988), resulting in an increased receptor occupancy (DA competes with the radiotracer for D2R binding) and thus, reduces the availability of receptors for radiotracer binding. There is extensive imaging evidence that amphetamine, by promoting DA release, reduces the in vivo binding of imaging radiotracers that are either D2R agonists ($^3$H-N-propynorapomorphine) (Kohler et al., 1981) or antagonists ($^{123}$I-IBZM, $^{11}$C-raclopride) (Carson et al., 1997; Jongen et al., 2008), but the counteracting effect of ALC was never reported.
Using a rat model of MDMA exposure, our group observed that pre-treatment with a single dose of ALC (100 mg/kg) was effective in preventing a marked serotonin loss in all assessed brain regions, indicating a clear ALC protective effect (Alves et al., 2009). Although ALC treatment seems not to affect healthy subjects, where neurotransmitter levels are kept mostly within the normal ranges (Adriani et al., 2004), in regions like the hippocampus or the ventral mesencephalon, our group observed increased levels of serotonin in rats treated only with ALC (Alves et al., 2009). The physiologic significance of this remains unclear.

In the present study, when cells were treated with a range of ALC doses, but not exposed to METH, intermediate doses of ALC led to increased levels of intracellular DA. Interestingly, this was observed either at 24h or 72h, although the degree of response varied with different doses of ALC. Moreover, at both time points, the extracellular levels of DA were unaltered throughout tested conditions, which support the idea that ALC treatment, in the absence of a neurotoxic event, may lead to increased synthesis of DA. ALC has long been reported to affect the cholinergic, serotonergic, dopaminergic, glutamatergic and GABAergic systems, but the mechanisms involved were mostly unknown (Tolu et al., 2002). Recently, Scafidi et al. using $^{13}$C-ALC, demonstrated that the acetyl moiety of ALC is used as an energy source by both astrocytes and neurons and rapidly incorporated into glutamate and GABA synthesis (Scafidi et al., 2010b). This clarified the direct involvement of ALC in increased glutamine, glutamate and GABA levels, via the TCA cycle. Importantly, the biosynthesis of both tyrosine and L-tryptophan, as well as DA and 5-HT precursors, involves either a transamination reaction with glutamate or the participation of glutamine as an amine donor (Yudkoff, 1997), which may explain the action of ALC over monoamine production. Noteworthy, glutamate is also the precursor of glutathione biosynthesis (Griffith and Mulcahy, 1999), an enzyme implicated in the antioxidant properties of ALC, as explained in chapter 1.

The action of METH over PC12 cells was extensively characterized. It was demonstrated that increased endogenous DA is required for METH-induced formation of ubiquitin and α-synuclein positive lamellar bodies (Fornai et al., 2004). These inclusions are not necessarily associated with neurodegeneration and are thought to represent a defense mechanism, against higher levels of oxidized and misfolded proteins. This seems to be confirmed in PC12 cells, where exposure to 1.0 µM of METH dose, produces the highest number of inclusions without cell death, while after exposure to 100 µM METH, inclusions are not visualized and massive cell damage occurs (Fornai et al., 2004). Interestingly, DA synthesis may promote inclusion formation either directly or via alternative metabolic
pathways, since production of these inclusions can be avoided both by DA depletion or other processes, such as administration of the iron chelator, antioxidant agent S-apomorphine (Fornai et al., 2004). In this work, in accordance with previous reports, a 24h exposure to METH 1.0 µM did not affect DA production and release. However, at 72h, both intra- and extracellular DA levels were increased. Pre-treatment with ALC (except for the 0.5 mM dose) led to intracellular DA levels that were no longer significantly higher than in the control condition. Moreover, while METH exposure induced a marked release of DA, pre-treatment with ALC seems to interfere with DA release, decreasing the amount of extracellular DA. These results were obtained for in vitro assays with PC12 cells as well as for in vivo with C57BL/6J male mice, in which a single dose of METH (10 mg/kg) led to a significant increase in DA release into the synaptic cleft, which then competes with $^{123}$I-IBZM for binding to D2R. In striatal neurons, increased extracellular or extravesicular DA levels were shown to occur in concomitance with increased levels of ROS (Cadet and Brannock, 1998; Lazzeri et al., 2007). In the brain, catecholamines are indeed an important source of free radicals as its autoxidation induces the production of quinones and, subsequently, the generation of free radicals and damage to proteins (Cadet and Brannock, 1998).

Administration of METH 1.0 µM to the cell culture medium corresponds to an in vivo dose of 5 mg/kg, which leads to a striatal concentration of about 1.0 µM (Fornai et al., 2007). As already mentioned in the first chapter, DA release in PC12 cells is much lower than in striatal cells due to poorer storage and synthesis. Furthermore, while increasing METH dose in vivo leads to loss of striatal terminals but not to soma loss, increasing METH dose in PC12 cells induces both necrosis and apoptosis (Fornai et al., 2007), which renders it difficult to directly compare in vivo exposure with PC12 cells exposure to METH. On the other hand, PC12 cells, like DA neurons, present the type A isoform of MAO, providing a good model for the study of altered redox events related to DA release. Therefore, increasing exposure from 24h to 72h, in PC12 cells, mimics the increase in ROS formation induced in vivo by METH exposure and provides an interesting model for the study of ALC properties.

In this study, the challenge with a single dose of 10 mg/kg of METH decreased striatal D2R binding ratios relative to the control group between 20% and 30%, depending on the time point of image acquisition. Similar results were obtained by Jongen et al., who observed a reduction in striatal D2R binding ratios of 27% in mice after the administration of 2.5 mg/kg of amphetamine (Jongen et al., 2008). In another study, in which amphetamine and the dopamine reuptake inhibitor methylphenidate were compared,
microdialysis assays showed that amphetamine (2.5 mg/kg) induced a 1400% increase in extracellular DA, while methylphenidate (5 mg/kg) increased it in 360%. This four-fold difference was not paralleled by synaptic DA levels, because reductions of striatal D2R binding after the administration of amphetamine and methylphenidate were 24 and 21%, respectively, as measured by $^{11}$C-raclopride (an antagonist D2R radiotracer) displacement (Schiffer et al., 2006). This may be due to the distinct pharmacologic mechanisms underlying these drugs actions. While methylphenidate action is confined to the synaptic cleft, by blocking the passive reuptake of DA, amphetamine binds to DAT, thus entering the presynaptic terminal and actively releasing high amounts of vesicular DA, inducing a massive increase in the ratio of extracellular to synaptic DA, comparing to methylphenidate (Schiffer et al., 2006). In human studies, METH was able to reduce D2R availability in the caudate (16%) and in the putamen (11%), similarly to observations for cocaine abusers (Volkow et al., 2001d). In schizophrenic patients with schizotypal personality disorder, METH also induced a significant decrease (12%) in $^{123}$I-IBZM striatal binding ratios, which was more pronounced during illness exacerbation (24%) (Abi-Dargham et al., 2004). Another interesting aspect of METH action is related with altered membrane permeability. Recent studies have confirmed that METH inhibits potassium-dependent DA release, likely caused by diminished vesicular DA uptake (Chu et al., 2010). Conversely, increased membrane permeability was long implicated in the acute effects of METH and massive leakage of albumin was shown to occur across the BBB in the cerebral cortex of METH-treated mice, concomitantly with nerve cell damage (Sharma and Ali, 2006; Martins et al., 2011). Whether decreased radiotracer binding to D2R after METH administration is due to competitive inhibition with DA or to D2R internalization (Sun et al., 2003; Skinbjerg et al., 2010) or even to changes in receptor affinity (Ginovart, 2005) is a matter of controversy, although the competitive inhibition theory has been the most consensual (Jongen et al., 2008). The intracellular milieu has lower pH and sodium ion concentration comparing to the extracellular environment. In these conditions, the affinity of benzamide-derived D2R radiotracers is lower, explaining the lower radiolabelled benzamides uptake when the receptors are internalized due to the action of DA (Shotbolt et al., 2012). Nevertheless, decreases on radiotracer binding to D2R relative to baseline provide reliable estimations concerning increases on synaptic DA concentration (Nikolaus et al., 2005).

In another study with baboons, pre-treatment with vigabatrin restored to normal levels the striatal $^{11}$C-raclopride (D2R antagonist) binding after acute cocaine administration (Dewey et al., 1997). Vigabatrin, similarly to ALC in the present study, was able to decrease the extracellular DA concentrations induced by cocaine (Dewey et al., 1997). The
administration of ALC to rats for seven consecutive days shown to increase DA in the nucleus accumbens shell which was associated to protective effect on acute stress exposure (Tolu et al., 2002).

A critical issue in brain receptor imaging is related to radiotracer specific activity (i.e., the radioactivity per mass unit). In order to assure that receptor occupancy is less than 5%, a high specific activity should be assured (Hume et al., 1998), otherwise significant receptor occupancy or eventually, pharmacological effects might occur, invalidating study results (Franc et al., 2008). In the present work, a radiopharmaceutical with a high specific activity (> 74 TBq/mmol) was used in order to avoid not only the occupancy of a great number of D2 receptors, but also pharmacological effects to occur. Additionally, the use of a high sensitivity micro-SPECT camera allowed the administration of rather low doses of $^{123}$I-IBZM.

A practical requirement related to in vivo animal imaging is that the animal has to remain still during the imaging procedure, requiring most often the administration of anesthetizing agents. However, it is reported that binding of radioactive tracers to brain target regions may be affected by anesthetics. Isoflurane was shown to induce changes in the dopaminergic system in nonhuman primates, possibly by modulating the availability of the dopamine transporter (Kobayashi et al., 1995; Tsukada et al., 1999). Furthermore, halothane anesthesia enhanced METH-induced DA release in the rat brain (Adachi et al., 2001). However, other studies exclude a significant effect of isoflurane anesthesia on D2R imaging (signal to noise ratio and radiotracer uptake) (Honert et al., 2004; Schiffer et al., 2006). Our protocol was based on a short inhalation period of a light dose of isoflurane (2%), which started immediately before the intravenous administration of the radiotracer and lasted only the time required for the injection procedure. After injection, animals recovered very quickly from isoflurane anesthesia and were returned to their home cage for the $^{123}$I-IBZM uptake period (70 minutes). Animals were anesthetized again for the imaging acquisition procedure. Since during the uptake period, animals were no longer under the effect of the anesthetic, we might assume that anesthesia did not have a significant effect on DA release.

The administration of repeated high doses of METH and amphetamine has been shown to not only affect receptors availability, but also to cause long lasting DAT depletion (Wagner et al., 1980). However, amphetamine seems to be more effective than METH in decreasing the number of DAT (Booij et al., 2006). Volkow et al. observed a significant DAT reduction in human METH abusers that persisted at least for 11 months after detoxification. Moreover, in these subjects, DAT reduction was associated with motor and
cognitive impairment (Volkow et al., 2001c). DAT are crucial for the termination of dopaminergic transmission through the reuptake of DA from the synaptic cleft and thus, have an important role in the spatial and temporal regulation of synaptic DA (Booij and Kemp, 2008). In the recent years, some authors came up with the idea that amphetamines might promote the fast internalization of DATs with the consequent reduction on the surface expression to bind to the DAT radiotracer (ex: 123I-ioflupane) (Saunders et al., 2000). In this study, we have used a single and relatively low dose of METH, which might be insufficient to induce such significant changes on DAT.

A limitation of this study is related with the low number of imaged animals per group (n=3). In order to increase the statistical significance of the study, and thus the respective power, the number of animals per group should have been, ideally, at least 5. Moreover, in order to validate these in vivo imaging results, it would be worth to use another imaging technique, the ex-vivo autoradiography (this is an ongoing task, since we have already started it). This would allow the comparison between the striatal binding ratios obtained by both imaging techniques. Also, the evaluation of DA levels in the synaptic cleft by microdialysis technique would bring valuable information concerning the competitive inhibition and the receptors internalization theories. Due to the possible effects of ALC on D1 receptors (D1R) and the influence of D2R on these receptors, the evaluation of those receptors could be interesting. However, so far, the availability of radioactive tracers to assess D1R is very limited. Furthermore, the evaluation of intra- and extracellular DA levels (and its metabolites, such as 3,4-dihydroxyphenylacetic acid, DOPAC, and homovanillic acid, HVA) in primary cultures of neurons would allow to complement and validate data obtained in PC12 cells.

3.5. CONCLUSION

The present study demonstrates a possible effect of ALC over METH-induced DA release. Our results show that ALC by itself increases intracellular levels of DA, which might be a consequence of the ALC role in tyrosine biosynthesis. However, in vivo, pretreatment with ALC was shown to decrease D2R occupancy subsequent to METH administration. The exact mechanisms underlying the action of ALC over DA release and membrane transport remain unclear. However, it is possible that ALC may exert its protective role through the direct regulation of cytoskeleton properties, which may interfere with the conformation of membrane receptors and therefore affect its binding affinity to different compounds. Alternatively, ALC may induce changes in the expression of DA receptors, and particularly
the D2R, since this receptor is crucial for the regulation of DA release, and consequently may play an important role in the induction of dopaminergic toxicity.
CHAPTER 4

GENERAL DISCUSSION
In order to better understand the mechanisms underlying the action of ALC, we have focused our work on two main aspects: a) the role of ALC in mitochondrial functionality \textit{in vitro} and glucose uptake, both \textit{in vitro} and \textit{in vivo}; b) the role of ALC over the dopaminergic function, both \textit{in vitro} and \textit{in vivo}.

Carnitine has been described as having a key role in cellular bioenergetics not only by facilitating the transport of fatty acids across the mitochondrial membrane into the matrix, where they undergo $\beta$-oxidation, but also by controlling the intramitochondrial ratio of acetyl-CoA/free CoA and consequently, modulating glucose oxidation. Considering these roles in cellular bioenergetics, allied to the reported putative neuroprotective action, we proposed to study the mechanisms involved in ALC action, particularly those related to mitochondrial functionality and dopaminergic function. Since the brain relies in glucose oxidation as the main source of energy, we started by assessing the role of ALC in neuronal metabolism in the mice brain \textit{in vivo}, under several experimental conditions. We used a radioactive analogue of the glucose molecule ($^{18}$F-FDG) to image C57BL/6J young male mice. On one hand, the expression of IGF1 seems to modulate glucose uptake through the indirect promotion of GLUTs translocation to the plasma membrane, augmenting glucose transport into the cell. On the other hand, carnitine supplementation activates the IGF1 signaling cascade and, consequently, promotes glucose utilization. These assumptions led us to investigate the expression of both Igf1 and Igf1R in the striatum, where the most relevant changes in glucose uptake were observed. To complement \textit{in vivo} data, we used a dopaminergic cell line to better understand the role of ALC in neuronal glucose uptake under the action of METH, in glucose-deprived conditions. Regarding glucose uptake, our results showed:

- a generalized increase in glucose uptake in several brain regions, immediately after the administration of METH when mice were preconditioned with ALC. This was particularly evident in regions receiving a strong dopaminergic input and therefore, more vulnerable to METH action (the striatum and the PFC);
- an increase in Igf1 levels in animals treated with ALC and a decrease in those treated with METH;
- an upregulation of Igf1R levels in ALC/METH group, matching the increase in glucose uptake and mismatching the Igf1 levels, that were unaltered;
- that the lowest tested dose of ALC (0.01 mM) increased glucose uptake, when administered alone or in combination with METH.

According to our observations, the administration of either ALC or METH alone did not show to have immediate (1-2 hours after injection) effects on glucose uptake. These
findings were not surprising since the effect of an acute single low dose is not comparable to a chronic exposure, which in the case of METH is known to induce significant reductions in glucose metabolism in several brain regions. Conversely, the combined administration of these drugs, increased glucose uptake in the mice brain, particularly high in regions with important dopaminergic inputs. Although not addressed in the present work, behavioral tests were held in the lab and animals that performed poorly in water maze test, were those treated with ALC/METH, reflecting memory impairments. Accordingly, Igf1R levels in the striatum of these animals were also increased 24h after drug administration. However, the enhancement in Igf1R levels was not paralleled by increases in Igf1 transcript levels. It is possibly that the systemic IGF1 may directly cross the BBB and this may be the reason for unaltered Igf1 levels in the striatum. If this is the case, IGF1 levels would also be increased. Increased Igf1 expression induced by ALC may be an attempt to promote GLUTs translocation to the outer cell membrane, thus augmenting glucose uptake by the brain, which seems to be relevant for the action of ALC.

We reason that the combined action of ALC and METH over the dopaminergic and adrenergic receptors and transporters, associated to neurotransmitters synthesis promoted by ALC, may be in the basis of the observed increase in glucose uptake. Moreover, our results indicate that the activation of IGF1 signaling cascade, namely the increase in the IGF1R levels and the eventual indirect promotion of GLUTs translocation to the plasma membrane, are also contributing factors.

To further explore the role of ALC in mitochondrial function, a dopaminergic cell line (PC12 cells) exposed to METH in deprived glucose conditions (LG) was used. Here, we assessed parameters such as cell viability, mitochondrial function and ATP synthesis. The ability of ALC to protect cells from loss of mitochondrial membrane potential and prevent the activation of the apoptotic cascade was also explored. Our results indicate that METH significantly decreases mitochondrial function for a 72h exposure, which was partially prevented when cells were preconditioned with ALC 0.01 mM. The combined use of this dose of ALC and METH, which revealed a significant increase in glucose uptake at 24h, is associated to decreased cell viability and ATP synthesis. On the other hand, when cells were exposed to equal concentrations of ALC and METH in a high-glucose medium, ATP synthesis was much higher. This suggests that if glucose is available, ALC promotes glycolysis for energy production, but if it is scarce, it may promote alternative metabolic pathways.
Our findings are not clear concerning the activation of the apoptotic cascade, as we observed no changes in the ratio of activated vs. total Bax. However, fluorescence images showed that ALC/METH, led to the translocation of active Bax to the nucleus, paralleled by decreased mitochondrial membrane potential (Δψm) and increased ROS formation. Our results suggest that increased ROS formation, loss mitochondrial membrane potential and Bax translocation to the nucleus or mitochondria are likely to be early events in the activation of the apoptotic cascade. Of note, we have also assessed the activation of caspase 3 without observing any significant change.

In order to accomplish our goals, we further evaluated the role of ALC over the dopaminergic functionality, both in vitro and in vivo. The above mentioned dopaminergic cell line and animal model were used and exposed to the same experimental conditions. Intra- and extracellular DA levels were evaluated at 24h and 72h in PC12 cells. For animal studies, an antagonist of D2R labeled with a radioactive isotope, was used to measure in in vivo, the availability of these receptors. Our results reveal that, in vitro, ALC can interfere with DA release, decreasing the amount of extracellular DA in the presence of METH. These data corroborate with those obtained in mice in which ALC was able to counteract the effects of METH on D2R occupancy by increasing the receptor displacement of METH. ALC by itself increased intracellular levels of DA, which might be a consequence of the ALC role in tyrosine biosynthesis. In vivo, pre-treatment with ALC showed to decrease D2R occupancy by DA, released as a consequence of METH administration. Possibly ALC exerts a protective effect through the direct regulation of membrane channels or transporters or by inducing changes in the conformation or in the expression of DA receptors, particularly the D2R, since they are crucial for the regulation of DA release.

Our group previously reported that animals administered with ALC presented increased levels of 5-HT (Alves et al., 2009) in the same brain regions that in the present study displayed the highest glucose uptake. The administration of repeated high doses of METH has been shown to not only affect receptors availability, but also to cause long lasting DAT depletion. In this study, we have used a single and relatively low dose of METH, which might be insufficient to induce such significant changes on DAT. In the future, it would be interesting to study if ALC exerts some action over the DAT as it does over D2R. To our knowledge, this aspect was not explored yet.

In vivo imaging requires that the animal does not move while is being scanned. This usually involves the administration of anesthetizing agents. A matter of concern and controversy is whether anesthetics modify radiotracers biodistribution and uptake level in
the brain. There is evidence in the literature that those agents reduce $^{18}$F-FDG uptake in several brain regions and may enhance the release of DA into the synaptic cleft. In the present study we consider that isoflurane anesthesia might have had a minor or even no effect in the radiotracers uptake due to the use of a low dose and a short period of administration (limited to the time required for the intravenous injection procedure) of isoflurane anesthesia, after which the animal recovered very promptly. During the radiotracers uptake period (45 minutes for $^{18}$F-FDG and 70 minutes for $^{123}$I-IBZM) the animals were no more under anesthesia.

A potential limitation of the present study is related with the low number of imaged animals. In order to increase the power of the study, each group should have included at least 5 animals. Despite this limitation, the obtained data presented a rather good correlation with the remaining assays. Moreover, we think that the assessment of the striatal levels of GLUTs and IGF1 would strengthen the data related to the Igf1, IGF1R and glucose uptake. Regarding the dopaminergic function, we think that in the future, it could be interesting to explore the role of ALC in D1R as well as in DAT (so far, these aspects remain unexplored).

Overall, these data demonstrate that ALC exerts its action by increasing glucose uptake and Igf1R levels in brain regions with strong dopaminergic input, such as the striatum after an acute insult such as the exposure to METH. ALC by itself enhances the levels of Igf1. Concerning neurotransmitter systems, ALC showed not only to increase the intracellular levels of DA, but also to improve the availability of D2R by promoting METH displacement. Although further studies are needed to support the use of ALC as a therapeutic agent, based on our data and from others, we think that in the future, it might be possible to use this compound as an adjuvant therapeutic agent in the treatment of some neurodegenerative diseases.
REFERENCES
REFERENCES


Brown GC, Borutaite V (2011) There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion.


Cifone MG, Alesse E, Di Marzio L, Ruggeri B, Zazzeroni F, Moretti S, Fumagdi L, Steinberg SM, Vullo E, De Simone C (1997) Effect of L-carnitine treatment in vivo on apoptosis and...


Schinder AF, Olson EC, Spitzer NC, Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16:6125-6133.


