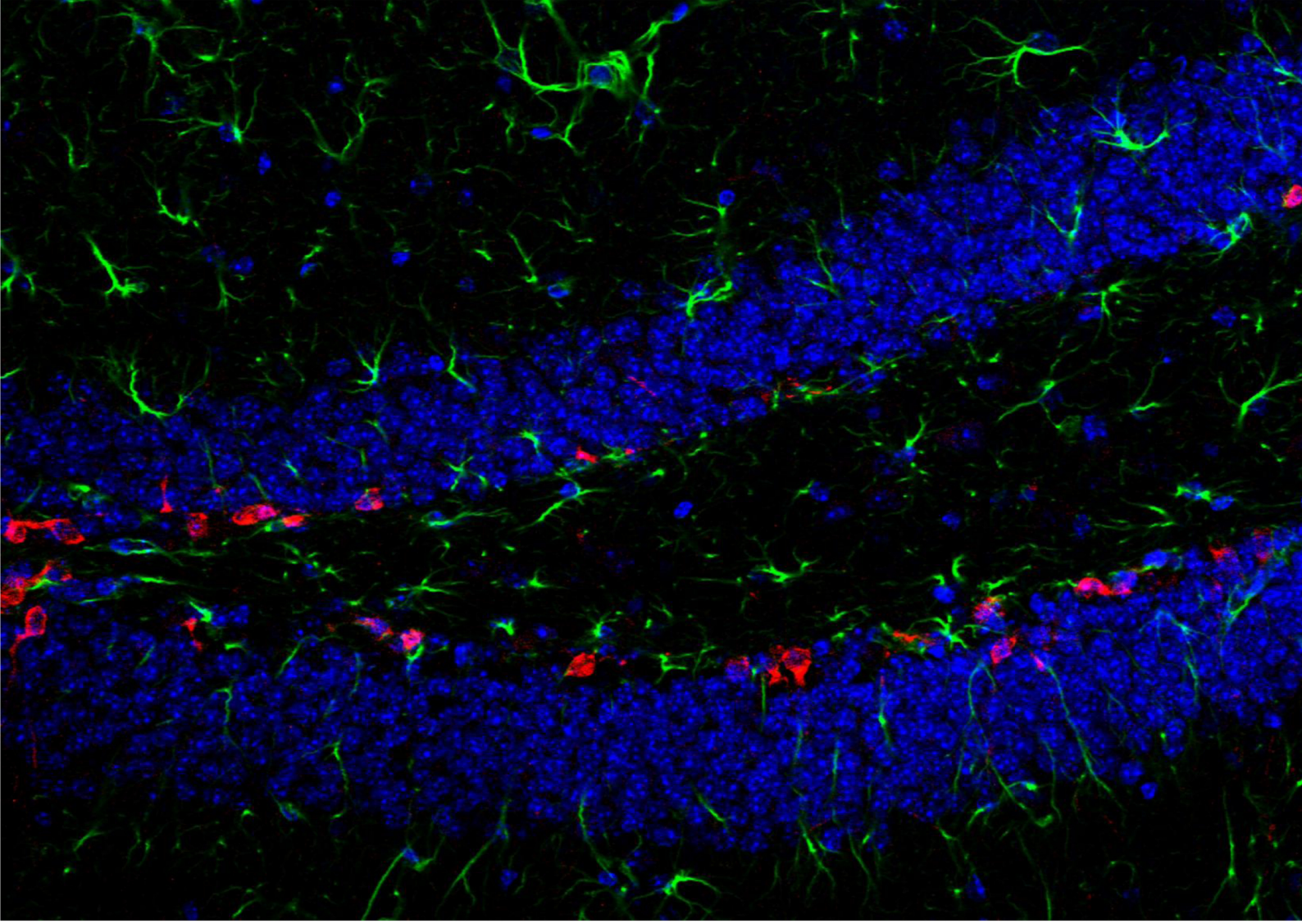

Effects of exposure to eslicarbazepine acetate and to other antiepileptic drugs on neurotoxicity and hippocampal development

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UNIVERSITY OF COIMBRA

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Cover image: confocal microscopy image of the dentate gyrus of the hippocampus from a juvenile male mice after exposure to eslicarbazepine acetate *in uteru* and during nursing. Cells were stained for doublecortin (DCX, red), glial fibrillary acidic protein (GFAP, green), and neuronal nuclei marker (NeuN, blue). Scale bar 50 μm.

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“Deus quer, o homem sonha, e a obra nasce!”

Fernando Pessoa

Aos meus avós,

Bábá, Simão e Palmira

INDEX

Abbreviations

7-AAD	7-actinomycin D
Ach	Acetylcholine
AEDs	Antiepileptic drugs
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BrdU	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
CA	Cornu Ammonis
CBZ	Carbamazepine
CK	Creatine kinase
CLAP	Chymostatin, leupeptin, antiparin, pepstatin A.
CNS	Central nervous system
CREA	Creatinine
DBS	Deep brain stimulation
DCX	Doublecortin
DG	Dentate gyrus
DNAse	deoxyribonuclease I
DTT	Dithiothreitol
ECF	Enhanced Chemifluorescence
EDTA	Ethylenediamine tetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
ESL	Eslicarbazepine acetate
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FST	Forced swimming test
GABA	Gamma-aminobutyric acid

GFAP	glial fibrillary acidic protein
GL	Granular layer
Glut	Glutamate
HBSS	Hank's balanced salt solution
i.p.	Intraperitoneal
IBE	International Bureau of Epilepsy
IGZ	Inner granular zone
ILAE	International League Against Epilepsy
LEV	Levetiracetam
LTG	Lamotrigine
LV	Lateral ventricle
MCM	Major congenital malformations
NeuN	Neuronal nuclei
NGS	Normal goat serum
NHS	Normal horse serum
NIH	National Institutes of Health
NMDA	Methyl-D-aspartate
NOR	Novel object recognition
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OB	Olfactory bulb
OGZ	Outer granular zone
OXC	Oxcarbazepine
PARP	Poly ADP ribose polymerase
PB	Phenobarbital
PGB	Pregabalin
PHT	Phenytoin
PMSF	Phenylmethylsulphonyl fluoride
PSA-NCAM	Poly-sialated neural cell adhesion molecule
PTZ	Pentylenetetrazole
PVC	Polyvinyl chloride
PVDF	Polyvinylidene difluoride
R-Lic	R-licarbazepine
SAPK-JNK	Stress-activated protein kinase/c-Jun NH2-terminal kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gels
SEM	Standard error mean

Abbreviations

S-Lic	Eslicarbazepine
SVZ	Subventricular zone
TC	Total cholesterol
TG	Triglycerides
TPM	Topiramate
VGSC	Voltage-gated sodium channels
VNS	Vagus nerve stimulation
VPA	Valproate

Summary

Eslicarbazepine acetate (ESL) is a new antiepileptic drug (AED), which is used as add-on therapy for adult patients with partial-onset seizures, with or without secondary generalization, since its approval in Europe in 2009. ESL is structurally related to carbamazepine (CBZ) and oxcarbazepine (OXC), and after oral administration to humans is rapidly and extensively hydrolyzed to its major and active metabolite, eslicarbazepine (S-Lic). S-Lic is responsible for the anticonvulsant action of ESL and, like CBZ and OXC, blocks voltage-gated sodium channels. In addition to S-Lic, there are two other metabolites present in the plasma after administration of ESL: R-licarbazepine (R-Lic), the R-enantiomer of S-Lic, and OXC, both without contribution to the anticonvulsant action of ESL. To date, ESL was shown to be more effective and less toxic than OXC and CBZ. To date, there are no studies regarding ESL administration during pregnancy and nursing on women with epilepsy and the potential consequences of *in utero* exposure to the offspring.

To address some of these aspects, we used an animal model, CD1 mice, which are known to be the species most similar to humans regarding the pharmacokinetics and oral biodisposition of ESL. Thus, this study had three main goals: a) to study the impact of AED exposure during pregnancy and during nursing on the biochemical parameters of blood and serum of the females progenitors, as well as, the effects of AED exposure on the formation of new cells in the adult hippocampus; b) to study the impact of AED exposure during pregnancy and during nursing on cognitive and non-cognitive behaviour of the progeny, as well as, the effects of AED exposure on the formation of new neurons in the adult hippocampus, and c) to study the possible effects of ESL and of other AEDs on the activation of cell death/neuroprotective mechanisms in cultures of embryonic hippocampal neurons; and, as well as, the effects of AEDs on the basal proliferation and cell cycle phases of neural stem cell cultures from the subventricular region (SVZ) of rats.

In vitro experiments were performed in cultured hippocampal neurons of embryonic rats, which were exposed to different concentrations of AEDs [ESL, S-Lic, R-Lic, CBZ, OXC, lamotrigine (LTG) and sodium valproate (VPA)]. We observed that neither ESL nor its metabolites reduced cell viability or induced the appearance of markers of cell death. However, exposure to OXC and VPA did induce the appearance of markers related to cell

death by apoptosis, and OXC was the most toxic drug. ESL, S-Lic and OXC decreased the levels of phospho-ERK1/2 and phospho-Akt but did not change the levels of phospho-SAPK/JNK, when compared to control situations, whereas CBZ decreased phospho-SAPK/JNK and phospho-Akt levels. However, LTG, and to some extent VPA, increased the phosphorylation levels of SAPK/JNK. Altogether, the results suggest that ESL and its metabolites are less deleterious to neuronal cells than the other AEDs studied.

In the studies performed *in vivo,* CD1 mice were used as a model. We observed that after a daily intake of the AEDs (ESL, CBZ, OXC and VPA), at therapeutic doses, during pre-gestation period, pregnancy and nursing, females treated with ESL and CBZ did not show changes in the evaluated biochemical parameters of blood and serum (glucose, total cholesterol, triglyceride, alanine and aspartate aminotransferase, creatinine and creatine kinase), whereas OXC and VPA decreased creatinine levels by 30%, and VPA also decreased the levels of triglycerides by 50%, when compared to untreated females. We also assessed the effects of AED exposure on the formation of newly born cells in the hippocampus of CD1 female mice. We observed that both CBZ and VPA decreased by 40% the number of cells positive for BrdU in the subgranular zone (SGZ) of the dentate gyrus (DG), while ESL and OXC did not change these values when compared to untreated females.

Then we studied the effects of AED exposure *in utero* and during nursing on the cognitive performance (memory and learning), locomotion, the possible anxiogenic vs. anxiolytic effects, and also pro-depressive effects, in the progeny of CD1 females. The performance of males and females was evaluated separately in the behavioural tests, which were conducted twice, at one month and at four months of age. Overall, we observed that the animals exposed to ESL did not show deficits in the performance of cognitive or non-cognitive tasks, but we observed a slight increase in locomotor activity of males, at one month of age. Animals exposed to CBZ, OXC and VPA showed a decrease in performance of some tasks associated with memory and learning. These effects were observed mostly in young animals, and few effects were kept into adulthood.

Next, we evaluated the effects of exposure to AEDs *in utero* and during nursing, on the basal proliferation and formation of new neurons in the DG of the hippocampus of CD1 mice born. The basal proliferation was evaluated by incorporation of EdU into neural

progenitor cells found in DG, and we observed a decrease of about 40% of EdU-positive cells in the SGZ of males, but not in females, exposed to OXC when compared to the untreated animals. Neither ESL, CBZ nor VPA induced changes in the basal proliferation of the DG. Regarding the formation of new cells/neurons, we quantified the BrdU-positive cells in the DG or those that co-localize with other neuronal markers, such as doublecortin (DCX) and the neuronal nuclei (NeuN) marker. Interestingly, we observed that in both males and females exposed to OXC the number of BrdU-positive cells in SGZ was decreased by 54% and 47%, respectively; in males exposed to VPA that number was decreased by 60%, when compared to control animals. Except for males exposed to CBZ, which increased by 16% the number of mature neurons in the SGZ, none of the other groups of males had changes in the number of mature or immature neurons in the DG. Regarding females, we observed that those who were exposed to OXC had less mature and immature neurons in the SGZ compared with the control group. ESL, CBZ or VPA did not induce changes in the formation of new neurons in the DG of CD1 females.

We also performed experiments *in vitro*, with cultured neural stem cells isolated from the SVZ of rats, to assess the effect of AED exposure (ESL, S-Lic, R-Lic, CBZ, OXC, LTG and VPA), on the basal proliferation and on the different cell cycle phases (G0/G1, S and G2/M). We used different concentrations of the AEDs and observed that the active metabolite of ESL, S-Lic, did not induce any change in basal proliferation or on cell-cycle phases. The other AEDs tested decreased basal proliferation at different concentrations, but the highest effect was caused by exposure to VPA (1 or 3 mM), which decreased basal proliferation by 85% or 95%, respectively. Regarding the cell cycle, we observed different effects of the AEDs, at different concentrations. We observed an increase (65%) in the percentage of cells in G2/M phase, after exposure to the OXC (0.3 mM), whereas VPA (3 mM) exposure caused a decrease in the percentage of cells in G2/M phase of about 70%. We also evaluated the effect of AEDs on cell death by apoptosis in the SVZ cultures, and observed that the VPA (3 mM) and OXC (0.3 mM) significantly increased the percentage of apoptotic cells.

According to the results obtained from both in the *in vivo* and in the *in vitro* approaches, ESL emerges as potentially less risky AED to be used by pregnant women with epilepsy and during nursing, since ESL did not have impact on the biochemical parameters

of blood and serum of the pregnant CD1 females, and no adverse effects were detected in cognitive and noncognitive functions of their progeny. Moreover, no changes were observed in the formation of new cells and neurons in the adult hippocampal DG of the pregnant CD1 females and their progeny, respectively. In addition, S-Lic, the active metabolite of ESL was not toxic to cultured hippocampal neurons, and it did not affect basal proliferation and cell cycle phases of the neural stem cell cultures from rat SVZ.

In conclusion, ESL is a safe and effective anticonvulsant with potential to be used in the treatment of pregnant women with epilepsy, while AEDs that have negative effects on cognitive performance, such as OXC, and also have a negative impact on the formation of new cells and adult neurons in the hippocampus, should be avoided during the pre-gestation, pregnancy and nursing periods,.

Resumo

O acetato de eslicarbazepina (ESL) é um novo fármaco antiepiléptico (AE) aprovado na Europa em 2009, e desde então é usado na terapêutica adjuvante de doentes adultos com crises epilétpicas parciais, com ou sem generalização secundária. O ESL é estruturalmente relacionado com a carbamazepina (CBZ) e a oxcarbazepina (OXC) e, após administração oral em humanos é rápido e extensamente hidrolisado no seu metabolito principal e activo, a eslicarbazepina (S-Lic). A S-Lic é maioritariamente responsável pela acção anticonvulsiva do ESL e, tal como CBZ e OXC bloqueia os canais de sódio dependentes da voltagem. Para além da S-Lic, há dois outros metabolitos presentes no plasma após a ingestão do ESL: a R-licarbazepina (R-Lic), enantiómero da S-Lic, e a OXC, contudo ambos sem contributo para acção anticonvulsiva do ESL. Até à data, o ESL tem-se revelado mais eficaz e menos tóxico do que a OXC e CBZ. Contudo, não existem estudos sobre os efeitos do ESL na mulher com epilepsia durante a gravidez e amamentação, e as consequências da exposição aos AEs *in utero* na descendência.

Para estudar estas situações recorreu-se a um modelo animal de murganhos (CD1), uma vez que é a espécie mais semelhante ao homem no que diz respeito à farmacocinética e biodisposição oral do ESL. Desta forma, este trabalho teve três principais objetivos: a) estudar os efeitos da exposição dos AEs durante o período pré-gestacional, gestacional, e amamentação sobre os parâmetros bioquímicos do sangue e soro das progenitoras, assim como os efeitos da exposição dos AEs na formação de novas células no hipocampo adulto; b) estudar o impacto da exposição dos AEs durante a gestação a amamentação no comportamento cognitivo a não cognitivo da descendência, assim como os efeitos desta exposição na formação de novos neurónios no hipocampo adulto, e c) estudar os possíveis efeitos do ESL e outros AEs na activação dos mecanismos de morte/neuroprotecção celular em culturas de neurónios do hipocampo embrionário, assim como os efeitos dos AEs na proliferação celular basal e fases do ciclo celular em culturas de células estaminais neurais da região subventricular de ratos (SVZ, do inglês *subventricular zone*).

Nas experiências *in vitro*, culturas primárias de neurónios de hipocampo de embriões de ratos foram expostas a diferentes concentrações dos AEs [ESL, S-Lic, R-Lic, CBZ, OXC, LTG (lamotrigina) e valproato de sódio (VPA)]. Observámos que nem o ESL nem os

seus metabolitos alteraram a viabilidade celular e também não induziram a aparecimento de marcadores de morte celular. No entanto, nas culturas expostas à OXC e ao VPA identificámos marcadores de morte celular por apoptose, e a OXC mostrou ser o AE mais tóxico. ESL, S-Lic and OXC diminuíram os níveis de fosforilação das cinases ERK1/2 e Akt, mas não alteraram os níveis de fosforilação da cinase SAPK/JNK, quando comparados com as situações controlo; enquanto a CBZ diminuiu os níveis de fosforilação das cinases SAPK/JNK a Akt. Porém, a LTG e o VPA aumentaram os níveis de fosforilação da cinase SAPK/JNK na mesma amplitude/extensão. No total, estes resultados sugerem que o ESL e os seus metabolitos são menos prejudiciais do que os restantes AEs estudados para as células neuronais.

Nos estudos *in vivo* usámos como modelo os murganhos fêmeas CD1. Nós observámos que após a ingestão diária de doses terapêuticas dos AE (ESL, CBZ, OXC e VPA), durante o período pré-gestacional, e durante a gravidez e a amamentação, as fêmeas tratadas com ESL e CBZ não apresentaram nenhuma alteração nos parâmetros bioquímicos serológicos e do sangue que foram avaliados (glucose, colesterol total, triglicerídeos, alanina e aspartato aminotransferase, creatinina e creatina cinase), enquanto as fêmeas tratadas com a OXC e o VPA apresentaram uma diminuição nos níveis da creatinina em cerca de 30%, e o grupo tratado com VPA também apresentou os níveis de triglicerídeos diminuídos em cerca de 50%, quando comparado com o grupo de fêmeas não tratadas. Também avaliámos os efeitos da exposição aos AE na formação de novas células no hipocampo das fêmeas CD1, e observámos que tanto a CBZ como o VPA diminuíram em cerca de 40% o número de células positivas para BrdU, na região subgranular (SGZ, do inglês *subgranular zone*) do giro denteado (DG, do inglês *dentate gyrus*) do hipocampo, enquanto o ESL e a OXC não alteraram estes valores quando comparados com as fêmeas não tratadas.

Estudámos em seguida os efeitos da exposição aos AE *in utero* e durante a amamentação, no desempenho cognitivo (memória e aprendizagem), na locomoção, nos possíveis efeitos ansiogénicos vs ansiolíticos, e ainda nos efeitos pro-depressivos na progénie das fêmeas CD1. A performance das fêmeas e dos machos foi avaliada em separado com testes comportamentais, duas vezes com um e com quatro meses de vida. Em geral observámos que animais expostos ao ESL não apresentaram deficiências no desempenho das tarefas cognitivas nem nas não-cognitivas, mas observámos um ligeiro aumento da

actividade locomotora nos machos com um mês de idade. Os animais expostos a CBZ, OXC e VPA apresentaram um desempenho diminuído em algumas tarefas relacionadas com a memória e a aprendizagem. Estes efeitos foram observados em sua maioria nos animais jovens, e apenas alguns dos efeitos se mantiveram até à idade adulta.

Em seguida, avaliámos os efeitos da exposição aos AE *in utero* e durante a amamentação na proliferação basal e formação de novos neurónios no DG do hipocampo de murganhos nascidos das fêmeas CD1. A proliferação basal foi avaliada pela incorporação de EdU nas células neurais progenitoras que se encontram no DG, e observámos uma diminuição do número de células positivas para o EdU em cerca de 40% na SGZ dos machos expostos à OXC, mas não nas fêmeas, quando comparados com os respectivos animais controlo. Nem o ESL, CBZ ou VPA causaram alterações na proliferação basal do DG. Relativamente à formação de novas células/neurónios, quantificámos as células no DG positivas para BrdU ou que co-localizavam com outros marcadores neuronais, como a "*doublecortin*" (DCX) e o marcador dos núcleos neuronais (NeuN). Curiosamente, observámos que os animais expostos à OXC, tanto os machos como as fêmeas, apresentaram uma diminuição em cerca de 54% e 47%, respectivamente, de células positivas para BrdU na SGZ, e ainda que os machos expostos ao VPA apresentaram uma diminuição em cerca de 60%, quando comparados com os respectivos animais controlo. Com excepção dos machos expostos à CBZ, onde observámos um aumento (16%) dos neurónios maduros ($BrdU^+/NeuN^+$) na SGZ, nenhum dos restantes grupos dos machos teve alterações no número de neurónios maduros ou imaturos. Já em relação às fêmeas, observámos que as que foram expostas à OXC apresentaram menos neurónios maduros e imaturos na SGZ quando comparadas com o grupo controlo. Nem o ESL, CBZ ou VPA induziram alterações na formação de novos neurónios no DG das fêmeas CD1.

Fizemos também uma abordagem *in vitro* com culturas de células estaminais neurais isoladas da região subventricular de ratos para avaliar os efeitos dos AEs (ESL, S-Lic, R-Lic, CBZ, OXC, LTG e VPA) na proliferação basal e nas diferentes fases do ciclo celular (G0/G1, S and G2/M). Foram usadas diferentes concentrações dos AEs, e observámos que o metabolito activo do ESL, S-Lic, não causou qualquer alteração na proliferação basal ou nas fase do ciclo celular. Todos os restantes AEs estudados originaram alterações na proliferação basal ou nas fases do ciclo celular, mas os efeitos maiores foram observados nas culturas expostas ao

VPA (1 e 3 mM), onde a proliferação basal foi diminuída em cerca de 85% e 95%, respectivamente. Em relação ao ciclo celular, constatámos diferentes efeitos dos AEs, para diferentes concentrações. Foi observado um aumento (65%) na percentagem de células na fase G2/M após a exposição à OXC (0.3 mM), e a exposição ao VPA (3 mM) causou uma diminuição em cerca de 70% na percentagem de células na fase G2/M. Também avaliámos o efeito dos AE na morte celular por apoptose nas culturas de SVZ, e apurámos que o VPA (3 mM) e a OXC (0.3 mM) aumentaram a percentagem de células apoptóticas.

De acordo com os resultados obtidos em ambas as abordagens, *in vivo* e *in vitro*, o ESL emerge como um AE com menor risco para as mulheres grávidas com epilepsia e durante o período de amamentação, uma vez que não causou alterações nos parâmetros bioquímicos do sangue e do soro que foram avaliados em murganhos fêmeas CD1 grávidas, assim como não foram detectados efeitos adversos sobre as funções cognitivas e não cognitivas da progénie. Para além disso, não foram observadas alterações na formação de novas células e neurónios no DG do hipocampo das fêmeas CD1 progenitoras e também da sua progénie, respectivamente. Por outro lado, S-Lic, o metabolito ativo da ESL não teve efeitos tóxicos nas culturas de neurónios de hipocampo, e não alterou a proliferação basal e as fases do ciclo celular das culturas de células estaminais neurais isoladas da SVZ de ratos. Em conclusão, o ESL é um anticonvulsivo seguro e eficaz com potencial para ser usado na terapia das mulheres grávidas e com epilepsia, enquanto AEs como a OXC que induziram efeitos negativos nos desempenhos cognitivos, como a memória e aprendizagem, e ainda causaram um impacto negativo na formação de novas células e neurónios no hipocampo, devem ser evitados como opção de tratamento durante o período pré-gestacional, gestacional e de amamentação.

Chapter 1
General Introduction

1. General Introduction

1.1. Epilepsy

1.1.1. Definition, facts and classification

Epilepsy is a poorly defined brain disorder. Actually, its definition has been updated several times during the last two centuries. The last definition dates from 2005 and was accepted by the International League Against Epilepsy (ILAE) and the International Bureau of Epilepsy (IBE): "*Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequence of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure"* (Fisher et al. 2005). Necessarily, it is also important to mention the accepted definition of epileptic seizure, defined as "*a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain"*(Fisher et al. 2005).

Epilepsy affects 50 million people worldwide and 85% of these live in the developed countries. Annual incidence of epilepsy in the world ranges from 30 to 57 cases per 100,000 humans; in North America incidence is approximately 50 cases per 100,000 (Theodore et al. 2006), while in Europe the number of cases varies between 28.9 and 47 per 100,000 (Cross 2011). Actually, one in twenty people might have one epileptic seizure in their lifetime; moreover, epilepsy occurs predominantly in infancy, childhood, adolescence and in elderly people (Forsgren et al. 2005). Although the prevalence of epilepsy is higher in men than in women (Hauser et al. 1993; Banerjee et al. 2009), and some studies propose that women with epilepsy have fewer children than healthy women, one to two in 200 pregnancies are in women with epilepsy (Cross 2011). Indeed, if those women have the right clinical follow up, proper therapy and correct information, common risks due to the disease and treatment should be controlled and they will have healthy children.

There are different types of epilepsy, and the classification and terminology of seizures and epilepsies were last standardized officially in 1981 and 1989, respectively (Berg et al. 2010). However, due to lack of uniformity and misunderstanding between the scientific and the medical community, during the last decade ILAE made an effort to attain international uniformity and a better classification. Finally, in 2009, ILAE proposed a review on terminology for organization of seizures and epilepsies. The new classification and

terminology of seizures is described in Fig. 1.1. The list of forms of epilepsy (electroclinical syndromes and other epilepsies) was updated in the 2006 (Engel 2006), but, as in the case of seizures, its nomenclature was revised in order to simplify concepts that better describe the existing understanding of these syndromes. The list of forms of epilepsy is described in Fig. 1.2.

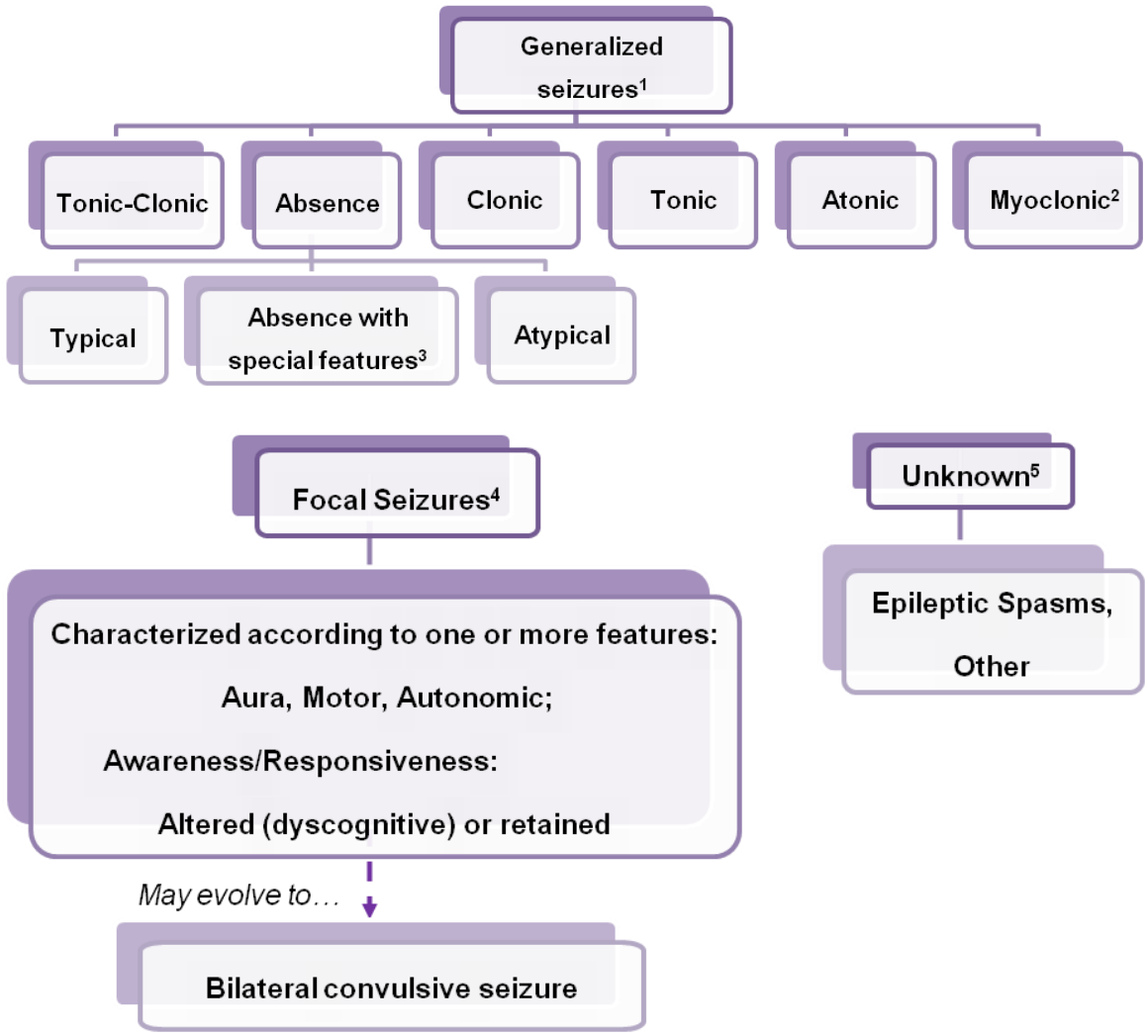

Figure 1.1. Diagram of revised classifications of seizures in 2009 (adapted from Berg et al., 2010 and ILEA, 2010). [1]Arising within and rapidly engaging bilaterally distributed networks; [2]Myoclonic and Myoclonic-atonic; [3]Myoclonic absence and Eyelid Myoclonia; [4]Originating within networks limited to one hemisphere; [5]Insufficient evidence to characterize as focal, generalized or both.

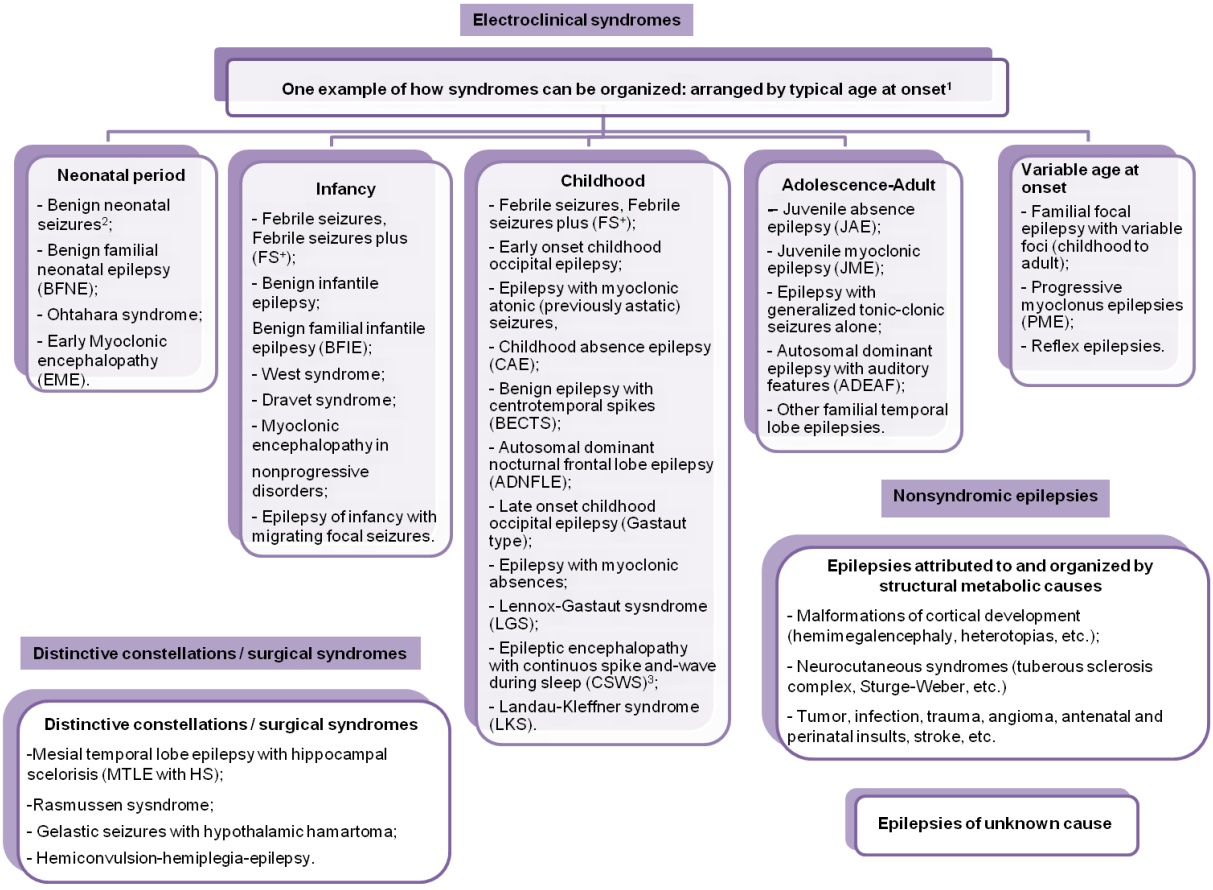

Figure 1.2. Diagram of revised classifications of epilepsies in 2010 (adapted from Berg et al., 2010 and ILEA, 2010). [1]The arrangement of electroclinical syndromes does not reflect etiology; [2]Not traditionally diagnosed as epilepsy; [3]Sometimes referred to as electrical *Status Epilepticus* during slow sleep (ESES); [4] Forms of epilepsies not meeting criteria for specific syndromes or constellations.

1.1.2. Treatment

People with epilepsy that are not clinically followed and treated tend to have an unacceptable quality of life in many aspects, including social, familiar and personal. Nowadays patients with a diagnosed epilepsy syndrome should have access to some kind of therapy and, with the correct therapeutic management; patients with epilepsy can have good quality of life. The first line of therapeutics for epilepsy consists of using a single antiepileptic drug (AED) therapy (monotherapy), while the second line (when first fails) uses a combination of AEDs (polytherapy). However, both therapeutic approaches with AEDs may become inadequate and ineffective for seizures control, and may have inherent side-effects (French et al. 2004). Approximately 20% of patients with epilepsy are drug-

resistant and may be potential candidates for non-pharmacological treatments, such as neurosurgical interventions, stimulation techniques, or the ketogenic diet.

Neurosurgical interventions have become increasingly applied with the improvement of surgical methods, and have turned out to be successful as a treatment for seizure-free conditions. Hence, operated patients are able to recover their quality of life (Wiebe et al. 2001; Schmidt and Stavem 2009). There are two types of surgeries with different approaches and goals, the curative or definitive and the functional or palliative. Briefly, curative surgery removes the brain tissue which caused the onset of seizures; it includes lobectomy, lesionectomy and multilobar or hemispheric surgery (hemispherectomy). Functional surgery is a treatment to change the neuronal pathways responsible for seizure onset. It improves or reduces the occurrence of seizure and includes intervention at corpus callosum and multiple subpial transections (reviewed by Pati and Alexopoulos 2010). In the case of patients that are drug-resistant and are not candidates for epilepsy surgery, these may benefit from neurostimulation techniques, such as vagus nerve stimulation (VNS, for some clinicians it is a palliative treatment), deep brain stimulation (DBS), responsive neurostimulation, and transcutaneous magnetic stimulation (reviewed by Fisher 2012). VNS and DBS have not been approved yet, but first trials showed significant improvement of patients regarding seizure frequency (Rolston et al. 2012). However, an effort should be done towards creating pivotal trials to validate long-term safety and efficacy of these electrical stimulation therapies. Furthermore, other therapies have been emerged and hold promise for therapeutic strategies, such as local drug therapy and cell and gene therapies, however only few experiments have been conducted in animals (reviewed by Pati and Alexopoulos 2010). Finally the ketogenic diet, which has been used for nearly a century, is a significant nonpharmacologic therapy for pediatric epilepsy cases with noncontroled seizures. It consists in a high fat content (>90%) regimen, adequate content in proteins and low in carbohydrates , in order to imitate the effects of starvation; treatment with ketogenic diet can have a duration of 2-3 years and frequently results in a ≥ 90% decrease or elimination of seizures, although the mechanisms underlying these effects remain unknown (reviewed by Pati and Alexopoulos 2010).

1.2. Antiepileptic drugs

Antiepileptic drugs (AEDs) are usually designated as: 1) "old generation AEDs", which refers to AEDs commercialized before the 1990s, such as phenytoin (PH), carbamazepine (CBZ), sodium valproate (VPA), phenobarbital (PB), and 2) "new generation AEDs", which refers to AEDs commercialized after 1990s or later, such as oxcarbazepine (OXC), lamotrigine (LTG), pregabalin (PGB), levetiracetam (LEV), and more recently eslicarbazepine acetate (ESL) (Elger et al. 2009).

1.2.1. Mechanisms of action

Antiepileptic drugs are an heterogeneous group of drugs used as therapy in some brain diseases such as epilepsy, mood disorders, migraine, neuropathic pain, among others. They have different chemical structures, but may share some chemical properties like lipophilicity, and chirality. Moreover, AEDs share molecular targets of their mechanisms of action. The main molecular targets of AEDs are voltage-gated ion channels (sodium, calcium and potassium), neurotransmitter metabolizing enzymes (e.g. GABA transaminase); neurotransmitter transporters (e.g. GABA re-uptake proteins); ligand-gated ion channels (GABA and NMDA, AMPA and kainate glutamate receptors), non-specific cation channels regulating intrinsic membrane properties, and proteins influencing synaptic function (reviewed by Mula 2009; Brodie et al. 2011; Lason et al. 2011). In general, there are three major categories of AEDs according to their mechanisms of action: 1) AEDs that act through modulation of voltage-gated ion channels; 2) AEDs that are enhancers of synaptic inhibition; 3) AEDs that are inhibitors of synaptic excitation (see table 1.1 for detailed information of AEDs and mechanisms of action).

Table 1.1. Mechanisms of action of antiepileptic drugs studied in this work, based on main targets at therapeutic concentrations.

AED	Na^+ channel	Ca^+ channel	GABA potentiation	GABA-A receptor	Antiglutamate neurotransmission
CBZ	++	+	+/-	-	+ (NMDA)
OXC	++	+	+/-	-	+ (NMDA)
ESL	++	-	-	-	+/-
LTG	++	+	+	-	++ (NMDA/AMPA)
VPA	+/-	+/-	+	+/-	+ (NMDA)

++ = primary action; + = secondary action; - = no activity; +/- =controversial. (Adapted from Mula, 2009)

Ion channels work as modulators of membrane excitability, however they w may induce abnormal excitability. The most important ion channels that represent molecular targets for seizure control by AEDs are voltage-gated sodium channel (VGSC), voltage-gated calcium channel and voltage-gated potassium channel (reviewed by Mula 2009). We will now describe in some detail the VGSC as they are the target of some of the AEDs used in the present study, as illustrated in table 1.1.

VGSC are transmembrane channels that mediate transient and fast sodium currents, which produce action potentials, and also mediate persistent or late sodium currents, which are responsible for enhanced repetitive neuronal firing, facilitating hyperexcitability. Depending upon the membrane potential, the functional state of VGSC varies between resting state (closed), activated state (open) or inactivated state (closed). In response to stimulation (change in voltage of the cell membrane), the resting state is converted into the activated state that, in a while, becomes inactivated. The inactivation state stays for milliseconds until the cell membrane repolarizes and can then be activated again for the next action potential (reviewed by Stafstrom 2007). Different AEDs bind to the VGSC at different states of the channel inhibiting its function, thus suppressing normal or high-frequency and repetitive neuronal firing and avoid hyperexcitability. For example, ESL has much higher affinity for the inactivated state of the VGSC, while CBZ and OXC have higher affinity for resting states of the channel ((Almeida and Soares-da-Silva 2007).

Next, we will address in some detail the AEDs used in this study.

1.3. Antiepileptic drugs used in the present study

1.3.1. Carbamazepine and oxcarbazepine

Carbamazepine (CBZ) (5*H*-dibenz[*b,f*]azepine-5-carboxamide) is one of the most widely used AEDs. It was approved in USA in 1974 but has been used in the UK since 1965. It was first used to treat trigeminal neuralgia, but nowadays, in addition to treatment of epilepsy, is also used for treatment of bipolar disorder, manic episodes, psychiatric disorders and neuropathic pain (Albani et al. 1995; Elger and Bauer 1998).

Chemically, CBZ is a dibenzazepine (tricyclic structure) with a double bond between C-10 and C-11, lacks a saturated carbon atom and has an amide linked to its heterocyclic ring (Fig. 1.1) (Elger and Bauer 1998). It is extensively metabolized in the liver by epoxidation to its toxic metabolite, carbamazepine-10,11-epoxide which has the same anti-

convulsive properties of CBZ, and only 1% of the total administered drug is excreted in the intact form. CBZ induces the hepatic cytochrome P-450 enzyme system activity, namely microsomal enzyme system CYP3A4, and hence it induces its own metabolism. It interacts with other drugs, such as other AEDs, contraceptives and antibiotics. 75-85% of the drug binds to plasma proteins and 17-31% of free compound is detected in the cerebrospinal fluid (CSF). The main targets of action of CBZ and its metabolite are VGSC, which are inhibited by CBZ binding. Moreover, CBZ has also other targets, such as calcium and potassium ion channels, and modulation of the respective ions currents, receptors and signalling pathways. Modulation of these targets by CBZ is also involved in its anticonvulsive properties (reviewed by Ambrosio et al. 2002; Chong and Bazil 2010; Bialer 2011).

Carbamazepine has several adverse effects at therapeutic doses that have been identified, such as ataxia, sedation, dizziness, vomiting, nausea and diarrhea, hyponatremia, changes in plasma lipids and sex hormones concentrations (Tateishi et al. 1999). Furthermore, regarding cognitive deficits, CBZ exposure *in utero* did not show adverse effects on cognitive functions in human progeny (Meador and Zupanc 2004), while in animal models CBZ has been reported to have minor effects on memory performance (Shannon and Love 2004; Shannon and Love 2005; Shannon and Love 2007).

Oxcarbazepine (OXC) (10,11-dihydro-10-oxo-carbamazepine) is an improved CBZ derivative, approved in some European countries in the nineties and in USA in 2000. It is a second generation AED to CBZ, used as first-line therapy in the treatment of epilepsy; furthermore, it is also used in the treatment of neuropathic pain and mood disorders (Elger and Bauer 1998; Landmark and Johannessen 2008).

Structurally, OXC has few differences from CBZ in the dibenzazepine ring, with a ketone in place of the carbon double bond (Fig. 1.1). After oral administration, OXC is absorbed almost completely and its metabolism takes place in the liver, but is less affected by inducible cytochrome CYP3A4-mediated oxidative metabolism. Instead, it is rapidly transformed by presystemic metabolic 10-keto reduction to the main active metabolite, 10-hydroxy-carbazepine (MHD) or licarbazepine; both are responsible for the pharmacologic actions of OXC. Thus, without formation of epoxide, OXC is less toxic to the system than CBZ and has better tolerability. However, both share the same anticonvulsive properties (for review see: Ambrosio et al. 2002; Chong and Bazil 2010; Bialer 2011). Protein binding of OXC is 38% of total drug and may induce cytochrome P450 enzymes (CYP450). The targets of OXC and MHD action are mostly the same of CBZ, and the main mechanism of action is the

inhibition of VGSC. However, OXC also inhibits several types of voltage-gated calcium channels (reviewed by Schmidt and Elger 2004; Landmark and Johannessen 2008).

Oxcarbazepine at therapeutic doses has several adverse-effects, such as somnolence, headache, dizziness, hyponatremia, rash, fatigue, ataxia and alopecia are the most reported. Regarding cognitive effects, few studies exist in adults; however, and although scarce information is available, OXC has not been associated with impairment of neuropsychological functions (Aikia et al. 1992; Salinsky et al. 2004).

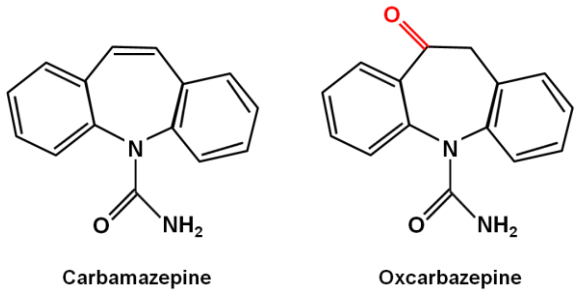

Fig. 1.1. Chemical structure of carbamazepine (CBZ) and its second generation derivative, oxcarbazepine (OXC). OXC differs from CBZ with a ketone in place of the carbon double bond (red). (Adapted from Bialer 2011).

1.3.2. Eslicarbazepine acetate

Eslicarbazepine acetate (ESL) (BIA 2-093, S-(-)-10-acetoxy-10,11-dihydro-5H-dibenz[*b,f*]azepine-5-carboxamide) is a novel once-daily antiepileptic drug completely developed by BIAL (BIAL-Portela e Companhia, SA, S.Mamede do Coronado, Portugal), as third generation to CBZ. ESL was specifically developed in order be less toxic than CBZ by preventing toxic metabolites production, and to have a better tolerability and efficacy in the treatment of epilepsy (Benes et al. 1999). ESL was approved in Europe since 2009 and it has been found to be well tolerated and effective in adults as adjunctive treatment for partial-onset seizures (Elger et al. 2009). Furthermore, pharmacokinetic studies have shown that ESL is well tolerated in children with epilepsy (Almeida et al. 2008a); ESL did not require dosage adjustment in patients with moderate hepatic impairment (Almeida et al. 2008b); ESL has similar pharmacokinetics in elderly and in young healthy subjects (Almeida et al. 2005); and pharmacokinetics of ESL were not affected by food intake (Maia et al. 2005) or by gender (Falcao et al. 2007).

Eslicarbazepine acetate is chemically related to CBZ and OXC; however, it presents some differences within its structure, which results in differences in the metabolism, allowing ESL to be less toxic than the other AEDs. ESL shares the dibenzazepine nucleus with the 5-carboxamide substituent with CBZ and OXC, but differs at the 10,11-position (Fig, 1.2). After oral administration, ESL is rapidly absorbed and its metabolism takes place in the liver through a hepatic first-pass hydrolytic metabolism by liver esterases. ESL does not yield toxic metabolites like CBZ, which is metabolized to CBZ-10,11-epoxide; thus, ESL does not cause enzyme induction or autoinduction (Almeida and Soares-da-Silva 2007). Recently it was shown that ESL is rapidly and extensively metabolized to the main active metabolite eslicarbazepine (S-Lic), and then, by an oxidation to OXC, undergoes a minimal chiral inversion to R-licarbazepine (R-Lic). Both R-Lic (4.5%) and OXC (0.5%), are minor metabolites detectable in plasma (Perucca et al. 2011). S-Lic is also a metabolite of OXC, but, as the active entity of ESL, its bioavailability after ESL and OXC administration is close to 100% and 70%, respectively (reviewed by Bialer and White 2010; Bialer 2012). Protein binding of S-Lic is 30% and is concentration-independent up to 100 mg/L; furthermore, after an oral dose, more than 90% of ESL metabolites are excreted by the kidneys, two-thirds as free form and one-third as the glucuronide conjugate. However, in patients with epilepsy, S-Lic clearance is increased by CBZ, PB and PHT, while other AEDs, such as LTG, topiramate (TPM) and VPA do not induce changes on plasma concentrations of S-Lic. In addition, AEDs used concomitantly with ESL (except for PHT), have not their clearance affected by ESL. ESL has minimal or no inhibitory effect on CYP450 enzymes, as well as on other liver enzymes such as epoxide hydrolase and UGT1A1 and UGT1A6. However, ESL may have some interactions with non-AED medication, for instance, ESL may reduce the efficacy of oral contraceptives, and may interfere with Simvastatin, and thus both need dose adjustment when used concomitantly with ESL (reviewed by Bialer and Soares-da-Silva 2012).

Eslicarbazepine acetate, like CBZ and OXC, was found to inhibit VGSC, namely by binding preferentially to the inactivated state of the channel. ESL and its metabolites reduce repetitive neuronal firing by preventing the inactivated sodium channel to return to the active state. In fact, it was shown *in vitro* that the affinity of ESL for the inactivated VGSC was higher than the affinity for the resting state (Bonifacio et al. 2001). Other plausible targets of ESL are secondary to the main mechanism of action of ESL. For instance, *in vitro* it was observed that ESL inhibits endogenous glutamate release from hippocampal synaptosomes

(Ambrosio et al. 2001) and from striatal slices (Parada and Soares-da-Silva 2002), while *in vivo* ESL did not affect glutamate release. However, these effects are not likely to contribute to the anticonvulsant properties of ESL (reviewed by Ambrosio et al. 2001).

Eslicarbazepine acetate also shares adverse effects with other AEDs, which are dose-dependent. The most common are dizziness, somnolence, abnormal coordination, headache, rash, disturbed attention, blurred vision, diplopia, vertigo, vision, diarrhea, nausea and vomiting. Recently, Milovan and colleagues observed that OXC-treated healthy volunteers had more adverse events than ESL, but both had similar cognitive profiles, and changes on performance of cognition tasks did not have clinical significance (Milovan et al. 2010). However, further studies should be done regarding cognitive effects of ESL on patients, at different ages as well as on women with epilepsy and their progeny.

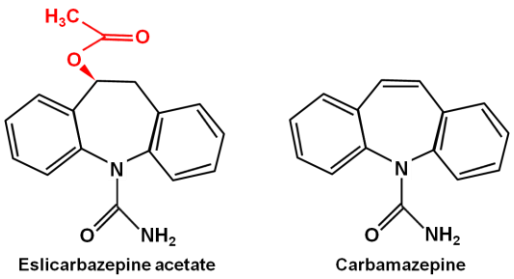

Fig. 1.2. Chemical structure of eslicarbazepine acetate (ESL) a third generation to CBZ. ESL differs from CBZ in the 10,11-position with a acetate group (red). (Adapted from Bialer 2011).

Eslicarbazepine and R-licarbazepine

It is known that ESL is extensively metabolized to eslicarbazepine (S-Lic), the active metabolite mainly responsible for the anticonvulsive properties of ESL. However, R-Lic, the minor metabolite of ESL and enantiomer of S-Lic (produced by chiral transformation through oxidation of OXC, (Fig. 1.3) (Perucca et al. 2011), is almost devoid of activity (Hainzl et al. 2001). Other particular difference between the enantiomers is the transport through the blood-brain barrier (BBB). In mice, R-Lic is a potential substrate to be transported by P-glycoprotein in BBB, which may difficult its entry into brain, which decreases its brain concentration. On the ot her hand, S-Lic is not transported by P-glycoprotein mediated transport (Fortuna et al. 2012). In fact, in mice, after oral administration of both

enantiomers, biodisposition of S-Lic in brain-plasma was almost twice as that of R-Lic (Alves et al. 2008).

-Lic is also an active metabolite of OXC, however, due to its favourable pharmacokinetics (its water solubility compared to CBZ and OXC is >10 fold higher), as well as its higher plasma availability compared to R-Lic, makes S-Lic a better anticonvulsant. Increasing daily doses of OXC, besides increasing bioavailability of S-Lic, lead to an increase of hepatotoxicity. Furthermore, oral bioavailability of S-Lic after OXC administration is near 70% while after ESL administration is near 100%. Therefore, the use of ESL as pro-drug of S-Lic makes its favourable pharmacokinetic properties possible to being used as active entity in ESL therapy. Moreover, ESL is a better AED when compared to CBZ in treatment of patients with epilepsy (Bialer 2011). In conclusion, clinical evidence suggests that ESL should preferentially be used in the treatment of epilepsy (reviewed by Bialer and Soares-da-Silva 2012).

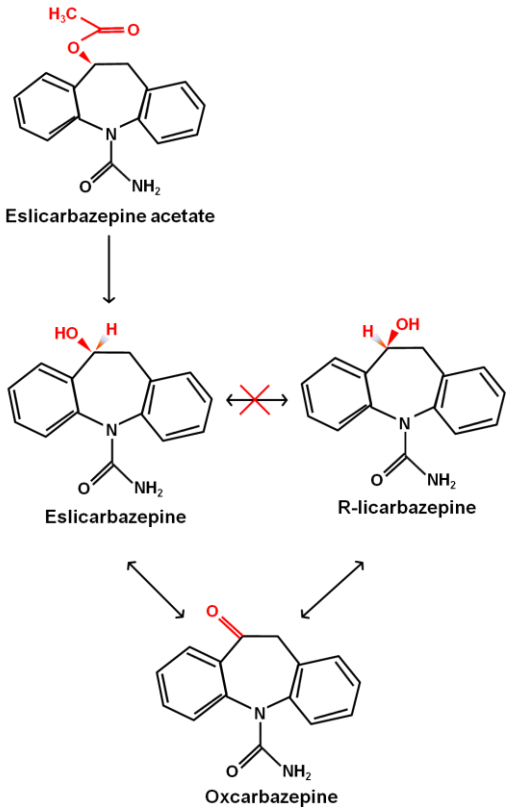

Fig. 1.3. Chemical structure and metabolic pathways of eslicarbazepine acetate (ESL) and its metabolites, Eslicarbazepine (S-Lic), R-licarbazepine (R-Lic) and oxcarbazepine (OXC). ESL is rapidly and extensively metabolized to S-Lic, which undergoes chiral inversion to R-Lic (presumably through oxidation to OXC). (Adapted from Perucca et al. 2011).

Evidences of better efficacy and tolerability

Eslicarbazepine acetate has been shown to have better efficacy and tolerability compared to other AEDs, mainly CBZ and OXC. Its metabolism and pharmacokinetics in healthy volunteers and special populations have been well studied over the last decade: in healthy people, pharmacokinetics of ESL was linear and dose-dependent after single and multiple dosing (Almeida and Soares-da-Silva 2004); age and gender did not affect ESL pharmacokinetics, as well as food (Almeida et al. 2005; Maia et al. 2005); clearance of ESL metabolites were decreased in patients with moderate to severe renal impairment (Maia et al. 2008), but not in patients with mild to moderate hepatic impairment (Almeida et al. 2008b). Furthermore, in people with epilepsy, clinical studies showed that clearances of ESL metabolites in children (2-16 years) had an inverse correlation with age (Almeida et al. 2008a); in a phase III study with adult patients with partial-onset seizures, ESL was found be well tolerated and efficacious as once-daily dose add-on treatment in patients that were refractory to treatment with one or two AEDs (CBZ, LTG and VPA). In fact, in this study 1,200 mg/day reduced seizure frequency and had the highest responder rate when compared to placebo (Elger et al. 2009); in addition, other approach was done with single doses (1,800 and 1,200 mg/day) and evidence corroborated previous studies (Gil-Nagel et al. 2009); recently, in other phase III study with adults on chronic treatment with ESL (more than one year) and with more one or two concomitant AED (CBZ and VPA), S-Lic was the first active metabolite found in plasma, and its plasma levels were dose-dependent (Perucca et al. 2011). In conclusion, ESL is an alternative to existing AEDs with some benefits for treatment of partial-onset seizures in patients resistant to treatment with 1-2 concomitant AEDs.

Several studies were performed *in vitro* and *in vivo* which showed that ESL is less toxic and more efficacious than CBZ and OXC: ESL was shown to be an effective anticonvulsant against seizures induced by maximal electroshock, and was similar or more efficacious in protecting rats from seizures induced by metrazol (Benes et al. 1999), and inhibited the release of glutamate induced by veratridine from striatal slices better than CBZ and OXC (Parada and Soares-da-Silva 2002). In cultured hippocampal neurons, ESL was shown to be less toxic than CBZ and OXC: ESL at high concentrations induced slight nuclear condensation but in less extent than CBZ and OXC and did not induce apoptotic markers such as caspase-3 (Ambrosio et al. 2000); in addition it did not induce degeneration and swelling of neurites neither produced ROS or decreased ATP levels in hippocampal cultured

neurons (Araujo et al. 2004). However, less is known about the possible neurotoxic effects of ESL metabolites, S-Lic and R-Lic in cultured hippocampal neurons, as well as in neural stem cell cultures, and we have addressed some of these aspects in the present thesis.

1.3.3. Lamotrigine

Lamotrigine (LTG) [6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine] is a phenyltriazine which makes it unrelated to other AEDs (Fig. 1.4). It was licensed for therapeutic use in 1991 and approved in USA in 1994 for the treatment of partial seizures. Moreover, it has been an important adjunctive treatment of Lennox-Gastaut syndrome in pediatric and adult patients. Besides its use in different epilepsy syndromes, LTG was also approved for treatment of bipolar disorders since it acts as an effective mood stabilizer; however, even off-licence, it has been used for treatment of migraine, neuropathic pain, psychosis, schizophrenia, peripheral neuropathy and trigeminal neuralgia (reviewed by Johannessen Landmark and Patsalos 2010).

After oral ingestion, LTG is quickly absorbed and has a bioavailability close to 100%. With 55% of protein binding, this AED is metabolized by hepatic glucuronidation via UDP-glucuronosyltransferase (UGT1A4) to various metabolites which are pharmacologically inactive (reviewed by Johannessen Landmark and Patsalos 2010), and excreted by kidneys. It is able do induce auto-induction at higher doses and is affected by other AEDs (CBZ, OXC, PB, PH and primidone), which induce LTG metabolism. On the other hand, VPA inhibits its metabolism; hence plasma levels of LTG are increased by two fold. This pharmacodynamic interaction with VPA increases therapeutic efficacy of both but, on the other hand, aggravates the side-effects (Brodie and Yuen 1997).

The main mechanism of action of LTG is the same of CBZ and OXC, it inhibits VGSC, thus reduce neuronal firing activity. It has also as targets the voltage-gated calcium channels; moreover, it increases dendritic hyperpolarization-activated cation current, which decreases action-potential generation (reviewed by Johannessen Landmark 2008).

Lamotrigine has some side-effects at therapeutic doses, such as rash, psychosis, somnolence, insomnia, ataxia, blood dyscrasias, diplopia, headache, tremor, hypersensitivity reactions, and gastrointestinal disorders. This AED has been vastly used during pregnancy, and the existing data shows that LTG does not have a risk of inducing major malformations but has an increased risk of causing orofacial clefts (Meador and Penovich 2008).

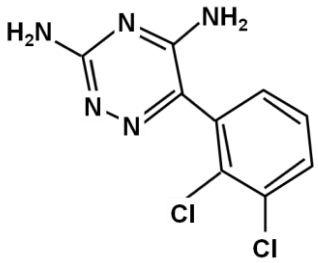

Fig. 1.4. Chemical structure of lamotrigine (LTG). LTG is structurally different from other AEDs. (Adapted from Bialer 2011).

1.2.5. Sodium Valproate

Sodium valproate (VPA) (2-propylvaleric acid, 2-propylpentanoic acid or n-dipropylacetic acid) is a branched short-chain fatty acid that does not possess a cyclic ring or a nitrogen atom, and it is structurally different from other AEDs (Fig. 1.5). It was first reported as an AED in 1964, and it was first approved for marketing in France (1964) and after that it began to be used worldwide. However, it was only approved by US-FDA in 1978 (reviewed by Chateauvieux et al. 2010). VPA is the most prescribed AED because of its wide spectrum in the treatment of several epilepsy syndromes and seizures types. VPA is also an efficient mood stabilizer, has beneficial effects in depression, migraine headaches, neuropathic pain and schizophrenia (reviewed by Landmark 2007).

After oral administration, VPA is rapidly absorbed and has a bioavailability near to 100%. A high percentage of the administered VPA (85-95%) binds to plasma proteins and the unbound fraction is only 7-9%. However, its serum concentration is dose-dependent and protein binding decreases at higher levels (> 100 mg/mL), as well as in hepatic and renal patients. Moreover, its levels are decreased by enzyme-inducing drugs, such as CBZ. VPA is metabolized in the liver by beta-oxidation and glucuronidation to its hepatotoxic metabolites 4-ene-VPA and 2,4-diene-VPA, and less than 4% is excreted unchanged by the kidneys (reviewed by Trojnar et al. 2004).

This AED has several mechanisms of action. VPA blocks voltage-gated sodium channels, in addition to potassium and calcium channels; however these mechanisms may not have an important role in seizure prevention (Loscher 1992). On the other hand,

GABAergic neurotransmission is modulated by VPA. VPA decreases excitability by enhancing GABA release, as well as by increasing GABA synthesis (Loscher 2002). Moreover, VPA affects intracellular signaling pathways by modulation of ERK, inositol metabolism, glycogen-synthase-3, protein kinase C, and several functional pathways in the brain by affecting early inducible genes (reviewed by Landmark 2007).

Valproate was classified as a Histone Deacetylase Inhibitor (HDACI), thus VPA has a direct action on gene transcription by inhibiting histone deacetylation, which allows transcription sites to be more accessible for transcription. Furthermore, VPA has been investigated and used in clinical trials for treatments in AIDS (as an antiviral complement), cancer treatments and neurological disorders, such as Amyotrophic Lateral Sclerosis and Dementia (reviewed by Chateauvieux et al. 2010).

However, although VPA has had a wide clinical use, it also has a vast list of side effects, mainly due to its hepatotoxicity, teratogenicity and adverse effects on cognition, mainly in young patients. Some of the side-effects reported for VPA are: weight gain, drowsiness, nausea, unsteadiness, spina bifida, anencephaly, cardiac defect, dysmorphic features, valproate syndrome (decrease of intrauterine growth), decrease fertility, craniofacial, skeletal or limb defects, autism spectrum, decrease of IQ, child hepatotoxicity, thrombocytopenia, platelet dysfunction, aplastic anemia (reviewed by Chateauvieux et al. 2010).

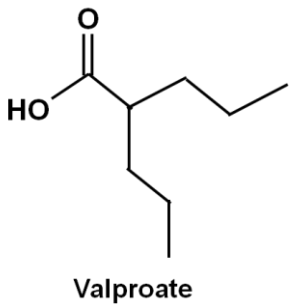

Fig. 1.4. Chemical structure of Valproate (VPA). VPA is a branched short-chain fatty acid that does not possess a cyclic ring or a nitrogen atom, and it is structurally different from other AEDs. (Adapted from Bialer 2011).

1.4. *Antiepileptic drugs and neurotoxicity*

Antiepileptic drugs are essentially used to prevent or interrupt epileptic seizures by acting on its possible onset. Furthermore, it is expected that they have a neuroprotective role on the brain to face neuronal damage induced by seizures. However, AEDs may also exert unfavourable effects, like neurotoxicity, and since the 1970s neurotoxic effects of AEDs have been recognized, particularly the pro-apoptotic effects occurring during brain development. Patients that are more vulnerable to neurotoxicity are infants, children and fetus subject to *in utero* exposure to AEDs. During development of the brain there is a transitory stage of rapid growth, the "brain growth spurt" period, which is the period that brain weight increases in average 5% to 10% (reviewed by Ikonomidou and Turski 2010). During this period there is an excess of neurons and occurs a significant growth of neural network arborization with elongation and branching of dendrites and neurons, which allows an enhancement in brain functioning (Bittigau et al. 2002). The "brain growth spurt" period takes place at different times in human and rodents due to intrinsic differences, but both periods are comparable (Bayer et al. 1993). In mice and rats this period occurs postnatally, with peak growth rate at P7-P10 until third week, while in humans this period starts prenatally during the third trimester and has its first peak of growth rate at birth, (Ikonomidou 2010); however, other growth peaks happen at different stages of brain development. Parallel to this event in the developing brain, there occurs programmed cell death (physiological apoptosis), which is not transitory, but has an even more important role during the "brain growth spurt" period. Physiological apoptosis will eliminate the excess or unsuccessful newly-born neurons from the different brain areas and distinct sub-populations of neurons, as well as damaged neural cells (reviewed by Ikonomidou and Turski 2010). There are several endogenous regulators of apoptosis, such as neurotransmitters, growth factors and cytokines, as well as different executers of this process, such as caspases and calpains (Henderson 1996; Ikonomidou et al. 2001). Accordingly, any factor or agent that acts on apoptosis regulators, or even at the executers level, may trigger non-physiological apoptosis and hence may end in death of neurons that should not be eliminated (Webb et al. 2001). Actually, AEDs may upset neuroprotective mechanisms in the brain, which in turn imbalance the opposite and neurodestructive systems. Thus, AEDs may induce sensitive neurons to undergo apoptotic death in the developing brain, by unbalancing the programmed neuronal cell death (Bittigau et al. 2002).

A loss in the number of newly-formed neurons may compromise the growth of neural network arborization, and hence impair brain function in structures such as hippocampus and neocortex (Faiella et al. 2000; Kaindl et al. 2006) which in turn cause malformations and cognitive dysfunctions, among other adverse-effects.

Assessing neurodegeneration in the developing brain is not easy to be conducted in humans, but it can be done in animal models. In fact, it was reported that administration of AEDs at clinical doses in the developing brain of rodents may trigger widespread apoptotic neurodegeneration and neurotoxicity was also shown to be exacerbated by combinations of AED therapy (Bittigau et al. 2002; Olney et al. 2004; Ikonomidou 2010). Some other studies showed that following VPA (50-400 mg/kg on P7) or PB (20-100 mg/kg, P7) treatments dose-dependent neurodegeneration was observed (Bittigau et al. 2002); CBZ (100 mg/Kg, P8) and PHT (50 mg/kg, P8) also induced cell death (Kim et al. 2007). In addition toxicity induced by AEDs was also observed *in vitro,* in cultured neurons. For instance, CBZ caused apoptosis in cultured cerebellar granule cells (Gao et al. 1995; Nonaka et al. 1998), as well as in cultured hippocampal neurons, and the same was true for OXC (Ambrosio et al. 2000). More recently, it was observed that OXC was the most toxic drug in these cultures, while the new VGSC blocker, ESL, did not induce apoptosis nor caused structural damage to the neuritic network (Ambrosio et al. 2000; Araujo et al. 2004). However, the effect of ESL metabolites, S-Lic and R-Lic, on the viability of neuronal cells, had not been addressed yet, and we aim at clarifying these aspects in the present work.

Apoptosis, rather than necrosis, has been reported as the main mechanism of cell death induced by AEDs. Necrosis is also a mechanism of cell demise; however the AEDs studied in this work, even the most toxic (OXC), do not seem to cause necrosis. For this reason, necrosis will not be further detailed in this work.

In previous *in vitro* studies, our group showed that CBZ and OXC at high concentrations (0.3 mM) cause apoptosis-like features, like condensed chromatin nuclei, and activated caspase-3 (in the case of OXC) (Ambrosio et al. 2000; Araujo et al. 2004). Furthermore, OXC (0.3 mM) increases reactive oxygen species and decreases ATP levels, although without changes on energy charge (Araujo et al. 2004). These data suggest that AEDs cause neuronal cell death by apoptosis, by activation of caspase-3-like enzymes. Caspases are a vast family of proteolytic enzymes which are present in the cells as inactive precursors. They are strictly related with cell death and may be activated by two pathaways, the extrinsic pathway (at membranar receptors) or the intrisic pathaway (at mithocondria).

Both pathways may be triggered by the same stimuli, for example, elevated intracellular calcium, but then each pathway has different effectors which culminate in the activation of caspase-3, the main apoptotic effector. In turn, active caspase-3 has several substrates such as DNA repair enzyme (e.g. poly-ADP-ribosome-polymerase, PARP) (reviewed by Timmer and Salvesen 2007), cytoskeletal proteins (e.g. alphaII-spectrin) (Bernath et al. 2006), anti-apoptotic proteins (e.g. Bcl-2)(Kirsch et al. 1999).

Bittigau and colleagues reported that AEDs induce neuronal death by apoptosis by reducing the levels of the active phosphorylated forms of extracellular signal regulated kinase (ERK 1/2) and protein kinase B (AKT), as well as by mechanisms involved in the synthesis of brain derived neurotrophic factor and neurotrophins 3 and 4. Actually, both kinases, ERK and AKT, are involved in two pathways, the MEK/ERK 1/2 and PI3K/AKT, respectively, which are involved in neuronal survival (Bittigau et al. 2002). In addition, Hetman and Godz (2004) showed that ERK1/2 has a prosurvival activity in neurons (Hetman et al. 1999; Hetman and Gozdz 2004), but, depending on cell type and on the signal that triggers cell death, it also has a role in apoptosis; indeed persistent activation of ERK 1/2 contributes to apoptosis in primary cortical neuronal cultures (Satoh et al. 2000; Stanciu et al. 2000). Regarding PI3K/AKT, this pathway has different mechanisms that play a role in surviving of neurons (Yao and Cooper 1995; Dudek et al. 1997). Actually, activated AKT inhibits apoptosis (Brazil and Hemmings 2001), whereas its deactivation accompanies cell death which may be induced by many different agents (Luo et al. 2003). Thus, changes in these signalling pathways reflect an imbalance between neuroprotective and neurodegenerative mechanisms in the brain, which can lead to apoptotic cell death (Bittigau et al. 2003; Asimiadou et al. 2005).

It is therefore of utmost importance to explore whether neurodegeneration caused by some of the AEDs used in the present study exert their effects by affecting some of the signalling pathways mentioned above.

1.5. Neurogenesis

Mammalian brain, including human brain, has the ability of producing new neurons and glial cells throughout life, in a balanced way (Eriksson et al. 1998). Neurogenesis is an automated program of neuronal differentiation that occurs on a tightly regulated way

involving endogenous interveners such as neurotransmitters, neurotrophic factors, hormones, and neuromodulators, which may be changed in particular situations such as pregnancy (Shingo et al. 2003), injury to the brain (Zhao et al. 2003), or voluntary exercise (van Praag et al. 1999). Since the nineties there has been a boom of data from rodents, used as the animal model, demonstrating the occurrence of neurogenesis namely in two particular regions of the adult mammalian brain, the olfactory bulb (OB) and the hippocampus, both located in the telencephalon. Within these regions there are apparently particular environments which offer optimal conditions for occurrence of the several stages of adult neurogenesis: 1) proliferation of adult neural stem cells (NSCs) or progenitors; 2) fate determination/migration; 3) differentiation/maturation; 4) survival and 5) integration in the existing neuronal network of newborn neurons (reviewed by Ming and Song 2005). Moreover, in the OB and in the hippocampus there are specific neurogenic niches (microenvironment), the subventricular zone (SVZ) and the subgranular zone (SGZ), respectively. A neurogenic niche hosts active NSCs or progenitors, as well as different cell types and tissue components, such as progeny of progenitor cells, astrocytes, oligodendrocytes, microglia, immune cells, vasculature and its basement membrane (Mercier et al. 2002). Moreover, it is also important the presence of proteins, such as the ciliary neurotrophic factor (Emsley and Hagg 2003), and basic fibroblast growth factor (bFGF) (Zheng et al. 2004). In the mammalian brain, both neurogenic regions are close to one another in each hemisphere; actually the hippocampus outlines the medial wall and bottom of the lateral ventricle. Though, developmental patterns of neurogenesis are different in each region (van Praag et al. 2002; Overstreet et al. 2004), as described in next sections.

It was only in 2002 that Carlen and colleagues demonstrated that the newly formed neurons within the OB and the DG in the adult brain are functional and integrate into the pre-existing neuronal network (Carlen et al. 2002). Noteworthy, in the last decade there has been emerging evidence of neurogenesis in other brain regions, namely in the spinal cord (Yamamoto et al. 2001), striatum, amygdala (Bernier et al. 2002), mesencephalon (Zhao et al. 2003), hypothalamus (Gould et al. 2001; Xu et al. 2005), neocortex (Dayer et al. 2005), and dorsal vagal complex (Bauer et al. 2005). These evidences are mainly supported by *in vitro* studies using primary cultures of hypothalamus, cerebellum, spinal cord, cortex and optic nerve, which differentiate into neurons and macroglial cells (Kirschenbaum et al. 1994; Palmer et al. 1999; Kondo and Raff 2000; Laywell et al. 2000; Nunes et al. 2003;

Markakis et al. 2004; Lee et al., 2005a). Adult neurogenesis has only been reported *in vivo* in the SVZ and in the SGZ of the dentate gyrus (reviewed by Ma et al. 2009) and, more recently, in the hypothalamus, within the ependymal layer on the base of the third ventricle(Lee et al. 2012). However, efforts are being developed so that experimental support of neurogenesis may be obtained in other brain regions, especially with *in viv*o studies.

1.5.1. Neural stem cell proliferation and differentiation in the SVZ

The SVZ is a thin layer adjacent to the ependymal cell layer which lines the lateral wall of the lateral ventricles. Its proliferative neural stem cells may become neurons or glia (Altman 1969; Doetsch et al. 1997). Within SVZ there are four types of cells: ependymal cells; glial fibrillary acidic protein (GFAP)-positve progenitors (type B, also referred as SVZ astrocytes); transit amplifying cells (type C), and migrating neuroblasts (type A) (Doetsch et al. 1999; Alvarez-Buylla and Lim 2004; Garcia et al. 2004) (Fig. 1.5.II). It has been accepted that the resident adult neural stem cells in this neurogenic niche are SVZ astrocytes which express GFAP and are morphologically similar to astrocytes from other brain regions (Doetsch et al. 1999; Garcia et al. 2004). Stem cells (type B) proliferate and give rise to transient amplifying cells (type C), which rapidly proliferate and generate neuroblasts (type A). Neuroblasts migrate as chains through the rostral migratory stream (RMS) into the OB. Immature neurons that reach OB are attached/sheated by astrocytes and migrate radially into outer cell layers. After located, they differentiate into GABAergic granule interneurons in the granule cell layer or in periglomerular dopaminergic interneurons in the glomerular layer. The fate of newly formed neurons in the OB may be decided before neuroblasts leave SVZ, however the mechanisms underlying this process are still unknown (Merkle et al. 2007) (Fig. 1.5.III). Neural stem cells from SVZ can be cultured *in vitro* as neurospheres (floating aggregates). These cultures were described for the first time by Reynolds and Weiss (1992), and are characterized by the capacity for self-renewal and multipotency assigned to NSCs. Using a brain from an adult mice, they obtained its first culture by isolating the striatal region, which contained SVZ, and originated an undifferentiated population of cells that expressed nestin (intermediate filament). Nestin has been considered a marker of adult stem cells and adult neural progenitor cells (Reynolds and Weiss 1992). Once obtained, stem cells and progenitors cells from SVZ are kept in a serum-free medium, supplemented with epidermal growth factor (EGF); cells grow, proliferate and constitute floating aggregates, the neurospheres. In the present work, neurosphere cultures

derived from SVZ were used as the model to study the effects of AED exposure on the proliferation and on cell cycle distribution of NSCs, as described in chapter 6.

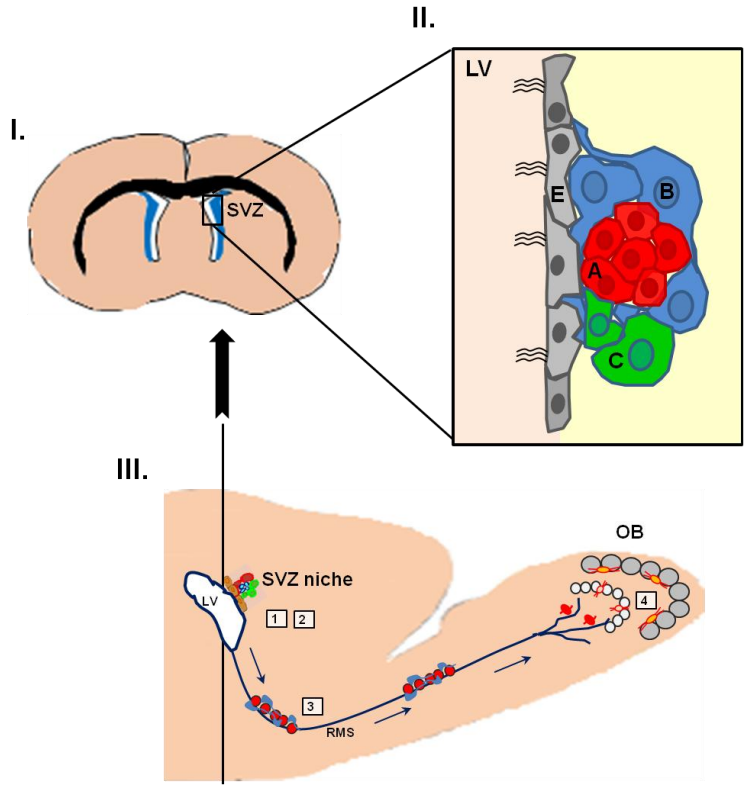

Fig. 1.5. Architecture of the subventricular zone (SVZ) in the lateral ventricles of mouse brain and neurogenesis in the olfactory system. I. Cross section of the adult mouse brain showing the SVZ next to the walls of the lateral ventricles (LV). **II.** Detail and schematic illustration of the SVZ cell types: E cells (gray) – multiciliated ependymal cell; B cells (blue) – SVZ astrocytes; C cells (green) - rapidly dividing precursors; and A cells - neuroblasts (red). **III.** Different steps of neurogenesis into the OB: 1 – Proliferation (SVZ astrocytes originate rapidly dividing precursors), 2 – Fate specification (rapidly dividing precursors differentiate into neuroblasts), 3 – Migration (neuroblasts migrate from SVZ to OB through the rostral migratory stream, RMS), 4 – Synaptic integration (newly neurons that reach OB migrate radially and incorporate into the neuronal network of the OB as interneurons. SVZ – subventricular zone; LV – lateral ventricle; RMS – rostral migratory stream; OB – olfactory bulb. (Adapted from Ming and Song 2005)

As mentioned before, during brain development, there is a period of growth spurt, characterized by an increase of proliferation with the production of an excess of new neurons. Changes in any stage of neurogenesis may induce neuronal migration disorders, which in turn may negatively affect newly formed neurons and synaptogenesis (Bittigau et al. 2003; Clancy et al. 2007; Hofmann 2010). Neurotransmitters like GABA and glutamate have a crucial role as modulators of neurogenesis, since their signalling pathways regulate proliferation, neuroblast migration and differentiation (reviewed by Kempermann 2011). However, these neurotransmitters are in turn indirectly modulated by external agents that act on their receptors; GABA and NMDA receptors are targets of some AEDs' mechanism of action. In *in vitro* models there is evidence showing that changes in receptor activity show effects on the neuronal cultures. For instance, GABA receptor antagonists were shown to change migration (Behar et al. 2000), while NMDA receptor activation changes radial neuronal migration (Monti et al. 2002; Fukuda et al. 2003).

1.5.2. Differentiation in the hippocampus: studies in cultured hippocampal neurons and in the dentate gyrus of the hippocampus

The SGZ is a thin cell layer of proliferative cell progenitors between the granular cell layers and the hilus of the dentate gyrus (DG) (Fig. 1.6.B). The dentate gyrus is a substructure of the hippocampus, C-shaped, constituted by small, round granule cells which form the fascia dentata, and a core, the hilus. Within the hippocampus there is other substructure physiologically distinct, the *cornu Ammonis* (CA), which is composed by three subfields: CA1, CA2 and CA3 (Fig. 1.6.B); each subfield contains pyramidal cells that are distinguished by their size, afferent input and efferent projections. It is through CA1 pyramidal cells that the DG and *cornu Ammonis* connect (Fig. 1.6.B).

Like in the SVZ, in the SGZ of the DG there are different types of cells according to their stage in the differentiation process, namely two different neural progenitor cells (NPCs): type one (vertical astrocytes) and type two (intermediate precursors); type three cells (intermediated neuroblast) and neurons are also present (Fig.1.6.C). NPCs can be distinguished by their morphology and expression of particular molecular markers. Type one progenitors express nestin, Sox-2, and GFAP (Fukuda et al. 2003; Garcia et al. 2004; Suh et al. 2007); its soma is triangular-shaped and extend a strong apical process into the molecular layer; they are abundant in the SGZ and do not divide often (Filippov et al. 2003). Type one progenitor cells may result in type two but do not express GFAP. Type two

progenitor cells express Sox-2, a transcription factor that is also expressed in embryonic stem cells and NSCs from the SVZ, and is important for cells to keep their properties, such as "stemness". They also express nestin and proteins of neuronal line as doublecortin (DCX) and poly-sialated neural cell adhesion molecule (PSA-NCAM); moreover, they are morphologically different from type I. Type three cells covers a transition of a neuroblast with slowly proliferation rate, to a post mitotic immature neuron. These cells may express neuronal markers (DCX, PSA-NCAM, NeuroD), but lack glial markers, and may present a highly variable morphology (Ehninger and Kempermann 2008).

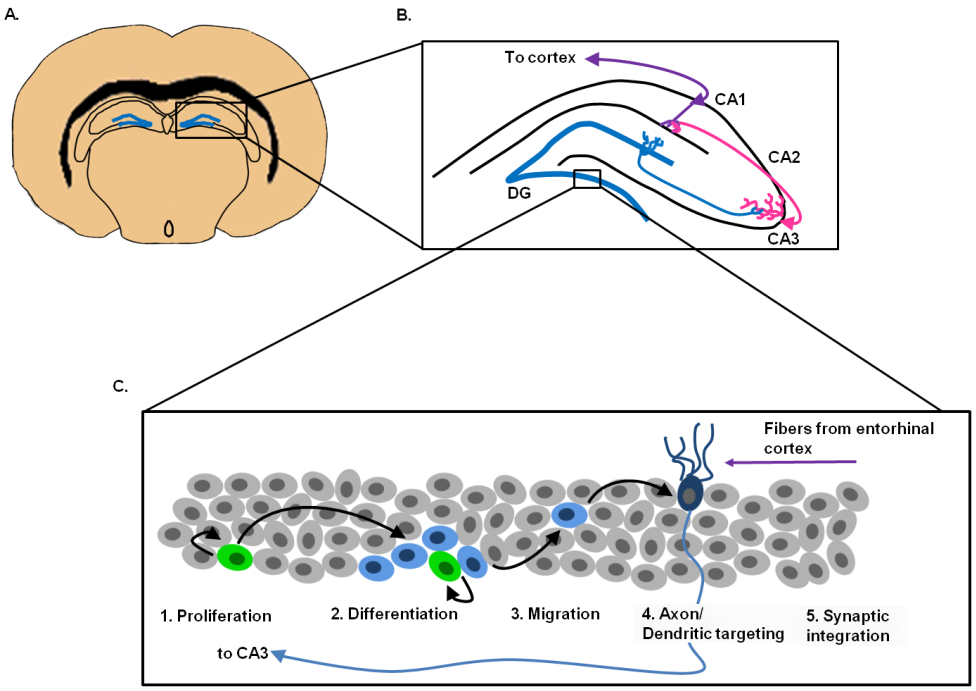

Fig. 1.6. Schematic representation of the hippocampus and of the steps of neurogenesis.
A) Illustration of an hippocampal slice and their substructures, dentate gyrus (DG) and *cornu Ammonis* and the three subfields, CA1, CA2 and CA3. Granule cells of the DG extend axons from the inner granule zone to the dendrites of pyramidal cells in the CA3 layer. CA3 pyramidal neurons extend their axons to CA1 dendrites and these cells extend axons to multiple neural areas, in particular to the cortex. **B)** Adult neurogenesis in the dentate gyrus of the hippocampus occurs in five steps: 1 – Proliferation (stem cell located within the subgranular zone (SGZ) in the DG give rise to transient amplifying cells); 2 – Differentiation (transient amplifying cells differentiate into immature neurons; 3 – Migration (immature neurons migrate into tge granule cell layer); 4 – Axon/dendritic targeting (immature neurons project their axons towards the CA3 pyramidal cell layer and their dendrites in the opposite direction into the molecular cell layer; 5 – Synaptic integration (new granule neurons receive and integrate input signals from the entorhinal cortex and gyrus region.) (Adapted from Deng et al. 2010).

Daily, it is estimated that about 9000 newly formed neurons are generated in the adult rat hippocampus (Cameron and McKay 2001), however most of them die. After division of progenitors (type one), daughter cells differentiate into immature neurons (type two) and those that survive after the first two weeks become mature, migrate a short distance as neuroblasts (type three) through the granular layers and differentiate as granule neurons. Their dendritic trees increase in size and complexity and are projected to the molecular cell layer while their respective axons reach the pyramidal cells of the CA3 region in the opposite direction. Finally, new granule neurons join the hippocampal neural circuitry where they receive and integrate input signals from the entorhinal cortex and send output signals to the CA3 and hillus regions. The newly formed neurons are morphological and electrophysiological similar to mature granule cells of dentate gyrus (Fig. 1.6.C) (reviewed by Ming and Song 2005). Those new neurons have an important role in memory and learning processes (van Praag et al. 2002), and/or in mechanisms of specific memory acquisition (Rochefort et al. 2002).

In the developing brain of children, neurogenesis proceeds at a higher rate than in the mature brain of the adult, which may explain the higher plasticity and hence, the enormous ability to learn observed in children (Qiu et al. 2007). Thus, a reduction in proliferation or loss of NPCs in the dentate gyrus during early brain development may affect structurally and functionally the hippocampus which may explain *in vivo* the observed defects on cortical development and may account for cognitive dysfunctions (Marsh et al. 2006). These alterations may be due to changes on the neuromodulators, intrinsic or extrinsic factors, such as neurotransmitters. As it was mentioned before, neurotransmitters are affected by the action of AEDs through their receptors, thus, AEDs may interfere with neurogenesis.

The hippocampus was chosen in the present work as an *in vitro* (cultured hippocampal neurons) and as an *in vivo* model in which to study the effects of AED exposure on neurotoxicity and on neurogenesis, respectively. Indeed, hippocampus is one of the brain structures most extensively studied due to its functions, namely learning and memory, and due its vulnerability to be damaged by extrinsic and intrinsic agents. Experimentally, *in vitro* and *in vivo* models of the hippocampus meet the requirements for representativeness of

various cell types, as well as functional information processing properties in the hippocampus.

In 1993 Ray and colleagues obtained from embryonic brain, hippocampal precursor cells *in vitro* (Ray et al. 1993). Later, it was demonstrated *in vitro* that adult hippocampal precursors originate neurons, astrocytes and oligodendrocytes due to its multipotency (Palmer et al. 1999; Babu et al. 2007). Beyond cultures of NPCs from the hippocampus, culturing neurons as differentiated cells is also usually performed. In these cultures, we may obtain a system with mature neurons with neuronal morphologies, as axons, complex dendritic trees, and synapses, which are electrophysiological active; altogether allow a suitable model for studying neuronal differentiation, neurotoxicity, neurosurvival, among other mechanisms (Vicario-Abejon 2004).

In *in vivo* models, adult neurogenesis in the dentate gyrus may be assessed by immunohistochemistry, enabling us to understand the neuronal development and neurogenesis that occurs within the adult hippocampus. Given that neurogenesis is a phenomenon of neuronal differentiation, starting with proliferation of PSCs and comprises few developmental steps, it is possible to identify these steps by using different neuronal markers during the different steps of differentiation as stated by Kempermann and colleagues, 2004: "*there is a progression from expression of immature to mature markers combined with suggestive changes in morphology"* (Kempermann et al. 2004) (Fig. 1.6).

1.6. Maternal exposure to antiepileptic drugs

It has been reported that among all pregnancies there is a prevalence of 0.7% pregnant women with epilepsy (Holmes et al. 2001). Although the majority of babies born to women with epilepsy are healthy (Adab et al. 2004), these women have an increased risk of having an abnormal pregnancy due to either the AED treatment or the occurrence of seizures, and having a safe gestation is not always easy to manage. Actually, it is not advisible that women with epilepsy discontinue AED therapy during pregnancy, as seizures may occur and cause injury to the fetus and the mother. On the other hand, AED exposure *in utero* and during nursing may induce major or minor anomalies or teratogenic effects to the progeny (Lindhout and Omtzigt 1992; Morrell 1996; Palmieri and Canger 2002; Sankar 2007; Tomson et al. 2011). Besides the adverse effects to the fetus due to AED exposure during pregnancy, mothers may also be negatively affected.

It is known that during pregnancy several psychological and physiologic changes occur, which may alter pharmacokinetics of AEDs and contribute to the decline in AED levels: 1) volume of distribution of AED increases during pregnancy; 2) protein binding of AEDs may increase, which decreases AED levels; 3) drug clearance increases during pregnancy, which decreases AED levels (Anderson 2005). Overall, these changes may affect availability of AEDs in the plasma, which in turn may decrease its efficacy in seizures control (reviewed by Pennell 2003; Tomson et al. 2011). In addition, several AEDs, mostly older-generation AEDs, such as CBZ, VPA, and PH, have different effects on metabolism and serologic parameters of patients, which have been associated with an increase of markers of vascular risk (Chuang et al. 2012). Moreover, pregnant women with epilepsy have a higher risk of obstetrical complications, such as premature rupture of membranes, eclampsia, cesarean section, induced labor, *abruptio placentae*, hyperemesis gravidarum, vaginal bleeding and anemia (Yerby 2000).

There is also evidence showing that in humans with epilepsy the fertility rate is reduced by 15% to 30% compared to healthy people. Fertility is affected by both epilepsy itself with interictal and ictal effects, and by AEDs, which may affect levels of sexual hormones and hence deregulate their systems (reviewed by Pennell 2009). Valproate is one of the most prescribed and studied AED. It has been correlated with the occurrence of polycystic ovaries and polycystic ovarian syndrome, as well as with body-weight gain, and increasing insulin resistance in childbearing women (reviewed by O'Brien and Gilmour-White 2005). Besides VPA, CBZ has also been associated with obesity (reviewed by Biton 2003), while treatment with ESL, OXC, or LTG did not affect body-weight in epileptic patients (Elger et al. 2007; Cansu et al. 2011), but there is a lack of information for pregnant women.

For pregnant women with epilepsy on AED treatment there is little information available, beyond the expected changes in pregnancy, such as the changes in hormonal profile occurring during pregnancy which induces variations in serum lipid levels; in fact, cholesterol and triglycerides levels normally increase during a normal gestation (Herrera 2002). Other serologic parameters, such as alanine and aspartate aminotransferase (ALT and AST, respectively), and creatine kinase (CK) are not normally changed during the gestational period. Studies reporting effects of AEDs on these parameters during pregnancy are not many or are absent. However, the direct effects of AEDs on some of these parameters are already known. Per example, CBZ and OXC negatively affect cytochrome

P450 system (CYP450) by inducing their enzymes. Thus, alterations on CYP450 system lead to changes on metabolic pathways of other important molecules, such as hormones, vitamins and cholesterol (Patsalos et al. 2002; Mintzer et al. 2009; Lopinto-Khoury and Mintzer 2010). In patients taking CBZ, it was found that this AED increased the concentration of lipids in serum, such as total cholesterol (TC), while VPA decreased both TC and triglycerides (Nikolaos et al. 2004). However, studies by Lakshmi and Sunanda (2008), have reported effects of CBZ on the values of ALT and AST in females treated with this AED during pregnancy, when compared to controls (Lakshmi S. and K. 2008). However, there are other parameters that are significantly changed during pregnancy, such as creatinine (CREA). During pregnancy glomerular filtration rate is enhanced, which increases creatinine clearance. Some AEDs may also increase creatinine clearance, such as OXC, in children, but not in adults (Flesch 2004), while others, such as CBZ or LTG do not affect either CREA's production or its clearance.

In animal models, namely in rodents, opposite data exist regarding effects on serologic parameters due to pregnancy or due to AED treatment. Briefly, mice fertility (C3H/He) was not changed neither by CBZ treatment (25 mg in 10 g food, daily) (Rayburn et al. 2004), nor by VPA treatment (160-180 mg/kg, daily) (Chapman and Cutler 1984); however, in rats VPA treatment (300 mg/kg/day) decreased fertility by 25% (Ubeda-Martin et al. 1998). Regarding the effects of AEDs on body weight during pregnancy, Christensen and colleagues found no changes in body weight of pregnant C3H/He mice after treatment with CBZ (Christensen et al. 2004). As for the effects of AED treatment on serum biochemical parameters, subchronic VPA treatment (100 or 500 mg/kg/day) has been associated with changes in lipid metabolism, namely at gene transcription level. VPA increased total TC and TG levels (Lee et al. 2008) but did not change ALT and AST activities (Lee et al. 2007). However, there is a lack of information regarding the effects of AEDs during gestation or nursing periods in mice models. Thus, it is imperative to develop studies in order to understand the specific effect of each AED on female mice throughout maternity and then, depending on the results obtained, and clinical trials should be conducted in humans for those AEDs which cause less adverse effects in the mice model.

1.6.1. Treatment with AEDs during pregnancy: risks of monotherapy and of polytherapy

During pregnancy there are changes in the systemic disposition of AEDs that may decrease their plasma levels and, in turn, this may affect seizure control (reviewed by

Battino and Tomson 2007). For these reasons it is essential to adjust dosages to prevent seizures, or change therapy in order to avoid the potentiating of toxicity effects, which are dose-dependent for most AEDs (Meador et al. 2006). However, for women with epilepsy, it is not easy to manage a safe pregnancy given that almost all commercialized AEDs have been associated with major congenital malformations (MCM) (Meador et al. 2008). However, monotherapy has been the best choice in alternative to interrupting AED therapy, which is not advisable for pregnant women.

It is known that polytherapy exposure has a higher risk than monotherapy of inducing adverse outcomes (like MCM) in the progeny. The frequency of MCM in the progeny is reported to be 4.5% for monotherapy and 8.6% for polytherapy (Holmes et al. 2001). In addition, during the last decade other authors have also reported that polytherapy has a higher risk of incidence of MCM than monotherapy (Dean et al. 2002; Artama et al. 2005; Morrow et al. 2006). These data led clinicians to adopt monotherapy during preconception planning phase and then, during gestation and nursing (Pennell 2003; Meador et al. 2008). However, some AEDs, even in monotherapy, should be avoided during this vulnerable period for the mother and for the future baby. For instance, VPA, which has been recognized as teratogenic for a long time now (Meador et al. 2006; Jentink et al. 2010), or OXC, which was associated with a high risk for failing to prevent convulsive seizures (reviewed by Pennell 2008), are AEDs that should not be used to control seizures during gestation and nursing.

Prescription of an AED should be done after the assessment of the risk-to-benefit of the drug, taking into account possible teratogenic effects and cognitive adverse outcomes of that particular AED. Nevertheless, there is a lack of information about the pharmacokinetics of the AEDs during pregnancy, which does not allow to prescribe an optimal dose free of deleterious effects. However, there are some guidelines by the American Academy of Neurology (1998), the American College of Obstetricians and Gynecologists (1997) and the ILAE (1993) that should be recommended to childbearing and pregnant women with epilepsy. Briefly: "*1) optimized treatment prior to conception, 2) use of monotherapy, if possible, 3) choose the most effective AED for seizure type and syndrome, 4)use the lowest effective dose, 5) supplement with folate prior to conception and during pregnancy (folic acid plays an important role in nucleic acid metabolism, thus it may avoid teratogens effects of AEDs), 6) treatment of children at birth with vitamin K, and possibly treatment of the mother with vitamin K late in pregnancy*" (in Meador et al. 2006). Nowadays, among all

commercialized AEDs, none of them was shown to be completely safe and efficacious for mothers and progeny. To date, ESL has not yet been studied as an option in pregnant women with epilepsy. However, previous data from clinical trial studies (Almeida and Soares-da-Silva 2004; Elger et al. 2009; Gil-Nagel et al. 2009) or data from studies in animal models (Benes et al. 1999; Parada and Soares-da-Silva 2002; Araujo et al. 2004) , suggest that ESL may be a better choice for the mothers and for the future babies due to its safer profile.

1.6.2. Teratogenicity of antiepileptic drugs

Critical periods of human development occur at embryonic and fetal periods, where specific organs are most susceptible to damage from teratogens. Teratogenic agents interact with DNA, and may be a genetic agent, a physical condition, an infectious agent, a drug or chemical that induces *per se* or in a multifactorial way, negative changes on morphology of an organ or on its function (Brent and Beckman 1990). Antiepileptic drugs may be teratogens; several authors have already described these effects, namely, it was observed that children born to mothers on AED treatment during gestation, have a higher risk (two-threefold increase) for congenital malformations (anatomical teratogenesis) or developmental delay (behavioural teratogenesis) (Pennell 2003; Perucca 2005; Meador et al. 2006; Meador et al. 2007). The mechanisms underlying their teratogens effects are under investigation; there is a proposed mechanism of anatomical teratogenesis, which involves free radicals formed during AED metabolism and that may induce oxidative macromolecular damage (Wells et al. 1997). However, this mechanism may not explain behavioural teratogenesis, which has been associated with apoptosis induced by AEDs (Bittigau et al. 2003; Asimiadou et al. 2005).

Teratogen exposure may result in major congenital malformations (MCMs) or in minor congenital malformations. MCMs occur when teratogens affect any vulnerable organ during the embryonic period (DiPietro 2005), causing either functional limitation and/or may need clinical intervention, and normally result in physical or mental disability. The most common organs subject to the action of teratogens are the heart, the eyes, the limbs, the ear, the palate, external genitalia or the central nervous system (CNS), and the most common MCMs associated with AEDs are urogenital defects, clef/lip palate, neural tube defects and cardiovascular and musculoskeletal defects (Meador and Penovich 2008;

Meador et al. 2009). Neural tube defects (NTDs) are caused by defective neurulation or abnormal development of the neural tube, and cause malformations on the nervous system and its membranes; these malformations happen between the third and fourth week of gestation, at time when neural tube closure takes place. Most of these defects are difficult to prevent due to late pregnancy detection (reviewed by Pennell 2008). Minor congenital anomalies result from action of teratogens mainly during fetal period (from 8th week of development until birth) (DiPietro 2005). They have a minor clinical relevance since they consist in malformations without medical or surgical consequences; on the other hand they have a higher incidence than MCMs (Leppig et al. 1987). The most common minor malformations may occur in the sensory organs, cardiovascular and digestive system, musculoskeletal system, limbs and external genitals (Compton et al. 2011). However, the CNS is the most sensitive system to teratogens, since it may suffer effects throughout embryonic and fetal development and their morphological or physiological abnormalities have long-term consequences (DiPietro 2005).

Worldwide there are reports about AED use during pregnancy registries and data regarding MCMs as effects of *in utero* exposure to VPA, LTG, CBZ, PH, and PB, which are considered the main effectors of anatomic defects (Meador et al. 2009). In human studies, CBZ is one of the most common AED used as monotherapy by pregnant women with epilepsy. Most studies have reported that CBZ (as monotherapy) is the AED with the lowest rate of MCM in infants (4.62% and 2.28% for healthy women) (Meador et al. 2009). However, the MCMs that have been associated to CBZ are namely microcephaly and growth retardation (Holmes et al. 2001), neural tube defects, cardiovascular and urinary tract anomalies, cleft palate (Matalon et al. 2002), and cleft lip (Hernandez-Diaz et al. 2012).

Information on OXC is particularly scarce and the number of outcomes is inadequate to formulate consistent conclusions, and some of the data are from polytherapy situations. However, it was observed one isolate case of cardiac malformation from an argentine study (polytherapy with OXC), while infants born to mothers at OXC monotherapy were healthy (Kaaja et al. 2003; Meischenguiser et al. 2004); in Denmark, one prospective study reported two infants with ventricular septal defect in 37 pregnancies exposed to OXC (Sabers et al. 2004); and other studies reported urogenital malformation (Artama et al. 2005).

Valproate is one the most studied AED; moreover, it is the AED with the highest rate of incidence of MCMs when compared to other AEDs such as CBZ, LTG, PH and PB, and when

compared to healthy women (2.28%) (Meador et al. 2009). Over the last few years several studies have reported data about VPA and are consistent with the patterns that were observed from prospective pregnancy registries. The most common morphological anomalies observed in infants born to mothers on VPA monotherapy included cardiac anomalies, NTDs, hypospadias, polydactyly, bilateral inguinal hernia, dysplastic kidney and equinavarus club foot (Arpino et al. 2000; Vajda et al. 2004; Artama et al. 2005; Wyszynski et al. 2005; Meador et al. 2006; Morrow et al. 2006). Children exposed to VPA *in utero* normally exhibit a pattern of anomalies denominated as "fetal valproate syndrome", which consist namely in facial features (e.g. infraorbital groove, medial deficiency of eyebrows, flat nasal bridge, small downturned mouth) and specific congenital malformations (e.g. neural tube defects, congenital heart disease, genitourinary malformations, arachnodactyly) (Clayton-Smith and Donnai 1995).

Lamotrigine is a third generation AED with the highest number of reported studies during pregnancy. Lamotrigine has been used as treatment in pregnant women with epilepsy, however it has a big disadvantage compared to other AEDs, its clearance is increased by 150% due to its metabolism. During pregnancy UGT1A4, the isoenzyme responsible for LTG hepatic glucuronidation, is highly activated, which increases LTG clearance (Petrenaite et al. 2005). Actually, several studies have reported that pregnant women at LTG monotherapy were presented seizure worsening (Pennell et al. 2004; Vajda et al. 2006). Thus, higher doses of LTG have to be administered in order to prevent seizures. Nonetheless, studies have shown that LTG is safer to be used during pregnancy than the older and the newer AEDs. The rate of incidence of MCM due to LTG treatment is 2.91% (compared to 2.28% for healthy women)(Meador et al. 2009). Meador and colleagues (2006) in a study with 647 cases, observed four cardiac defects, one facial cleft, one neural tube defect, two skeletal defects, three gastrointestinal tract defects, and six hypospadias/genitourinary tract defects in children born to women on LTG treatment (Meador et al. 2006). Other authors have also reported lower incidence of adverse outcomes (Perucca 2005; Tatum 2006). However, all AEDs used during pregnancy have in common two effects: 1) rate of MCMs increases with individual doses; and 2) each AED at polytherapy has higher incidence rate of MCM than at monotherapy (even at doses lower than in monotherapy) (Meador et al. 2006; Morrow et al. 2006). Regarding animal studies, VPA teratogenicity has been extensively studied among fetuses of mice and rats, and a pattern of abnormalities have been observed: spina bifida, failure of neural tube closure,

syndactyly and oligodactyly (Nau et al. 1991; Ehlers et al. 1992). In addition, Manent and colleagues (2007) observed that rats exposed *in utero* to vigabatrin and VPA showed hippocampal and cortical dysplasias and were associated with disrupted neuronal migration; later, the same group observed that LTG caused hippocampal and neocortical alterations in a dose-dependent manner, while CBZ, TPM and LEV did not induce malformations (Manent et al. 2007; Manent et al. 2008). Reduced brain weight after birth is other common negative effect that was observed in animals exposed to AEDs, such as PB, PHT, and VPA during development, (reviewed by Meador et al. 2007). As was already mentioned, to date there are not data reporting effects of ESL exposure *in utero* and its respective outcomes.

1.6.3. Behavioural consequences of antiepileptic drug treatment during gestation and nursing

In addition to major and minor congenital malformations, AEDs have also been associated with negative outcomes related do cognitive and non-cognitive functions in humans, which have a higher impact on patient performances in the case of AED polytherapy (Brodie et al. 1987). Actually, since 1970s there are data about AED exposure *in utero* and their potential effects on cognition and, since then, "older" and also "newer" AEDs have been associated with an increased risk of changes in behaviour. In fact, none of the commercialized AEDs are totally safe and free of adverse-effects on cognitive and non-cognitive functions of progeny born to mothers with epilepsy and on AED treatment. "Older" AEDs such as CBZ, VPA and PHT have been associated with a greater risk of cognitive and behaviour impairment while "newer" AEDs have been reported as having more modest adverse effects in cognitive and behaviour (Loring et al. 2007). In addition, last year the international registry of AEDs and pregnancy strengthened that not only VPA has a higher risk associated with dose-dependent anomalies, but the same applies to LTG, CBZ and PB (Tomson et al. 2011). Behavioural teratogenesis is mostly detected postnatally and may account for impaired cognitive functions like memory and/or learning.

Cognition is a class of behaviour, and behaviour is a complex process which combines different functions that cover sensory and motor experiences. Cognition is also related with more than one function dependent of mental processes. It covers attention, learning, coordination, memory, linguistic, arithmetic skills, and speed of intellectual functioning. Thus, cognitive dysfunctions are strictly associated with brain areas involved in

these functions (Cavanna et al. 2010). Although AEDs have been related with these dysfunctions, we should consider that behaviour is also a final conjugation of different factors (genetic, environment, social conditions, pathology), which does not allow clinicians to correctly predict the behavioural/cognitive profile of each AED or mechanism of action. Furthermore, the mechanisms of action that have been described for each AED may not explain all the behavioural effect of the AEDs (Perucca 2005; Cavanna et al. 2010).

As a result of the effort that clinicians have done in the last two decades, data is available from prospective observational studies in humans about behavioural teratogenesis of *in utero* exposure to AEDs (Bromley et al. 2010). Among the "older" AEDs, CBZ has been shown as a relatively safe drug without effects on QI of the progeny (Wide et al. 2002; Gaily et al. 2004; Meador and Zupanc 2004), but on the other hand, CBZ is able to induce minor effects on memory performance and motor delay (Bromley et al. 2011). Regarding VPA, besides adverse effects on neurological development and the observed fetal valproate syndrome, VPA is also associated with impairment of cognition and behaviour (Clayton-Smith and Donnai 1995; Shepard et al. 2002; Zaki et al. 2010). Low verbal IQ has been observed in children exposed to VPA *in utero* and its impact is higher than that induced by CBZ, LTG or PHT (Adab et al. 2004; Meador et al. 2009). Recently, Bromley and colleagues strengthened that VPA impairs cognitive development in children exposed to VPA as compared to control children (Bromley et al. 2010).

In the case of OXC and LTG, as "newer" AEDs, fewer studies on cognitive dysfunctions are available. However, Bromley and colleagues recently observed that regarding performance on nonverbal abilities and on hand and eye coordination tasks, children exposed to LTG *in utero* had an increased risk for poorer outcomes compared to control children (Bromley et al. 2010). Very limited or absent information is available for OXC exposure *in utero*. However, some studies have reported opposite effects in adult patients treated with OXC. Curran and Java (1993) observed that patients on OXC treatment had improved performance on behavioural tasks (Curran and Java 1993), while Salinsky and colleagues (2004) observed that OXC induced negative neuropsychological effects (Salinsky et al. 2004), and recently Milovan and co-workers (2010) observed that in healthy volunteers OXC tends to impair some cognitive functions, but the effects were not considered clinically relevant (Milovan et al. 2010). Regarding ESL, few data has been

reported about cognitive effects of ESL treatment in healthy humans. Milovan and colleagues (2010) observed that healthy adults treated with a single dose of ESL did not have changes in performance of cognitive abilities as compared with placebo, and ESL treatment caused improved word fluency (Milovan et al. 2010).

There is also evidence of behavioural teratogenesis in animal models in studies aiming at determining the effects of AEDs in development. Briefly, in non-epileptic rats, Shannon and Love (2004) showed that CBZ produced more modest impact on working memory tasks than GABA modulators, which reduced performance in this task; CBZ was also shown to cause disrupted attention and, at higher doses, CBZ also impaired learning (Shannon and Love 2004; Shannon and Love 2005; Shannon and Love 2007). However, in a study similar to ours, but performed in a different animal model (C3H/he mice), it was observed that prenatal exposure to CBZ slightly decreased locomotion activity, but did not change cognition or anxiety behaviour as compared to placebo (Rayburn et al. 2004). There is a vast list of studies in rats focusing on the effects of VPA exposure *in utero*, and mostly have reported impacts on cognitive and other behaviours, as follows: reduced exploratory behaviour (Schneider and Przewlocki 2005), increased activity in a novel open field, increased anxiety-like behaviour, no significant effects on learning and memory (Schneider et al. 2007; Markram et al. 2008; Schneider et al. 2008), and reduced sociability (Kolozsi et al. 2009). For OXC and LTG there is lack of data from studies using animal models. However, other studies showed that OXC did not alter performance of mice in elevated plus maze test and passive avoidance test (using pentylenetetrazole-kindling mice) (Agarwal et al. 2011), while LTG did not disrupt working memory (Shannon and Love 2004). To our knowledge, information about the effects of ESL in cognitive and non-cognitive behaviour of mice is not available.

Overall, in this thesis we present for the first time data using CD1 mice as the animal model to explore the effects of *in utero* and postnatal exposure to ESL on cognitive abilities, anxiety-behaviour and pro-depression mood of the progeny. In addition, we also identified for the first time the main effects of a long-term treatment with ESL in CD1 pregnant females during prenatal period, gestation and nursing. All data was simultaneously obtained and compared with the most commonly commercialized AEDs, CBZ and VPA, with the most

similar AED, OXC, and with the most used AED in the last decade in pregnant women with epilepsy, LTG.

1.7. Objectives

In recent years, pharmaceutical companies have done an effort to develop new antiepileptic drugs in order to overcome the adverse effects of currently available ones due to reduced potency, unsafely and unfavourable toxic profile such as hepatotoxicity or toxicity to central nervous system (reviewed by Loring et al. 2007; Pennell 2008; Johannessen Landmark and Patsalos 2010). Furthermore, adverse effects of AEDs become even more important to clinician that prescribed AED therapy for pregnant women with epilepsy. Women are not advisable to interrupt AED therapy due to the occurrence of seizures, but on the other hand AEDs are teratogens, and children born to women with epilepsy have a higher risk of incidence of malformations or presenting peculiar deficits on the cognitive skills

ESL is a novel once-daily antiepileptic drug completely developed by BIAL (BIAL-Portela e Companhia, SA, S.Mamede do Coronado, Portugal). Eslicarbazepine acetate is chemically related to CBZ and OXC; however it has strict differences within its structure, which results in differences in the metabolism, allowing ESL to be less toxic than the other AEDs. Actually, ESL was specifically developed in order be less toxic than CBZ by preventing toxic metabolites production, and to have a better tolerability and efficacy in the treatment of epilepsy (Benes et al. 1999). In fact, it was observed that in a phase III clinical trials in adults patients with onset seizures, they benefited from ESL treatment compared to CBZ and other AEDs. ESL was more efficacious and well tolerated and reduced seizure frequency. ESL was approved in Europe since 2009. It has been found to be well tolerated and effective in adults as adjunctive treatment for partial-onset seizures (Elger et al. 2009).

Within this scenario, we propose to identify the effects of ESL and its metabolites on the mechanisms that are involved in the neurotoxicity and neuroprotection pathways in cultured hippocampal neurons; and the effects of exposure to AED during pregnancy and during nursing in the serological parameters of the progenitor, and performances in cognitive and non-cognitive of the progeny, as well as the effects of exposure on the

formation of new neurons in adult hippocampus (*in vivo*) and in the basal proliferation and cell cycle stage of *in vitro* stem cell cultures from subventricular region (SVZ) of rats.

The aim of the specific chapters are presented as follows:

In chapter 3 we compared the neurotoxicity profile of ESL and of its metabolites S-Lic and R-Lic to those of the structurally-related CBZ and OXC, and to LTG and VPA. We investigated the presence of cell death markers and cell viability parameters, as well as the activation of prosurvival intracellular signalling pathways in primary cultures of hippocampal neurons exposed to the AEDs.

In chapter 4 we focused on the effects of long-term treatment with AEDs (ESL and of the other AEDs), on the pre-gestation period, gestation and nursing, in blood serum biochemical parameters of progenitors. In addition, we investigated how previous AED treatment affected the generation of newly born cells in the dentate gyrus of the adult hippocampus of CD1 female mice.

In chapter 5 we investigated the effects of AED exposure *in utero* and nursing on behaviour of CD1 mice, including cognitive (memory and learning skills) and non-cognitive (locomotion, the possible anxiogenic vs. anxiolytic effects, and even pro-depressive effects in the progeny of CD1 female). We evaluated separately males and females and focused on if AED exposure affected in the some way juvenile and adult mice regarding outcomes on the behaviour performance.

In chapter 6 we aimed to study the effects of AEDs on neural stem cell proliferation and hippocampal neurogenesis in the adult brain of CD1 mice, after *in utero* and nursing exposure. We also studied, in an *in vitro* model, the effects of AEDs on the proliferation, cell cycle distribution and cell death of neural stem cell cultures from SVZ of juvenile Wistar rats.

Chapter 2

Methods and Materials

2. Methods and Materials

2.1. *In vitro* experiments

2.1.1. Animals

Adult female Wistar rats were obtained from Charles River (Barcelona, Spain). Animals were maintained in Eurostandard type III H cages (Tecniplast, Ultragen, Porto, Portugal), with 2 animals each. Each cage was provided with standard corncob litter (Mucedola, Ultragen) and a piece of tissue paper. Rodent pellets (4RF25/C, Mucedola) and water (pH 2.5-3.2) were provided *ad libitum* in a 12 h dark:light cycle. The animals were kept in our animal facilities (Faculty of Medicine, Coimbra, Portugal) in a room with controlled temperature (21°C±1°C) and humidity (55%). All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

2.1.2. Rat primary hippocampal cultures

Adult female Wistar rats were sacrificed by cervical dislocation. The E18/19 embryos were removed and kept in calcium- and magnesium-free Hank's balanced salt solution (HBSS, 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $Na_2PO_4.2H_2O$, 4.16 mM $NaHCO_3$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4). After decapitation, hippocampi were detached and hippocampal neurons were dissociated with trypsin (2.0 mg/mL, 15 min, 37°C) and deoxyribonuclease I (DNAse, 0.15 mg/mL) in HBSS. The cells were cultured in serum-free Neurobasal medium, suplemented with B27 supplement, glutamate (Glut, 25 μM), gentamicin (0.12 mg/mL), as described previously (Ambrosio et al. 2000; Araujo et al. 2004), and GlutaMAX™ (0.5 mM). Cells were then plated on 48-well plates coated with poli-D-lysine (0.1 mg/mL), at a density of $0.1x10^6$ cells/cm^2, for analysis of cell viability or on 6-well plates for preparation of lysates. For immunocytochemistry assays, cells were plated on 16-mm diameter glass coverslips coated with poli-D-lysine (0.1 mg/mL) at a density of $0.075x10^6$ cells/cm^2 (12-well plates). Cultures were kept at 37°C in a humidified incubator in 5% CO_2/95% air, for 7-8 days, the time required for maturation of hippocampal neurons.

2.1.3. Rat primary neural stem cell cultures

Wistar rats (P7-8) were decapitated and brains kept in HBSS. After removing olfactory bulbs, meninges and cerebellum, a single coronal cut was done exposing the lateral ventricles and SVZ (adapted from Lorincz and Zawistowski 2009). Neural stem cell (NSC) cultures were obtained from the SVZ, whose dissected fragments, encompassing both ependymal and subependymal layers, were digested in 0.025% trypsin and 0.265 mM EDTA, for 20 min at 37°C, and dissociated by gentle trituration. Following dissociation, cells were ressuspended in fresh Dulbecco's Modified Eagle's Medium:F-12 nutrient mixture (D-MEM/F-12) with GlutaMAX™-I, supplemented with 1% B27, 1% antibiotic (10,000 units/mL of penicillin, 10 mg/mL streptomycin), 10 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF). Single cells were then plated on uncoated Petri dishes at a density of 3,000 cells per cm^2. SVZ stem cells were then allowed to develop as floating aggregates (primary neurospheres) in a 95% air-5% CO_2 humidified atmosphere at 37°C, for 7 days, as described previously (Carreira et al. 2010). Neurospheres were passaged four times before being collected. Then, neurospheres were mechanically dissociated and seeded on 12-well plates coated with poly-L-lysine (0.1 mg/mL) for flow cytometry assays, and kept in the same medium as above. Cell cultures were used 3-4 days after plating, and then exposed to AEDs for 24h. For immunocytochemistry assays cells were plated at a density of $0.050x10^6$ cells/cm^2 on 16-mm diameter glass coverslips coated with poli-L-lysine (0.1 mg/mL) (12-well plates), and kept in the same medium as above.

2.1.4. Exposure of primary cultures to the drugs

According to the experimental approach, hippocampal neurons and neural stem cell cultures were exposed to AEDs at different concentrations (0.01, 0.03, 0.1 and 0.3 mM for ESL, S-Lic, R-Lic, CBZ, OXC and LTG; and 0.01, 0.03, 0.1, 0.3, 0.5, 1 and 3 mM for VPA) for different periods of time, as indicated in figure legends and in the text of the Results (Chapter 3). AEDs were prepared in DMSO (except VPA, which was prepared in water). Exposure of the cells to the AEDs was performed by taking 200 µl aliquots of culture medium from each well, in which the AEDs were homogenized, and then added back gently to the correspondent well. Same volume of DMSO was used in control conditions.

2.1.5. Analysis of cell viability

The resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) reduction assay was used for the assessment of cell viability following treatment of cultured hippocampal neurons with different concentrations of AEDs, for 24h. When taken up by living cells, resazurin, a nonfluorescent blue dye, is reduced to resorufin (O'Brien et al. 2000), a pink dye which is fluorescent when activated spectrophotometrically at 570 and 600 nm. Briefly, culture medium was removed and then 300 microliters of 10% resazurin (in Krebs buffer) were added to the cultures and incubated for 2 h at 37°C, in the incubation chamber. The absorbance of the culture media was quantified for each well at 570 and 600 nm. All experiments were carried out in triplicate, and the results were expressed as percentage of that observed in control conditions.

2.1.6. Samples preparation for immunocytochemistry studies

Cultured hippocampal neurons were used for immunocytochemistry assays. Different procedures were followed for sample preparation, as described below:

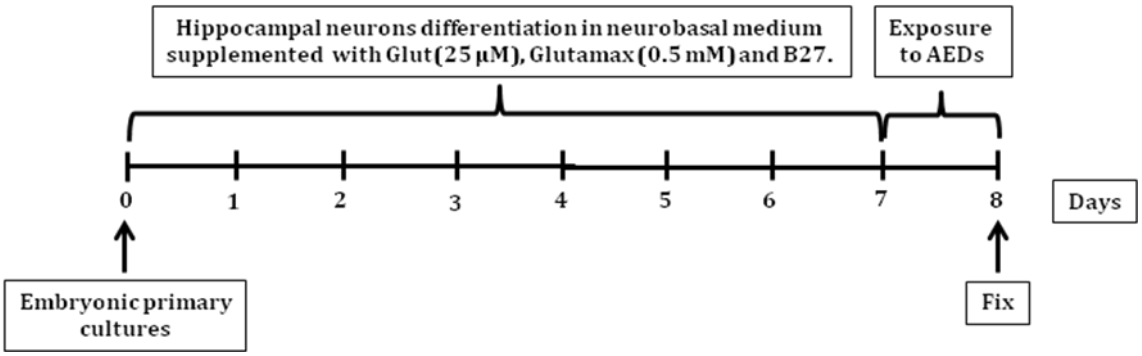

Fig. 2.1. Experimental protocol used to study the effect of AEDs on cell death mechanisms of hippocampal neurons, following 24 hours of treatment. The concentrations tested were 0.3 mM for ESL, S-Lic, R-Lic, CBZ, OXC and 3 mM for VPA. After exposure to the AEDs, the cells were fixed (Fix) as described in the text. The antibodies used were anti-cleaved caspase-3, anti-cleaved PARP, and anti-AIF.

2.1.6.1. Immunocytochemistry

Following treatment, cell cultures were rinsed three times with 0.1 M phosphate-buffered saline (PBS; pH 7.4), and then fixed with 4% paraformaldehyde and 4% sucrose in PBS at room temperature, for 20 min. Then cells were washed three times with PBS, followed by permeabilization with 1% Triton X-100 for 5 min and washed three times with

PBS. Non-specific binding was blocked with 3% bovine serum albumin (BSA) in 0.2% Tween-20 in PBS, for 1 h, at room temperature. The incubation with the primary antibodies was performed in blocking solution for 90 min, at room temperature, or overnight at 4°C (see Table 2-I for detailed description of the antibodies used). Cells were then rinsed with PBS and incubated with the appropriate secondary antibodies, for 1 h (1:200, anti mouse, anti rabbit or anti guinea pig IgGs conjugated with Alexa Fluor 488, 594 or 633), at room temperature. Nuclei were labelled with Hoechst 33342 (1 μg/mL) for 3 min. Coverslips were mounted in glass slides with DAKO fluorescence mounting medium. Images were acquired in a laser scanning confocal microscope LSM 510 META (Zeiss, Jena, Germany).

Table 2-I. Primary antibodies used for immunocytochemistry experiments.

Antibody	Host	Dilution	Company
Cleaved caspase-3	Rabbit	1:100	Cell Signalling (Fisher Scientific)
Cleaved PARP	Rabbit	1:100	
AIF	Rabbit	1:100	

2.1.6.2. Confocal microscopy imaging and analysis of cultured hippocampal neurons

In each coverslip, the cells of 7-10 randomly selected fields were counted, which represents approximately 900 – 1,200 cells per coverslip. The number of cells labelled for the protein of interest (Table 2.I) was counted in each coverslip (using Photoshop, version CS3; ASI, CA, USA). Data was expressed as percentage of the total number of nuclei, counterstained with Hoechst 33342. A minimum of 3-4 independent experiments was analyzed for each condition tested.

2.1.7. Samples preparation for protein lysates of cultured hippocampal neurons

Hippocampal cultured neuron lysates were prepared and used in Western blotting experiments. Different procedures were followed for sample preparation, as described below:

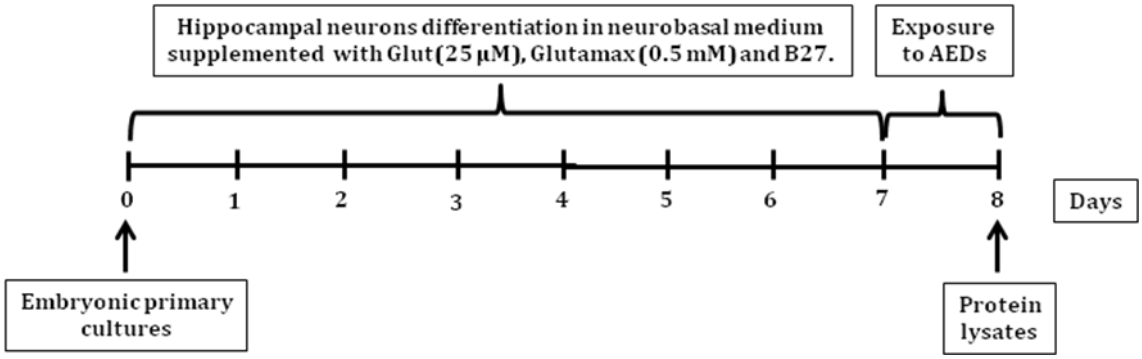

Fig. 2.2. Experimental protocol to study the effect of AEDs on cell death mechanisms of hippocampal neurons, following 24 hours of treatment. The concentrations tested were 0.3 mM for ESL, S-Lic, R-Lic, CBZ, OXC and LTG and 3 mM for VPA. The antibodies used were anti-cleaved caspase-3 and anti-cleaved PARP.

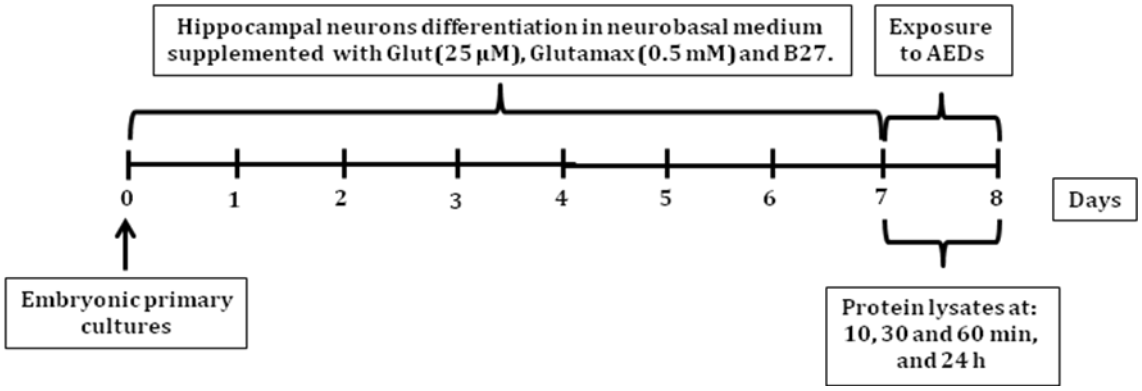

Fig. 2.3. Experimental protocol to study the effect of AEDs on different signalling pathways involved in mechanisms of toxicity or neuroprotection. The concentrations tested were 0.3 mM for ESL, S-Lic, R-Lic, CBZ, OXC and LTG and 3 mM for VPA, during 10, 30 and 60 min, and 24 h. The antibodies used were anti-phospho ERK 1/2, anti-ERK 1/2, anti-phospho Akt (Thr308), anti-phospho Akt (Ser473), anti-Akt (pan), anti-phospho SAPK/JNK (Thr183/Tyr185), and anti-SAPK/JNK.

2.1.7.1. Preparation of cell lysates of cultured hippocampal neurons for Western blotting experiments

Following treatment with AEDs, the medium of hippocampal cultures was removed and cells were washed and lysed at 4°C in lysis buffer with 50 mM KCl, 50 mM PIPES, 10 mM EGTA, 2 mM $MgCl_2$, 0.5% Triton X-100, supplemented with 100 µM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antiparin, 5 µg/mL pepstatin A (CLAP), 1 µg/mL orthovanadate, 10 µg/mL NaF, pH 7.4, at 4° C. Protein concentration was determined by the bicinchoninic acid (BCA) method. Samples were denatured following addition of 6x concentrated sample buffer (0.5 M Tris,

30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue). After heating for 5 minutes at 95° C, the samples were frozen at -20° C until analysis.

2.1.7.2. Western blotting

Following lysis and denaturation of samples, equal amounts of protein were separated by electrophoresis on SDS-polyacrylamide gels (SDS-PAGE), and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes, previously activated with methanol (1 min). Membranes were then blocked, for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) and 5% low-fat milk (or 3% BSA for the analysis of phosphorylated proteins). Incubations with primary antibodies (see Table 2-II for detailed description of the antibodies used) were performed in TBS-T 1% low-fat milk (or 1% BSA for phosphorylated proteins), overnight at 4°C (see Table 2-II for detailed description of the antibodies used). After extensive washing in TBS-T (three times), the membranes were incubated, for 1 hour at room temperature, with an alkaline phosphatase-linked secondary antibody (anti-mouse or anti-rabbit IgG 1:20,000) in TBS-T 1% low-fat milk (or 0.5% BSA for phosphorylated proteins). Finally, membranes were washed with TBS-T (for 1 h, changing the medium every 20 min). Protein immunoreactive bands were visualized by Enhanced Chemifluorescence (ECF) substrate in a VersaDoc 3000 imaging system (Bio-Rad Laboratories, Lda., Amadora, Portugal), following incubation of the membrane with ECF reagent for 5 min. Protein controls were performed using antibodies against either the total protein in study (e.g. total Akt), or against mouse anti-α-tubulin (1:10,000). The immunoreactive bands were analyzed with the QuantityOne software (version 4.6.9, Bio-Rad Laboratories, Lda., Amadora, Portugal).

Table 2-II. Primary antibodies used for Western blotting.

Antibody	Host	Dilution	Source
Cleaved caspase-3	Rabbit	1:1,000	Cell Signaling
Cleaved PARP	Rabbit	1:1,000	
Phospho-p44/p42 MEK (Erk1/2) (Thr202/Tyr204)	Rabbit	1:1,000	
Phospho- SAPK/JNK (Thr183/Tyr185)	Rabbit	1:1,000	
SAPK/JNK	Rabbit	1:1,000	
Phospho-Akt (Thr308)	Rabbit	1:1,000	
Phospho-Akt (Ser473)	Rabbit	1:1,000	
Akt (pan)	Rabbit	1:1,000	
p44/p42 MEK (Erk1/2)	Mouse	1:2,000	

2.1.8. Detection of cell proliferation and cell cycle analysis by flow cytometry

2.1.8.1. EdU incorporation, cell harvesting and fixation

To analyze the proliferation of neural stem cells, 10 µM of 5-ethynyl-2'-deoxyuridine (EdU) was added to the cultures 4 h prior to harvest and fixation. Cells were detached and harvested to flow tubes, after 15-20 min of treatment with Accutase™ at 37°C (10 mL/75 cm^2). After harvesting, cells were pelleted by centrifugation at 200 x *g* for 20 minutes. The supernatant was removed and the pellet was ressuspended and washed once with 1 mL PBS. Samples were pelleted again (200 x *g* for 15 minutes), and the supernatant was removed. Fixation of the pelleted cells was performed by resuspension in 1 mL of 70% ethanol and incubation overnight, at 4°C. This procedure of ethanol fixation was used with neural stem cells after we realized that paraformaldehyde fixation was not applicable to NSC cultures (for detailed protocol description, see Appendix 2).

2.1.8.2 EdU detection and identification of cell cycle phases

EdU incorporation was used to assess cell proliferation by flow cytometry using a commercially available kit from Invitrogen (Click-iT® EdU Alexa Fluor® 488 Flow

cytometry Assay kit, Invitrogen, Life Technologies, Madrid, Spain), as described previously (Carreira et al. 2010). Detection of EdU incorporation was based on click chemistry, a copper-catalysed reaction between an azide (conjugated to a fluorophore) and an alkyne (EdU) (Rostovtsev et al. 2002). Fixed cells were centrifuged at 245 x *g* for 20 min, the supernatant was removed and cells washed with 1 mL PBS (centrifugation at 200 x *g*, for 15 min). After discarding the supernatant, cells were incubated for 30 min at room temperature, protected from light, with "cocktail reaction" (Alexa Fluor® 488 azide, copper sulphate and 1x Click-iT reaction buffer additive, all available from the kit). Then, cells were centrifuged (200 x *g* for 15 min) and washed twice with 1 mL PBS, the supernatants removed, and 1 mL PBS was added. Next, cells were incubated with ribonuclease A and the nuclear dye 7-actinomycin D (7-AAD, also available in the kit) for 30 min, at room temperature, protected from light. Samples were kept in ice and protected from light until being analysed by flow cytometry on the same day.

2.1.8.3 Flow cytometry and instrument standardization

Cells were analyzed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 15 mW, air-cooled, 488 nm argon-ion laser for excitation of AF® 488 and 7-AAD. AF® 488 fluorescence was detected through a 530/30-nm bandpass filter and displayed on four-decade log scales. Fluorescence emission from 7-AAD was detected through a 670 nm longpass filter and using linear amplification. Photomultiplier tube voltage and spectral compensation were established using cells single-stained with Alexa Fluor 488 alone, 7-AAD alone, or incubated with EdU alone. For doublet discrimination, in addition to FL3 heights (7-AAD fluorescence), FL3 areas and widths were measured. Measurements of, at least 50,000 events, were collected per sample. Analysis of the multivariate data was performed with BD CELLQuest™ Pro software (version 5.2). Cell cycle analysis of DNA histograms was performed with WinMDI 2.8 software developed by Dr. J. Trotter (freely available at http://facs.scrips.edu/software.html). A clear dual parameter contour density, 7-AAD vs AF® 488 was obtained. 7-AAD was detected in a linear scale (X axis), while the AF® 488 azide was detected in 10^1 decade log scale (Y axis). In this study AF® 488 azide-positive events were localized always above the 10^1 decade log.

2.2. *In vivo* studies

2.2.1. Adult animals and husbandry

Adult breeder CD1 mice (30 females and 10 males) 8 weeks old, of conventional microbiological status, were purchased at Charles River (Barcelona, Spain). Animals were maintained in Eurostandard type II L cages (Tecniplast, Ultragen, Porto, Portugal), with a maximum of 2 females or 1 male per cage. Each cage was provided with standard corncob litter (Mucedola, Ultragen) and a piece of tissue paper. Rodent pellets (4 RF21, Mucedola) and water (pH 2.5-3.2) were provided *ad libitum* in a 12 h dark:light cycle. The animals were kept in our animal facilities (Faculty of Medicine, Coimbra, Portugal) in a room with controlled temperature (21 ±1°C) and humidity (55%). All experiments were performed in accordance with institutional and European guidelines (86/609/EEC) for the care and use of laboratory animals.

Females became familiar with the researcher by handling each two days, at least one week prior to the beginning of the experiment. This procedure decreased the animals stress and, at the same time, the researcher was also able to learn the normal behaviour of each animal identifying easily possible changes.

2.2.1.1. Mating

The females were assigned to five groups (6 females per group) in the beginning of the experiment: Control group (Ctrl), and ESL, CBZ, OXC or VPA treated groups, and were maintained two per cage, physically separated by creating two compartments with a PVC board (Fig. 2.4), until birth of litters. Before mating, females had ovulation induced and synchronized (these manipulations were guided by the main technician of the animal facilities). Matings were carried out in the proportion of 4 females per male, during 7 days and the cages were maintained in a restricted area of the animal facilities. On the day the litters were born, females were separated and kept one per cage until the day they were sacrificed, after weaning of the pups.

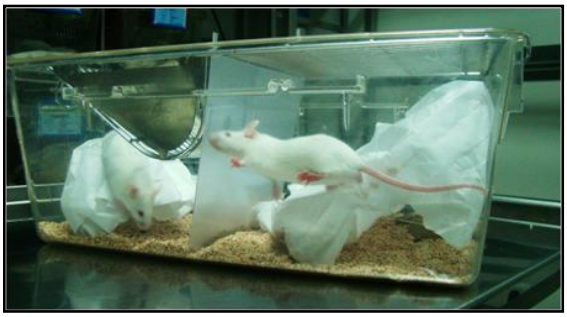

Fig. 2.4. Picture of a cage showing the PVC board used to create two compartments in each cage. The PVC board separated the females and ensured that each female ingested the daily dose of AED administered.

2.2.1.2. Procedures for exposure of CD1 female mice to AEDs

Exposure to AEDs started 30 days before mating and finished at weaning (see Fig. 2.5 in section 2.2.2 for scheduled procedures with animals).

During 70 days, females were fed daily on fresh apple cubes (1 cm^3) injected with each of the AEDs (ESL, CBZ and OXC 30 mg/Kg, and VPA 300 mg/Kg, or vehicle for the control group). The PVC board that divides the cage into two compartments ensured that each female ingested the daily dose of AED administered. During treatment, the females were weighted every week, and blood glucose levels were also measured every week, after weighting the animals, in a drop of blood collected from the tail using Accu-Chek® Aviva Nano (Roche Diagnostics, Roche, Amadora, Portugal). The blood glucose levels were determined after four hours of fasting. Weight and glucose levels were not measured during the mating period and seven days following birth of the litters, to avoid perturbation of the animals.

Following weaning of the pups (20 days after birth), all females were deeply anesthetized with sodium pentobarbital (Braun, 120 mg/Kg, i.p.), and blood samples were quickly collected by cardiac puncture. Serum was separated by centrifugation (2 x *g*, 15 min) and sent for analysis. Serum biochemical parameters analysed included total cholesterol (TC), triglycerides (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA) and creatine kinase (CK). These analyses were perfromed in a Syncron CX3 autoanalyser (Beckam Coulter) at the Clinical Laboratory of the Faculty of Pharmacy, Coimbra, Portugal. After sacrifice and cardiac puncture, the animals

were submitted to transcardiac perfusion and the brains were collected and storage at 4°C until further analysis (see next section for detailed description of transcardiac perfusion).

2.2.1.3. Transcardiac perfusion of CD1 females

After deeply anesthetized females were perfused transcardially with 4% paraformaldehyde in PBS and the brains were removed and kept in paraformaldehyde overnight at 4°C. Then, the brains were dehydrated in 20% sucrose in PBS and kept at 4°C until being sectioned. Coronal sections were cut in 8 series of slices from the striatum and hippocampus on a freezing microtome at 30 µm of thickness, and collected in antifreeze solution for storage at 4°C until further use for immunohistochemical studies.

2.2.2. New born animals and husbandry

Following birth of litters 100 CD1 mice, 50 females and 50 males, were maintained in our animal facilities under the same conditions described above in section 2.2.1. The pups were exposed to AEDs during gestation and nursing. After weaning they were separated by gender, each gender divided in five groups: control, ESL, CBZ, OXC and VPA, and each group with 10 animals randomly distributed in two cages (5 each).

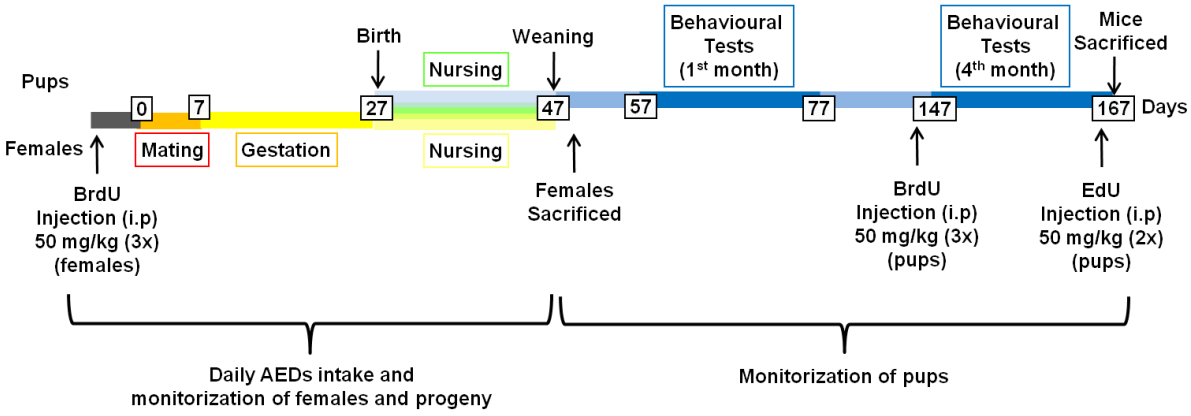

Fig. 2.5. Experimental approach to study the effects of AEDs exposure during gestation and nursing on females and offspring. Several parameters were measured and evaluated in both groups of animals. For detailed description of each experiment, see respective sections in 2.2.

2.2.2.1. Behaviour tests and procedures with offspring

All animals (100) performed behaviour tests at 1 and 4 months of age. During the periods of experimentation all animals were maintained near the testing room, inside the respective cages, in a ventilated cabinet (12 h dark:light cycle), and provided with food and

water *ad libitum*. Animals became familiar with the researcher by handling the animals every other day for at least one week prior to the beginning of the experiments, and were transferred to the "new" place (testing room) two days before the behaviour tests to, become familiar with the environment. This procedure decreased the stress of the animals.

The experiments were conducted between 9:00 a.m. and 6:00 p.m. and the testing room was illuminated with dim red light and with controlled temperature (21°C±1°C). The mazes were cleaned with wet (ethanol 10%, v/v) and dry cloths between animals usage. The behaviour tests performed by pups at 1 and 4 month of age are described below.

Open-field test

The open-field test was used to access and evaluate the animal locomotor activity. This task was monitored in an acrylic open-field arena (30 x 30 cm, divided in 9 squares, 30 cm tall) and the exploratory behaviour of the animals was evaluated for 8 min by counting the total number of line crossings (Walsh and Cummins 1976)(Fig. 2.6).

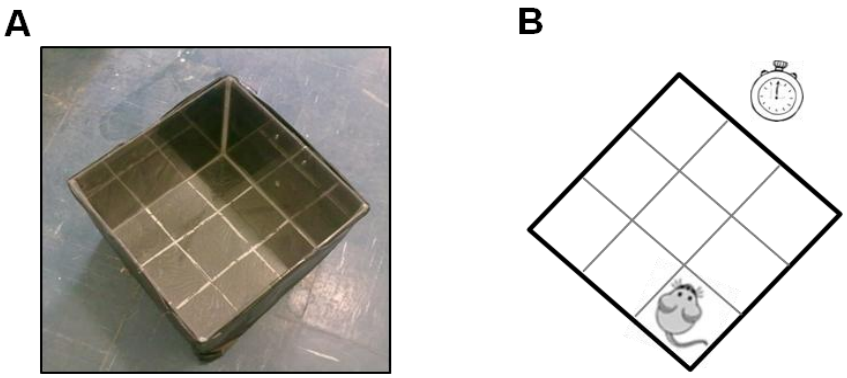

Fig. 2.6. Arena of open-field test. A) Acrylic open-field arena used to evaluate locomotor activity; **B)** Schematic illustration of open-field arena. Locomotor activity was evaluated for 8 min.

Object recognition test

The object recognition test was used to evaluate memory performance. This task was evaluated in two 8-min sessions in the open-field arena (after the open-field test). First, the animals had access to explore two identical objects for 8 min – training session. Two hours later, animals had access to explore two different objects during 8 min (a familiar and a novel one). Object Recognition time was calculated by the ratio of the time exploring the novel object over the total exploration time of both objects (Bevins and Besheer 2006) (Fig. 2.7).

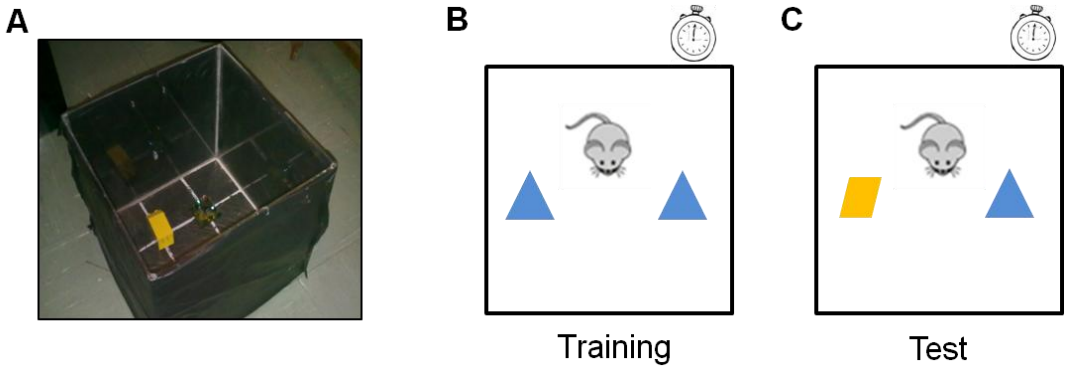

Fig.2.7. Object recognition test. A) Acrylic open-field arena and objects used to evaluate memory performance; **B-C)** Schematic illustration of object recognition test **B)** training: two identical objects, and **C)** test: two dissimilar objects. Each session lasts 8 min and is separated by 2 h inter-trial interval.

Y Maze test

The Y Maze test was used to evaluate spatial memory performance. This task is evaluated in a Plexiglas apparatus consisting of 3 arms in a Y-shape maze, separated by equal angles. Animals were subjected to two 8-min sessions, training and test, separated by 2 h inter-trial interval. During training, animals are allowed to explore two arms while a small wall makes the third one inaccessible. In the test session the wall was removed and animals could explore the entire maze (Fig. 2.8). Memory performance was measured by the percentage of time spent exploring the novel arm over time spent exploring all arms (Dellu et al. 1992).

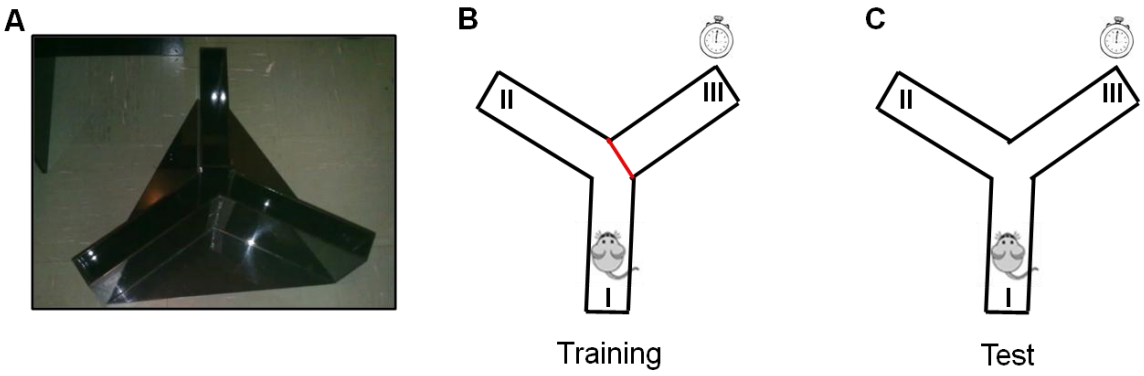

Fig.2.8. Y Maze test. A) Plexiglas apparatus used to evaluate spatial memory performance; **B-C.** Schematic illustration of Y maze test test: **B)** training: third arm is inaccessible (red line), and **C)** test: animals could explore all maze. Each session lasts 8 min and is separated by 2 h inter-trial interval.

Inhibitory (passive) avoidance test

The inhibitory avoidance test was used to evaluate aversive memory (learning). This task consisted in evaluating mice step-down latency, which is measured in a shock generator (Ch2001, Insight Equipamentos, Ribeirão Preto, Brasil). This training apparatus is a 50 x 25 x 25 cm plastic box with a 2 cm-high, 4 x 6-cm-wide platform at the box center. The floor of the apparatus was made of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart. The experiment consisted in two sessions, the training and the test sessions separated by 90 min inter-trial interval. During the training session the animal was placed on the platform and at the time they leave it, mouse received a 1 s foot shock (0.7 mA). In the test, the latency to step down the four paws on the grid was measured keeping a ceiling time of 180 s (Kazlauckas et al. 2005) (Fig. 2.9). Animals, whose latency was 180 s, were not included in the analysis.

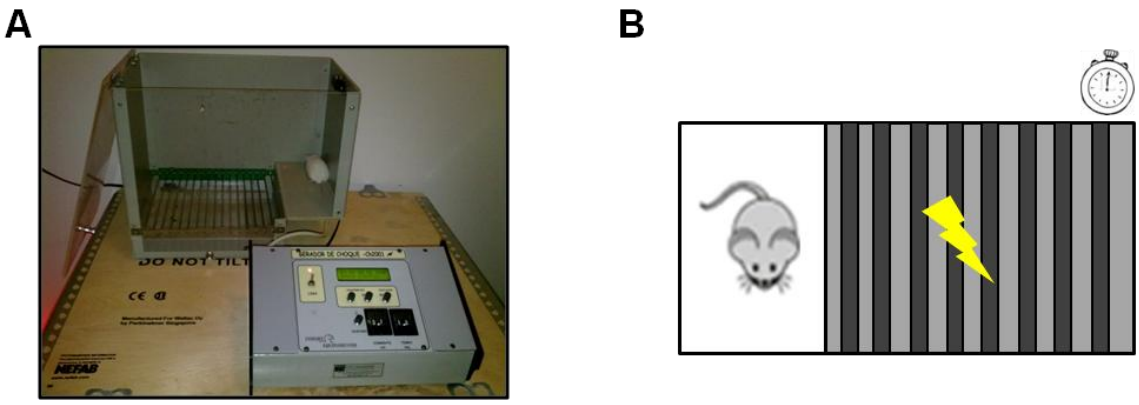

Fig.2.9. Inhibitory (passive) avoidance test. A) Shock generator used to evaluate aversive memory (learning associated); **B)** Schematic illustration of step-down latency test, which consists in two sessions (training and test sessions) separated by 90 min inter-trial interval.

Elevated Plus Maze test

The elevated plus maze test was used to evaluate locomotor behaviour and anxiety-related behaviour. This maze consisted in four arms (same size, 40 cm x 5 cm) arranged in the form of a cross and raised 50 cm above the floor. Two arms are open and the other two opposed arms are enclosed with a 30 cm high opaque Plexiglas walls, except for the entrance (Fig. 2.10). The animals were subjected to only one 5 min session to explore the maze. They were placed on the central square of the maze facing an enclosed arm. The

entries and the time spent exploring each arm was registered. Locomotor behaviour was expressed by the number of entries in the arms (open or enclosed) and anxiety was evaluated from the ratio of time spent exploring the enclosed arms over the total time, minus the time spent in central square. Any animal that fell off the maze was excluded from the analysis (Walf and Frye 2007) (Fig. 2.10).

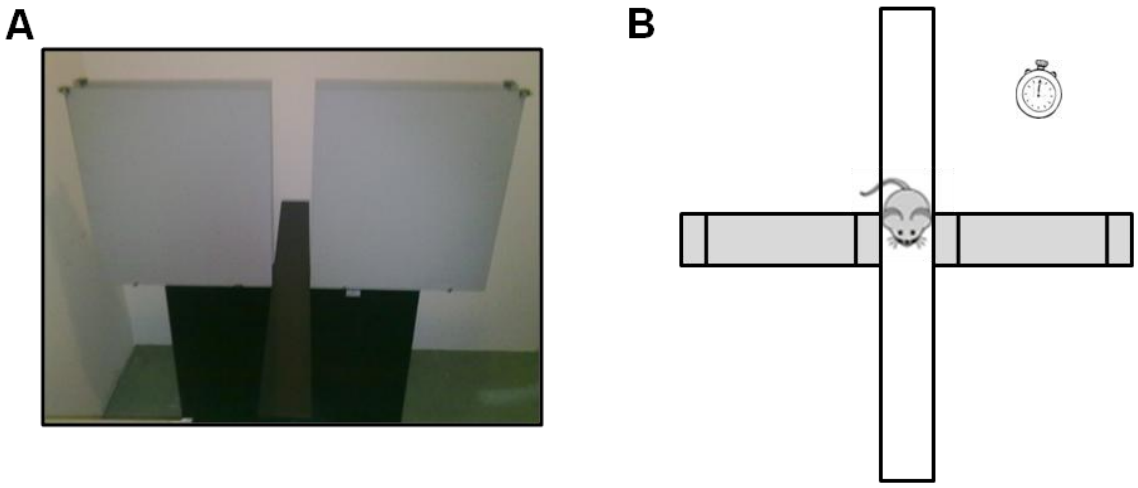

Fig. 2.10. Elevated plus maze test. A) Plexiglas apparatus of elevated plus maze used to evaluate locomotor and anxiety-related behaviour; **B)** Schematic illustration of elevated plus maze which consist in one session of 5 min. White arms – open arms; grey arms – closed arms.

Forced Swimming test

The forced swimming test was used to evaluate drug (AED) induced-depressive profile. In this task animals were evaluated after exposure to a situation to which it is not possible to escape. This is performed in a plastic cylinder tank with 10 cm diameter and 24 cm height, containing 19 cm height of water, at 25±1°C. The experiment lasts 6 min and the immobility time is recorded. The animal is considered immobile when floating or just make necessary movements to keep its head above the water (Porsolt et al. 1977) (Fig. 2.11).

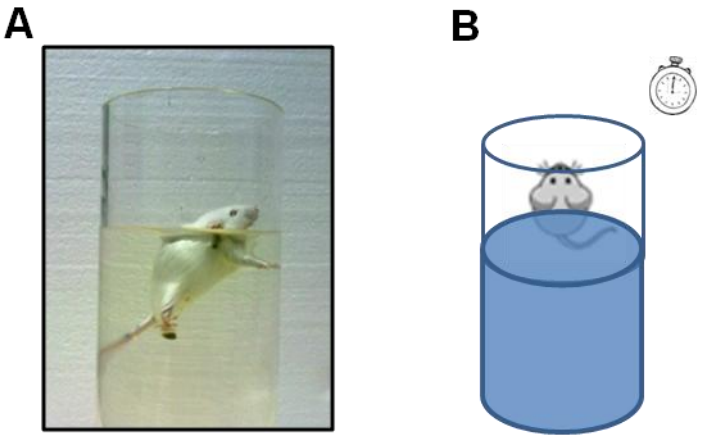

Fig. 2.11. Forced swimming test. A) Apparatus used to evaluate drug-depressive profile; **B)** Schematic illustration of forced swimming test, which lasts 6 min. A plastic cylinder containing 19 cm height of water at 25±1°C is illustrated.

2.2.2.2. Transcardiac perfusion of CD1 mice at 4 months of age

After deeply anesthetized, 50 animals (25 per gender, 5 from each group of treatment) were quickly weighed before being perfused transcardially with 4% paraformaldehyde in PBS. Then, brains were removed, weighed and kept in paraformaldehyde overnight at 4°C. After that, brains were dehydrated in 20% sucrose in PBS and reserved at 4°C until being sectioned. Coronal sections were cut in 8 series of slices (30 µm thick) for the striatum and hippocampus on a freezing microtome and collected in antifreeze solution for storage at 4°C until further use.

2.2.3. Immunohistochemistry

2.2.3.1. BrdU detection and of other neuronal markers

Neurogenesis in the dentate gyrus of hippocampus of CD1 pregnant females and their offspring was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation with other neuronal markers such as neuronal nuclei antibody (NeuN) and doublecortin antibody (DCX). BrdU, as thymidine analogue, can incorporate in the new DNA strand of the granule cells (hippocampus) during the S-phase of cell cycle, namely in the subgranular zone (SGZ), innergranular zone (IGZ) and outergranlar zone (OGZ) of the dentate gyrus. BrdU was delivered by intraperitoneal injection (i.p.) in three doses of 50 mg/Kg each (24 hours interval), 28-30 days before sacrificing the mice (females and offspring). Their brains were

cut in coronal sections (30 μm thick)and those free-floating brain sections were processed for detection by immunohistochemistry of BrdU, NeuN or DCX. Briefly, brain sections were treated with 1 M HCL for 20 min at 65°C, for DNA denaturation, and then blocked for 1 h with 5% normal goat serum (NGS), or normal horse serum (NHS) in 0.25% Triton X-100 in PBS. Slices were then incubated with the primary antibodies, rat anti- BrdU (1:50), mouse anti-NeuN (1:200), and goat anti-DCX (1:400), 48 h at 4°C with stirring (see Table 2-III for detailed description of the antibodies used). After rinsing with 0.25% Triton X-100 in PBS, the sections were incubated with goat or donkey anti-rat IgG conjugated with Alexa Fluor 488 (1:200), and goat anti-mouse IgG conjugated with Alexa Fluor 594 or 633 (1:200), and donkey anti-goat IgG conjugated with Alexa Fluor 594 (1:200) for 2 h at room temperature, with stirring. After rinsing with 0.25% Triton X-100 in PBS, the sections were mounted in 2% gelatin-coated slides with DAKO fluorescence mounting medium. BrdU-positive cells were counted in an epifluorescent microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) in the 5 mid sections of the hippocampus of at least 3-4 animals for each experimental group. Co-localization of BrdU with NeuN or DCX was analyzed by laser scanning confocal microscopy, in at least 50 cells from each coverslip in a LSM 510 META. Double-labelled cells were analysed by orthogonal reconstruction of sections scanned at 1 μm-thickness using Zeiss LSM Image Browser (version 4.0.0.157, Carl Zeiss Jena GmbH, Oberkochen, Germany).

Table 2-III. Primary antibodies used for immunocytochemistry experiments.

Antibody	Host	Dilution	Company
BrdU	Rat	1:50	AbD Serotec
DCX	Goat	1:400	Santa Cruz Biotechnology
NeuN	Mouse	1:200	Chemicon

2.2.3.2. EdU detection

Cell-proliferation in the dentate gyrus of hippocampus was detected by EdU incorporation. EdU is a thymidine analogue which can be incorporated by DNA during the S-phase of the cell cycle. EdU was delivered by i.p. injection in two doses of 50 mg/Kg each (12 h interval), one day before sacrificing the mice (offspring). Brains were cut in coronal sections and those free-floating brain sections were processed for detection by

immunohistochemistry using click-chemistry with Alexa Fluor 488 azide, according to the manufacturer instructions (Click-iT® EdU Alexa Fluor® 488 HCS Assay, Invitrogen, Life Technologies, Madrid, Spain). Briefly, brain free-floating sections were washed with 3% BSA in PBS and then were permeabilized with 0.5% PBS-Triton X-100 for 30 min at room temperature. Next, "cocktail reaction" (Alexa Fluor® 488 azide, copper sulphate and 1x Click-iT reaction buffer additive, all available from the kit) was added to the brain sections for 1 h, protected from light, at room temperature. After washed twice with 3% BSA in PBS, brain sections were incubated with Hoechst 33342 (1 μg/mL) for 10 min, protected from light, at room temperature. Finally, sections were rinsed with 3% BSA in PBS and mounted in 2% gelatine-coated slides with DAKO fluorescence mounting medium. EdU-positive cells were counted in an epifluorescent microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) in the 5 mid sections of the hippocampus, of at least 3-4 animals for each experimental group.

2.2.4. Cresyl violet staining

Brain sections from offspring were also stained with cresyl violet. Briefly, sections were mounted in 2% gelatine-coated slides and let dried overnight. Sections were sequentially immersed in an ethanol gradient (100%, 95% and 70%), washed with distilled water and then stained with 0.5% cresyl violet for 30-60 seconds. Afterwards, the sections were submitted again to dehydration in an ethanol gradient (70%, 95% and 100%), and finally immersed in xylene 100% (twice). After dried, the sections were coverslipped with DPX medium. Images were visualized and analyzed in a bright field microscope (Axioskop 2 Plus, Zeiss, Jena, Germany).

2.3. Statistical Analysis

The data are presented as means ± SEM. Statistical significance was determined by using one-factor analysis of variance (one-way ANOVA) or Kruskal-Wallis test (one-way analysis of variance by ranks, non-parametric method), as appropriate, followed by *pos hoc* Dunnet's or Dunn´s tests, respectively, and two-way analysis of variance (two-way ANOVA), followed by *post-hoc* Bonferroni's test, as indicated in the figure legends and in the text. Differences were considered significant when $p<0.05$.

2.4. Materials

All materials, reagents and consumables indicated in the sections 2.1 and 2.2 are listed below.

Antiepileptic drugs, such as ESL, S-Lic, R-Lic, CBZ and OXC were obtained from BIAL – Portela & Cª., S.A. (S. Mamede do Coronado, Portugal). Lamotrigine and Valproate (TOCRIS) were purchased from Biogen Scientifica S.L. (Madrid, Spain).

Trypsin, Neurobasal medium, B27 supplement, GlutaMAX™, Gentamicin, trypsin-EDTA solution (0.05%), D-MEM/F-12 with GlutaMAX™-I, and antibiot ic (10,000 units/mL of penicillin, 10,000 units/mL streptomycin), were from GIBCO® (Life Technologies™) and were purchased from Alfagene (Carcavelos, Portugal).

Growth factors, EGF and bFGF were purchased from Peprotech (London, UK).

DNAseI, poli-D-lysine, poli-L-lysine, glutamate, resazurin, PMSF, DTT, CLAP, orthovanadate, Accutase, cresyl violet and primary antibody mouse monoclonal Anti-α-Tubulin produced in mouse were purchased from SIGMA-ALDRICH (Madrid, Spain).

Monoclonal anti-BrdU produced in rat (AbD Serotec) was purchased from Alfredo Cavalheiro (Queluz, Portugal). Polyclonal anti-DCX produced in goat was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-NeuN produced in mouse (Chemicon), were purchased from Millipore Iberica (Madrid, Spain). All other primary antibodies were products of Cell Signaling and were purchased from Izasa (Carnaxide, Portugal).

Click-iT® EdU Alexa Fluor® 488 Flow cytometry Assay kit, Click-iT® EdU Alexa Fluor® 488 HCS Assay, Hoechst 33342, and all secondary antibodies (mouse, rat, and goat) IgGs conjugated with Alexa Fluor were products of Molecular Probes® (Life Technologies™) and were purchased from Alfagene (Carcavelos, Portugal)

NHS and NGS were purchased from Vector Labs (Baptista Marques, Lisboa, Portugal).

DAKO fluorescence mounting medium was purchased from Lusopalex (Queluz de Baixo, Portugal). BSA, chloroform, ethanol, isopropanol (MERCK), and xylene (MSD) were purchased from VWR (Carnaxide, Portugal).

BCA™ Protein Assay kit (Pierce, Thermo Scientific) was purchased from Fisher Scientific (Loures, Portugal).

PVDF membranes, alkaline phosphatase-linked secondary antibodies (mouse and rabbit) for Western blotting and ECF reagent (Amersham Pharmacia Biotech) were purchased from GE Healthcare (Carnaxide, Portugal). Other reagents used in immunoblotting experiments were purchased from BioRad (Sintra, Portugal).

Sodium pentobarbital was purchased from B.Braun (Queluz de Baixo, Portugal). Other reagents used in *in vivo* experiments were purchased from Reagente 5 (Porto, Portugal).

All equipment used in behavioural tests was "home-made" at the Physics Department, University of Coimbra, Portugal. The maze models were designed in accordance to the models described at Panlab Integrated Solutions (http://www.panlab.com/panlabWeb/portada.php).

Chapter 3

Activation of neurotoxicity and neuroprotection pathways by antiepileptic drugs in cultured hippocampal neurons

3.1. Summary

Antiepileptic drugs (AEDs) restore and balance synaptic flux in the brain in order to prevent seizures. However, some AEDs are unsafe and have unfavourable toxicity profiles, which is more frequent with older-generation AEDs, such as carbamazepine (CBZ) and valproate (VPA). Thus, it is crucial that new AEDs have a better safety profile than older ones. We compared the toxicity profile of eslicarbazepine acetate (ESL), a new AED developed by BIAL (Portugal) and of its metabolites, S-Licarbazepine (S-Lic) and R-Licarbazepine (R-Lic), to the toxicity of the structurally-related compounds CBZ and oxcarbazepine (OXC), and to the non-related AEDs lamotrigine (LTG) and VPA, in primary cultures of rat hippocampal neurons. We also assessed whether AEDs modulate pro-survival/pro-apoptotic pathways such as extracellular-regulated kinase (ERK1/2), Akt and stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling pathways.

We found that neither ESL, nor its metabolites S-Lic and R-Lic, as well as CBZ and LTG, caused toxicity to hippocampal neurons, up to 0.3 mM, for 24 h of exposure. OXC was the most toxic drug decreasing cell viability in a concentration-dependent manner, leading to activation of caspase-3 and PARP cleavage. VPA also caused the appearance of the apoptotic markers, but did not alter cell viability. Phosphorylation levels of ERK 1/2, Akt and SAPK/JNK were differently affected by AEDs. Briefly, ESL, S-Lic and OXC decreased the levels of phospho-ERK1/2 and phospho-Akt but did not change the levels of phospho-SAPK/JNK, when compared to basal levels, whereas CBZ decreased phospho-SAPK/JNK and phospho-Akt levels. However, LTG, and to some extent VPA, increased the phosphorylation levels of SAPK/JNK.

Altogether, the results suggest that ESL and its metabolites are less deleterious to neuronal cells than the other AEDs studied. Moreover, exposure to OXC and VPA did induce the appearance of markers related to cell death, and OXC was the most toxic drug.

3.2. Introduction

A large variety of antiepileptic drugs (AEDs) are available for the clinical treatment of epilepsy, bipolar disorder, migraine, mania, neuropathic pain, schizophrenia and anxiety (Rogawski and Loscher 2004; Stefan and Feuerstein 2007). However, some AEDs are less potent, and unsafe, with unfavourable and toxicity profiles, such as hepatotoxicity, toxicity to central nervous system (CNS) and complex drug/drug interactions (Loring et al. 2007; Pennell 2008; Johannessen Landmark and Patsalos 2010). In recent years, pharmaceutical companies have done an effort to develop new AEDs in order to overcome the adverse effects caused by currently available ones. Several studies have described the toxicity of AEDs to neuronal cells, *in vitro* and *in vivo*, (Bittigau et al. 2002; Landmark and Johannessen 2008; Manent et al. 2008; Chateauvieux et al. 2010) but the mechanisms underlying the toxic effects of AEDs in neuronal tissue are not well understood yet.

Carbamazepine is still one of the mostly prescribed AEDs, although it has a large number of side effects (Albani et al. 1995; Elger and Bauer 1998). In the last two decades, two CBZ derivatives were approved as anticonvulsants, first OXC in 1990 (Elger and Bauer 1998), and more recently eslicarbazepine acetate (ESL) in 2009 (Elger et al. 2009; McCormack and Robinson 2009). Both have been developed to improve tolerability and efficacy (Landmark and Johannessen 2008). These AEDs (CBZ, OXC and ESL) are structurally similar and exert their primary antiseizure activity by blocking voltage-gated sodium channels (VGSC) in neuronal membranes that are responsible for action-potential generation (Parada and Soares-da-Silva 2002). OXC and ESL were developed by structural variation of CBZ but there are striking species differences in their metabolism, mainly between CBZ and ESL. ESL is structurally different at the 10,11-position, and consequently, it is not metabolized to CBZ-10,11-epoxide and therefore not susceptible to enzyme induction or autoinduction (Bialer 2006). Moreover, ESL is a once-daily VGSC blocker (Almeida and Soares-da-Silva 2007) that is rapidly absorbed and undergoes extensive first-pass metabolism, in humans (Almeida et al. 2005) and mice (Alves et al. 2007), to its main active metabolite, S-Lic, which is responsible for approximately 95% of total systemic drug exposure, and to R-Lic and OXC, to a lesser extent (Almeida et al. 2005). Our group demonstrated previously that ESL is less toxic to cultured neurons than the structurally-related and widely used AEDs CBZ and OXC, namely at high concentrations (0.3 mM). While

ESL did not induce apoptosis nor causes structural damage to the neuritic network (Ambrosio et al. 2000; Araujo et al. 2004), CBZ is able to elicit apoptosis in cultured cerebellar granule cells (Gao et al. 1995; Nonaka et al. 1998) and OXC increases the activity of caspase-3-like enzyme (Ambrosio et al. 2000). In fact, in cultured hippocampal neurons, OXC was shown to be the most toxic drug (Ambrosio et al. 2000; Araujo et al. 2004). However, the effects of ESL metabolites, S-Lic and R-Lic, on the viability of neuronal cells, has not been addressed yet.

Lamotrigine also exerts its anticonvulsive action by blocking voltage-dependent sodium channels but is chemically unrelated to any of the above referred AEDs (Xie et al. 1995; Kuo 1998). LTG was approved in the United States in 1994 and is an effective and well-tolerated drug with few side-effects and low toxicity at the CNS. In the neonatal rat brain, LTG did not induce neuronal apoptosis (Katz et al. 2007) and has been reported to have neuroprotective capacity (Willmore 2005).

Valproate (VPA), first licensed in Europe in the early 1960s, has become the most prescribed AED worldwide despite of its described adverse-effect profile (Perucca 2002). It is a broad spectrum AED with a simple structure of a short branched fatty acid, and several mechanisms of action are proposed: enhances GABAergic neurotransmission (Loscher 2002; Johannessen and Johannessen 2003), modulates brain metabolism, decreases excitability by affecting intracellular signalling pathways, namely the extracellular-regulated kinase (ERK) pathway (Rogawski and Loscher 2004), and exerts effects on voltage-gated sodium, potassium and calcium channels (Johannessen and Johannessen 2003). VPA induces apoptotic neurodegeneration in the developing rat brain in a dose-dependent manner and reduces the levels of phosphorylated ERK 1/2 and Akt (Bittigau et al. 2003). Recently Wang and colleagues (2012) showed that VPA selectively induces apoptosis in neurons in mixed neuron-astrocyte cultures from human fetal neural progenitors (Wang et al. 2012). However, *in vitro* studies described opposite effects of VPA, showing that VPA promotes the phosphorylation of ERK 1/2, Akt and SAPK/JNK, which may ultimately be responsible for its neuroprotective effect (Mora et al. 1999; Chuang 2005; Di Daniel et al. 2005).

In the present study, we compared the neurotoxicity profile of ESL and of its metabolites S-Lic and R-Lic to those of the structurally-related CBZ and OXC, and to LTG and VPA. We assessed cell death and cell viability parameters, as well as the activation of prosurvival intracellular signalling pathways in primary cultures of hippocampal neurons exposed to the AEDs. We found that ESL and its metabolites S-Lic and R-Lic, as well as LTG,

were not toxic to hippocampal neurons and OXC was the most toxic drug studied. This work contributes to clarifying some of the mechanisms involved on the effects of AEDs on neuronal viability in cultured hippocampal neurons.

3.3. Results

3.3.1. Toxicity of antiepileptic drugs in cultured hippocampal neurons

We first investigated whether ESL and its metabolites, S-Lic and R-Lic, and the other AEDs, affected cell viability. Cultured hippocampal neurons were exposed to the AEDs for 24 h, at concentrations of 0.01, 0.03, 0.1 and 0.3 mM (for ESL, S-Lic, R-Lic, CBZ, OXC and LTG) and of 0.01, 0.03, 0.1, 0.3, 0.5, 1 and 3 mM for VPA. The viability of hippocampal neurons determined after exposure to ESL or its metabolites (for all the concentrations tested) was not significantly altered comparing to control conditions (Fig. 3.1.A-C) as assessed by the resazurin reduction assay. Furthermore, treatment of hippocampal neurons with CBZ and LTG, which is chemically unrelated with CBZ but has similar pharmacological targets, did not significantly affect neuronal viability for the concentrations tested (Fig. 1.D, F). VPA, which is both chemically and pharmacologically unrelated to CBZ derivatives, also did not alter the viability of the hippocampal cultures (Fig. 3.1.G). However, OXC significantly decreased the cell viability of hippocampal cultures, in a concentration-dependent manner, reducing cell viability to 93.2±2.3% ($p<0.05$) and 86.3±2.9% ($p<0.01$) of the control, for 0.1 and 0.3 mM, respectively (Fig. 3.1.E). Lower concentrations of OXC (0.01 and 0.03 mM) did not affect cell viability. The observed toxicity to hippocampal neurons caused by high concentrations of OXC is in agreement with previous studies by our group (Ambrosio et al. 2000; Araujo et al. 2007).

Nuclear condensation/fragmentation, which is a hallmark of cell death, was next analysed in cultured hippocampal neurons exposed to AEDs for 24 h, for the higher concentration tested in the resazurin assay (0.3 mM, for all AEDs, and 3 mM for VPA) . In control cultures, 21.4±1.4% of total cells presented condensed/fragmented nuclei, which is in agreement to previous studies showing similar basal levels of cell death in hippocampal cultures (Almeida et al. 2005; Araujo et al. 2007). Exposure to ESL, S-Lic, R-Lic, CBZ, LTG and VPA did not increase the percentage of condensed/fragmented nuclei ($p>0.05$) (Fig. 3.2.A).

However, treatment with OXC (0.3 mM) significantly increased the percentage of cells showing nuclear condensation/fragmentation to 37.1±6.0% ($p<0.01$) (Fig. 3.2.B).

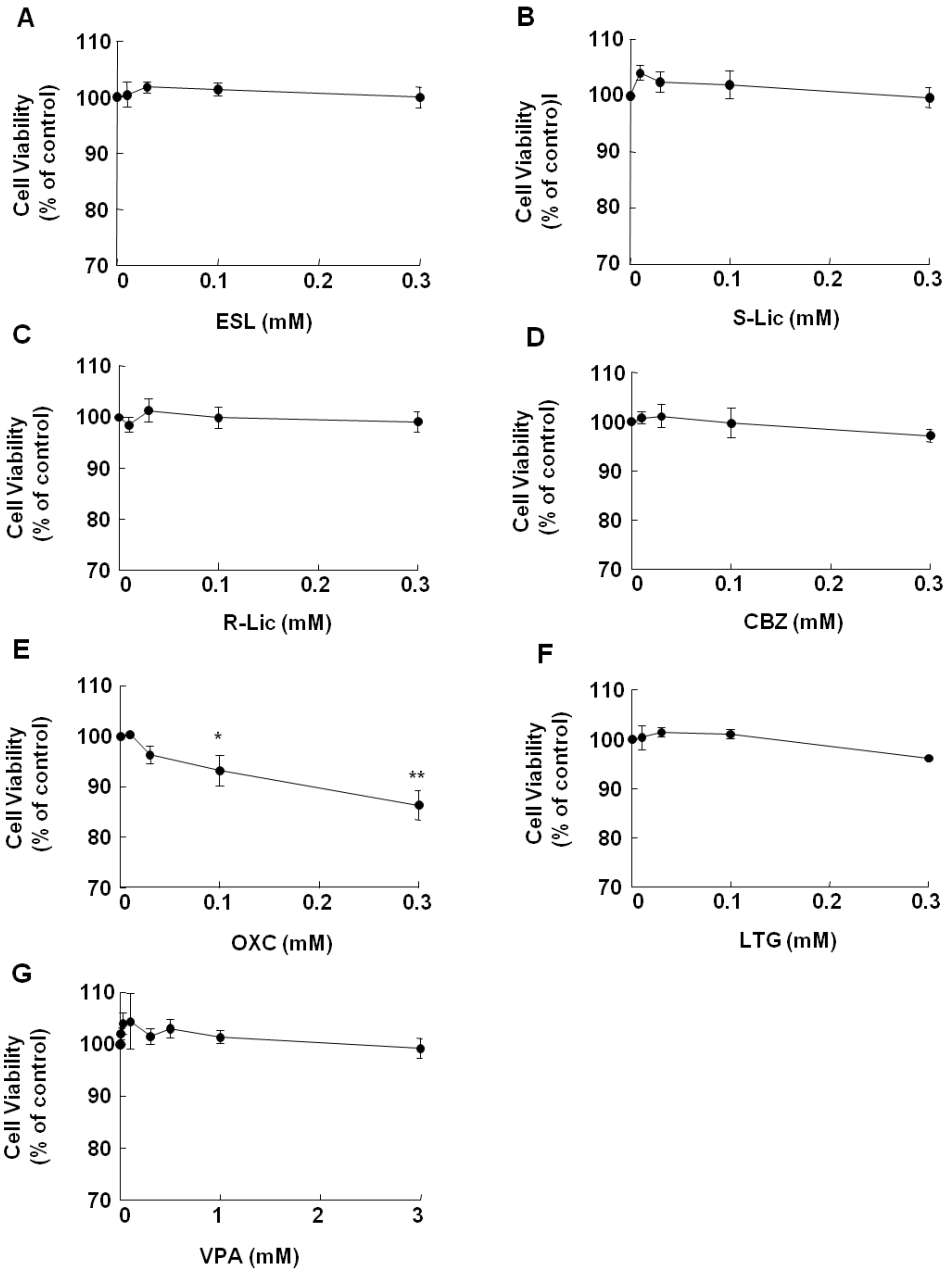

Figure 3.1. Effect of exposure of cultured hippocampal neurons to AEDs on their viability. Hippocampal neurons were exposed to increasing concentrations of **(A)** ESL, **(B)** S-Lic, **(C)** R-Lic, **(D)** CBZ, **(E)** OXC, **(F)** LTG, and **(G)** VPA, and cell viability was evaluated by the resazurin reduction assay. The results are presented as percentage of control (no drug), and represent the means ± SEM of at least four independent experiments performed in triplicate. *$p<0.05$; **$p<0.01$, significantly different from control, One-way ANOVA, Dunnett's post-test.

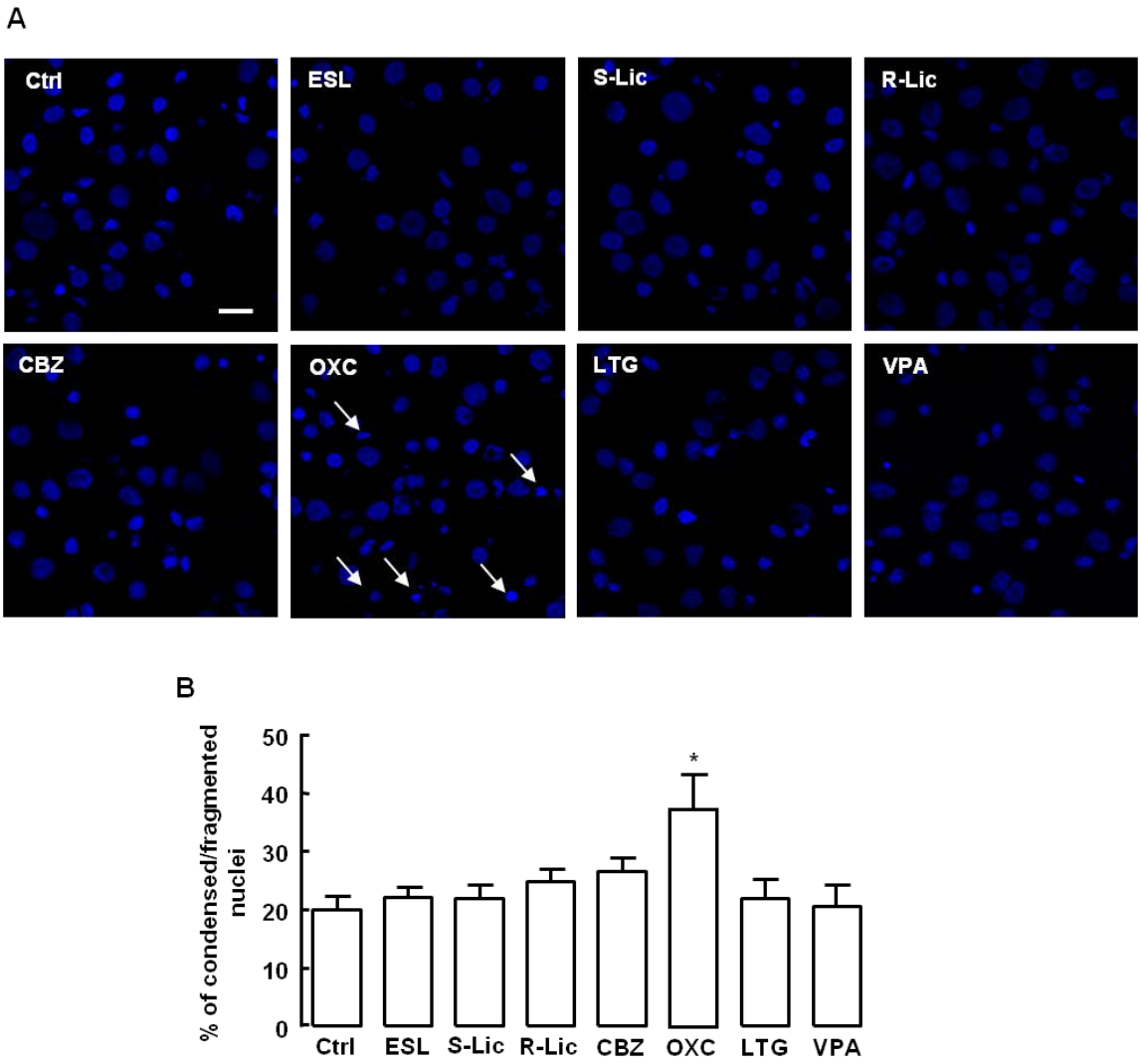

Figure 3.2. Effect of exposure to AEDs on nuclear condensation/fragmentation of hippocampal neurons. (A) Representative images of hippocampal neurons incubated for 24 h with AEDs (0.3 mM for all AEDs and 3 mM for VPA). Cell nuclei were labelled with Hoechst 33342. **(B)** Percentage of cells with condensed chromatin was significantly higher after exposure to OXC as compared to control, but was not changed for the other AEDs. The results represent means ± SEM of at least 4 independent experiments. **$p<0.01$, significantly different from control, One-way ANOVA followed by Dunnett's post hoc test.

3.3.2. Cell exposure to OXC or VPA increased apoptotic markers in primary hippocampal cultures

We next investigated the presence of apoptotic markers, such as activation of caspase-3, the main effector caspase in apoptotic cell death, which is activated by proteolytic cleavage of full-length caspase-3 (32 kDa), producing fragments of 17/19 kDa. The presence of these fragments can be used as a marker for caspase-3 activation. For this purpose, an antibody that selectively recognizes the breakdown products (17/19 kDa), but not full-length caspase-3, was used. Cultured hippocampal neurons were exposed to the AEDs for 24 h at 0.3 mM (except for VPA at 3 mM). We detected cleavage of caspase-3 in cultures exposed to OXC, as well as to VPA. Treatment with OXC caused a 5-fold increase in cleaved caspase-3 ($p<0.001$), while VPA caused a 2-fold ($p<0.01$) increase, when compared to the control (Fig. 3.3.). Exposure of the cells to ESL, S-Lic, R-Lic, CBZ or LTG, did not cause cleavage of caspase-3, as detected by Western blotting.

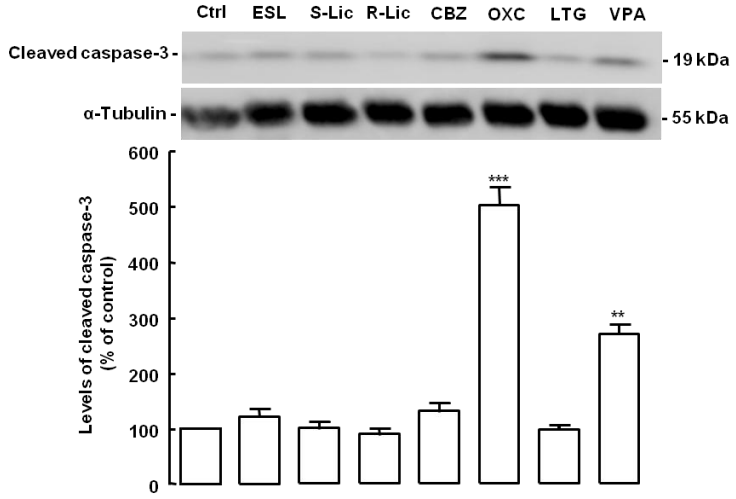

Figure 3.3. Levels of cleaved caspase-3 in hippocampal neurons following exposure to AEDs. Hippocampal neurons were exposed for 24h to AEDs (concentrations for each AED were described in Fig. 3.2). Representative Western blots and quantification of the levels of the 17/19 kDa cleavage product of caspase-3 are shown. Levels of cleaved caspase-3 were significantly higher in cultures treated with OXC and VPA than in control (Ctrl). α-Tubulin (55 kDa) was used as a loading control. The results are presented as percentage of control, and represent means ± SEM of at least 4 independent experiments. ***$p<0.001$, significantly different from control, Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

PARP is a downstream substrate of caspase-3 and a powerful effector of cell death, causing ATP depletion. Next, we investigated the profile of PARP cleavage in neurons treated with the AEDs. PARP is a target of proteolytic cleavage by active caspase-3, and the presence of 89 kDa fragment is indicative of PARP cleavage. Cell exposure to OXC or VPA, but not to the other AEDs, caused PARP cleavage, which increased to 212.1±39.1% ($p<0.001$) and 218.0±23.2% ($p<0.01$) of the control, respectively, as evaluated by Western blot analysis (Fig. 3.4).

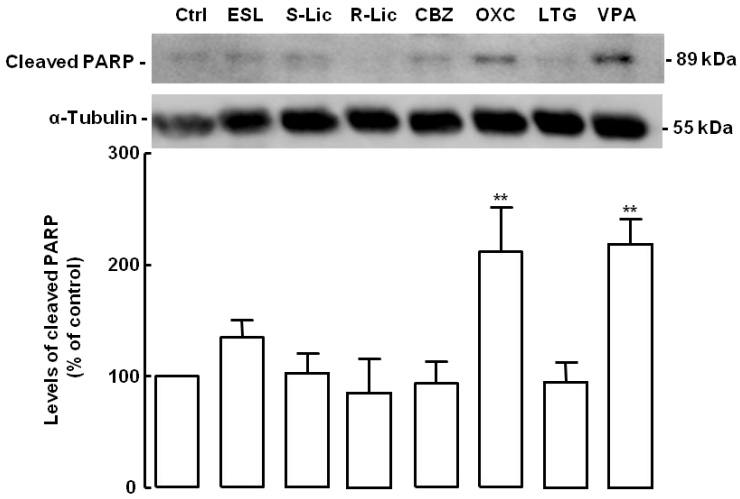

Figure 3.4. Levels of cleaved PARP in hippocampal neurons following exposure to AEDs. Hippocampal neurons were exposed for 24h to AEDs (concentrations for each AED were described in Fig. 3.2). Representative Western blots and quantification of the levels of 89 kDa cleavage product of PARP are presented for each AED. Levels of cleaved PARP were significantly higher in cultures treated with OXC and VPA than in control. α-Tubulin (55 kDa) was used as a loading control. The results are presented as percentage of control, and represent means ± SEM of at least 4 independent experiments. ***$p<0.001$, **$p<0.01$, significantly different from control, Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

Translocation of AIF from the mitochondria to the nucleus may occur during neuronal death. In AED-treated cultures, AIF was detected by immunocytochemistry in the cytoplasm of either viable or dying neurons. Cells treated with ESL and its metabolites, or the other AEDs, did not show evidence of nuclear translocation of AIF in any case. This factor had a cytosolic localization even though condensed/fragmented nuclei were quite evident in OXC-treated cells (0.3 mM) (Fig. 3.5). Thus, AIF does not appear to be involved in cell death triggered by exposure of hippocampal neurons to OXC.

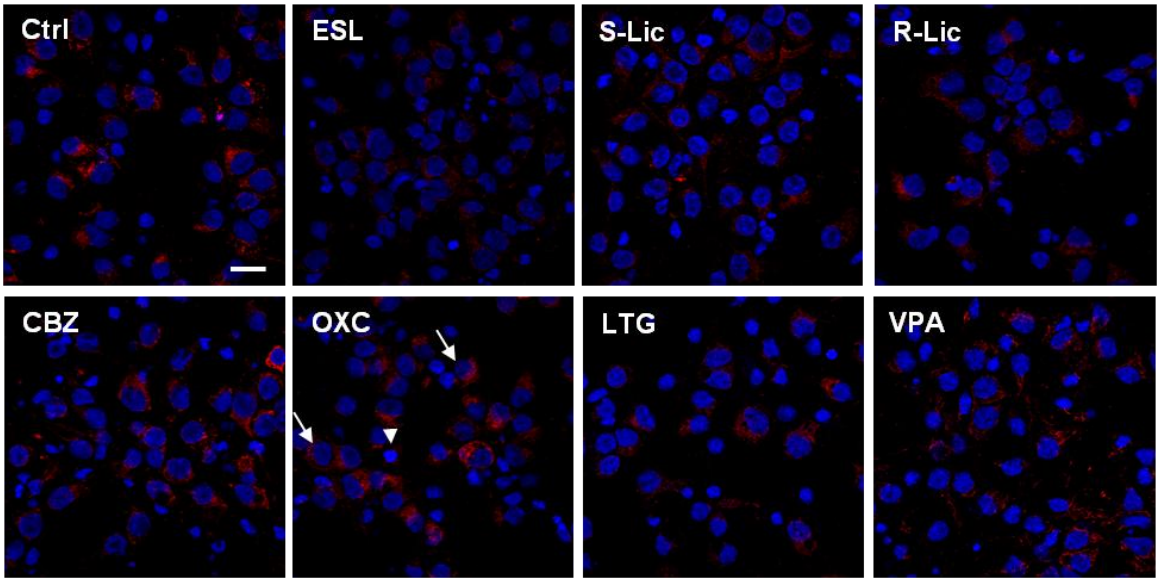

Figure 3.5 AIF did not translocate into the nucleus of dying neuron after exposure to AEDs. Cytosolic localization of AIF in viable (arrow) or dying (arrowhead) hippocampal neurons following exposure for 24h to AEDs (0.3 mM for all AEDs and 3 mM for VPA). Representative images of cultured hippocampal neurons without translocation of AIF from cytosol to the nuclei in any of the conditions tested. Nuclei were labeled with Hoechst 33342 (blue). Scale bar: 20 µm. .

3.3.3. Effects of antiepileptic drugs on signaling pathways related to neuronal injury/survival

In order to examine whether the effects of the AEDs presented above are associated with changes in pro-survival/pro-apoptotic pathways in cultured hippocampal neurons, we next studied the activation of extracellular-regulated kinase (ERK 1/2; p44/p42), Akt and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK; p54/p46). The phosphorylation levels of these kinases were analyzed by Western blot at different time points, for short-term exposures, up to 60 min, and for a longer period of 24 h of incubation with each of the AEDs (0.3 mM for ESL, S-Lic, R-Lic, CBZ, OXC and LTG; 3 mM for VPA). The phosphorylated forms of those kinases were differently affected, as described below.

In cultured hippocampal neurons exposed to ESL or S-Lic, the levels of phospho-ERK 1/2 were decreased as compared to control conditions (Fig. 3.6.A, B). The levels of phospho-ERK 1/2 were decreased to 39.7±8.4% of the control for ESL, and to 54.0±9.8% of the control for S-Lic ($p<0.01$), 60 min following exposure to the drug. Phosphorylation of ERK 1/2 was still decreased 24 h after treatment with ESL, but was back to control levels 24 h

after exposure to S-Lic. Exposure to OXC for 30 or 60 min also caused a significant decrease in the levels of phosphorylated ERK 1/2 (to 61.62±4.75 or 58.27±6.01% of the control, respectively, $p<0.05$) (Fig. 3.6.E). In contrast, 60 min after exposure to VPA, there was an increase in the levels of phospho-ERK 1/2 to 144.4±2.6% of the control ($p<0.05$) (Fig. 3.6.G). The other AEDs, R-Lic, CBZ and LTG did not cause significant changes on the levels of phospho-ERK 1/2 (Figs. 3.6.C, D and F).

The active form of Akt results from the combined phosphorylation of serine 473 and threonine 308 residues. In the present study, only the Thr308 residue showed altered levels of phosphorylation following exposure of hippocampal neurons to AEDs (Fig. 3.7). ESL caused a significant decrease in the levels of phospho-Thr308 to 50.2±4.6% of the control (Fig. 3.7.A; $p<0.05$) after 1 h treatment. S-Lic produced a similar effect, but earlier, at 30 min (Fig. 3.7.B; $p<0.05$). R-Lic only decreased phosphorylation of Thr308 after 24 h of exposure to the drug (Fig. 3.9.C; $p<0.01$). CBZ decreased Thr308 phosphorylation (47.9±16.8% of the control, $p<0.01$) (Fig. 3.7.D) after 30 min of exposure. Exposure to OXC caused a time-dependent decrease in phospho-Thr308, up to 24 h of exposure (45.12±6.5%, of the control, $p<0.01$) (Fig.3.9.E). LTG did not alter Akt phosphorylation (Fig.3.7.F). However, VPA significantly increased the phosphorylation of Thr308 after 60 min of exposure (202.3±25.9%, of the control, $p<0.001$), but this effect was lost at 24 h of exposure to VPA (Fig. 3.9.G).

We also analyzed the levels of phospho-SAPK/JNK. It was observed that neither ESL nor its metabolites caused changes in the phosphorylation levels of either isoform of SAPK/JNK, p54 and p46 (Figs. 3.8.A-C). The same was observed in cells exposed to OXC (Fig. 3.8.E). On the other hand, CBZ induced a decrease in the levels of phospho-p46 by approximately 70% up to 60 min after exposure ($p<0.01$) (Fig. 3.8.D). Exposure to LTG for 60 min caused a strong increase in the phospho levels of both isoforms (137.2±10.5% of the control, for phospho-p54, and 137.5±9.8% of the control, for phospho-p46, $p<0.05$), and it remained elevated by 2-fold at 24 h following treatment (194.0±17.6% of the control for phospho-p54, and 198.8±28.4% of the control, for phospho-p46, $p<0.001$) (Fig.3.8.F). Exposure to VPA for 24 h caused an increase in phospho-p46 to 136.2±16.8% of the control ($p<0.05$) (Fig.3.8.G).

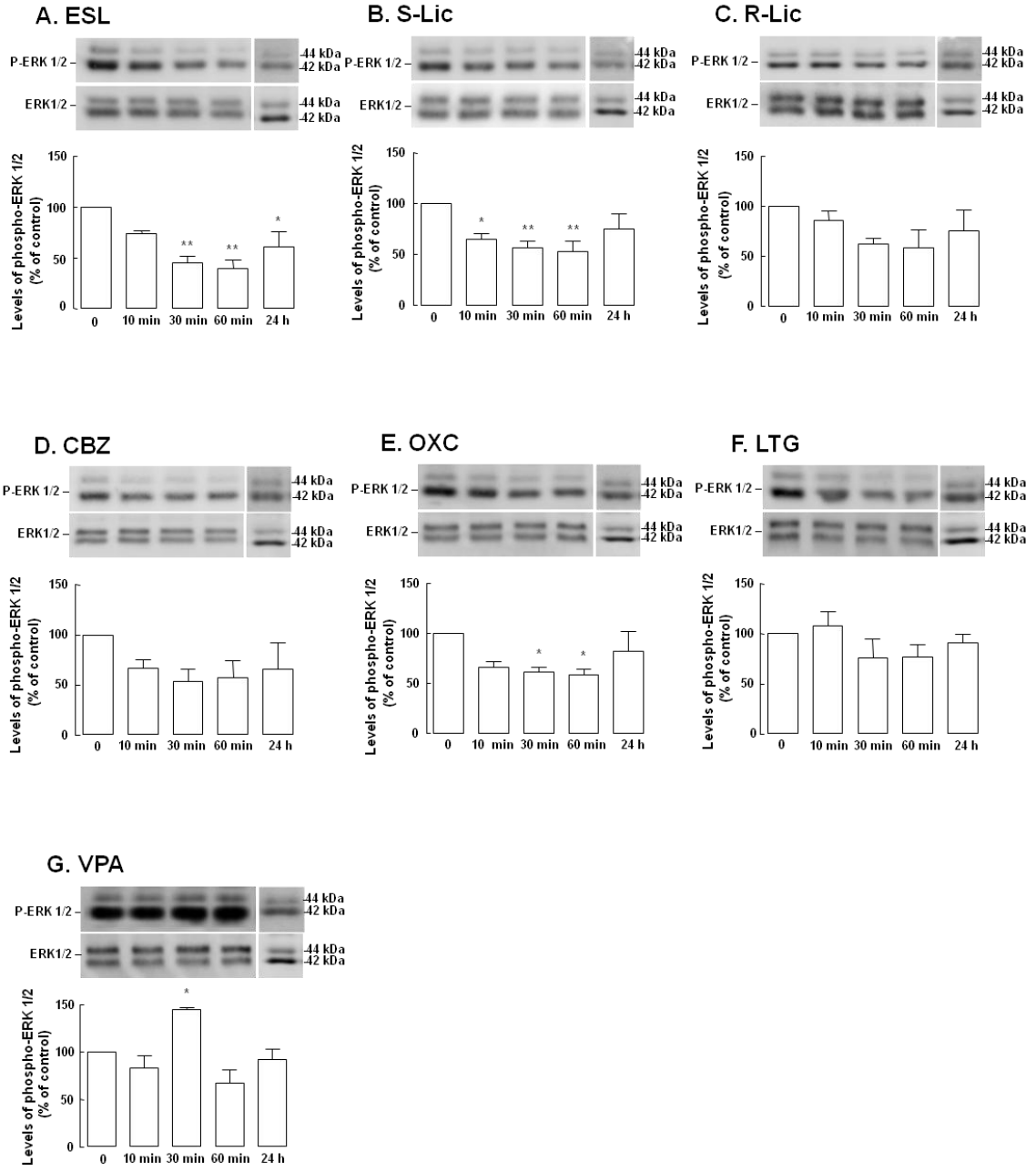

Figure 3.6. Analysis of phospho-ERK 1/2 (p44/p42) levels in hippocampal neurons following exposure to AEDs. Representative Western blots for phospho-ERK 1/2 and total ERK 1/2 are shown, as well as quantification of the levels of phospho-ERK 1/2 following exposure to AEDs for the periods indicated. The concentrations for each AED were the same as those described in Fig. 3.2. The results are presented as percentage of control, and represent means ± SEM of at least 3 independent experiments. *p<0.05, **p<0.01, significantly different from control, one-way ANOVA, Dunnett's post-test.

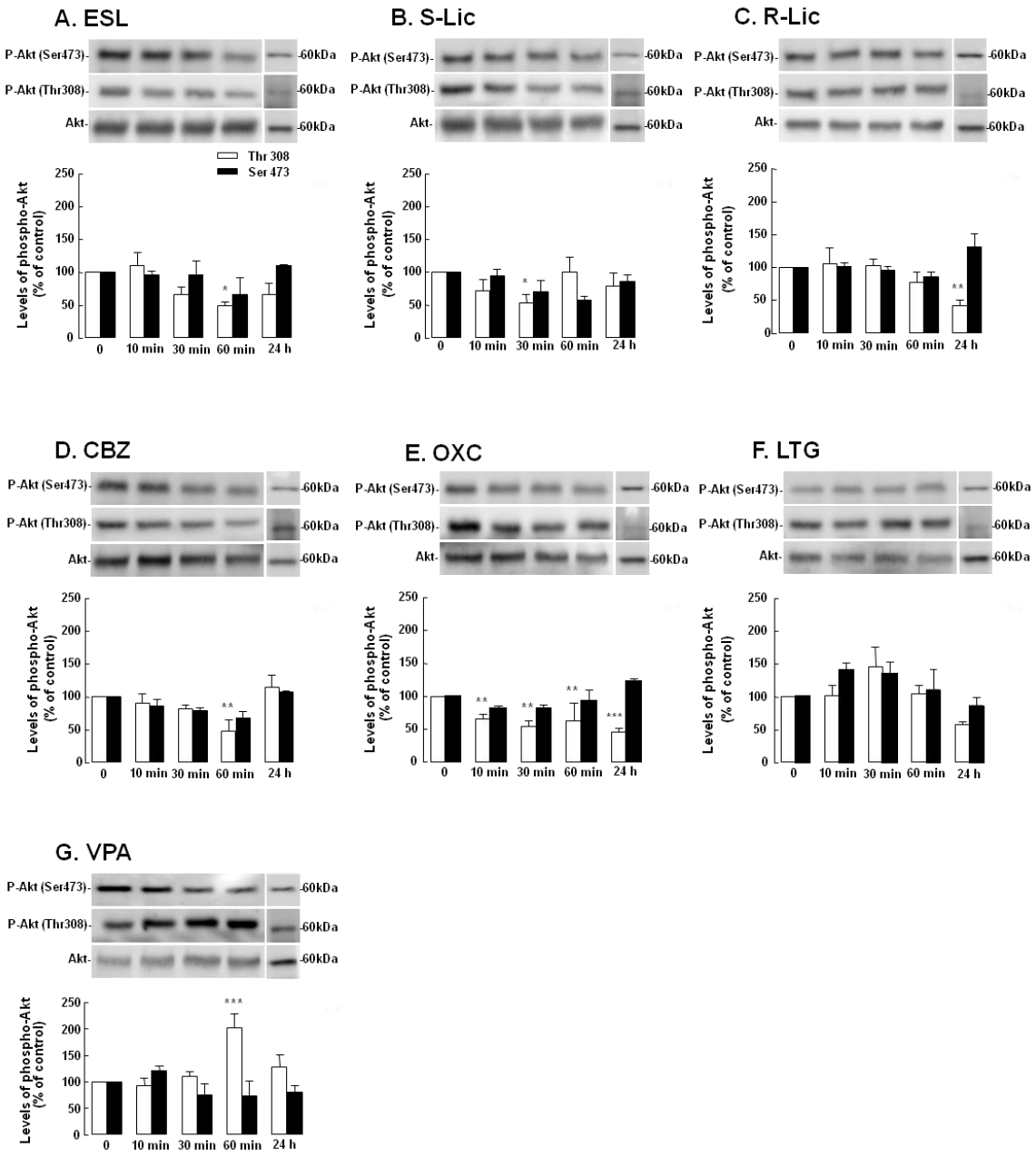

Figure 3.7 Analysis of phospho-Akt (Thr308/Ser473) in hippocampal neurons following exposure to AEDs. Representative Western blots for phospho-Akt and total Akt are shown, as well as the quantification of the levels of each Akt phospho-residue (Thr308 and Ser473) following exposure to AEDs for the periods indicated. The concentrations for each AED were the same as those described in Fig. 3.2. The results are presented as percentage of control, and represent means ± SEM of at least 3 independent experiments. *p<0.05, **p<0.01,***p<0.001, significantly different from control, two-way ANOVA (time and residue as factors), Bonferronis's post-test.

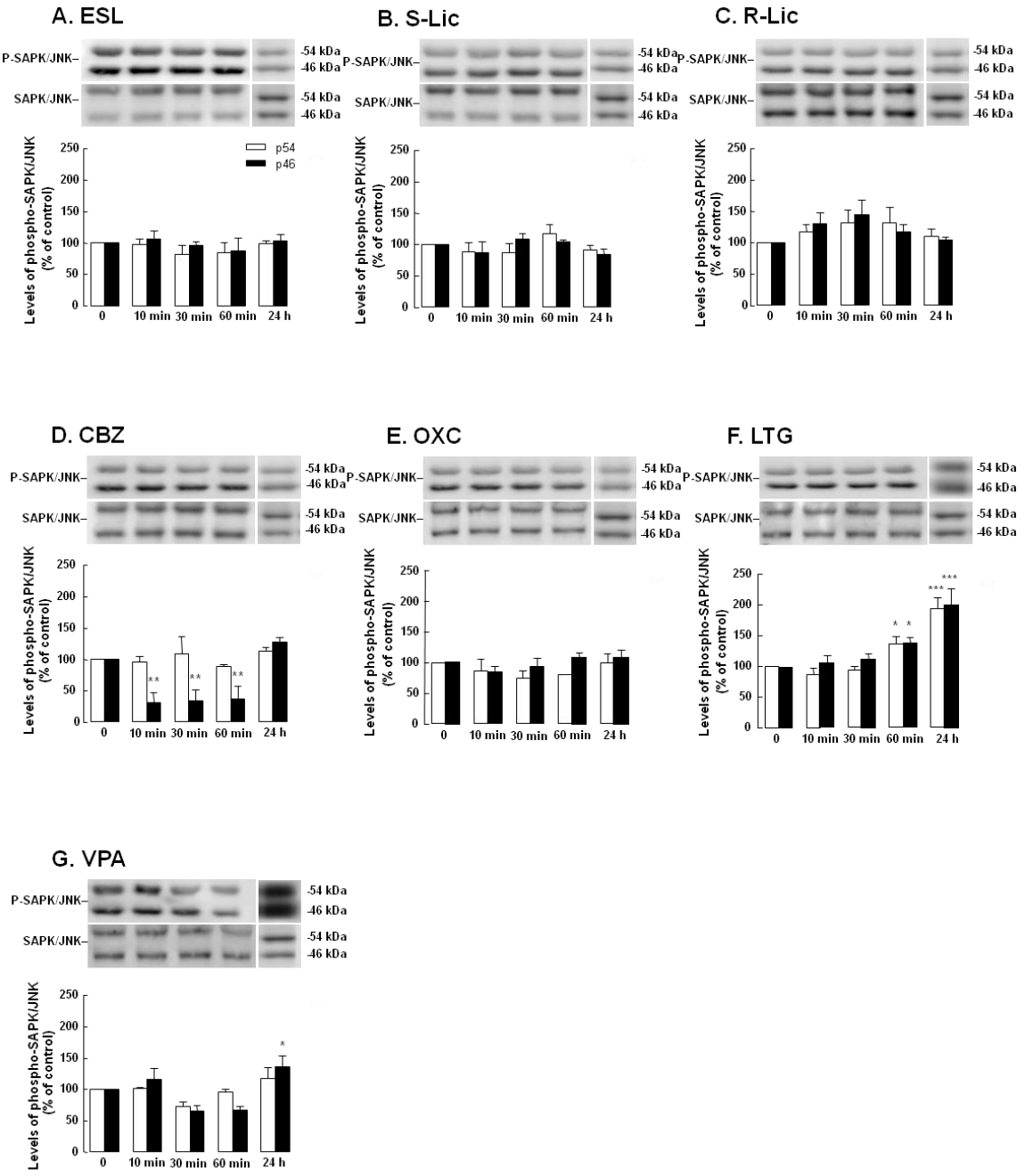

Figure 3.8 Analysis of phospho-SAPK/JNK in hippocampal neurons following exposure to AEDs. Representative Western blots for phospho-SPAK/JNK and total SAPK/JNK are shown, as well as the quantification of the levels of phospho-p46 and phospho-p54 following exposure to AEDs for the periods indicated. The concentrations for each AED were the same as those described in Fig. 3.2. The results are presented as percentage of control, and represent means ± SEM of at least 3 independent experiments. *p<0.05 and **p<0.01, ***p<0.001, significantly different from control, two-way ANOVA (time and isoforms as factors), Bonferroni's post-test.

3.4. Discussion

Antiepileptic drugs interact primarily with neurotransmitters receptors or ion channels, and they may directly or indirectly interfere with intracellular proteins and signalling systems with which they are associated. AEDs can also induce neuronal cell death by triggering apoptotic neurodegeneration in developing brain (Ikonomidou et al. 2000; Bittigau et al. 2002; Olney et al. 2002). There is no information about the potential toxic effects triggered by ESL metabolites or by LTG in cultured hippocampal neurons. The present data show that neither ESL nor its metabolites, S-Lic and R-Lic, neither LTG, caused any neurotoxicity to cultured hippocampal neurons. OXC was the most toxic drug in all assays, particularly for high concentrations, and VPA also induced the appearance of apoptotic markers in hippocampal cultures.

Exposure to OXC (0.1 and 0.3 mM) for 24 h increased cell death in hippocampal neurons. Activation of caspase-3 and PARP cleavage was also evident following treatment with OXC. ESL, S-Lic, R-Lic or LTG had no effect, up to 0.3 mM, in the viability of cultured hippocampal neurons, for the parameters evaluated in this study. OXC stands out as the most toxic drug evaluated in this study, which is in agreement with previous studies in hippocampal neurons (Ambrosio et al. 2000; Araujo et al. 2004). In a previous study, we found that treatment with OXC (0.3 mM) for 24 h triggered a loss of the mitochondrial membrane potential. Since the release of AIF from the mitochondria and its translocation to the nucleus occurs after mitochondrial depolarization (Zhang et al. 2002), we examined whether AIF could be a player in the toxic effect of OXC in hippocampal neurons. Interestingly, the translocation of the AIF to the nuclei of dying hippocampal neurons exposed to OXC was not observed (nor in cells treated with the other AEDs), even though apoptotic nuclei were quite evident in OXC-treated cells. This protein does not seem to be involved in the cell death triggered by exposure to OXC in hippocampal neurons. Thus, accordingly to our results cell death observed after exposure to OXC is mainly caspase-dependent.

Cell exposure to OXC (0.3 mM) or VPA (3 mM) caused an increase in caspase-3 activation, and also in PARP cleavage. However, cell death was not detectable with VPA when looking at nuclear condensation/fragmentation, and cell viability was not decreased by this drug. One possible explanation for the lack of cell death (despite some indication of apoptosis such as the presence of cleaved caspase-3) is that caspase-3 cleavage occurs

earlier in the cell death cascade than nuclear condensation and changes in the redox capacity of the cells. Thus, although a clear decrease in cell viability is not yet detected by these methods, caspase-3 has already been activated and the cell death cascade put in motion. Furthermore, stimulation of cell survival pathways concomitantly with cleavage of caspase-3 may prevent cell death; this being the case, following exposure to VPA, although caspase-3 cleavage is detectable, it is not associated to cell demise.

Mitogen-activated protein kinases (MAPKs) are activated by diverse stimuli such as neurotransmitters, cellular stress and growth factors (Liu et al. 2007). ERK 1/2 (extracellular signal-regulated kinase) and Jun-N-terminal kinase (JNK) also known as stress-activated protein kinase (SAPK) are two subfamilies of MAPKs. The MAPK/ERK pathway among other features is involved in neuronal survival. Hetman and Godz (2004) showed that ERK1/2 has a prosurvival activity in neurons (Hetman et al. 1999; Hetman and Gozdz 2004). On the other hand, it also has a role in apoptosis, which depends on cell type and on the signal that triggers cell death. It was observed that persistent activation of ERK 1/2 contributes to apoptosis in primary cortical neuronal cultures (Satoh et al. 2000; Stanciu et al. 2000). The SAPK/JNK is a central mediator of stress and is essential for the regulation of physiological and pathological processes (Johnson and Nakamura 2007). It mediates apoptosis by regulation of pro- and anti-apoptotic activity of members of Bcl-2 family and gene transcription (Burke 2007). PI3K/AKT signalling pathway also plays a role in surviving of neurons by different mechanisms (Yao and Cooper 1995; Dudek et al. 1997). Akt is a serine/threonine kinase involved in this pathway and when activated it mediates cell survival by inhibition of apoptosis, proliferation and transcription, among other functions (Brazil and Hemmings 2001), and thus deactivation of Akt accompanies cell death induced by many different agents (Luo et al. 2003). Any changes in these signalling pathways reflect an imbalance between neuroprotective and neurodegenerative mechanisms in the brain, and this imbalance can induce apoptotic death (Bittigau et al. 2003; Asimiadou et al. 2005).

In this study, we observed that CBZ did not increase neuroprotective signaling, and actually strongly decreased the levels of phospho-SAPK/JNK, and to a lesser degree, also decreased the levels of phospho-Akt. Possibly, for a longer exposure, the toxic effect of CBZ would be more evident and detectable by morphological criteria such as nuclear condensation/fragmentation, as we found in previous studies (Ambrosio et al. 2000; Araujo et al. 2004). Moreover, CBZ derivatives like OXC, ESL and ESL metabolites R-Lic and S-Lic

also decreased the levels of phosphorylated forms of ERK 1/2, Akt and SAPK/JNK. OXC strongly decreased the phosphorylation status of ERK 1/2 and Akt. A previous work by Bittigau et al. (2002) suggested that AEDs can induce apoptosis in neurons by inhibiting and decreasing the signaling of survival pathways such as ERK 1/2 and Akt (Bittigau et al. 2002). ESL, S-Lic and R-Lic also decreased the levels of phosphorylated ERK 1/2 and Akt, but less strongly and less robustly than OXC, and these drugs did not induce any other sign of neurotoxicity as OXC did, suggesting that ESL metabolites are less deleterious to neuronal cells because they affect the survival pathways to a lower extent than other AEDs. On the other hand, one may speculate that these effects of ESL and its metabolites on ERK 1/2 may constitute a compensatory response mechanism to the strong increases in ERK activation observed at the time of spontaneous seizures. In fact, in an *in vivo* study, phospho-ERK levels were shown to increase after a behavioural seizure detection (Houser et al. 2008). Inhibiting ERK signaling might be useful for seizure control (Nateri et al. 2007).

Unless metabolic impairment or hepatic failure occurs, it is not likely that OXC will persist in high levels in the plasma, and thus it is not expected that it will impact on the overall survival of neuronal cells *in vivo*. In fact, at least in humans this AED is readily converted to S-Lic and R-Lic (Almeida and Soares-da-Silva 2007). Regarding the fact that OXC is a minor metabolite of ESL in humans, OXC accounts for less than 5% of metabolized ESL, and it is unlikely that high concentrations of OXC as those used in this study will reach the brain.

Interestingly, we observed that LTG strongly increased the phosphorylation of SAPK/JNK in hippocampal neurons, which may indicate that LTG promotes neuronal survival by this pathway, as suggested but not fully explored by Chang and colleagues (Chang et al. 2009). Another study has also demonstrated that LTG does not affect the signaling of ERK 1/2 or Akt (Aubry et al. 2009), but its effect on the SAPK/JNK pathway had up to now remained unaddressed. Moreover, LTG was shown to be able to prevent staurosporine-induced caspase-3 activation in a neuronal cell line (Li et al. 2002), for a concentration similar to the one used in this study (0.3 mM). Our results support the possibility that LTG may be a neuroprotective agent, acting through the JNK pathway.

Finally, we observed that VPA stimulated the phosphorylation of ERK 1/2, Akt and SAPK/JNK, which may trigger neuroprotective mechanisms. Our data are in agreement with earlier findings showing that treatment with VPA activates ERK 1/2, Akt and SAPK/JNK,

which are ultimately responsible for its neuroprotective effects (Mora et al. 1999; Chuang 2005; Di Daniel et al. 2005).

In summary, we showed in this study that ESL and its metabolites, S-Lic and R-Lic, up to 0.3 mM, do not induce neurotoxic effects in hippocampal neurons *in vitro*, and their inhibitory effects on MAPK/ERK pathway may be beneficial *in vivo* to face the overactivation of ERK1/2 that may occur during spontaneous seizures. CBZ and LTG were not toxic to cultured neurons at the concentrations tested (0.03-0.3 mM), and LTG may exert neuroprotective effects through the activation of the JNK pathway. Treatment with OXC or VPA induced the appearance of markers related to cell death, and OXC was the most toxic drug. Thus, ESL may be a better alternative for the treatment of epilepsy than the other drugs studied here, since it is endowed with an improved safety profile.

Chapter 4

Exposure to antiepileptic drugs during prenatal period, gestation and nursing on CD1 female mice

4.1 Summary

Antiepileptic drugs (AEDs) were administered to CD1 female mice during the prenatal period, pregnancy and nursing, and the effects of AEDs on fertility, body weight and serum biochemical parameters were analysed. We also determined the effects of AED exposure on the newly born cells in the dentate gyrus of the hippocampus of the CD1 female mice.

The fertility rate was not changed by the AEDs, except that the group of animals exposed to oxcarbazepine (OXC) had the lowest number of pregnant females (3 out of 8 possible). Regarding the body weight of the pregnant females, it increased in the normal range from 26.4±0.6 g to 42.9±2.2 g in the control group, during the gestation and nursing periods, and a similar increase was observed in the experimental groups. Blood glucose levels showed slight differences between groups but they did not differ from the control values (104.19±4.74 mg/dL).

Eslicarbazepine (ESL) and carbamazepine (CBZ) did not change the biochemical parameters measured in blood serum of treated females, when compared to control females: 73.83±6.58 mg/dL for total cholesterol (TC); 106.00±12.75 mg/dL for triglycerides (TG); 62.57±10.26 IU/L for alanine aminotransferase (ALT); 169.00±15.08 IU/L for aspartate aminotransferase (AST); 0.46±0.03 mg/dL for creatinine (CREA); and $43x10^3$±$1.57x10^3$ IU/L for creatine kinase (CK). However, TG levels were decreased by 50% ($p<0.05$) due to VPA exposure, whereas the CREA levels were decreased by 30% ($p<0.05$) upon exposure of CD1 females to OXC or VPA, as compared to the control values.

Concerning the newly born cells in the dentate gyrus of the hippocampus of CD1 females after exposure to AEDs vs controls, the numbers of BrdU-positive cells ($BrdU^+$) were the following in control females: subgranular zone of the dentate gyrus (SGZ): 7.95±0.89 $BrdU^+$ cells; inner granular zone (IGZ): 4.05±0.53 $BrdU^+$ cells and outer granular zone (OGZ): 0.36±0.14 $BrdU^+$ cells. ESL and OXC did not change the number of $BrdU^+$ cells in the dentate gyrus. However, CBZ or VPA decreased by 40% ($p<0.05$) the number of $BrdU^+$ cells in the SGZ, as compared to the SGZ of control females, but they did not alter this number on the other layers of the dentate gyrus.

4.2. Introduction

Over the past few decades many tens of thousands of children have been born to mothers suffering from eplilepsy. Having a safe pregnancy however, while undergoing long-term therapy on antiepileptic drugs (AED) is not always easy. These mothers have to take into account, both the "disease" and the "treatment". On the one hand, it is not advisable that epileptic patients stop AED therapy during pregnancy, as seizures may occur and cause harmful effects on the fetus. Furthermore, AED therapy may have adverse effects on both the mother and the fetus. It is therefore imperative that we evaluate the drug-specific effect of AED therapy in healthy subjects, in order to predict its possible effects in epileptic patients.

Growing evidence suggests that older-generation AEDs, such as carbamazepine (CBZ), and valproate (VPA), and more recently oxcarbazepine (OXC), may have intense and distinct effects on metabolism and serologic parameters in humans (Chuang et al. 2012). These biochemical parameters are normally evaluated in blood serum and are indicators of liver or kidney function, as well as markers of risk factors of cardiovascular diseases, and can be used as indicators of the healthy status of the organism. It is known that CBZ and OXC induce enzymes of cytochrome P450 system (CYP450) affecting this system activity, which changes the metabolic pathways of other important molecules, such as hormones, vitamins and cholesterol (Patsalos et al. 2002; Mintzer et al. 2009; Lopinto-Khoury and Mintzer 2010). In patients taking CBZ, it was found that this AED increased concentration of lipids in serum, such as total cholesterol (TC), while VPA decreased both TC and triglycerides (Nikolaos et al. 2004). VPA has also been associated with both body-weight gain and reduced blood glucose levels (Morrell 1996; Pack and Morrell 2002). Eslicarbazepine acetate (ESL) is a non-inducing enzyme which is metabolized by noncytochrome P450 enzymes to eslicarbazepine (S-Lic) (Almeida and Soares-da-Silva 2003). However, there is no information on the effects of this new AED on indicators of organism status, namely serum lipid profile, liver enzymes, such as aminotransferases, and on indicators of kidney function, such as creatinine levels.. Thus, one of the objectives of this study was to determine the effects of ESL and of the other AEDs on the blood serum biochemical parameters discussed above in CD1 female mice treated with the AEDs during the prenatal period, gestation and nursing..

Concerning the effects of exposure to the AEDs on newly born cells on dentate gyrus of hippocampus in CD1 female mice during prenatal period, gestation and nursing, BrdU incorporation in the dentate gyrus was followed. BrdU is a thymidine analogue which is incorporated within DNA during its synthesis in S phase of the cell cycle, thus BrdU becomes part of the nuclear content of a new-born cell. These new-born cells are a product of cell proliferation, which is the first and slow step of neurogenesis (Kempermann et al. 2004). In the adult mammalian brain, there are two particular and distinct areas where neurogenesis occurs: in the lateral wall of the lateral ventricles, known as subventricular zone (SVZ), and in the subgranular zone (SGZ) of the dentate gyrus (DG). These neurogenic areas are susceptible to changes in the surrounding environment, such as imbalance in the glutamate and GABA systems (Laeng et al. 2004), and some AEDs are known to act in neurotransmition, which may change GABA and glutamate availability. VPA was shown to inhibit proliferation and to induce neuronal differentiation of adult hippocampal neural progenitor cells *in vitro* and *in vivo* (Hsieh et al. 2004). However, recently, Chen and colleagues (2009) analysed some of the commonly used AEDs, such as CBZ and VPA, and did not observe differences on cell proliferation and neurogenesis in the hippocampal dentate gyrus of rats that received chronic administration of these AEDs during early postnatal life (Chen et al. 2009).

In the present study we focused our attention on the generation of newly born cells in the dentate gyrus of the adult hippocampus of CD1 female mice, where progenitor cells proliferate in the subgranular zone (SGZ), and then migrate towards the granular layer (GL), where they differentiate into neurons and integrate in the existing circuits. The possible alterations in cell proliferation, survival and neurogenesis may be a potential mechanism underlying brain impairment associated with treatment with certain AEDs, such as VPA, CBZ and LTG (Chen et al. 2009; Boku et al. 2011). In this respect, we tested not only some of these AEDs (CBZ and VPA), but also ESL and OXC.

4.3. Results

As described in detail in the Methods, 40 CD1 female mice were assigned to five groups, with 8 females per group: Control group (Ctrl), ESL, CBZ, OXC and VPA. During the prenatal period, during gestation and during nursing each female was fed once daily with the following doses of the AEDs: ESL, CBZ and OXC, 30 mg/Kg; VPA, 300 mg/Kg, or vehicle

(DMSO) for the control group. After weaning, all females were euthanized after pentobarbital anesthesia and blood samples were collected by cardiac puncture for analysis of biochemical parameters in the serum (see figure 2.7 for detailed description of the experimental protocol). Then, females were submitted to transcardiac perfusion, and the brains were collected and stored at 4°C until they were sectioned for immunohistochemistry analysis. BrdU incorporation was quantified in the 5 central slices of each hippocampus.

In summary, the objective of the study described in this chapter was to determine whether the long-term administration of the AEDs to CD1 females during prenatal, gestation and nursing may affect body weight, blood glucose levels, biochemical parameters of blood serum, as well as the number of newly born cells in the dentate gyrus of CD1 pregnant females, assessed by $BrdU^+$ incorporation in the three layers of the dentate gyrus of the hippocampus

4.3.1. Impact of long-term exposure to AEDs on the fertility of CD1 females

The fertility of females was evaluated by counting the number of litters per each group tested, out of 8 mated females: control – 7/8, ESL – 5/8, CBZ – 7/8, OXC – 3/8 and VPA – 7/8. Females treated with OXC had the lowest fertility rate, 37%.However none of the groups maintained the original number of born pups per litter (Table 4.1). We did not observe differences between each treatment group and the control group for the live and surviving offspring. We also observed that there were no differences between the gender's pups for each experimental group (Table 4.1).

Table 4-I. Effects of AEDs on fertility of CD1 females and on survival and gender of the offspring.

		Born pups		Surviving pups					
Group	**Pregnant females (out of 8)**	**Total**	**Median/ Litter[1]**	**Total**	**Median/litter[1]**	**Males**		**Females**	
						Total	**Median/ litter[1]**	**Total**	**Median/ litter[1]**
Control	7	60	9.5 (8-13)	41	6.5 (5-10)	23	4 (1-6)	18	2.5 (1-5)
ESL	5	55	12(9-12)	40	7 (7-10	14	3.5 (2-5)	26	5 (3-7)
CBZ	7	77	11 (9-13)	61	9 (8-10)	36	5 (4-7)	25	4 (1-6)
OXC	3	36	11 (9-16)	29	11 (6-12)	14	5 (3-6)	15	6 (1-8)
VPA	7	80	12 (7-14)	52	7 (2-9)	21	3 (2-4)	31	5 (2-7)

CD1 females were exposed daily, during the whole experimental period, to 30 mg/Kg of ESL, CBZ, or OXC or to 300 mg/Kg of VPA. Control females were exposed to the vehicle in the same conditions.. [1]Results are expressed as median (min – max) of 3-7 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

4.3.2. Effects of AEDs exposure on body weight and biochemical parameters of blood serum

CD1 females were monitored every day of the experimental study to confirm the ingestion of the apple piece with the respective AED. Body weight and serum glucose levels were measured weekly, except during mating and after birth (Fig. 4.1). The remaining parameters were measured in the blood serum after sacrifice of the females, at the end of the nursing period.

4.3.2.1. Changes in body weight and blood glucose levels of CD1 female mice during prenatal period, gestation and nursing

Body weight and serum glucose levels were measured weekly except during mating and after birth, as described in the Methods. Body weight increased in the normal range from 26.4±0.6 g to 42.9±2.2 g in the control group during the gestation and nursing periods, and a similar increase was observed in the treatment groups (Fig.4.1A).

A normal glucose level in the blood is one of the most important factors during gestation to ensure a successful pregnancy. We found that blood glucose levels (determined

at least after 4 h of fasting) in the CD1 females did not change during prenatal period, gestation and nursing, and only slight differences were observed between treated group and control group; however they did not differ significantly from the control (104.19±4.74 mg/dL) (Fig. 4.1B).

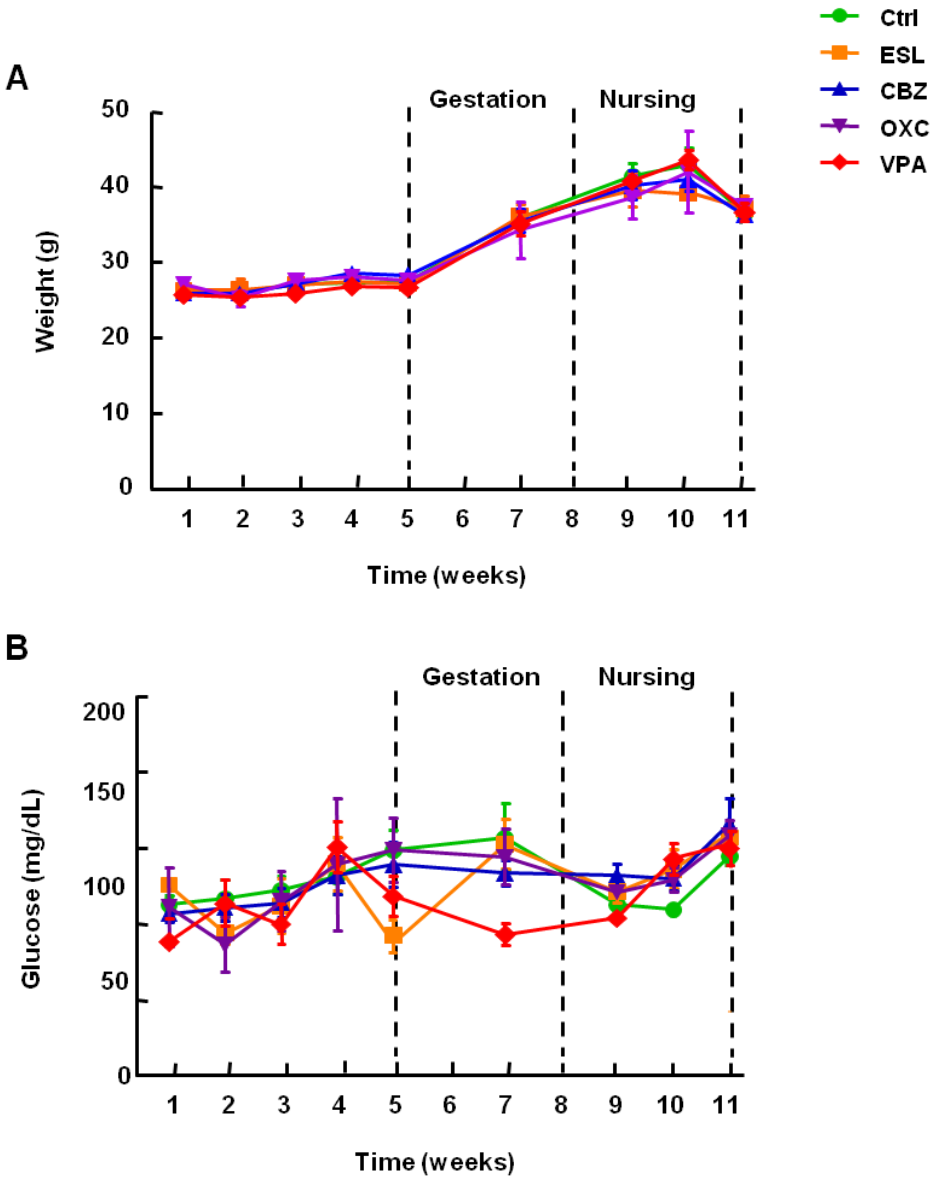

Figure 4.1. Changes in the body weight and blood glucose levels of CD1 females exposed to AEDs during prenatal (5 weeks), gestation (3 weeks) and nursing (4 weeks). (A) Monitorization of body weight in CD1 female mice. **(B)** Monitorization of fasting blood glucose levels in CD1 female mice. The total number of pregnant females per treatment was: control (7), ESL (5), CBZ (7), OXC (3) and VPA (7). The results are presented as means ± SEM of 3-7 independent measurements. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

4.3.2.2. Changes in biochemical parameters measured on blood serum due to CD1 female exposure to AEDs

Total cholesterol and triglyceride levels in blood serum reveal the quality of blood and may be associated with the risk of cardiovascular diseases. In the present study we observed that CD1 females of the control group had 73.83±6.58 mg/dL of total cholesterol, which was not altered in the other experimental groups treated with the AEDs (Fig. 4.2.A). Regarding the triglyceride values, this parameter was reduced in blood serum of animals that were treated with VPA (54.00±5.08 mg/dL, $p<0.05$), when compared to the control group (106.00±12.75 mg/dL). The other AEDs did not change the triglyceride concentrations (Fig. 4.2.B).

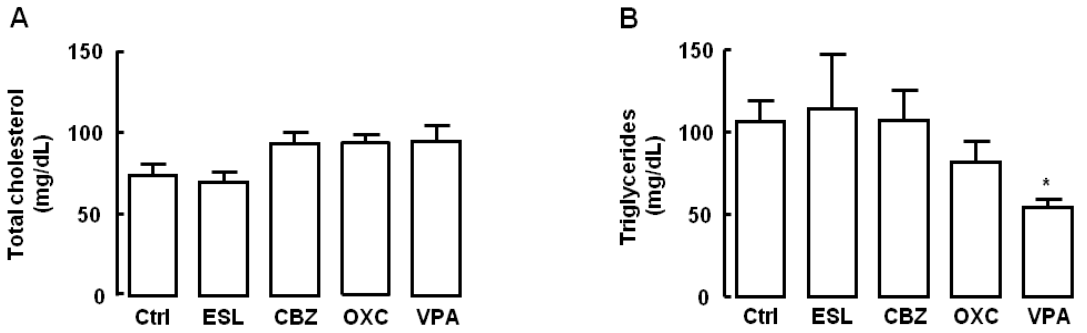

Figure 4.2. Effects of AEDs on total cholesterol and triglyceride concentrations measured in blood serum of CD1 female mice at the end of AED treatment. **(A)** Total cholesterol; **(B)** Triglycerides. The number of serum blood samples for each AED treatment corresponds to the number of gestant females monitored during the experiment, as described in legend of figure 4.1. The results are presented as means ± SEM of 3-7 independent measurements. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *$p<0.05$, significantly different from control.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are found in different organs, such as liver. Changes in these parameters may be indicators of liver damage, which is responsible for the metabolism of several AEDs. In control females, the concentrations measured for ALT and AST were 62.57±10.26 and 169.00±15.08 IU/L, respectively, (Fig.4.3. A-B). These concentrations in the treated groups did not differ from the control group.

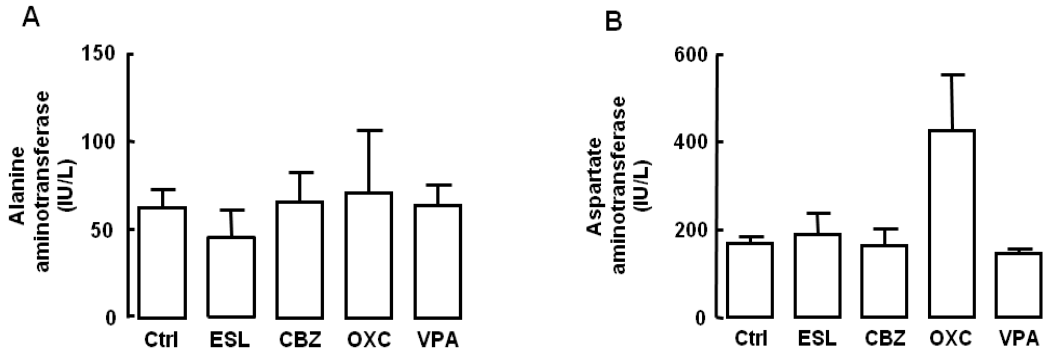

Figure 4.3. Alanine aminotransferase and aspartate aminotransferase concentrations measured in blood serum of CD1 female mice at the end of AED treatment. (A) Alanine aminotransferase; **(B)** Aspartate aminotransferase. The number of serum blood samples for AED group corresponds to the number of pregnant females monitored during the experiment, as described in legend of figure 4.1. The results are presented as means ± SEM of 3-7 independent measurements. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

Creatinine (CREA) is a fairly reliable indicator of kidney function. In the case of dysfunction due to long-term exposure to the therapeutic drugs, the creatinine levels in the blood may raise. In this study, CD1 females treated with ESL and CBZ showed CREA levels which were similar to those of control females (0.46±0.03 mg/dL), while the females exposed to OXC or VPA had concentrations of CREA significantly lower than the control females (0.33±0.03 and 0.33±0.04 mg/dL, respectively, $p<0.05$) (Fig. 4.4.A).

Creatine kinase (CK) is mainly found in the brain, skeletal muscle and heart. Its elevated value is associated to the damage of the heart muscle, for instance in cases of heart attacks. This parameter may be elevated also in conditions that produce injury to the skeletal muscles or brain. In this study, we measured concentrations of CK of $1.48x10^3 \pm 6.57x10^3$ IU/L in control animals and none of the treated groups differ from the control group (Fig.4.4.B).

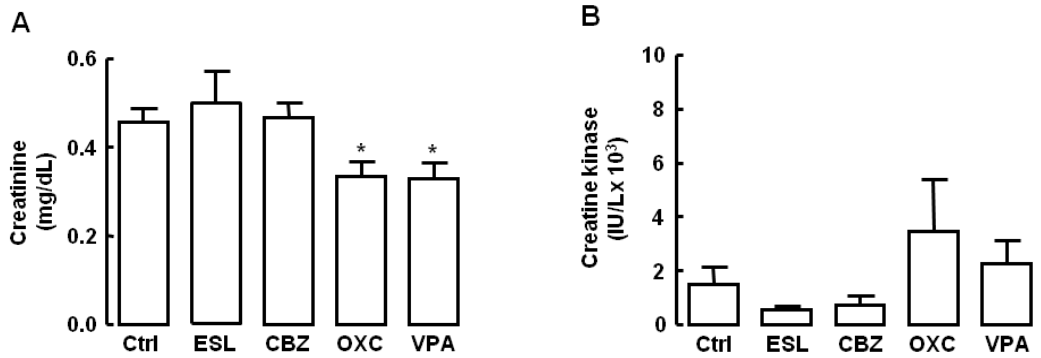

Figure 4.4. Effects of AEDs on creatinine and creatine kinase concentrations measured in blood serum after euthanasia of CD1 female mice at the end of AED treatment. (A) Creatinine (CREA); **(B)** Creatine kinase (CK). The number of serum blood samples for AED group corresponds to the number of pregnant females monitored during the experiment, as described in legend of figure 4.1. The results are presented as means ± SEM of 3-7 independent measurements. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

Overall, in this part of the study we show that the long-term administration of ESL and CBZ during prenatal period, pregnancy and nursing did not alter the concentrations of blood glucose, TC, TG, ALT, AST, CREA and CK when compared to control females, while treatment with VPA decreased the concentrations of TG and CREA, and OXC decreased CREA values.

4.3.3. Long-term exposure to AEDs differently affects the number of newly born cells on the dentate gyrus of hippocampus of CD1 females

BrdU-positive cells ($BrdU^+$) were counted 6 weeks after BrdU injection, to investigate their distribution across the DG of the hippocampus in the experimental and control groups of CD1 female mice. Newly generated cells, which incorporate BrdU, are "born" in the sugranular layer (or zone) (SGZ) of the dentate gyrus and normally they migrate into the granule cell layer, which is divided in two more layers (or zones), the inner granular zone (IGZ, above SGZ) and an external layer, the outer granular zone (OGZ) (Fig. 4.5).

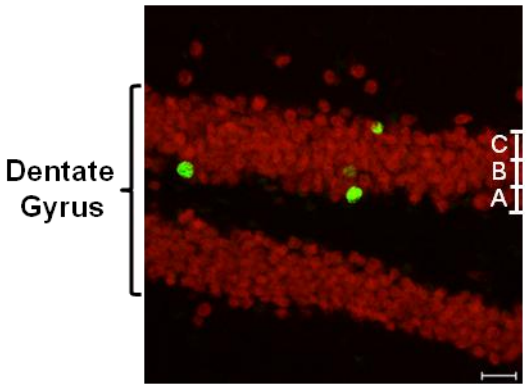

Figure 4.5. Representative image of the dentate gyrus obtained from a control CD1 female hippocampus with BrdU-positive cells in the different layers. The brain sections were labelled with mouse anti-NeuN (red), a neuronal marker, and rat anti-BrdU (green), a proliferation cell marker. A – Subgranular zone (SGZ: 2 $BrdU^+$ cells); B – Inner granular zone (IGZ: 1 $BrdU^+$ cells); C – Outer granular zone (OGZ: 1 $BrdU^+$ cells). Scale bar – 20 µm.

We observed that the number of $BrdU^+$ cells in females treated with ESL and OXC was not changed in the three layers of the dentate gyrus, when compared to the control (SGZ: 7.95±0.89; IGZ: 4.05±0.53 and OGZ: 0.36±0.14 $BrdU^+$ cells) (Fig. 4.6.A). However, the number of $BrdU^+$ cells detected in SGZ of CD1 females exposed to CBZ and VPA was significantly decreased by 40%, when compared with the control group (CBZ: 4.65±0.50 and VPA: 4.75±0.69 $BrdU^+$cells; $p<0.05$). In addition, the number of $BrdU^+$ cells detected in the other granular zone did not change with treatment with these AEDs (CBZ or VPA) (Fig.4.6B).

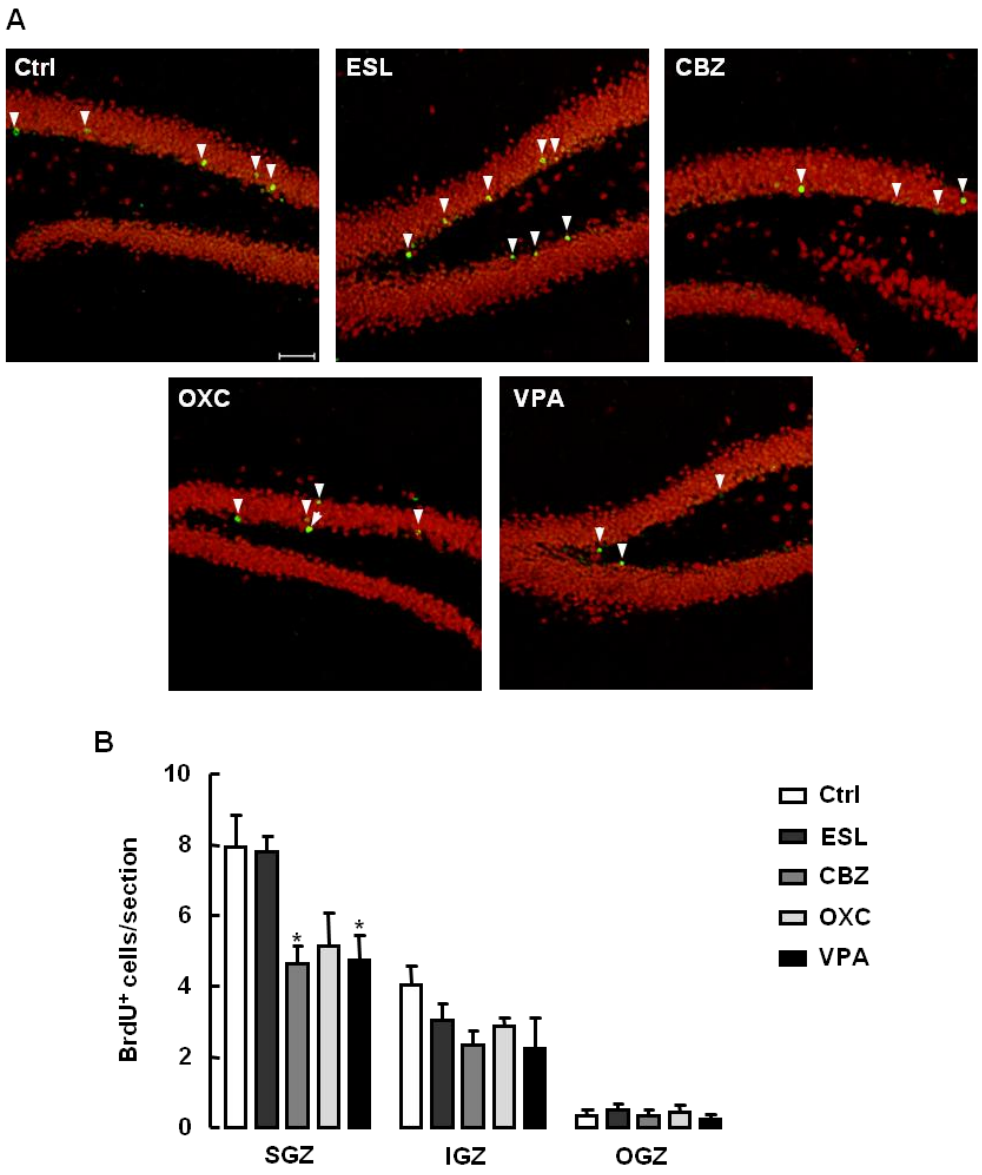

Figure 4.6. Changes in BrdU incorporation in the dentate gyrus of CD1 females after long-term exposure to AEDs. BrdU was administered 6 weeks before animals were sacrificed and its incorporation was assessed in all layers of dentate gyrus. **(A)** Representative images of the dentate gyrus of control and treated females (ESL, CBZ, OXC, and VPA). Arrow-heads indicate $BrdU^+$ cells.. Scale bar – 50 µm. Red – NeuN; Green .- BrdU **(B)** Quantification of $BrdU^+$ cells per brain section in each experimental group. ESL or OXC did not alter the number of BrdU-positive cells in the three layers of the dentate gyrus in CD1 females, whereas CBZ and VPA treatments significantly decreased the number of $BrdU^+$ cells in SGZ by 40% ($p<0.05$), when compared to the respective control. No changes were observed due to treatment with ESL or OXC in the distribution of $BrdU^+$ cells across other cell layers of the dentate gyrus. The results are presented as means ±SEM of at least 3-4 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, statistically different from respective control.

4.4. Discussion

Selection of an appropriate AED to be used during pregnancy in patients with epilepsy involves evaluation of the side effects of the AEDs, including those that are primarily associated with teratogenic effects in the offspring. However, the potential impact of AED therapy on pregnant mothers must not be overlooked, mainly regarding metabolic side effects which can lead to obesity, metabolic syndrome, cardiovascular disease or hepatotoxicity. Biochemical parameters in the blood serum are used as health indicators of the organism. In fact, it has been described that AEDs may affect the metabolic pathways in a negative way (Patsalos et al. 2002; Mintzer et al. 2009; Lopinto-Khoury and Mintzer 2010). Therefore, it is important to understand the individual effect of an AED monotherapy and all the possible benefits/drawbacks for the future mothers.

In the present study we determined the possible effects of long-term administration to CD1 female mice of ESL, CBZ, OXC and VPA, during prenatal period, gestation and nursing on various important parameters, as described in the Results. The CD1 mice lineage was chosen because the metabolism of ESL is similar to the metabolism of the AED in humans (Alves et al. 2008).

Concerning the effects on fertility, CD1 females treated with OXC had the lowest fertility rate (37%), but administration of the other AEDs (ESL, CBZ or VPA) did not affect fertility, when compared with females that were not exposed to AEDs. Previous studies regarding mice fertility, showed that C3H/He mice treated with CBZ (25 mg in 10 g food) did not differ from placebo group in relation to litter size and duration of gestation (Rayburn et al. 2004), while VPA (160-180 mg/kg daily) was also reported as not affecting fertility (Chapman and Cutler 1984). On the other hand, in rats, VPA (300 mg/kg/day) decreased fertility by 25% (Ubeda-Martin et al. 1998); thus our results are in agreement with the observations made in mice regarding CBZ and VPA. In the present study, we tested for the first time the effects of ESL and OXC on the fertility of CD1 females and we observed that ESL did not affect fertility of CD1 females after a long-period of treatment, whereas OXC may

reduce fertility. Our data also suggested that the AEDs tested do not have impact on offspring survival, as well as on pups gender.

The use of VPA and CBZ has been associated with weight gain in humans (Biton 2003), while the use of ESL and OXC did not show the same effect (Elger et al. 2007; Cansu et al. 2011). In rodents, Christensen and colleagues did not observe changes in body weight of pregnant C3H/He mice after treatment with CBZ (Christensen et al. 2004) and Brown and colleagues did not observe changes in body weight of adult males CD1 mice treated with VPA (Brown et al. 2008). In our study, although weight increased as expected for all pregnant females during gestation and nursing, we did not observe differences in body weight between treated groups and the control group. The association between VPA and weight gain has been attributed to the ability that this AED has to inhibit lipid oxidation and consequent decline of basal metabolism, thus patients who have taken VPA spend less energy than average (Gidal et al. 2003). Mice are about 3,000 times smaller than humans, and their basal metabolic rate is seven times greater (Demetrius 2004); thus, it may be inadequate to expect in mice similar effects of weight gain with VPA to those observed in humans.

Regarding the blood serum parameters, CD1 females treated with ESL, CBZ, OXC or VPA did not show any significant changes in serum glucose levels, when compared to control females. Valproate was previously shown to lower blood glucose levels (Luef et al. 2003). One possible explanation given by the authors of this study was that VPA, as a fatty acid derivative, competes with long chain fatty acids for binding sites in the plasma, such as albumin, which would increase the availability of free fatty acids and stimulates insulin production, thus reducing blood glucose levels (Luef et al. 2003). In our study we did not observe significant changes in the blood glucose levels between females treated with ESL or CBZ or OXC, and and the control group through the whole period of treatment, even during gestation and nursing. However, we observed a slight decrease of blood glucose in the females exposed to VPA during gestation and beginning of lactation, but the values were not statistically different from controls.

It is well described that AEDs may influence serum lipid profile via inhibiting or stimulating CYP450 iso-enzymes, since these are important in the metabolism of many drugs, particularly those in this study: CBZ, OXC and VPA (Patsalos et al. 2002; Pylvanen et al. 2003; Mintzer et al. 2009). ESL has been described as having no effect on CYP450 enzymes

(Almeida and Soares-da-Silva 2003). Additionally, it is known that in humans changes of the hormonal profile during pregnancy induce changes in serum lipid levels, and an increase was expected in cholesterol and triglycerides levels during a normal gestation (Herrera 2002). Although we did not observe significant changes in the concentration of TC, we can observe a trend to increase in total cholesterol levels after treatment with CBZ, OXC and VPA. However, the expected increase in TC may be less important in mice in comparison to humans, since maternal cholesterol is primarily utilized during pregnancy for steroid synthesis in the mice fetus, while in human the fetus is almost completly responsible for producing its own cholesterol (Yoshida and Wada 2005).

Concerning triglycerides levels in humans, there are contradictory results in the literature: Morrell and colleagues (2003) observed that TG levels tend to be higher in obese men taking VPA (Morrell 1996); in a study involving women with epilepsy, Luef and colleagues found serum TG tended to be increased in women taking VPA (Luef et al. 2002). On the other hand, Kim and Lee (2007) did not find differences in lipid profiles between different AED treatment groups (Kim and Lee 2007). On the contrary, Nikolaos and colleagues (2004), found significantly lower TG values in epileptic patients on VPA (Nikolaos et al. 2004). Regarding triglycerides levels in mice, Lee and colleagues (2008) observed that in ICR mice, after treatment with VPA (100 or 500 mg/kg/day), TG concentration in the serum significantly increased while no changes in the liver enzymes were observed. These authors suggest that abnormalities in lipid metabolism may be related to genes that are responsible for increased biosynthesis of cholesterol and triglycerides, and which may be affected by subchronic VPA treatment (Lee et al. 2008). Our data show that pregnant CD1 female mice treated with VPA for a long-period presented reduced triglyceride levels on blood serum. However, we do not have a convincing explanation for this effect yet, and further studies should be done.

ESL has not been associated with hepatic or renal dysfunction mainly due to its metabolism. ESL is completely reduced by esterases in the liver to eslicarbazepine. Thus, without the formation of carbamazepine-10, 11-epoxide is not susceptible to auto-induction (Bialer 2006). Moreover, there is not information about effects on aminotransferases and creatinine in humans and mice, except for one isolated case of a moderate elevation of creatine kinase in patients (Elger et al. 2007). As an enzyme-inducer of P450 system, it was

expected that CBZ produced changes in the levels of ALT and AST, which are markers of hepatotoxicity. VPA is also known to cause hepatotoxicity (Dreifuss et al. 1987). In fact, Tutor-Crespo and colleagues (2004) showed that both aminotransferases are less affected by chronic treatment with CBZ and VPA among others AEDs (Tutor-Crespo et al. 2004). Other authors did not observe any effects of either CBZ, OXC or VPA on the serum concentrations of ALT and AST of children after a long-term AED treatment (Babayigit et al. 2006). In contrast, Attilakos and colleagues showed that children receiving VPA monotherapy had serum aminotransferases increased (Attilakos et al. 2007). In mice serum, however, ALT and AST levels were unchanged after treatment with a single dose of VPA (100 or 1000 mg/kg) (Lee et al. 2007) and also other AEDs have not been associated with changes in these enzymes in mice.

Alanine and aspartate aminotransferase have normal values during pregnancy in humans (Bacq et al. 1996), although one study reported elevation of ALT solely at postpartum (Schrocksnadel et al. 2003). However, studies reporting effects of AEDs on these parameters during pregnancy are not many. Briefly, Lakshmi and Sunanda (2008) did not observe any changes in the values of ALT and AST in females treated during pregnancy with CBZ, when compared to control group (Lakshmi and Sunada. 2008); OXC had not been associated with changes on ALT and AST during pregnancy either; and VPA induced effects that were observed solely in the progeny after prenatal exposure, where serum levels of aminotransferases were unchanged (Bambini-Junior et al. 2011). In pregnant mice, effects of these AEDs on ALT and AST have not been reported yet. Although we did not observe significant changes in both, ALT and AST, between females treated with AEDs and the control group, we observed a trend to higher values of AST in females treated with OXC. The absence of statistical significance may be due to the small number of females (three) in the OXC treated group..

Creatinine as a waste molecule generated from muscle metabolism that can be used as a marker of renal dysfunction leading to elevated levels of CREA in the serum; thus its detection does not suffer direct effects from AEDs but reflects how AEDs affect renal function. ESL does not induce its own metabolism or clearance, which is dependent of renal function in humans (Maia et al. 2008); CBZ increased clearance of some proteins but not creatinine in humans (Giessmann et al. 2004), while OXC increased creatinine clearance in children but not

in healthy adults (Flesch 2004); finally, VPA clearance is dependent of renal function and of its free drug concentration (Reith et al. 2001). Creatinine production in humans during pregnancy are unchanged, however increased blood volume and kidney function resulting in lower levels of serum creatinine (Chames et al. 2003). In one study conducted with pregnant women treated with CBZ, creatinine levels were unchanged (Lakshmi and Sunada. 2008). In mice, AEDs have not been associated with changes on serum creatinine levels. We showed that CD1 females treated with OXC and VPA presented decreased levels of CREA, while ESL and CBZ treatments did not show any effects, in comparison with the control group. Thus, it suggests that CREA levels either reflect changes induced by pregnancy or increased clearance caused by OXC or VPA.

Creatine kinase (CK) is an enzyme found in different organs and tissues, such as brain, skeletal and heart muscle, among others, where CK catalyzes the reversible formation of ATP and creatine from ADP and phosphocreatine (McLeish and Kenyon 2005). In women, elevated creatine kinase serum levels may be associated with muscular damage in ectopic pregnancy (Develioglu et al. 2002), but in general serum CK did not differ significantly between normal pregnant and non-pregnant women (Zatz et al. 1982). In mice, CK is also found in several tissues and organs and does not show significant changes during gestation of these animals.

Studies on the effects of AEDs on CK are rarely found in the literature. Tiihonen and colleagues reported that patients receiving long-term CBZ therapy did not show differences of serum CK activity as compared to control patients (Tiihonen et al. 1995). Also, OXC and VPA have not been associated with changes on CK. In mice, one study reports that CK activity was not affected by treatment with VPA (Knapp et al. 2008).Accordingly, we also observed in mice that none of the AEDs tested (ESL, CBZ. OXC or VPA) induced changes on CK activity in CD1 pregnant females. Therefore none of the treatments caused impairment of tissues or organs where this kinase is found.

Our data reinforces that the decision of the type of AED administered during pregnancy, besides taking into account their adverse effects on the fetus, should also take in consideration the impact of the treatment on all serum biochemical parameters throughout pregnancy and nursing periods. Lipid and protein profile, liver enzymes and renal function

are important indicators of healthy status of the mother and indirectly, of the fetus. In summary, our data indicates that of all the AEDs tested in this study the less harmful was ESL since this AED did not alter serum biochemical parameters and the BrdU incorporation of dentate gyrus of the CD1 female mice, as compared to control females.

Chapter 5

Impact of in utero exposure to antiepileptic drugs during gestation and nursing on the behaviour of CD1 mice offspring: changes in cognitive and motor functions during development

5.1. Summary

Exposure to AEDs in early-life, namely *in utero, during gestation,* and then during nursing, may have long-term effects on cognitive and/or on non-cognitive functions in humans. In this work we explored the effect of AEDs exposure during gestation and nursing on brain function, by evaluating general motor activity, memory, learning, anxiety and depression in CD1 mice offspring. All the behavioural tests were conducted in animals (males and females) at one (juveniles) and at four months (adults) of age.

Locomotor activity was evaluated by the open-field test. As expected, in general, both males and females showed reduced locomotor activity in adult age, as evidenced by the fact that both genders reduced the number of line crossings for all experimental groups with age. The effect of ESL on locomotor activity was gender- and age-dependent, since it increased the number of line crossings only in juvenile males as compared to controls, while OXC increased the activity of juvenile males and of juvenile and adult females. However, CBZ and VPA treatment did not affect locomotor activity in CD1 mice.

Memory related to hippocampal function was evaluated by the object recognition test, whereas spatial memory was evaluated by the modified Y maze test. We found that ESL had no effects on the behaviour of CD1 mice offspring in both learning and memory tasks. Regarding the other AEDs, CBZ, OXC and VPA decreased the percentage of time that juvenile males spent exploring a novel object. This effect was lost with age in the case of CBZ and OXC treatments, but was maintained for VPA treatment. Adult females exposed to CBZ and OXC, spent more time exploring a novel object when compared to untreated animals. Regarding the modified Y maze test, males' behaviour in this task was not affected due to AED exposure, except that juvenile females exposed to CBZ spent less time exploring a novel arm, but this behaviour was lost with age. Aversive memory was tested by the inhibitory avoidance test, in which animals received a foot-shock stimulus. Both juvenile males and females exposed to CBZ showed decreased latency in relation to juvenile controls. This effect was lost with age in males but not in females. Moreover, juvenile females exposed to OXC, showed decreased latency and lost this behaviour in adulthood.

Anxiety was evaluated with the elevated plus maze. Males exposed to CBZ, spent more time in the open arm when adults, while ESL increased the number of total entries, indicating an increase in motor activity. Thus, an ESL-associated hyperactivity was observed with two different behaviour tests – the open-field and the elevated plus maze. However, this effect was lost with age. Females exposed to AEDs have no changes on their anxious profile, which was similar to control animals.

The propensity for depression was evaluated by the forced swimming test. Males exposed to OXC showed increased immobility time as juveniles, but this effect was lost with age. No effects were observed with the other AEDs in males, while none of the AEDs had a pro-depressive effect on female's behaviour.

Overall, ESL did not impair cognitive functions in CD1 mice, although a transient ESL-associated hyperactivity was observed with two different behaviour tests. CBZ, OXC and VPA caused slight changes in some of the brain functions assessed in this study. Most of the effects observed in one-month-old mice were lost with age, while others seem to be gender dependent. CBZ was the AED that most affected the parameters that were evaluated, namely memory and learning functions. In addition, when assessing brain morphology by cresyl violet staining, no changes were observed in the cortex and hippocampus of four month old CD1 mice after exposure to AEDs during gestation and nursing.

5.2. Introduction

In humans, *in utero* exposure to teratogens may cause major morphological abnormalities (within the embryonic period) and/or minor morphological changes (within the fetal period) and the central nervous system is susceptible to teratogenic effects throughout both periods (reviewed by DiPietro 2005). During brain development any changes in its formation and/or maturation may induce neuronal migration disorder or changes on synaptogenesis and neurogenesis (Bittigau et al. 2003; Clancy et al. 2007; Hofmann 2010). The neurological consequences that those effects may have on brain systems or structures such as the hippocampus, may account for harmful repercussions on cognitive functions like memory and/or learning which are detected postnatally as behavioural teratogenesis (Broadbent et al. 2004).

It is known that exposure to AEDs *in utero* and postnatally may induce major or minor anomalies of teratogenic effects (Lindhout and Omtzigt 1992; Morrell 1996; Palmieri and Canger 2002; Sankar 2007; Gidal and Tomson 2008; Tomson et al. 2011), however women with epilepsy should not interrupt AED therapy during pregnancy, or during nursing. Although the majority of babies born to women with epilepsy are healthy (Adab et al. 2004), AEDs may affect the developing brain on two separate occasions: prenatal (*in utero*) and postnatal (nursing), and both may have long lasting effects on intellectual and developmental disabilities and/or motor side effects (Loring et al. 2007). These effects may occur as AEDs may act on brain neurotransmitters during gestation, since AEDs cross through the placenta and may directly affect the fetus, or later, during nursing, since AEDs are transmitted to babies by breastfeeding, (Pennell 2003; Pennell 2008). Thus, it is imperative that, when assessing the risk-to-benefit ratio of prescribing an individual AED to pregnant mothers, to take into account its adverse cognitive effects.

Primary effects of AEDs on cognition were the first to be evaluated since research in humans has become focused on cognitive development as well as on physical development. Primary effects comprise reduction in psycho-motor processing speed, sustained attention and learning, and normally are detected when AED doses are within therapeutic recommendations, thus primary side effects are normally modest in case of monotherapy (Loring et al. 2007). The higher the dose, the greater the effect of the AED and therefore, when humans are exposed to polytherapy especially during pregnancy and early childhood, impacts on cognition are more accentuated than in monotherapy (Adab et al. 2004; Motamedi and Meador 2006).

In the last two decades there has been an increase in published information regarding the behavioural teratogenesis of *in utero* exposure to AEDs by prospective observational studies in humans. Other studies, such as case studies, may or may not be replicated and have brought conflicting results because of the methodological differences and lack of information in some of the studies; thus more adequately powered and prospective studies are necessary (Bromley et al. 2011).

Eslicarbazepine acetate, as a recently developed AED, has fewer published studies concerning cognitive effects or other behavioural features, than the older AEDs (CBZ and VPA). The existing studies were performed in healthy humans such as the study by Millovan and colleagues (2010), who showed that healthy adults administered with a single dose of ESL have a lower incidence of adverse effects in relation to OXC in the same conditions. No

impact was observed with a single dose of ESL on cognitive abilities, as compared with placebo. Moreover, ESL treatment caused improved word fluency on individuals (Milovan et al. 2010).

Regarding the effects of CBZ in humans we can find many reports in the literature most of them showing that CBZ is relatively safe in terms of cognitive outcomes in the progeny (Wide et al. 2002; Gaily et al. 2004; Meador and Zupanc 2004), but has minor effects on memory performance (Bromley et al. 2010). Other studies have been performed with CBZ using animal models. In non-epileptic rats, Shannon and Love (2004) showed that CBZ produced more modest impact on working memory tasks than GABA modulators, which reduced performance in this task; CBZ was also shown to cause disrupted attention and later, it was shown that CBZ impaired learning tasks at higher doses (Shannon and Love 2004; Shannon and Love 2005; Shannon and Love 2007). Most of the studies in mice exposed to CBZ were performed in seizured-animal models which showed that this AED had no significant side-effects on the performance in behavioral tests (Borowicz et al. 2000). However, in a study similar to ours, but performed in a different animal model (C3H/he mice), Rayburn and colleagues observed that prenatal exposure to CBZ slightly decreased locomotion activity, but did not change cognition or anxiety behaviour compared to placebo (Rayburn et al. 2004). In relation to OXC, very limited information is available on exposure to this drug *in* utero and there are only few reports about its effects in humans. Some studies in adult humans initially described its effect on cognition as a modest benefit (Aikia et al. 1992), and as having an improvement on the performance on some behavioral tasks (Curran and Java 1993). Later, Salinsky and colleagues (2004) observed similar effects between OXC and phenytoin as mild-to-moderate negative neuropsychological effects (Salinsky et al. 2004). Recently, in a study performed in healthy and adult humans, OXC was associated with a higher incidence of adverse effects events in relation to ESL. However, overall both had similar cognitive profiles with few significant effects in cognitive performance (Milovan et al. 2010). In animal studies, Agarwal and colleagues (2011) showed that OXC did not alter performance of mice in elevated plus maze test and passive avoidance test. However, this study was performed on pentylenetetrazole (PTZ)-kindling mice (Agarwal et al. 2011).

Regarding studies with VPA in humans, it has been asserted that maternal VPA exposure can induce fetal valproate syndrome among permanent adverse effects upon neurological and behavioral development (Clayton-Smith and Donnai 1995; Shepard et al. 2002; Zaki et al. 2010). Adab and colleagues (2004) showed an association between *in utero*

exposure to VPA and low Verbal IQ (Adab et al. 2004), while other group observed that exposure to VPA significantly impacted the IQ of children when compared to others that were exposed to CBZ, LTG and phenytoin (Meador et al. 2009). Recently it was shown that children exposed to VPA during pregnancy had a significantly poorer level of cognitive development when compared to controls and children exposed to other AEDs (Bromley et al. 2010). A vast list of studies in rats is found in the literature, focusing effects of VPA *in utero*, and mostly have reported impacts on cognitive and other behaviours, as follows: reduced exploratory behaviour (Schneider and Przewlocki 2005), increased activity in a novel open field, increased anxiety-like behaviour, no significant effects on learning and memory (Schneider et al. 2007; Markram et al. 2008; Schneider et al. 2008), and reduced sociability (Kolozsi et al. 2009).

The present study was the first approach using CD1 mice as the animal model, to explore the effects of *in utero* and postnatal exposure to ESL (as well as to other AEDs, for comparison) on cognitive abilities, anxiety-behaviour and pro-depression mood on progeny. The study was performed by conducting a battery of behavioural tests at the ages of one month old - juveniles, and four month old – adults, for both genders.

5.3. Results

5.3.1. Effects of AEDs on body and brain weights of the offspring of CD1 females

It is known that CD1 mice have sexual dimorphism regarding their body size and weight. Accordingly, we weighed all the animals (males and females) at the end of the experimental period to determine whether exposure to AEDs in early-life affects body or brain weights. We observed that the body weight of CD1 males that were exposed to ESL or CBZ did not differ from that of control males (41.46±0.85 g) while CD1 males exposed to OXC or VPA during early-life were significantly heavier (47.98±1.43 g, $p<0.01$ and 48.54±1.65 g, $p<0.001$, respectively) than the control animals (41.46±0.85 g) (Fig. 5.1A). . However, in the case of CD1 females that were exposed to AEDs in the same conditions of their siblings, their body weight did not differ from that of control females (30.87±0.99 g) (Fig. 5.1B). Therefore, the tested AEDs affected differently each gender's weight, thus

interaction between "AED" and "gender" is significant and accounted for 4.15% ($p<0.05$) of the total variance observed in the sample. Moreover, "AED" and "gender" as independent factors were extremely significant that accounted for 7.43% ($p<0.0003$) and 60.12% ($p<0.0001$), respectively, of the total variance.

Regarding the effects of AED exposure on brain weight, no effects were detected when comparing treated CD1 females to the respective control group values (males: 0.47±0.01 g and females: 0.49±0.02 g) (Fig. 5.2B). Therefore, interaction between "AED" and "gender" was not significant ($p>0.05$) and accounted for 3.06% of the total variance observed, while when "AED" and "gender" were considered as independent factors, they accounted for 1.94% and <0.1%, respectively, of total variance ($p>0.05$).

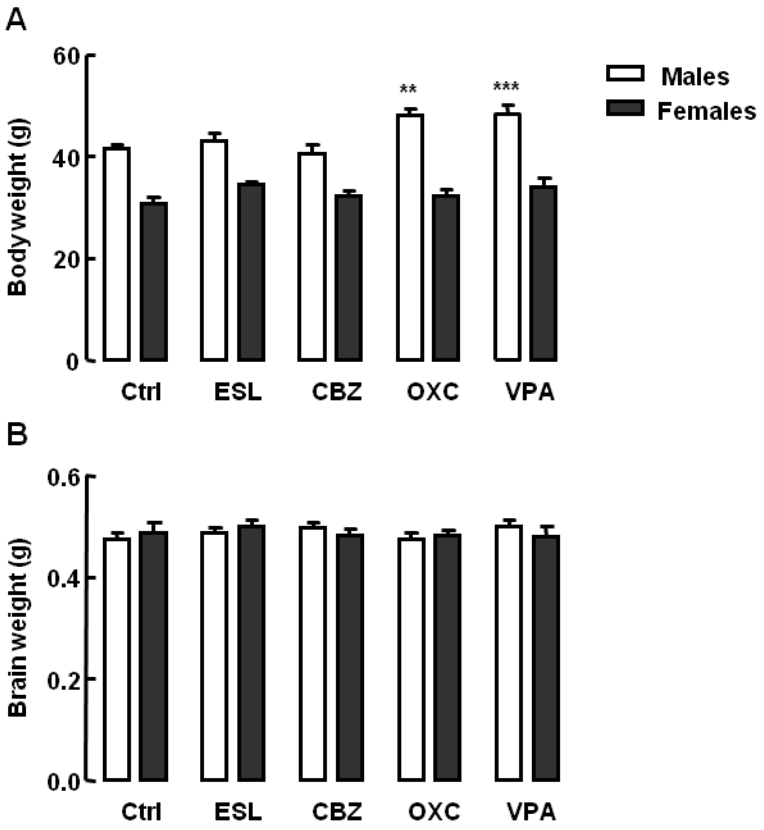

Figure 5.1. Effects of long-term exposure to AEDs *in utero* and after birth, during nursing, on body weight and on brain weight of adult CD1 mice (A) Body weight. OXC and VPA significantly increased body weight of males when administered during early-life, but ESL or CBZ had no effect. **(B)** Brain weight. Exposure to AEDs during gestation and nursing did not change the brain weight of adult CD1 mice. The results are presented as means ± SEM of at least 10 animals per treatment. Two-way factor ANOVA, followed by Bonferroni's post-test **-$p<0.01$ and ***-$p<0.001$, statistically different from correspondent Ctrl (by gender).

5.3.2. Effects of exposure to AEDs on brain functions in CD1 mice: locomotion, memory, learning, anxiety and depression

There are only few reports in the literature regarding the effects of *in utero* exposure to new generation AEDs on brain function during development (reviewed by Loring et al. 2007). In this part of the work we studied CD1 mice offspring of both genders at two different stages of development, at one and at four months of age, after long-term exposure (in utero and during nursing) of the mice to four AEDs: ESL, CBZ, OXC and VPA. For this purpose, we evaluated how exposure to AEDs affected the performance of the animals (treated groups vs control group) in a battery of behavioural tests, as described below. Data analysis was done with two-way factor ANOVA (with paired subjects).

5.3.2.1. Locomotor activity

Locomotor activity of CD1 mice was evaluated in the Open-field test, which is a sensorimotor task performed in a square Plexiglas box, as described in the Methods. First, we observed that exposure to AEDs affected in the same way juvenile and adult males, thus interaction between "AED" and "Age" as source of variation was not significant in the analysis ($p>0.05$), while "Age" and "AED" as independent factors were significant and accounted for 64.77% ($p<0.0001$) and 8.09% ($p<0.001$), respectively, of the total variance observed.

We observed that in juvenile males ESL exposure increased locomotor activity by 40% ($p<0.01$) when compared to the control (146±15.5 no. of lines crossed). However, this effect was lost with age, and adult males treated with ESL did not differ from control animals (71.0±3.6 no. of lines crossed). Similar results were obtained with OXC; on the other hand, neither CBZ nor VPA treatments induced changes on the locomotor activity of CD1 males in either juvenile or adult animals (Fig. 5.2.A).

Concerning females, we observed that each AED had a similar effect in these mice as juvenile or adult, thus interaction between "AED" and "Age" as source of variation was not significant in the analysis ($p>0.05$), while "Age" and "AED" as independent factors were significant and accounted for 58.33% ($p<0.0001$) and 7.00% ($p<0.001$), respectively, of the total variance observed. The data obtained show that ESL, CBZ or VPA exposure did not

change locomotor activity of either juvenile or adult females, as compared to control animals (juvenile: 167±12.6; adult: 92.6±6.1, no. of lines crossed), while females that were exposed to OXC were more active when compared to control animals, and the locomotor activity was increased in the OXC treatment groug by 40% ($p<0.01$) in juvenile females and by 30% ($p<0.05$) in adult females (Fig. 5.2.B).

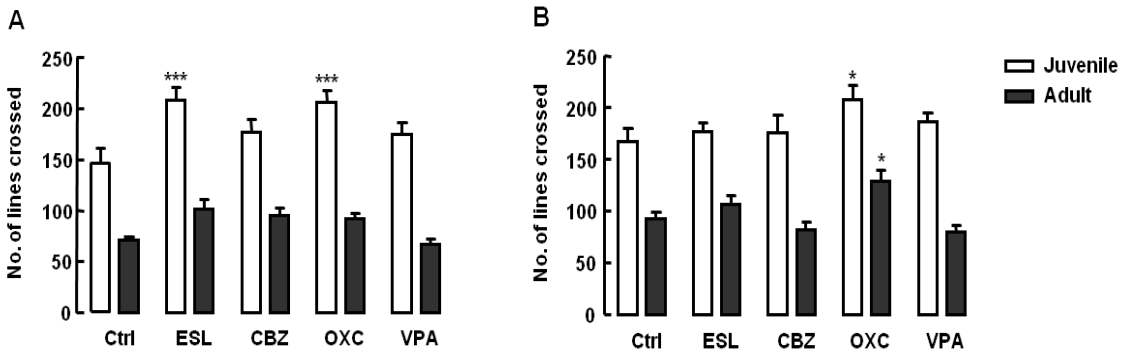

Figure 5.2. Effects of AED exposure on locomotor activity evaluated with the open-field test. (A) Males: juvenile males (one month old) exposed to ESL and OXC were more active than the controls. However, locomotor activity of adult males (four month old) was not affected by AED exposure **(B)** Females (either juvenile or adult) that were exposed to OXC were more active than controls, while other AED treatments did not change the performance of females in this task. The results are presented as means ± SEM of at least 10 animals. Two-way factor ANOVA, followed by Bonferroni's post-test. *-$p<0.05$ and ***-$p<0.001$, statistically different from correspondent Ctrl (by age).

5.3.2.2. Memory

Recognition memory was evaluated by the Novel Object Recognition (NOR) task, which is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. Exploring a novel object reflects the use of learning and recognition memory related to hippocampal functions.

It is observed that alterations induced by AEDs were similar in juvenile and in adult males, thus interaction between "AED" and "Age" as source of variation was not significant in the analysis ($p>0.05$), while "Age" and "AED" as independent factors were significant and accounted for 17.63% ($p<0.0001$) and 18.65% ($p<0.0001$), respectively, of the total variance observed. Thus, CD1 males exposed to ESL during early-life showed similar

performance in the NOR task as control animals (juvenile: 67.5±3.1%; adult: 71.7±3.6%). On the other hand, juvenile males were affected by exposure to CBZ, OXC or VPA, so that the time spent exploring a new object in relation to control animals was decreased by 20% ($p<0.001$ for CBZ and VPA, and $p<0.01$ for OXC). Adult males exposed to VPA kept a decrease of 17% ($p<0.01$) in the time spent exploring a new object when compared to control group, while the effects were lost with age for CBZ and OXC (Fig. 5.3.A).

Regarding the females, we observed that alterations induced by AEDs did not affect in the same way these animals when were juvenile or adult, thus interaction between "AED" and "Age" was significant ($p<0.01$) and accounted for 15.84% of the total variation observed, while the factors "Age" and "AED" as independent factors were not significant and only accounted for total variation by 1.60% and 1.34% ($p>0.05$). Bonferroni's post test showed that while juvenile females that were exposed to AEDs during early-life did not differ from control females (78.4±3.1%), adult females that were exposed to CBZ or OXC showed an increase of 30 or 25% ($p<0.05$) respectively, in the time spent exploring a new object compared to control animals (58.5±6.3%). ESL and VPA did not change exploration time in adult females (Fig. 5.3.B).

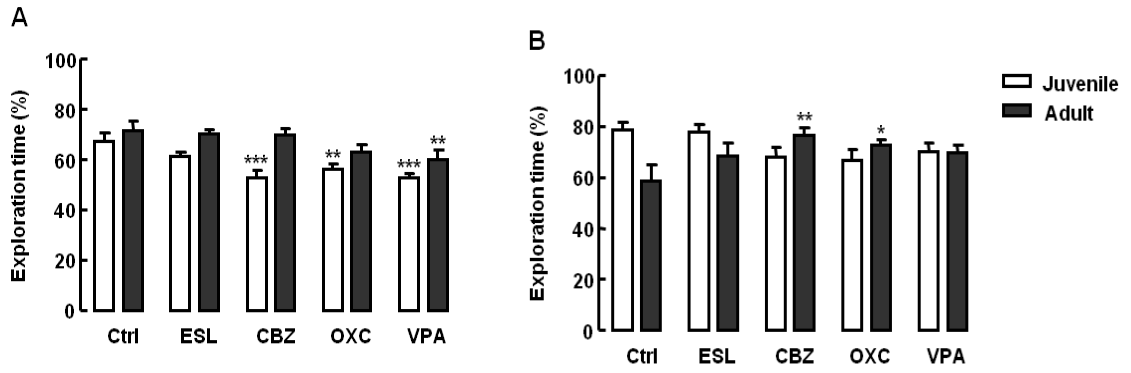

Figure 5.3. Effects of AEDs on memory performance evaluated with the Novel Object Recognition test. (A) Males: juvenile males exposed to CBZ, OXC or VPA spent less time exploring the novel object than control animals. In adult males only the VPA treatment group kept this effect. **(B)** Females**:** in juvenile females the exploring times were similar in all groups, but adult females that were exposed to CBZ or OXC showed increased exploration times, as compared to controls, while the other AEDs had no significant effects The results are presented as means ± SEM of at least 10 animals. Two-way factor ANOVA, followed by Bonferroni's post-test *-$p<0.05$; **-$p<0.01$ and ***-$p<0.001$, statistically different from correspondent Ctrl (by age).

Spatial memory is dependent on the integrity of hippocampus. The willingness to explore new environments was evaluated with Y Maze Modified test, since rodents prefer to

explore a new arm of the maze rather than the one that was previously explored. In this test, the males that were exposed to AEDs during early-life had similar performances on the Y Maze task, when compared to the control animals, either in juvenile or in adult age (37.3±3.0 and 33.1±2.2% of time in new arm, respectively). Thus interaction between "AED" and "Age" was not significant ($p>0.05$) and accounted only for 3.24% of the total variance observed, as well as "AED" and "Age" as independent factors, which were not significant and accounted for 2.31 and 3.31% ($p>0.05$) respectively, of the total variance (Fig. 5.4.A). Regarding the females, we observed that AEDs affected in the same way these animals either juveniles or adults, thus interaction between "AED" and "Age" as source of variation was not significant ($p>0.05$) and accounted for 7.02% of the total variation, while the factors "AED" and "Age" as independent factors were not significant in the total variance observed and accounted for 5.62 and 0.39% ($p>0.05$) respectively. In females, with Bonferroni's post-tests, we observed that in juvenile females exposure to CBZ decreased by 25% ($p<0.05$) the time spent in the new arm, in relation to controls (44.8±4.2% of time in new arm). Adult females exposed to the AEDs did not differ from control animals in this test (36.7±2.3% of time in new arm).(Fig. 5.4.B).

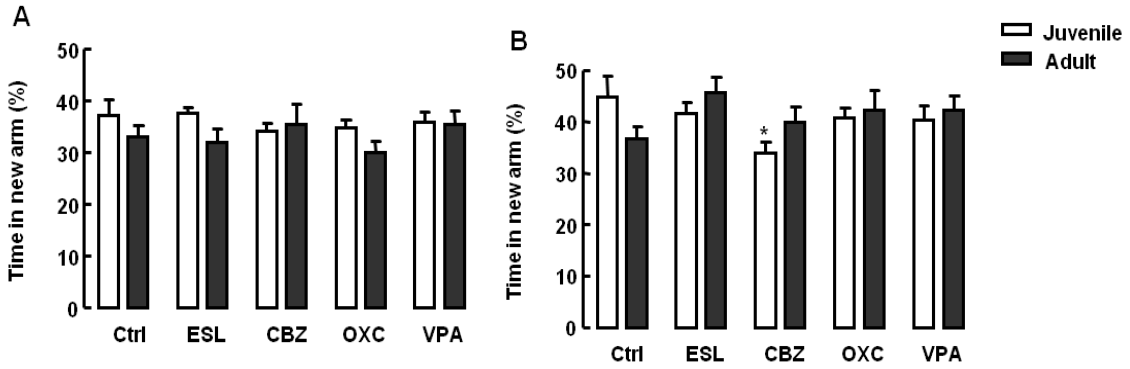

Figure 5.4. Effects of AEDs on memory performance evaluated with the Y Maze test. (A) Males**:** CD1 males (juvenile or adult) exposed to AEDs did not differ from controls. **(B)** Females: Juvenile females exposed to CBZ spent less time in new arm in relation to the control females, but this effect was lost with age. Other females kept their performance similar to the control animals. The results are presented as means ± SEM of at least 10 animals. Two-way factor ANOVA, followed by Bonferroni's post-test *-$p<0.05$, statistically different from correspondent Ctrl (by age).

To further evaluate learning and memory in CD1 mice exposed to AEDs during early-life we used the Passive (Inhibitory) Avoidance test, which is a fear-aggravated task. In this

task, animals learn to avoid a situation in which an aversive stimulus such as a foot-shock was previously delivered. We observed that AEDs affected in the same way juvenile and adult males, thus interaction between "AED" and "Age" as source of variation was not significant ($p>0.05$) and accounted for 6.56% of the total variation; additionally "AED" and "Age" as independent factors were not significant on the total variance observed and accounted for 5.49 and 3.37% ($p>0.05$) respectively. Though, Bonferroni's post test showed that ESL, OXC or VPA did not change the latency time, neither in juvenile nor in adult males, when compared to controls (juvenile: 78.7±16 s; adult: 81.7±29.8 s). However, juvenile males that were exposed to CBZ in early-life showed the latency time decreased by 63% ($p<0.05$) when compared to the control animals, but this effect was lost with age (Fig. 5.5.A). In CD1 females, we also found that AEDs affected in the same way juvenile and adult females, thus interaction between "AED" and "Age" as source of variation was not significant ($p>0.05$) and accounted for 2.37% of the total variation. However, "AED" and "Age" as independent factors were significant and accounted for 7.31% ($p<0.05$) and 41.69% ($p<0.0001$) respectively, of the total variance observed. Neither ESL nor VPA changed latency time of females exposed to these AEDs in early-life, as compared to the control females (juvenile: 79.4±16.8 s; adult: 144.5±20.1 s). However, females that were exposed to CBZ or OXC in early-life showed a decrease of the latency of 70 and 74% ($p<0.05$), respectively, in comparison to the control animals. This effect of OXC was lost with age but for CBZ the latency time was still decreased by 60% ($p<0.05$) in adult females when compared to the control adult females (Fig. 5.5.B).

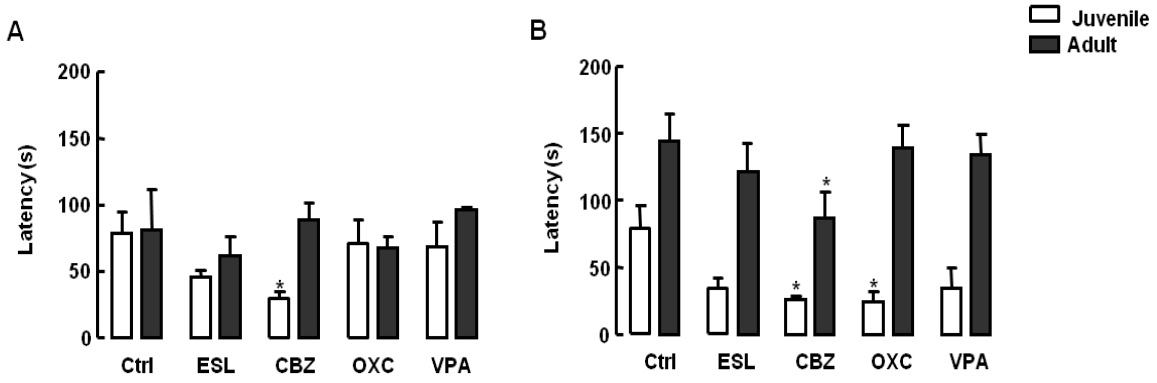

Figure 5.5. Effects of AEDs on memory performance evaluated with the inhibitory avoidance test. (A) Males: juvenile CD1 males exposed to CBZ showed decreased latency time in comparison to the control, but this effect was lost in adult males. None of the other AEDs changed the latency time in both juvenile and adult males. **(B)** Females: in females, exposure to CBZ decreased the latency time in both juvenile and adult animals Exposure to OXC decreased latency time in juvenile females but not in adults.. Neither ESL nor VPA induced changes on the latency time. The results are presented as means ± SEM of at least 10 animals. Two-way factor ANOVA, followed by Bonferroni's post-test *-$p<0.05$, statistically different from correspondent Ctrl (by age).

3.3.2.3. Anxiety

Anxiety-related behaviour is measured with the Elevated Plus Maze (EPM) test. The EPM apparatus has two opposite closed arms and two opposite open arms arranged to form a "+"-shaped maze elevated above the floor. Rodents have innate motivation to explore novel environments, thus they freely explore the maze and behaviours are recorded, namely the time spent in the arms and the number of entries that were made on the open and closed arms.

In the present test, we quantified % of time spent by the CD1 mice in the open arm to evaluate anti-anxiety behaviour induced by AEDs, and total number of entries (open and closed arm), to evaluate spontaneous motor activity. Regarding time spent in open arm, we observed that AEDs effected in a different way both ages, thus interaction between "AED" and "Age" as source of variance was considered significant ($p<0.05$) and accounted for 13.03% of the total variance observed, while "AED" and "Age" as independent factors were significant and not significant, respectively and accounted for 1.33% ($p>0.05$) and 9.54% ($p<0.05$), respectively of the total variance observed. Briefly, juvenile males that were exposed to AEDs in early-life had similar behaviour when compared to control animals

(18.5±3.3% time spent in open arm). In this test, adult males exposed to the AEDs, except for CBZ, kept similar performance to the controls (17.3±3.5% time spent in open arm), while exposure to CBZ increased by 60% ($p<0.05$) the time spent in open arm (Fig. 5.6.A).

Concerning total number of entries, we observed that AEDs affected in the same way juvenile and adult males, thus interaction between "AED" and "Age" as source of variation was not significant ($p>0.05$) and accounted for 3.53% of the total variation, while "AED" and "Age" as independent factors were significant and accounted for 11.52% ($p<0.05$) and 28.12% ($p<0.0001$), respectively, of the total variance observed. Thus, it is observed that males exposed to ESL in early-life increased by 20% ($p<0.05$) the total number of entries in relation to control animals (25.1±1.7 total of entries). This result is in agreement with the data observed in open-field task, where juvenile males that were exposed to ESL were more active than the controls (see figure 5.2.A.). However, the total number of entries in the case of adult males did not differ from controls (17.3±1.3 total entries). Other AEDs did not affect the total number of entries, which were similar in both ages of the males (Fig. 5.6.C).

CD1 females that were exposed to AEDs during early-life were not affected by AEDs in the prosecution of this task at both periods of testes. In respect to "time in open arm" (TOA) and then "total entries" (TE), we observed that AEDs affected in the same way juvenile and adult animals, thus interaction between "AED" and "Age" as source of variance was not considered significant ($p>0.05$) and accounted for 6.58% (in TOA) and 1.53% (in TE) of the total variance, while "AED" and "Age" as independent factors were significant ($p<0.0001$) and not significant ($p>0.05$), respectively, and accounted for 3.03% and 44.09% (in TOA), and 5.48 and 1.41% (in TE), of the total variance observed for both measures. Neither juvenile nor adult females differ from controls regarding TOA (juvenile: 21.4±3.1; adult: 20.26±1.8% time of time spent in new arm) (Fig. 5.6.B) as well as regarding TE (juvenile: 24.8±1.7; adult: 16.2±1.6 total entries) (Fig. 5.6.D).

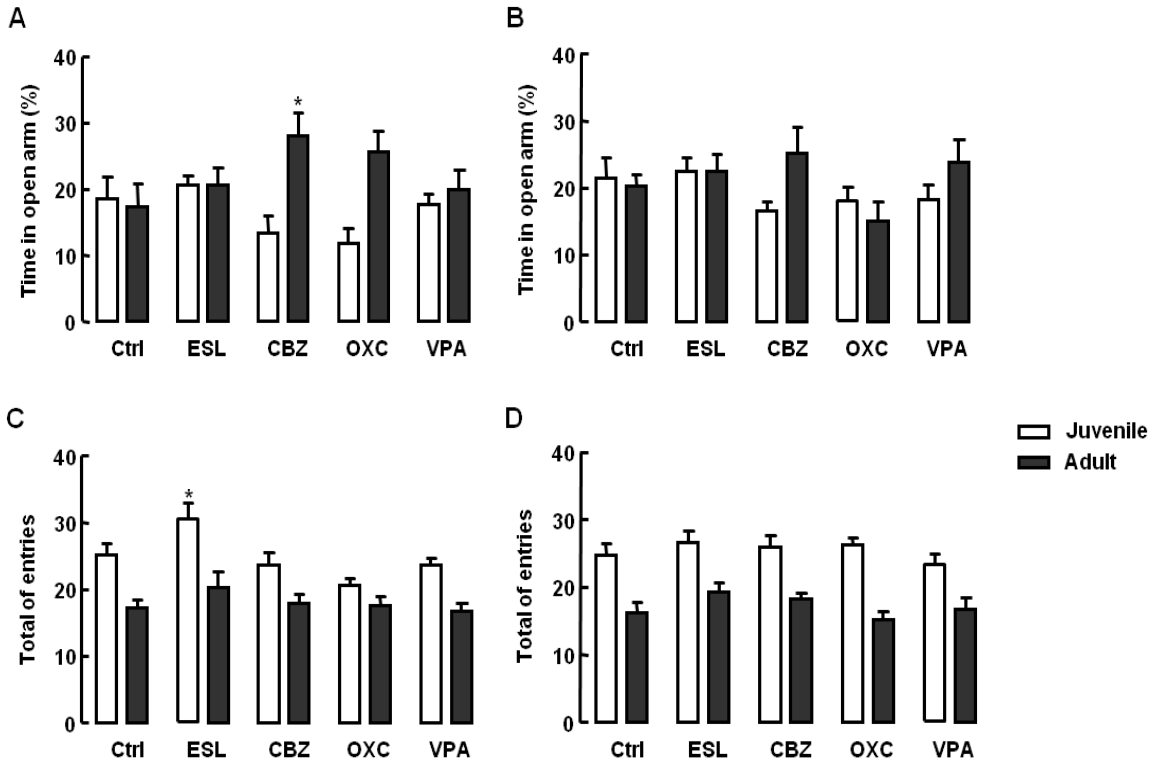

Figure 5.6. Effects of AED exposure on anxiety with the elevated plus maze test. (A) Males: CD1 adult, but not juvenile males, exposed to CBZ spent more time in the open arm as compared to the respective controls. None of the other AEDs changed the time spent in open arm by the animals.. **(B)** Females exposed to AEDs had similar behaviour when compared to control animals. **(C)** Males: ESL increased the number of total entries in juvenile but not in adult male mice.. None of the other AEDs changed activity of males. **(D)** Females exposed to AEDs had similar locomotor performance when compared to control animals at both ages. Two-way factor ANOVA, followed by Bonferroni's post-test *-$p<0.05$, statistically different from correspondent Ctrl (by age).

3.3.2.4. Depression

Forced swimming test (FST) is a fine task with predictive validity for screening an antidepressant effect of a drug or treatment. Regarding males, we observed that AEDs affected in a different way juvenile and adult males, thus interaction between "AED" and "Age" as source of variance was considered significant ($p<0.05$) and accounted for 6.64% of the total variance, while "AED" and "Age" as independent factors were significant and accounted for 8.07% ($p<0.05$) and 31.22% ($p<0.0001$), respectively, of the total variance observed. Thus, exposure to ESL, CBZ and VPA in early-life did not change immobility time

after 6 min test in males of both ages, when they were compared to controls (juvenile: 235.6±5.9 s; adult: 309.2±13.3 s). On the other hand, OXC increased by 15% ($p<0.05$) the immobility time when juvenile males performed the test, but the effect was lost in adult males (Fig. 5.7.A). Regarding CD1 females that were exposed to AEDs during early-life, we observed that AEDs affected in the same way juvenile and adult animals, thus interaction between "AED" and "Age" as source of variance was not considered significant ($p>0.05$) and accounted for 2.66% of the total variance, while "AED" and "Age" as independent factors were not significant and significant ($p<0.0001$) and accounted for 1.05% and 62.07%, respectively, of the total variance observed. Thus, we observed that AEDs did not change immobility time at both ages when compared to control animals (juvenile: 235.8±7.0 s; adult: 297.4±8.0 s) (Fig. 5.7.B).

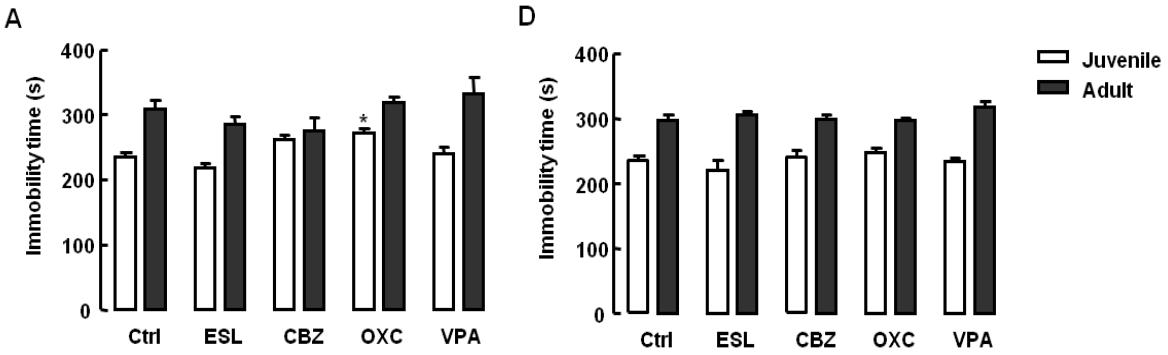

Figure 5.7. Effects of AED exposure on depression evaluated with the forced swimming test. (A) Males: CD1 juvenile males exposed to OXC showed increased immobility time as compared to the control, but lost this effect with age. None of the other AEDs changed this feature. **(B)** Females: treated females (juvenile or adult) had similar performance in the FST test to control females . The results are presented as means ± SEM of at least 10 animals. Two-way factor ANOVA, followed by Bonferroni's post-test *-$p<0.05$, statistically different from correspondent Ctrl (by gender).

5.3.3. Exposure to antiepileptic drugs in utero and during nursing did not affect brain morphology of offspring

Cresyl violet it is a basic stain that binds to the acidic components in the neuronal cytoplasm, such as ribosomes, and also to neuronal nuclei and nucleoli. A particular acidic component is the "Nissl bodies" which are aggregations of rich RNA endoplasmic reticulum. When neuronal injury occurs cresyl violet is lost, thus neurons are not stained (Paul et al. 2008).

We did not observe differences in the cresyl violet staining performed in adult control CD1 mice and adult CD1 mice exposed to AEDs *in utero* and during nursing (males or females). Both granule cell layers (dentate gyrus of the hippocampus) and the pyramidal cell layers (CA region of the hippocampus) showed well-stained neurons across all brain sections evaluated. We also evaluated sections of the brain cortex and did not find any changes in the architectures of their neurons, as compared to control animals (Fig. 5.8).

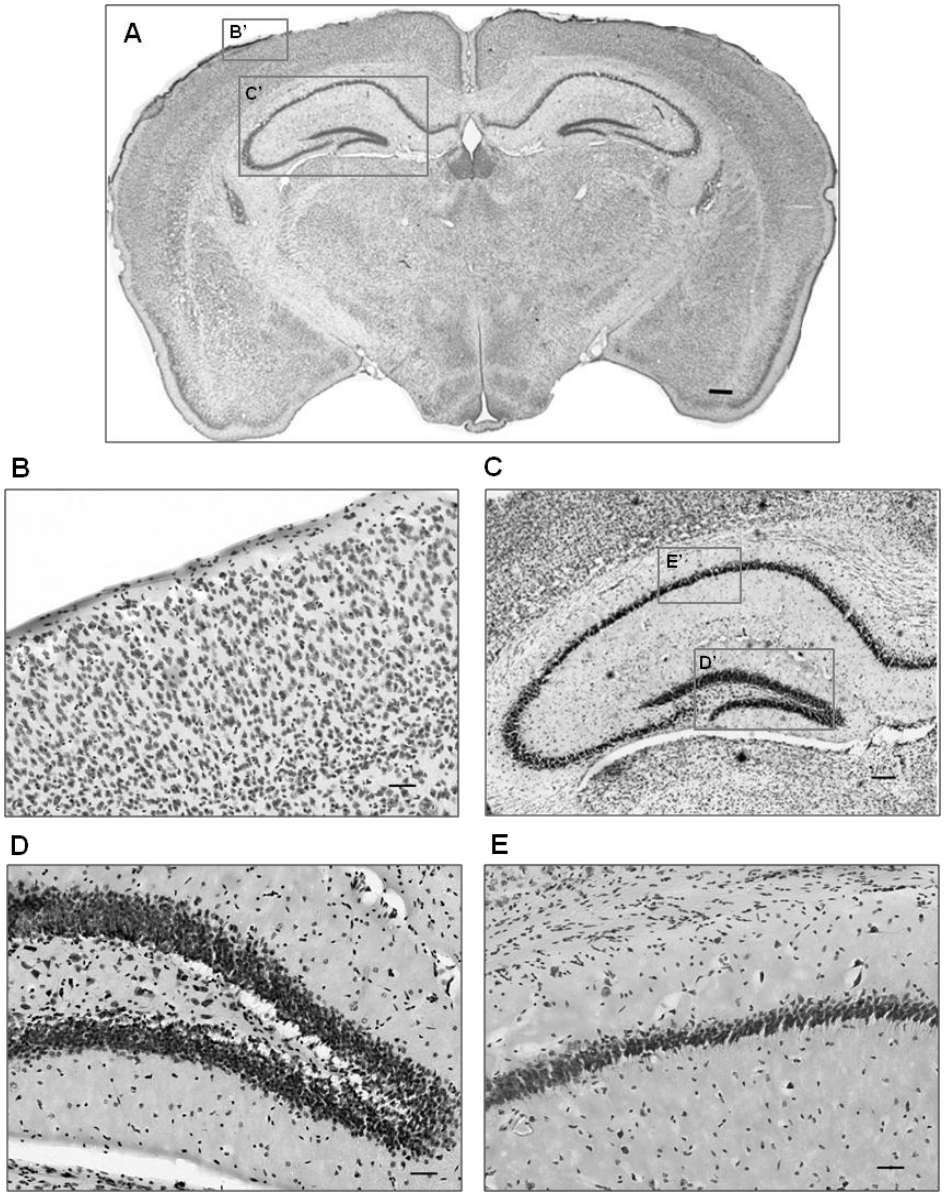

Figure 5.8. Images of adult CD1 mice brain sections stained with cresyl violet . Representative images of **(A)** coronal section of the brain; amplified images of **(B)** cortex; **(C)** hippocampus; **(D)** granule cells (dentate gyrus), and **(E)** pyramidal cells (CA). Scale bar: 20 μm (B, D and E), 50 μ, (A and C).

5.4. Discussion

This study focused on possible long-term effects of AEDs on cognitive and non-cognitive behaviours after exposure to the drugs during gestation and nursing, using as model of CD1 mice born to CD1 treated females. Our main results show some effects of CBZ, OXC and VPA on cognition. In the case of ESL, this AED showed a safer profile than the other AEDs tested, since exposure to ESL had none or only modest negative impacts on the brain functions evaluated by the behaviour tests. Moreover, the effects on memory, learning, motor activity and other behavioural traits were mostly lost with age, which suggests that these effects were transient, as those observed in children (Marsh et al. 2006). The behavioural profile associated with each AED is discussed below.

In the current study we weighed the brains of offspring that were exposed to AEDs in utero and during nursing at the age of four months. However brain-weight was not changed by the AEDs tested, in both genders. In brain, structural variations in cortical lateralization between genders are evident in both humans and animals (Galaburda 1991). Moreover, in rodents this begins to be seen during fetal development, mainly due to endocrine environment which is controlled by the actions of gonadal hormones (Diamond 1989; Diamond 1991; Berger-Sweeney and Hohmann 1997). In addition, different patterns of lateralization are also observed in other structures of the brain such as the hypothalamus (Madeira et al. 1999), the amygdala and the hippocampus (Hojo et al. 2006). In the hippocampus, it was previously observed that the dentate gyrus granule cell layer is larger in males than in females (Hojo et al. 2006), therefore, if behavioural and biochemical asymmetries observed in animals are due to impacts during prenatal or very early neonatal life (Afonso et al. 1993), it is reasonable to assume that the patterns of lateralization may be affected by external factors during brain development, such as exposure to AEDs. However, in the present study, we did not find sexual-dimorphism in the brain-weight due to AED exposure.

On the other hand, the current study showed differences between the body-weight of CD1 males and females, as expected. Moreover, we also observed that male progeny that were exposed to OXC and VPA were heavier than controls. VPA has previously been associated with body-weight increases in humans and rats (Biton 2003; Li et al. 2010), while in mice changes due to VPA in younger animals were not seen in adults (Brown et al. 2008); in fetuses, VPA exposure in utero decreased body-weight (Faiella et al. 2000). On the other

hand, OXC has not been associated with changes in body-weight in humans (Cansu et al. 2011), while in mice there is a lack of information.

Our study is the first, to our knowledge, that reports data about gestational and nursing exposure to ESL, CBZ, OXC or VPA and its long-term effects on cognition and other behavioural features of offspring during development.

In our study behaviour tests were performed in either males or females, at one and four months of age. Differences in pharmacokinetics regarding AEDs in males and females have been evidenced by drug-disposition studies, which are due to molecular and physiological factors of each gender; thus, its variations will be reflected in behavioural differences (Meibohm et al. 2002). In the literature, it has been described that learning and memory of male and female mice could be differentially affected by either their gonodal or thyroid hormones (van Haaren et al. 1990; Conrad et al. 2003; Hojo et al. 2006; Paus et al. 2010). Moreover, female mice may have increased variations due to their oestrous cycle which is inconsistent between individuals (Van Meer and Raber 2005). The mechanism for the sex-specific effects of AEDs was not a scope of our study but, since endocrine functions are distinct in the two genders and exert different effects on learning and memory (van Haaren et al. 1990), it is plausible that AEDs may affect in a different way male and female throughout life.

We investigated whether AEDs affect the performances of offspring at the age of 1 and 4 months in both male and female CD1 mice exposed to AEDs, as well as in controls, by performing the behaviour tests twice: in juvenile animals (1 month old) and in adult animals (4 months old). The order of the tests did not have any effect on the tasks' performance (Paylor et al. 2006).

General Motor activity

The main findings of this study in relation to animals exposed to AEDs showed that ESL had a stimulatory effect on locomotor activity in juvenile males, as compared to control animals, as evaluated in the open-field task, which is the most used test to assess general motor activity. The effect of ESL was both, gender-dependent, since it did not affect females, and age-dependent, since the effect was lost in adult males. OXC had also a stimulatory effect on locomotor activity of males and was age-dependent; furthermore, females exposed to OXC were also more active than controls, at both ages tested. In contrast to our observations, Beijamini and colleagues observed that, in rats treated with OXC (80 mg/kg,

three times per day), OXC did not change locomotor activity (Beijamini et al. 1998). However, differences in animal species, and experimental protocols utilized may account for the different results obtained

Furthermore, neither CBZ nor VPA affected general motor activity in the present study. Overall, none of the AED exposure in utero and during nursing, at the therapeutic doses (30 mg/kg: ESL, CBZ and OXC; and 300 mg/kg: VPA), caused a lack of motor efficiency due to physical impairment. The possible adverse effects of ESL on cognitive functions in humans have been previously studied by Milovan and colleagues, who demonstrated that ESL does not have impact on cognitive abilities of healthy patients (Milovan et al. 2010).

Memory and learning

Object recognition (OR) test encompasses spatial and non spatial aspects of declarative memory, which is hippocampus-dependent (Eichenbaum 2001). Moreover, the dentate gyrus of hippocampus has a role in the response of induced synapses when an novel object exposure occurs (Lee et al. 2005b), thus OR is commonly used to evaluate learning and recognition memory, based on natural behaviour of rodents to spend more time discovering a novel object than a recognizable one. Impairment in the performance of this test has been associated with a hippocampus-dependent memory trait (Clark et al. 2000; Broadbent et al. 2004), though others argue that it is an hippocampus-independent memory (Winters et al. 2004).

We observed that the effects of CBZ and OXC on memory and learning were age-dependent within each gender: both AEDs reduced the time that juvenile males spent exploring the novel object, but this behaviour was lost with age; however, CBZ and OXC increased the time that adult, but not juvenile females spent exploring the novel object. VPA had a gender-dependent effect in males. CD1 males exposed to VPA showed a worse performance on this task. VPA had a long-term impairment on features associated with recognition memory on CD1 males. Whether or not associated with the hippocampus, in our study we observed that learning linked to recognition memory was temporarily affected in CD1 male offspring in early-life, after a gestational-nursing exposure to the AEDs. On the other hand ESL exposure did not have any effect on this test.

Spatial memory is an hippocampal-dependent task (reviewed by Best et al. 2001) and we evaluated memory performance tasks with Y Maze test. We observed that the AEDs

did not affect the performance of males, since treated animals had similar behaviour to control group. However, CBZ had an age-dependent effect in females since this AED decreased time that juvenile females spent exploring new arm, and this effect was lost in adult females. Assuming that CBZ exposure may affect memory-associated brain structures, such as the hippocampus, in juvenile females, it is plausible that an eventual injury has been rescued by recruitment of the surrounding zones during development, and the effect observed was temporary and was not kept until adulthood.

Aversive memory is modulated by both hippocampus and amygdala, which have the function of inducing and strengthening this type of memory (Seidenbecher et al. 2003), which is normally assessed in screening drugs for cognitive enhancement. We evaluated aversive memory with a passive avoidance task, delivering an aversive stimulus (foot-shock) twice. In both genders CBZ decreased latency, it had age-dependent effect in males and kept its effects in females with age. Furthermore, the effect of OXC was gender and age-dependent since juvenile females exposed to OXC had significantly lower latency than adult females. Generally, it should be noted that data from the inhibitory avoidance test showed that CD1 females exposed to AEDs during gestation and nursing may have hippocampal-dependent features impaired either temporarily or permanently. Furthermore, latency times were higher in adult females than in juveniles, but this behaviour may be a result of less activity in adulthood.

Our results are in line with the literature concerning effects of CBZ in human progeny, which have been associated with poorer general memory performance (Bromley et al. 2010); and also in accordance with data from VPA in humans, which show that children exposed to this AED during pregnancy had impairment of cognition, namely verbal intelligence (Gaily et al. 2004; Bromley et al. 2010). Moreover, they are in agreement with observations in rats, which had low performance in spatial memory with VPA (Ingram et al. 2000). However, a study with C3H/He adult mice shows that prenatal exposure to CBZ did not affect cognition (Rayburn et al. 2004). In other studies in rats, CBZ had no effects on active avoidance learning task (Vorhees 1987).

The adverse-effects that we observed in the present study were mainly due to exposure of the CD1 mice to CBZ, which had a negative impact on the tasks' performance of juvenile CD1 males and females. Again, we believed that the impaired behaviour that we observed may be a result of temporary dysfunction in the brain structures, namely in

hippocampus which are responsible for learning and memory functions. Indeed, the majority of observed effects of AEDs occurred in animals as juvenile, which is a period of growth of body and the brain is still developing. In a brain in development, the structures that are more vulnerable and targets of harm are structures like striatum and hippocampus (Rodier 1994; Vinten et al. 2005; Meador et al. 2009). It is reasonable to assume that during these vulnerable periods of development, where maturation of the central nervous system is occuring, any exposure to an AED had a higher risk associated and undesirable to the fetus with long-term effect, which may be noticeable in behaviour in juvenile animals and then lost with age (Marsh et al. 2006). However, males exposed to VPA and females exposed to CBZ had negative effects that were kept in adulthood, namely on object recognition task and latency behaviour, respectively. In these cases, the existence of mechanisms, such as neuronal cell death and decline of neurogenesis in the hippocampus, may be underlying the permanent cognitive impairments, as it was observed in rodents with postnatally AED treatment; (Bittigau et al. 2003; Stefovska et al. 2008). Another possible hypothesis is the involvement of these AEDs on the cholinergic system, which is strictly associated with learning and memory (Hasselmo and Bower 1993; Hasselmo and Barkai 1995). Indeed CBZ and VPA was shown to induce an increase in brain acetylcholine (Ach) and a decrease in the choline level resulting on memory impairment (Nowakowska et al. 2007).

Although mechanisms for the sex-specific effects of AEDs are not well known, the differences that we observed between genders may be influenced by the way that AEDs affect hormonal environment in each gender. Overall, gestation and nursing exposure to CBZ, OXC and VPA had a negative impact on cognitive behaviour of offspring, (males and females), while ESL had no effects on both genders.

Anxiety

CBZ and VPA have been associated with anxiolytic and anxiety properties, respectively (Markram et al. 2008; Rezvanfard et al. 2009). Anxiety-related behaviour was evaluated on the elevated plus maze, which has been used to identify anxiolytic and anxiogenic drug effect in rodents (Pellow et al. 1985). General locomotor activity is also possible to be evaluated with this test by counting the number of total entries in the arms. In line to literature, we observed the anxiolytic effects of CBZ. However, CBZ effect was gender and age-dependent, since only affected adult males. None of the other AEDs had anxiety or anxiolytic-related effects in juvenile males or females. Moreover, regarding total arm

entries, we observed that ESL increased spontaneous motor activity in juvenile males. This observation is in agreement with the previous data from the open-field test, where with ESL exposure we observed that adults were less active than younger animals. Gestation and nursing exposure to ESL, OXC or VPA, did not induce anxiety or anxiolytic behaviour on CD1 mice offspring.

Depression

We also explored whether exposure to AED in utero and during the nursing period had long-term effects on depressive-behaviour of CD1 mice offspring. In order to evaluate this feature, animals were exposed to an unexpected and depressing situation using the forced swimming test, which is one of the most used behavioural tests to assess the anti-depressant effects of drugs (Petit-Demouliere et al. 2005). In our study we observed that treated females did not show any signs of depressive behaviour, except juvenile males that were exposed to OXC which showed an increased immobility time as compared to controls. With these results we conclude that OXC-specific effect as depressive-behaviour is not a false-positive since the same animals showed increased activity in the open-field test. However, in human studies, OXC had no impact in the mood of patients, while other studies show that OXC has potentially favourable outcome on depressive-behaviour (Mula and Sander 2007; Miller et al. 2008).

Thus, AEDs with negative effects on mood should be avoided since depressive-behaviour has been observed in patients with epilepsy and in pregnant women (reviewed by Gaynes et al. 2005; Kanner 2009; Mazarati et al. 2009) as well as in animal models.

Brain morphology

We also explored the possible occurrence of morphological alterations in the brain due to AED exposure, for instance cortical dysplasias in cortex or in the hippocampus. For this purpose, we used cresyl violet staining of brain sections obtained from control and from CD1 mice that had been exposed to the AEDs during gestation and nursing. Cresyl violet stains Nissl bodies inside the cells, thus neuronal cells when damaged loose this stain. Adult brains of offspring that were exposed to each of the AEDs, ESL, CBZ, OXC or VPA, were analyzed and compared to respective controls and we did not observe any differences. Thus, AED exposure did not have long-term effects on brain morphology of offspring during development. However, it has been reported that in rats exposure to VPA causes cerebellar anomalies (Ingram et al. 2000).

Our findings identified that CD1 mice offspring exposed to ESL had similar performances to control animals, while offspring exposed to CBZ, OXC and VPA had some negative long-term effects, namely on memory and mood, which were gender or age dependent. Thus, since treatment with ESL during gestation and nursing showed a safer profile for offspring, ESL may be considered as a good candidate for monotherapy in the treatment of pregnant women with epilepsy. However, despite the positive findings regarding ESL they were obtained in seizure naive CD1 mice and they should be confirmed not only in seizured animals, but also in healthy patients as well as in patients with epilepsy, since the negative impact of epilepsy per se should be considered.

Chapter 6

Effects of long-term exposure to antiepileptics on the proliferation of neural stem cells and on neurogenesis: in vivo and in vitro studies

6.1. Summary

The hippocampus, as a neurogenic area, is susceptible to changes in the surrounding environment during early-development of an individual, namely during gestation and nursing. Neurogenesis is controlled by several extrinsic and intrinsic agents, such as drugs, neurotransmitters, neurotrophic factors, among others. Thus, any disturbances in these agents may negatively affect neurogenesis and hence hippocampus-dependent functions (reviewed by Ikonomidou 2010).

It has been described that antiepileptic drugs (AEDs) may alter proliferation and neurogenesis in *in vitro* and in *in vivo* models. AEDs modulate neurotransmitters like GABA and glutamate which are involved in neurogenesis regulation. Furthermore, VPA, CBZ, OXC and LTG have histone deacetylase inhibitor properties which are associated with anti-proliferative effects (Beutler et al. 2005; Stettner et al. 2012).

In the study described in this chapter, we investigated the effect of AED exposure *in utero* and during nursing on the proliferation and neurogenesis in the dentate gyrus of the hippocampus in the adult CD1 mice born to treated females. We also studied the effects of AEDs on the proliferation, cell cycle and cell death of cultured neural stem cells isolated from the rat subventricular zone (SVZ).

Regarding the *in vivo* studies, we observed that basal proliferation, measured by EdU incorporation, in SGZ of adult CD1 males was decreased by exposure to OXC (40%), while in females no changes were found. Formation of newly born cells, measured by BrdU incorporation, was decreased in SGZ by OXC (54%) and by VPA (60%), in CD1 males, and by OXC (47%) in CD1 females. The number of newly immature or mature neurons was unchanged by AED exposure in males, except for CBZ which slightly increased the number of mature neurons in SGZ (16%). In females, OXC decreased the number of immature (62%) and mature (20%) neurons in SGZ, while other AEDs did not induce changes in neurogenesis.

Regarding *in vitro* studies, we observed that except for the main metabolite of ESL, S-Lic, other AEDs decreased proliferation as follows: ESL (0.3 mM) and R-Lic (0.01 mM),

decreased by 50% and 40%, respectively, CBZ (0.01-0.1 or 0.3 mM) decreased by 50% or 70%, respectively; OXC (0.03 or 0.3 mM) decreased by 60% or 75%, respectively; LTG (0.3 mM) decreased by 40%, and VPA (1 or 3 mM) decreased by 85% or 95%, respectively.

Regarding cell cycle analysis, CBZ (0.03 or 0.3 mM), LTG (0.01 mM) and VPA (1 mM) increased the number of cells in G0/G1 phase (by 20%, 15% and 20%, respectively); the number of cells in S phase was not changed by AEDs; ESL (0.1 mM) and VPA (1 or 3 mM) decreased the percentage of cells in G2/M phase (by 45%, 75% or 70%, respectively), while OXC (0.3 mM) increased this percentage by 65%. In addition, we quantified cell death by apoptosis and observed that VPA (3 mM) and OXC (0.3 mM) increased cell death, but VPA was more toxic than OXC in the SVZ cultures. The other AEDs tested (ESL, S-Lic, R-Lic, CBZ or LTG) did not cause cell death in the SVZ cultures.

In conclusion, ESL (*in vivo studies*) did not change basal proliferation and neurogenesis of SGZ in the hippocampus of the adult brain and its main metabolite, S-Lic, (*in vitro studies*) did not affect the proliferation of SVZ cultures. ESL or S-Lic did not induce cell death in both types of studies. On the basis of our findings, ESL may have potential advantages over other AEDs in AED therapy, namely during gestation and nursing, since ESL did not have adverse effects on basal proliferation in the adult brain of CD1 mice born to treated females, On the other hand, OXC decreased proliferation and neurogenesis in adult brain after exposure during gestation and nursing, while in the *in vitro* model of SVZ cultures it arrested cells in G2/M, which may be an indication of DNA damage. However, further studies should be performed in order to understand the mechanisms underlying the effects of AEDs on neurogenesis.

6.2. Introduction

In the adult mammalian brain, neurogenesis is a physiological process that extends from maturation of the central nervous system in the embryo until adulthood. In a *naive* brain, this physiological process occurs in a balanced way and is regulated by endogenous mechanisms which involve neurotransmitters, neurotrophic factors, hormones, and neuromodulators, among other intrinsic and extrinsic agents. It is known that neurogenesis mainly occurs in two distinct regions of the adult brain, the subgranular zone (SGZ) of the

dentate gyrus in hippocampus, and the subventricular zone (SVZ) of lateral ventricles. Neurogenic process comprises different stages, such as proliferation of progenitors and adult neural stem cells (NSCs), fate specification, migration, differentiation, maturation and integration in the neuronal network (reviewed by Kempermann 2011).

However, neurogenesis can be disturbed throughout life by several insults, such as seizures, or by different agents, as antiepileptic drugs (AEDs), which act as enhancers or inhibitors of different voltage-gated channels, and on neurotransmitters like GABA and glutamate. Since GABA and glutamate are modulators of the neurogenesis, this process may be affected by AEDs (Bittigau et al. 2002; Manent et al. 2007; Ikonomidou 2010). It is possible that AED exposure may interfere with the stages of neurogenesis, namely proliferation and migration, in the developing brain. Moreover, the neurogenic areas SGZ and SVZ are more vulnerable to effects of AEDs after birth, and a disorder on neurogenesis may account for cognitive dysfunctions in humans that have been exposed to AEDs during early-life (Marsh et al. 2006; Ikonomidou 2010).

There are various reports in the literature about the effects of AEDs on neural stem cell proliferation and neurogenesis using different rodent models. LTG and VPA have been shown to modulate seizure-induced neurogenesis in the adult hippocampus of rats (Jessberger et al. 2007; Chen et al. 2010), while, in contrast, another study shows that CBZ and VPA exposure does not induce changes on cell proliferation and neurogenesis in rats (Chen et al. 2009). Regarding *in vitro* studies, VPA was shown to induce neuronal differentiation in hippocampal neural progenitor cells cultures (Hsieh et al. 2004), and to promote neurogenesis in rat cortical or striatal primordial stem cells, while CBZ and LTG had no effect in the number of new neurons (Laeng et al. 2004). On the other hand, in a recent study using derived neural precursor cells from dentate gyrus, VPA and LTG increased the ratio of astrocytes and decreased that of neurons, while CBZ had the opposite effect (Boku et al. 2011); other studies showed that VPA inhibited proliferation of NSCs from the SVZ of adult mice (Zhou et al. 2011), and that VPA inhibited proliferation and enhanced differentiation of cortical neural progenitor cells (Jung et al. 2008). Since VPA has been considered a histone deacetylase inhibitor (HDACi), this property may explain its anti-proliferative properties (reviewed by Chateauvieux et al. 2010). However, there is a lack of information on this subject regarding OXC and eslicarbazepine acetate (ESL).

Some of these studies describe effects of AEDs in neural progenitor cells after stimulation (Brandt et al. 2006; Chen et al. 2010). Thus, it is important to understand the AED potential as proliferative or anti-proliferative agents in cultures and *in vivo,* namely theirs possible adverse effects on neurogenesis in the adult brain. Progeny of women with epilepsy and with AED therapy are exposed to the drugs *in utero* and during nursing, and it is known that these children have a great risk of teratogenic effects and cognitive dysfunctions throughout life. Cognitive dysfunction is strictly associated with impaired hippocampal functions, thus disturbance in SGZ neurogenesis may be linked to long-term side effects of AEDs (reviewed by Ikonomidou and Turski 2010).

Taking these findings in consideration, we aim to studying the effects of AEDs on neural stem cell proliferation and hippocampal neurogenesis in the adult brain of CD1 mice, after *in utero* and nursing exposure. We also studied, in an *in vitro* model, the effects of AEDs on the proliferation, cell cycle distribution and cell death of neural stem cell cultures from SVZ of juvenile Wistar rats.

6.3. Results

As described in the Methods, 50 CD1 males and 50 CD1 females were exposed to AEDs *in utero* and during nursing, at therapeutic doses of ESL, CBZ, OXC (30 mg/kg) and VPA (300 mg/kg) and were evaluated with behavioural tests at two different ages (one month and four months), as reported in Chapter 5. In the present chapter we will describe additional data obtained in the adult animals (four months old) submitted to the same protocol of AED exposure (as described in chapter 5), in which we evaluated the effects of AED exposure on basal proliferation and neurogenesis in the hippocampus, by assessing EdU and BrdU incorporation, respectively, by immunohistochemistry. Both EdU and BrdU are thymidine analogues that are incorporated into DNA during the S-phase of cell cycle. BrdU and EdU (50 mg/kg) were respectively injected in the animals, at one month and 24 h before animals being sacrificed by euthanasia, at four months of age. After euthanasia, with pentobarbital anaesthesia, all animals were submitted to transcardiac perfusion and the brains were collected and stored at 4°C until they were sectioned for immunohistochemistry analysis.

6.3.1. Long-term exposure to AEDs during gestation and nursing: effects on basal proliferation of the dentate gyrus of adult CD1 mice

We assessed EdU incorporation by immunohistochemistry with click-it reaction (see section 2.2.3.2. for detailed information). We observed that in adult CD1 males exposed to OXC a decrease in the number of EdU-positive cells (EdU^+) (3.42±0.62 EdU^+, $p<0.05$) in the SGZ of the dentate gyrus was detected, when compared to control animals (5.62±1.02 EdU^+). No changes were observed in the other layers of the dentate gyrus in these animals, as compared to controls (IGZ: 2.00±0.46 and OGZ: 0.35±0.24 EdU^+). Moreover, none of other AEDs changed the basal proliferation on dentate gyrus of CD1 males as shown in the representative images of EdU incorporation and quantification graph (Fig. 6.1.A-B). Adult CD1 females that were exposed to the AEDs in the same conditions of males did not show any changes on the number of EdU-positive cells in the three layers of the dentate gyrus, when compared to controls (SGZ: 6.08±0.83, IGZ: 1.96±0.57 and OGZ: 0.21±0.08 EdU^+) (Fig. 6.2.A-B).

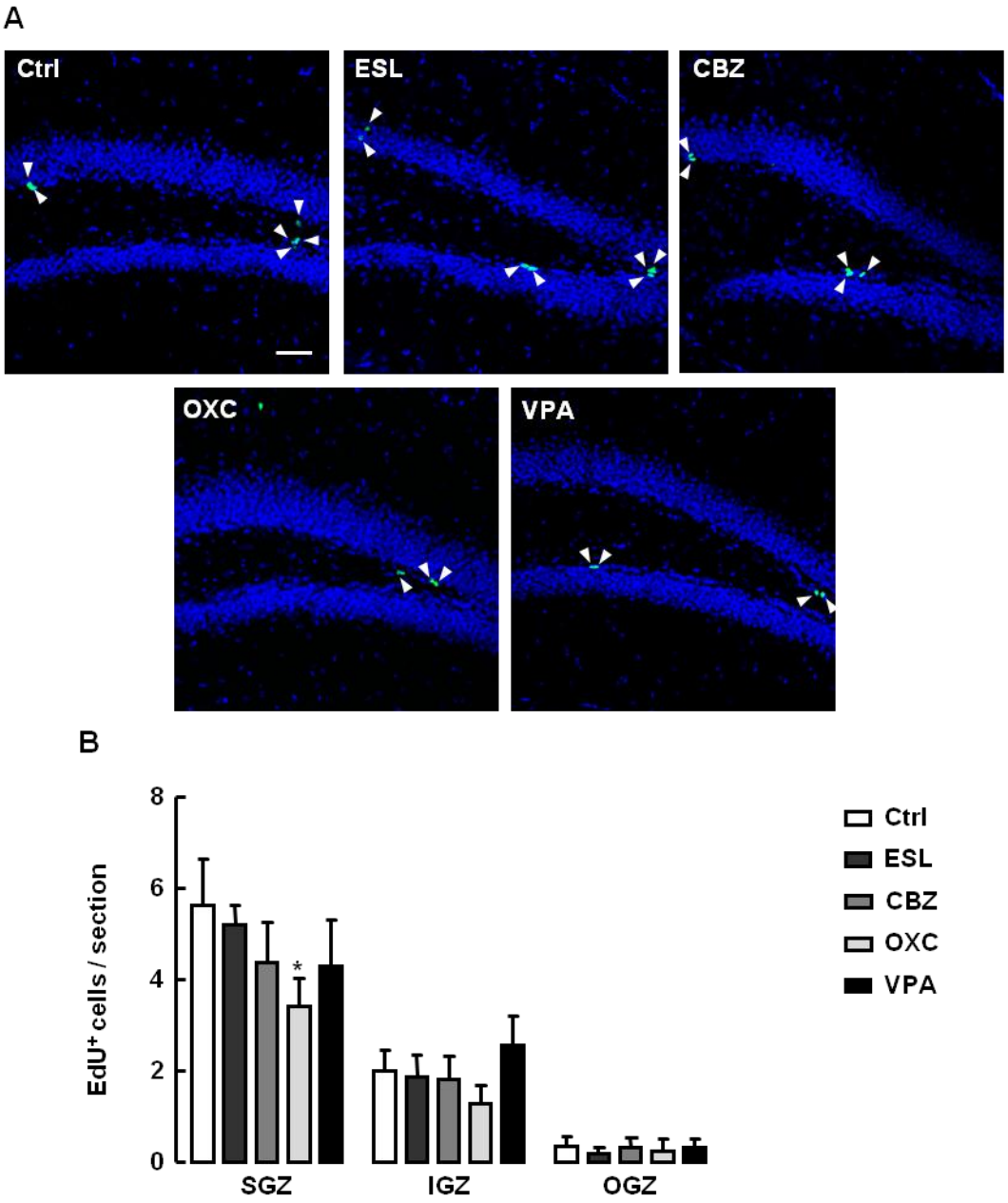

Figure 6.1. Effects of long-term exposure to AEDs on basal proliferation in the dentate gyrus of CD1 adult males. EdU was administered 24 h before animals were sacrificed and its incorporation was assessed by immunohistochemistry. **(A)** Representative images of dentate gyrus of control and of adult males that had been exposed to the AEDs (ESL, CBZ, OXC, or VPA). Arrow-heads indicate EdU positive cells (EdU⁺). Scale bar – 50 µm. Blue – Hoechst; Green- EdU **(B)** Quantification of EdU⁺ cells per five mid-sections of each brain, in controls and in treated animals. Exposure to OXC *in utero* and during nursing decreased the number of EdU⁺ cells by 40% ($p<0.05$) in SGZ, when compared to the respective control. No changes were induced by AED exposure on the number of EdU⁺ cells in IGZ and OGZ. The results are presented as means ± SEM of at least 3-4 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, statistically different from respective Ctrl (by zone/layer). SGZ – subgranular zone, IGZ – inner granular zone, OGZ – outer granular zone.

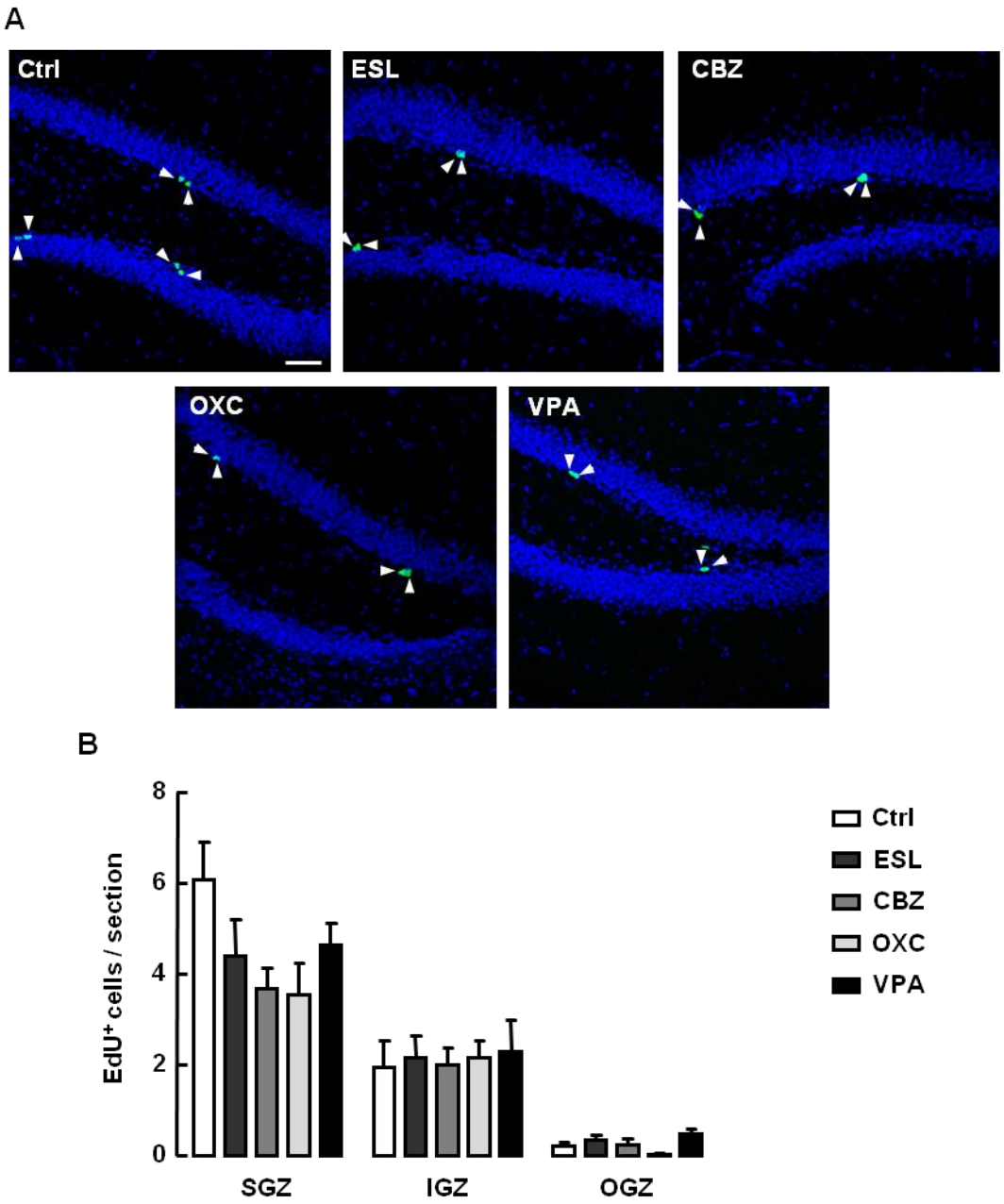

Figure 6.2. Effects of long-term exposure to AEDs on basal proliferation in dentate gyrus of CD1 adult females. EdU was administered 24 h before animals were sacrificed and its incorporation was assessed by immunohistochemistry. **(A)** Representative images of dentate gyrus of control and of treated adult females (ESL, CBZ, OXC, or VPA). Arrow-heads indicate EdU⁺ cells. Scale bar – 50 μm. Blue – Hoechst; Green- EdU; **(B)** Quantification of EdU⁺ per 5 five mid-sections of each brain, in control and in treated animals. The results are presented as means ± SEM of at least 3-4 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test. SGZ – subgranular zone, IGZ – inner granular zone, OGZ – outer granular zone.

6.3.2. Effect of long-term exposure to AEDs during gestation and nursing on the neurogenesis in the hippocampus of adult CD1 mice

We assessed BrdU incorporation by immunohistochemistry, as described before. We also identified and quantified cells that co-localize BrdU (BrdU-positive cells, $BrdU^+$) and doublecortin (DCX, $BrdU^+/DCX^+$), BrdU and neuronal nuclei (NeuN, $BrdU^+/NeuN^+$), and BrdU, DCX and NeuN ($BrdU^+/DCX^+/NeuN^+$), throughout the three zones of the DG: SGZ, IGZ and OGZ. Cells that co-localized BrdU with the other cell markers were expressed by percentage of fifty BrdU-positive cells. It is assumed that cells that express these neuronal markers and BrdU are new mature neurons that have differentiated in the adult brain (reviewed by von Bohlen und Halbach 2011). In this part of the work we evaluated how exposure to AEDs *in utero* and during nursing may affect neurogenesis in the hippocampus of adult CD1 mice born to treated females. The results obtained are described below.

In adult CD1 males we observed that ESL or CBZ exposure did not change the number of newly born cells in the SGZ, as evaluated by counting the number of $BrdU^+$ cells, while this number was decreased by OXC and VPA exposure (3.93±0.45 and 3.40±0.42 $BrdU^+$, respectively, $p<0.01$), as compared to controls (8.45±0.22 $BrdU^+$) (Fig. 6.3). Moreover, VPA decreased by 90% ($p<0.05$) the number of newly born cells in the OGZ, as compared to the control. Except VPA, no changes were found in IGZ and OGZ of treated animals when compared to the controls (IGZ: 3.25±0.57 and OGZ: 0.55±0.22 $BrdU^+$), as shown in the representative images of each experimental group (Fig. 6.3.A) and in the quantification graph (Fig. 6.3B). Regarding CD1 females, we observed that the number of newly born cells in SGZ was decreased by OXC exposure (3.84±0.45 $BrdU^+$, $p<0.01$, when compared to the control (7.10±0.89 $BrdU^+$), while in the IGZ and OGZ no changes in the number of newly born cells were detected, as compared to the controls (IGZ: 4.30±0.87 and OGZ: 0.45±0.22 $BrdU^+$) (Fig. 6.4.A-B). However, none of the other AEDs changed the number of cells that incorporated BrdU in the DG of CD1 females, when compared to controls, as shown in the representative images and in the quantification graph (Fig. 6.4.A-B).

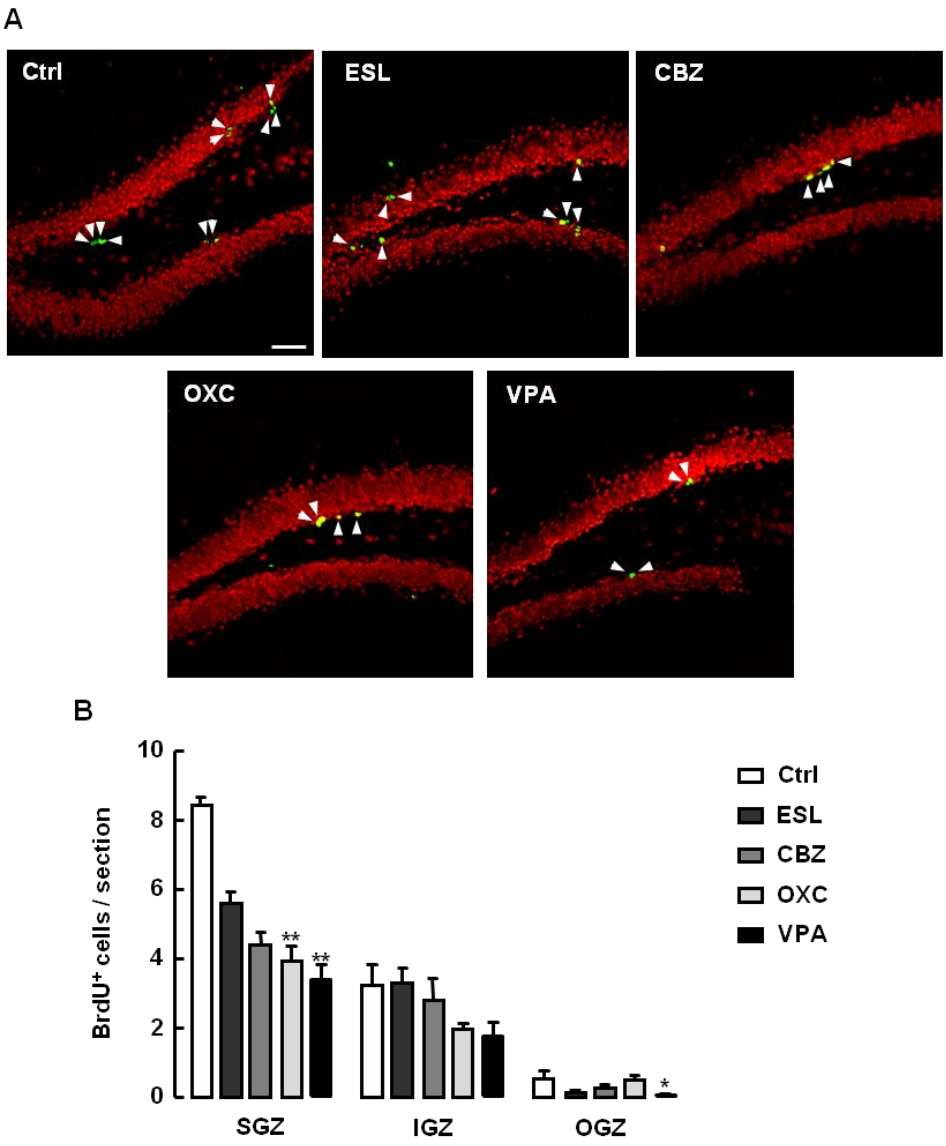

Figure 6.3. Effects of long-term exposure to AEDs on BrdU incorporation in dentate gyrus of CD1 adult males. BrdU was administered one month before animals were sacrificed and its incorporation in the dentate gyrus was assessed by immunohistochemistry. **(A)** Representative images of the dentate gyrus of control and treated adult males (ESL, CBZ, OXC, and VPA). Arrow-heads indicate BrdU⁺ cells in the three zones of DG: SGZ, IGZ and OGZ. Scale bar – 50 µm. Red – NeuN; Green- BrdU; **(B)** Quantification of BrdU⁺ cells per five mid-sections of the hippocampus in each brain. OXC decreased the number of BrdU⁺ cells in SGZ and VPA decreased the number of BrdU⁺ cells in both SGZ and OGZ. The results are presented as means ± SEM of at least 3-4 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$ and **-$p<0.01$, significantly different from respective Ctrl (per zone).

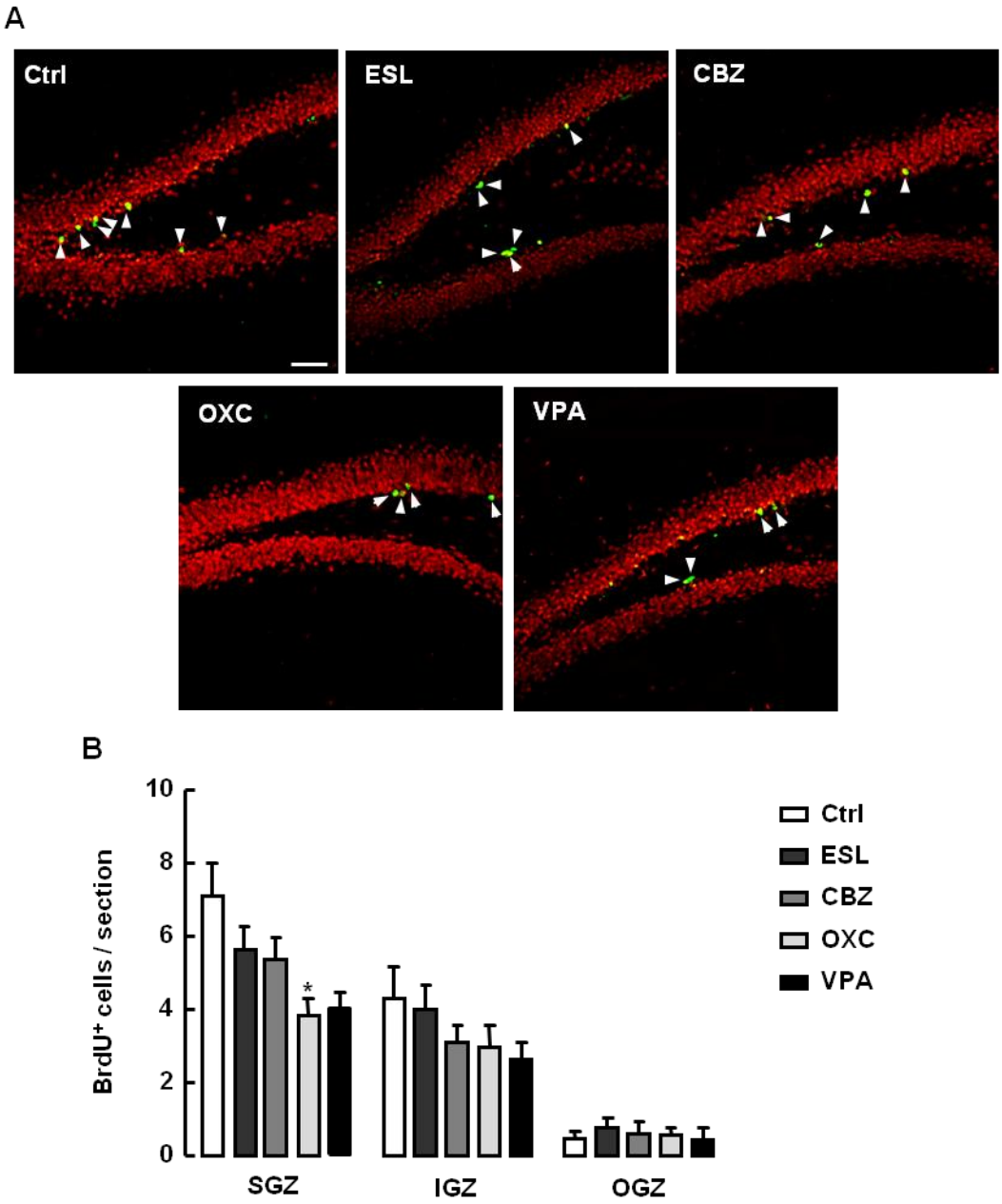

Figure 6.4. Effects of long-term exposure to AEDs on basal proliferation in the dentate gyrus of CD1 adult females. BrdU was administered one month before animals were sacrificed and its incorporation in the dentate gyrus was assessed by immunohistochemistry. **(A)** Representative images of dentate gyrus of control and treated adult females (ESL, CBZ, OXC, and VPA). Arrow-heads indicate $BrdU^+$ cells in the zones of DG: SGZ, IGZ and OGZ. Scale bar – 50 µm. Red – NeuN; Green- BrdU **(B)** Quantification of $BrdU^+$ per 5 mid-sections of the hippocampus in each brain. OXC decreased the number of newborn cells on SGZ but not in IGZ and OGZ. The results are presented as means ± SEM of at least 3-4 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, statistically different from respective Ctrl (per zone).

Newly born cells that incorporated BrdU may differentiate into neurons or keep as undifferentiated granular cells in the dentate gyrus. Once differentiated, these cells express specific neuron proteins such as doublecortin (DCX) and/or neuronal nuclei (NeuN), which are immunohistological markers of immature and mature neurons, respectively. We analysed fifty BrdU-positive cells throughout the DG per brain, in order to determine the number of these cells that are also DCX-positive or NeuN-positive or both. Newly born cells were classified as follows: cells that become differentiated into immature neurons ($BrdU^+/DCX^+$), and cells that become differentiated into mature neurons ($BrdU^+/DCX^+/NeuN^+$ and $BrdU^+/NeuN^+$) (Fig. 6.5).

In males we observed that CBZ exposure increased by 16% ($p<0.05$) the number of mature neurons ($BrdU^+/NeuN^+$) in the SZG of the DG, as compared to control (55.33±4.37%) (Fig.6.6.C), but did not alter the number of mature neurons in the other zones of the DG, as compared to controls (IGZ: 28.00±2.31% and OGZ: 12.67±1.76%). Exposure to the other AEDs (ESL, OXC and VPA) did not cause changes in the number of mature neurons, as well as in the other phenotypes as compared to controls, $BrdU^+/DCX^+$ (SGZ: 26.00±1.15%; IGZ: 14.00±3.46%; OGZ: 4.00±2.31%), and $BrdU^+/DCX^+/NeuN^+$ (SGZ: 24.67±1.76%; IGZ: 12.67±3.33; OGZ: 4.00±2.31%) (Fig. 6.6.A-B).

Regarding females, we observed that all $BrdU^+/DCX^+$ cells are $NeuN^+$, and that OXC exposure decreased the number of immature neurons by 62% ($p<0.05$) and mature neurons by 20% ($p<0.04$) in the SGZ, as compared to respective controls (24.67±4.81% and 56.00±6.00%, respectively) (Fig. 6.7.A-C). Neither IGZ nor OGZ presented any effects caused by OXC exposure. Moreover, none of the other AEDs changed the number of mature and immature neurons throughout DG (Fig. 6.7).

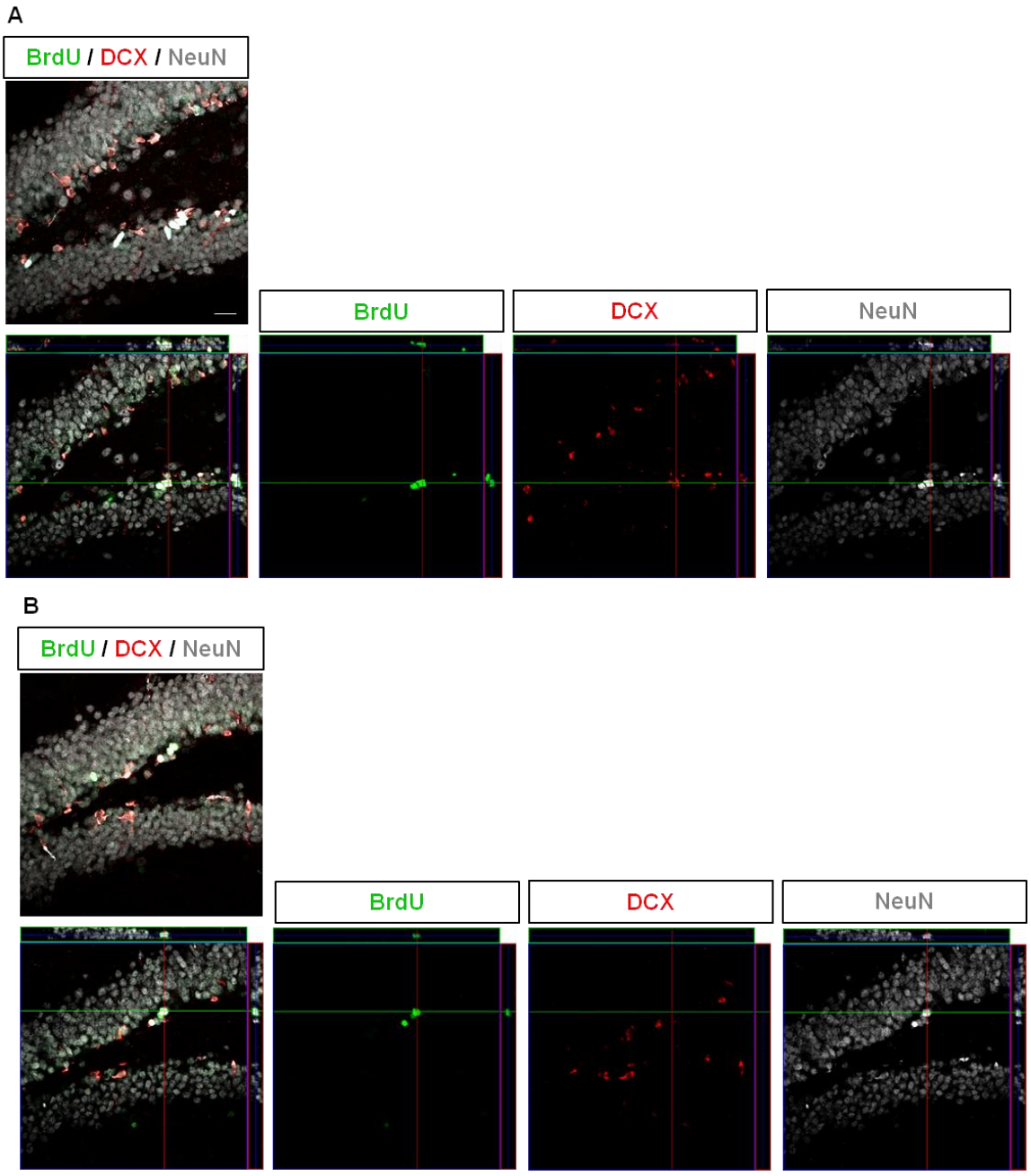

Figure 6.5. Colocalization of BrdU in DCX-positive cells or in NeuN-positive cells throughout the layers of the DG of the hippocampus of after long-term AED exposure. **(A)** $BrdU^+$ (green) colocalize with DCX^+ (red), and $NeuN^+$ (white) in the SGZ of the DG; **(B)** $BrdU^+$ (green) colocalize with $NeuN^+$ (white) but not with DCX (red) in the SGZ of the DG.. Representative images of DG of control CD1 mice showing mature and immature neurons. Scale bar: 20 µm. Grey – NeuN,Red – DCX; Green-BrdU.

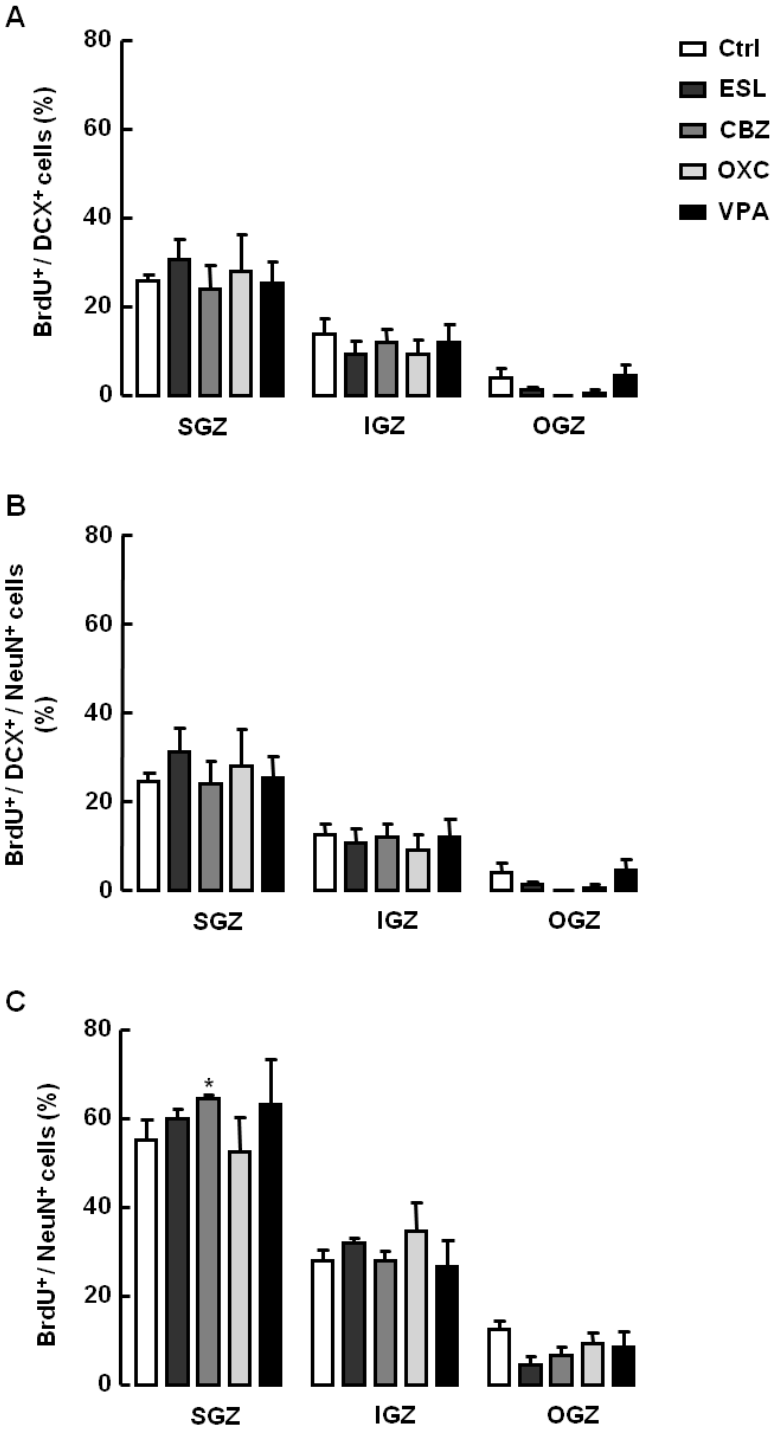

Figure 6.6. Effects of long-term exposure to AEDs on the distribution of immature and mature neurons in the DG of adult brain of CD1 males. **(A)** Quantification of $BrdU^+/DCX^+$ cells, **(B)** $BrdU^+/DCX^+$ /$NeuN^+$ cells, and **(C)** $BrdU^+$/ $NeuN^+$ cells per fifty $BrdU^+$ cells in each experimental group. The results are presented as means ± SEM of at least 3 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$ statistically different from respective control (by zone).

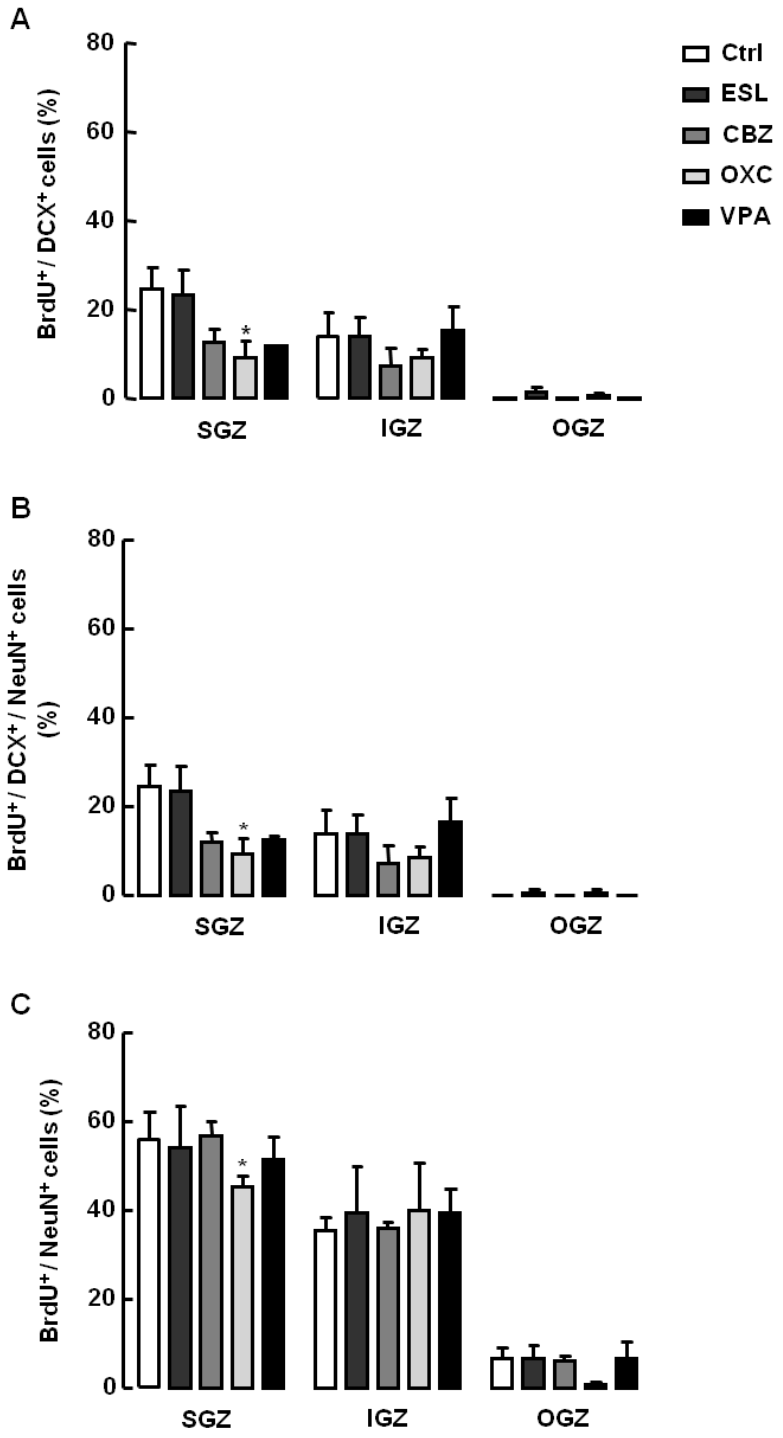

Figure 6.7. Effects of long-term exposure to AEDs on the distribution of immature and mature neurons in the DG of adult brain of CD1 females. **(A)** Quantification of $BrdU^+/DCX^+$ cells, **(B)** $BrdU^+/DCX^+$ /$NeuN^+$ cells and **(C)** $BrdU^+$/ $NeuN^+$,cells per fifty $BrdU^+$ cells in each experimental group. The results are presented as means ± SEM of at least 3 animals. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, statistically different from Ctrl.

6.3.3. Effects of AEDs in cell proliferation and cell cycle distribution of neural stem cell cultures from SVZ

In this chapter we also report data on the effects of the novel AED eslicarbazepine acetate (ESL) and of its metabolites, S-Lic and R-Lic, and of CBZ, OXC, LTG and VPA on the proliferation and cell cycle analysis of cultured neural stem cells isolated from the rat subventricular zone. The main objective was to determine whether the effects obtained *in vivo* and reported in the previous section somehow are also observed *in vitro*.

Cell proliferation was determined by the incorporation of EdU (10 μM) in SVZ cultures after 24 h exposure to a range of concentrations of each AED (0.01, 0.03, 0.1 and 0.3 for ESL, S-Lic, R-Lic, CBZ, OXC and LTG, and 0.1, 0.3, 1 and 3 mM for VPA). Cell cycle analysis consisted of measuring DNA content of the aforementioned cell cultures by quantifying the fluorescence of 7-aminoactinomycin D (7-AAD), which has a high affinity for DNA. EdU incorporation and the DNA content were analyzed by flow cytometry and then quantified in the histogram and respective contour plots (for detailed information see chapter 2, section 2.1.8 and appendix II) (Fig. 6.8. A-B).

In control conditions, the basal cell proliferation, determined from EdU incorporation, was 12.34±0.76% EdU-positive cells (Fig. 6.9). Regarding the effects of AEDs, cell proliferation was significantly decreased by 50% ($p<0.01$) for the highest concentration of ESL tested (0.03 mM) but was not affected for the lower concentrations of ESL (Fig. 6.9.A); S-Lic (up to 0.3 mM) did not affect basal cell proliferation (Fig. 6.9.B); R-Lic (0.01 mM) decreased cell proliferation by 40% ($p<0.05$) (Fig. 6.9.C); CBZ decreased cell proliferation by 50% ($p<0.05$) or by 70% ($p<0.01$) at 0.01-0.1 mM or at 0.3 mM, respectively (Fig. 6.9.D); OXC decreased cell proliferation by 60% ($p<0.05$) or by 75% ($p<0.001$) at 0.03 or 0.3 mM, respectively (Fig. 6.9.E); LTG (0.3 mM) decreased cell proliferation by 40% ($p<0.05$)(Fig. 6.9.F); and VPA (1 or 3 mM) decreased cell proliferation by 85% ($p<0.01$) or by 95% ($p<0.001$) respectively) (Fig. 6.9.G).

We also performed cell cycle analysis in order to detect possible alterations in the percentage of cells in each of the cell cycle phases caused by exposure to the AEDs. We observed that CBZ (0.03 and 0.3 mM) increased by 20%, ($p<0.05$) (Fig. 6.9D); LTG (0.01 mM) by 15% ($p<0.05$) (Fig. 6.9F); and VPA (1 mM) by 20% ($p<0.01$) (Fig. 6.9G), the

percentage of cells in G0/G1 phase, as compared to the control (73.00±4.79% G0/G1 cells). The percentage of cells in S phase was unchanged following exposure to the AEDs, as compared to control (4.19±0.74% S cells) (Fig, 6.9A-G). Furthermore, the percentage of cells in G2/M phase in basal conditions (7.14±0.63% cell) was decreased by 45% by ESL (0.1 mM, $p<0.05$) (Fig. 6.10A) and was decreased by 75 or 70% by VPA (1 mM, $p<0.05$ or 3 mM, $p<0.001$, respectively) (Fig. 6.10G) while OXC (0.3 mM) increased by 65% ($p<0.01$) the percentage of cells in G2/M phase, as compared to basal conditions (Fig. 6.10E). In summary, we found that the active metabolite of ESL, S-Lic did not induce any change on the basal levels of proliferation in cultured neural stem cells, and did not alter cell cycle distribution, while the other AEDs, at different concentrations, change the proliferation of neural stem cells and alter cell cycle distribution.

We also evaluated the possible changes caused by AED exposure on cell death by apoptosis in SVZ cultures, and this effect was assessed by flow cytometry. This data would help in understanding whether the changes observed in neural stem cell proliferation and cell cycle phases may be due to potential cytotoxicity of AEDs. We found that neither ESL, nor its metabolites (S-Lic and R-Lic), CBZ or LTG induced increased cell death, while exposure to VPA (3 mM) increased the percentage of apoptotic cells (14.10±3.10%, $p<0.05$) as compared to control cultures (4.27±1.32% of apoptotic cells) (Fig. 6.11A-G). OXC (0.3 mM) also tend to increasethe percentage of apoptotic cells in the SVZ cultures (7.55±2.52% of apoptotic cells), although this effect was not statistically significant.

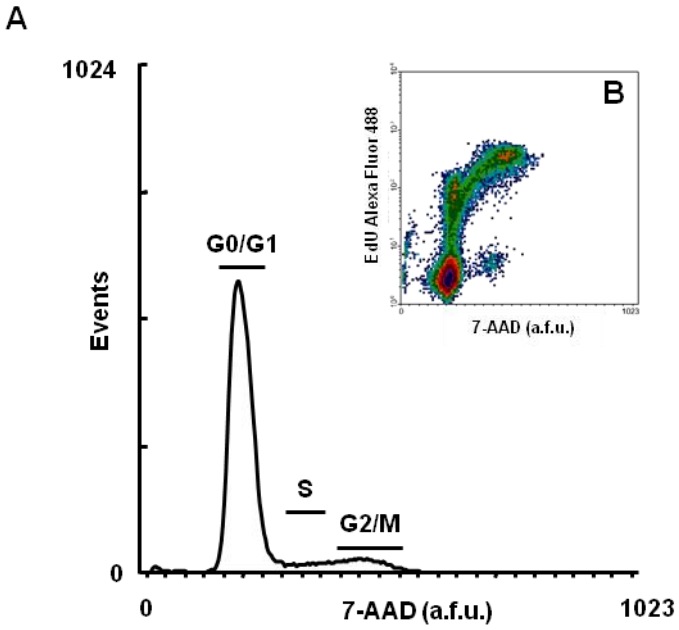

Figure 6.8. Evaluation of cultured SVZ cell proliferation and cell cycle analysis by flow cytometry.

(A) DNA histogram of SVZ cultured cells (control) and cell cycle phases identification as follows: G0/G1 (growth phase, 2N); S (DNA synthesis, 2N); and G2/M (DNA repairing and cell preparation for the coming mitosis). **(B)** Contour plot representing EdU and 7-AAD fluorescence of DNA content of an SVZ control culture. The cells were exposed to EdU for 4 h before being harvested, followed by fixation with 70% ethanol and then stained with Alexa Fluor 488® azide and 7-AAD. The cell cycle phases were clearly identified in the DNA histograms and respective contour plots a.f.u. – arbitrary fluorescence units.

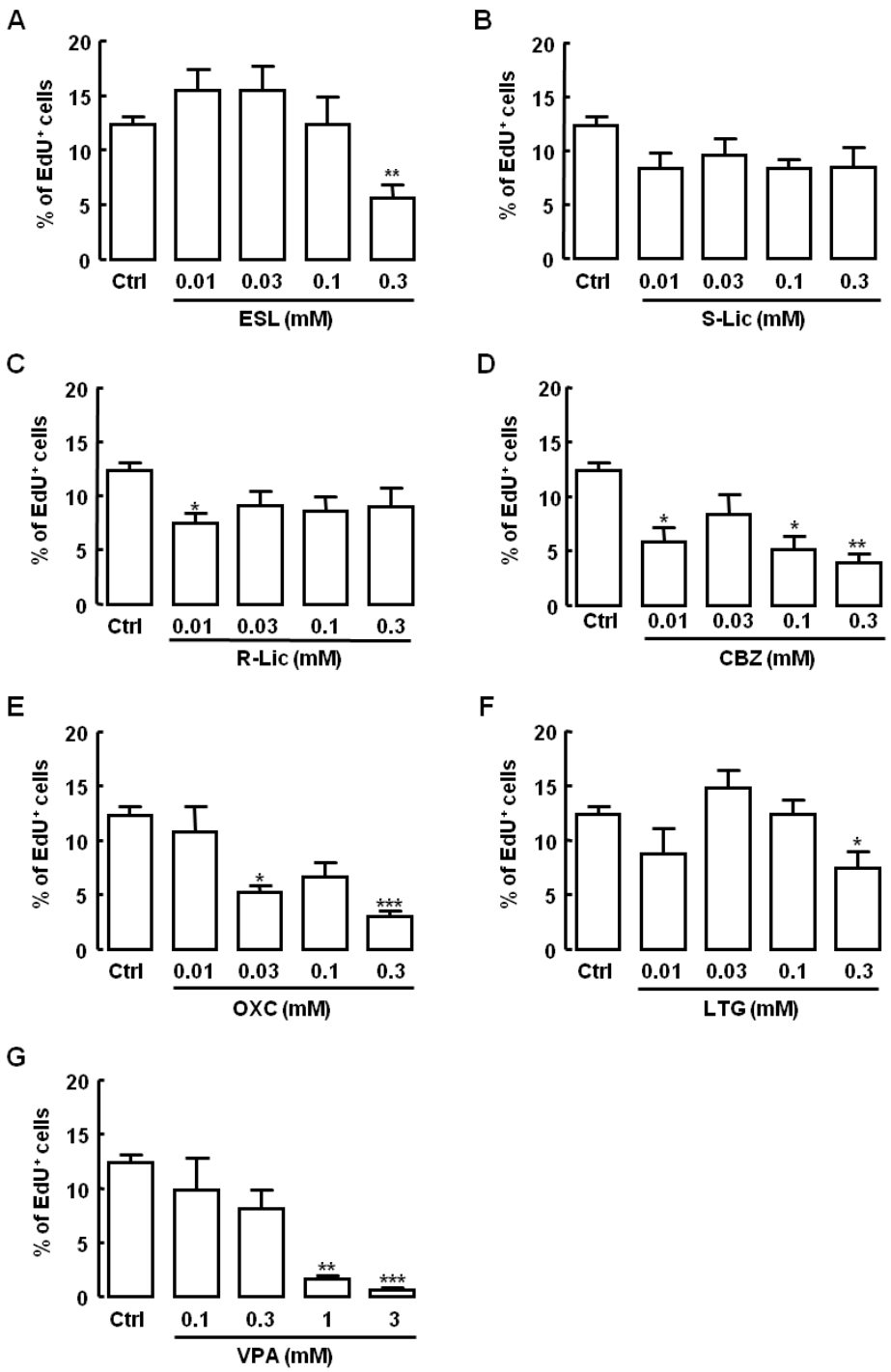

Figure 6.9. Effects of AED exposure on EdU incorporation in neural stem cells cultures of rat SVZ

(A) ESL, **(B)** S-Lic, **(C)** R-Lic, **(D)** CBZ, **(E)** OXC, **(F)**, LTG, **(G)** VPA. EdU incorporation was assessed by flow cytometry analysis after SVZ cultures were exposed for 24 h to each AED in the range of concentrations indicated in the graphs. Data are expressed as means ± SEM of at least 3 independent experiments. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, statistically different from Ctrl.

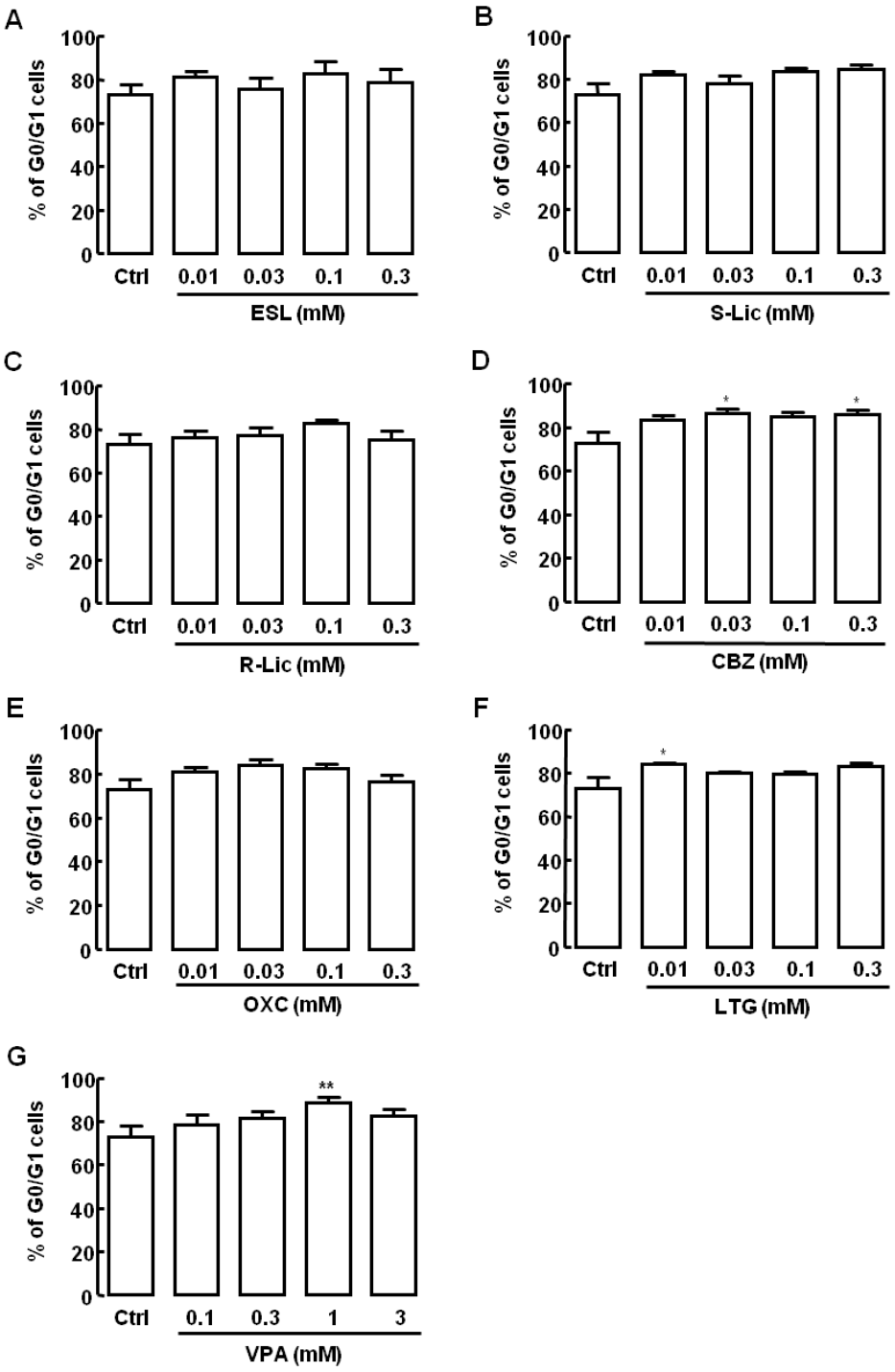

Figure 6.10. Effects of AED exposure on the percentage of cells in G0/G1 phase in neural stem cell cultures of rat SVZ.

(A-G) AEDs as described in the legend of figure 6.9. DNA content was assessed with 7-AAD by flow cytometry analysis after SVZ cultures were exposed to a range of concentrations of each AED as described in the graphs. Data are expressed as means ± SEM of at least 3 independent experiments. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test;
*-$p<0.05$, **-$p<0.01$, statistically different from Ctrl.

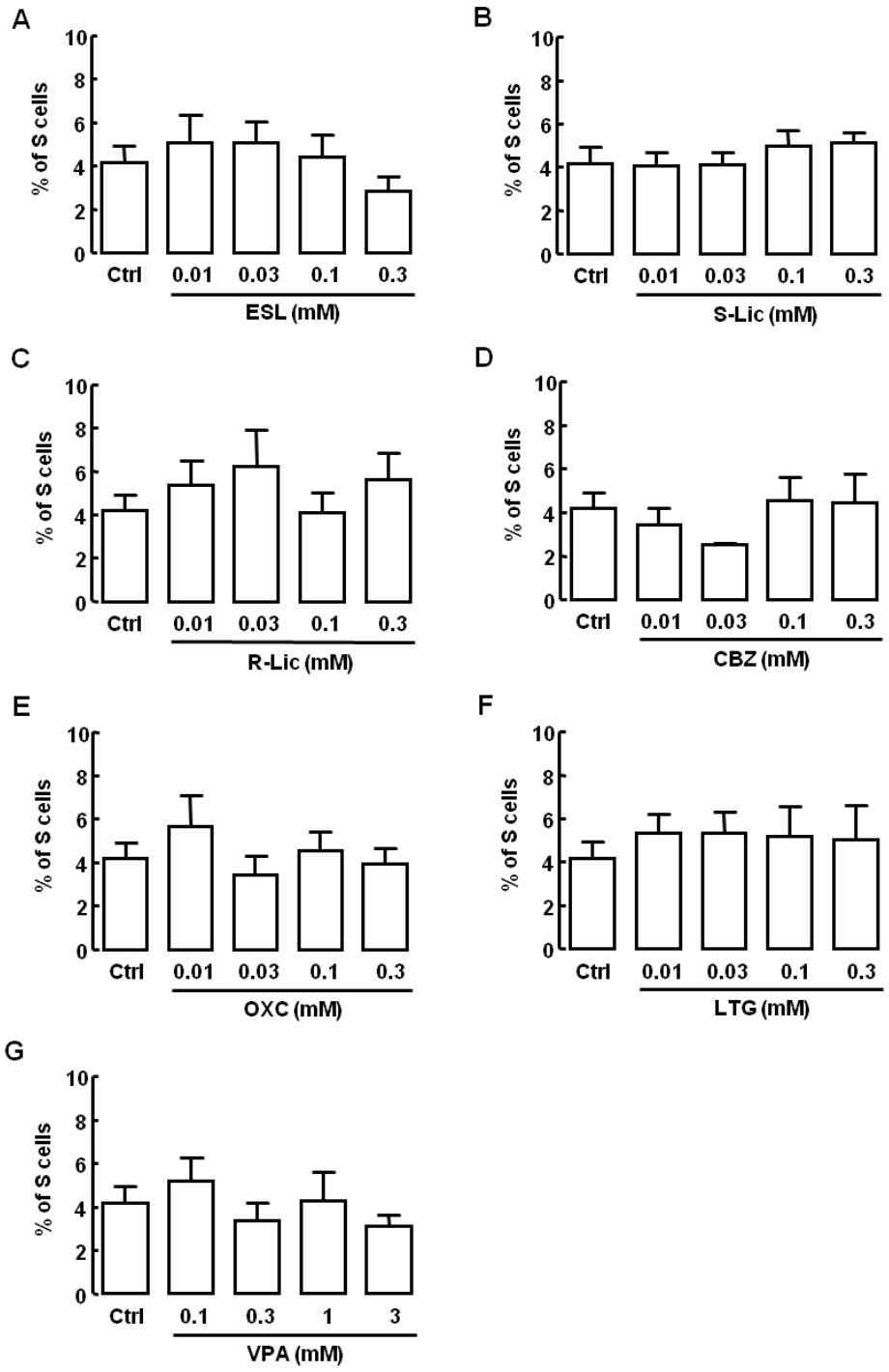

Figure 6.11. Effects of AED exposure on the percentage of cells in S phase of cell cycle in neural stem cell cultures of rat SVZ.

(A-G) AEDs as described the legend of figure 6.9. DNA content was assessed with 7-AAD by flow cytometry analysis after SVZ cultures were exposed to a range of concentrations of each AED as described in the graphs. Data are expressed as means ± SEM of at least 3 independent experiments. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test.

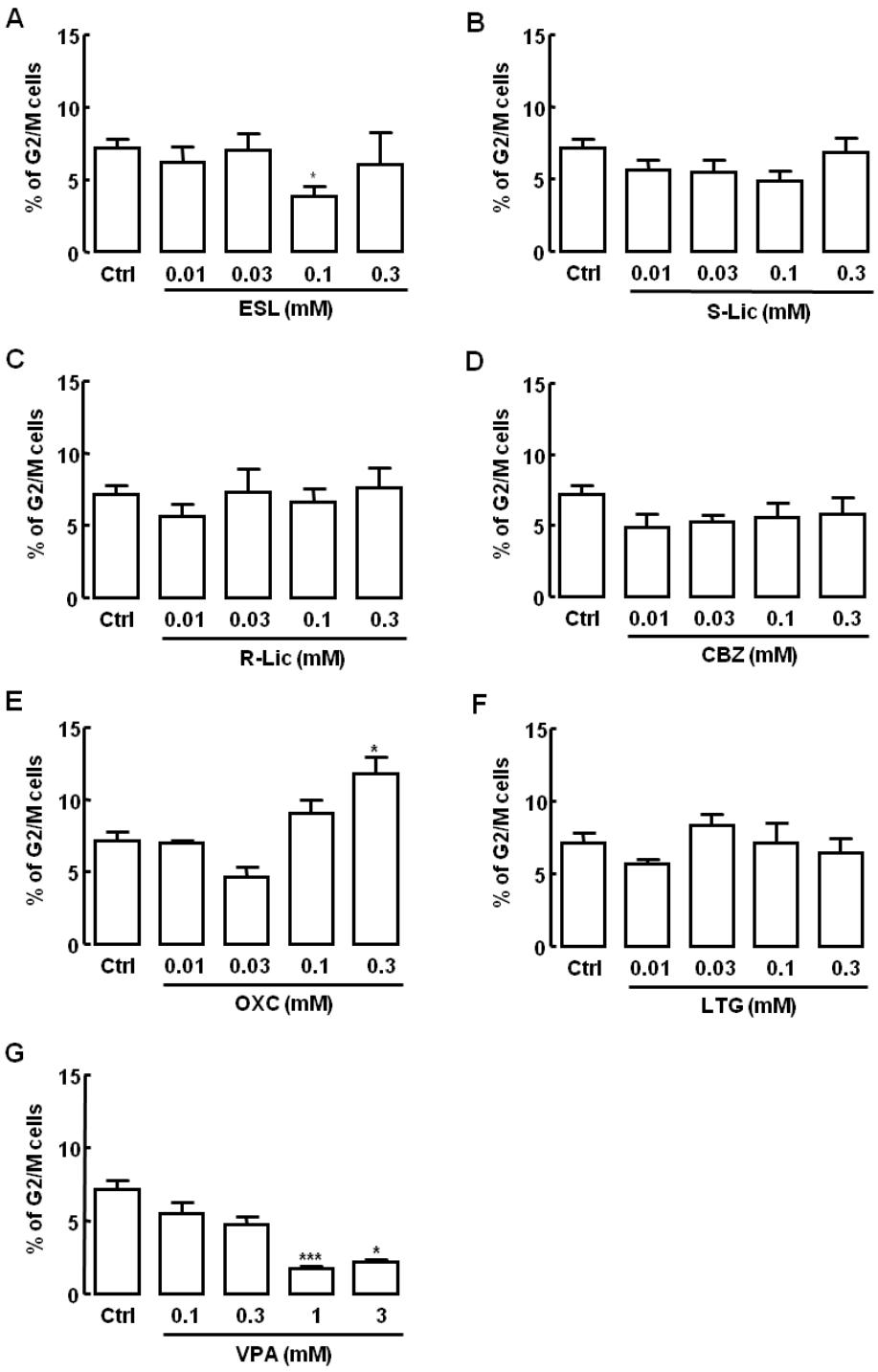

Figure 6.12. Effects of AED exposure on the percentage of cells in G2/M phase in neural stem cell cultures of rat SVZ.

(A-G) AEDs as described in the legend of figure 6.9. DNA content was assessed with 7-AAD by flow cytometry analysis after SVZ cultures were exposed to a range of concentrations of each AED as described in the graphs. Data are expressed as means ± SEM of at least 3 independent experiments. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test;
*-$p<0.05$, ***-$p<0.001$, statistically different from Ctrl.

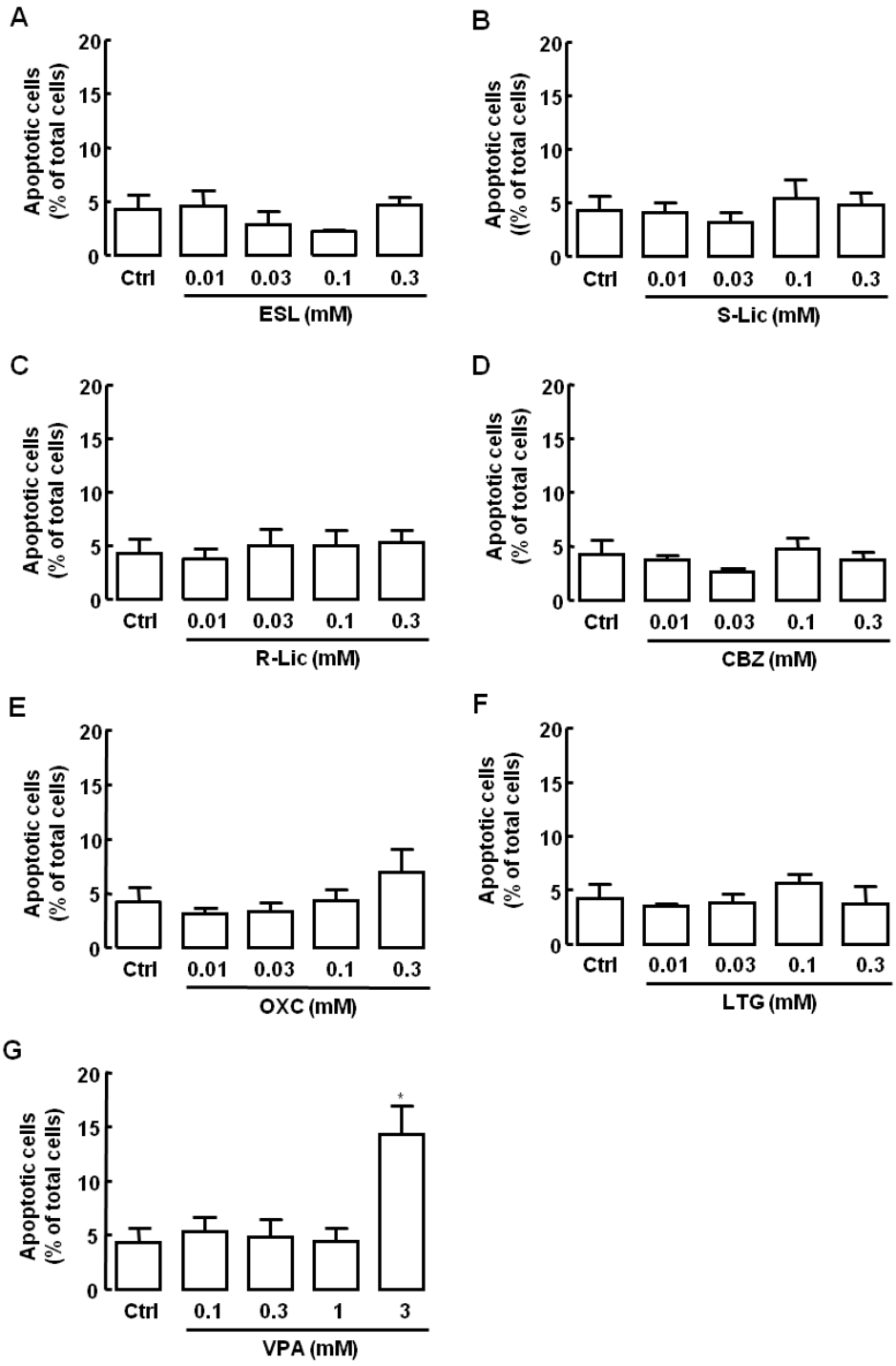

Figure 6.13. Effects of AEDs on cell death in neural stem cell cultures from rat SVZ (A-G) AEDs as described in the legend of figure 6.9. Cell death was assessed by flow cytometry analysis after SVZ cultures were exposed for 24 h to a range of concentrations of each AED as described in the graphs. Data are expressed as means ± SEM of at least 3 independent experiments. Kruskal-Wallis test (one-way analysis of variance by rank), followed by Dunn's post hoc test; *-$p<0.05$, statistically different from Ctrl.

6.4 Discussion

Hippocampus is one of the most vulnerable structures of a mammalian brain during early development (Ikonomidou and Turski 2010). This neurogenic area is susceptible to changes in the surrounding environment either in the prenatal or in the postnatal periods, since it is regulated by several extrinsic and intrinsic factors. These factors allow that neurogenesis occurs in a balanced manner, which is vital for safe neuronal subpopulations that are raised by cell proliferation in the adult brain. If newly born neurons are disturbed during the stage of formation/maturation, they won't be functional (reviewed by Kaindl et al. 2006). Among the intrinsic factors, GABA, glutamate, proteins coupled to ion channels and neurotrophic factors may in turn be modulated by AEDs since they are targets of their mechanisms of action. Thus, alterations on the aforementioned factors by AEDs may imbalance neurogenesis and may cause impairment of hippocampus-dependent functions, such as cognitive behaviour (Marsh et al. 2006; Ikonomidou and Turski 2010). Therefore, it is important to understand how long-term AED exposure *in utero* and during nursing, may affect basal neural stem cell proliferation and neurogenesis in the dentate gyrus of the hippocampus in the adult brain.

It is known that neurogenesis in males and females are differently affected by different steroid hormones that are characteristic of both genders (Galea 2008). In the present study we therefore evaluated cell proliferation and neurogenesis in the dentate gyrus of both males and females, at the age of four months, after having performed the behaviour tests (reported in Chapter 5). In the *in vivo* studies we observed that OXC exposure during gestation and nursing decreased the number of EdU-positive cells in the SGZ of the dentate gyrus of the hippocampus in CD1 male mice, while in CD1 females the effect of OXC on cell proliferation, evaluated by EdU incorporation, was not statistically significant. In general, we observed that AED exposure tended to decrease the number of EdU-positive cells compared to control, however we concluded that ESL, CBZ or VPA exposure *in utero* and nursing did not change basal proliferation through DG of both males and females. To our knowledge, this study reports for the first time effects of ESL and OXC in the basal cell proliferation. On the other hand, the lack of effects on SGZ proliferation after long-period exposure to CBZ or VPA are in agreement with prior findings, which reported that CBZ and VPA did not induce changes in cell proliferation in rats (Chen et al. 2009).

Regarding BrdU incorporation throughout the DG, we observed that AED exposure during early-life affected the formation of newly born cells in the adult brain. Both CD1 males and females exposed to OXC, had less newly born cells in the SGZ than controls,, while males exposed to VPA had less newly born cells than controls both in SGZ and OGZ. Although we observed that animals that were exposed to ESL and CBZ also had a decrease in the number of cells that incorporated BrdU, these differences were not significant different from the respective controls. With these findings, it became evident that AED exposure *in utero* and during nursing somehow changes basal proliferation and formation of newly born cells in the DG of adult hippocampus. Although the scope of this work did not focus on the mechanisms underlying the changes in cell proliferation in the hippocampus due to AED exposure, it is important to further elucidate which pathways are affected by AED exposure in early-life and how these changes cause long-term effects in events occurring later on, such as proliferation and formation of newly born cells in the dentate gyrus of adult brain.

We also studied effects of AED exposure on the formation of new neurons. In the dentate gyrus, newly born cells that survive may undergo maturation and become neurons. During this process, these newly granule neurons express specific markers according to its maturation stadium. We quantified BrdU-positive cells that also express doublecortin (DCX) and neuronal marker (NeuN). In CD1 males we have observed that OXC and VPA exposure decreased EdU and BrdU incorporation in the SGZ, however no effects were induced by both AEDs in the number of new neurons. On the other hand, we observed that CBZ exposure increases the number of mature neurons, but has no effect on the number of immature neurons in the SGZ. Regarding CD1 females, we observed that OXC exposure decreased the number of newly born cells. Additionally, OXC also decreased the number of both mature and immature neurons, in the SGZ. Regarding CBZ or VPA treated CD1 females, we did not observe statistically significant effects on the percentage of immature neurons. Moreover, we did not observe changes in the number of neurons through IGZ and OGZ in both CD1 males and females. Our results with VPA exposure are in agreement with those of Chen and co-workers, who also observed that VPA did not induce changes in neurogenesis (Chen et al. 2009).

Our main findings were that ESL exposure *in utero* and during nursing did not affect basal neural stem cell proliferation or neurogenesis, while OXC exposure affected proliferation (males) and neurogenesis (females). We also observed a decrease in the number of both mature and immature neurons after OXC exposure in females which may be

explained by the decrease of newly born cells in the SGZ of adult hippocampus. It is not clear whether OXC affects neurogenesis or if these effects are due to its toxic properties. Although OXC shares with ESL and CBZ the main mechanism of action, neither ESL nor CBZ exposure induced effects on neurogenesis in the DG as OXC did. OXC was shown as the most toxic drug in cultured hippocampal neurons (Ambrosio et al. 2000). Furthermore, we also observed in the study described in chapter 3, that OXC was the most toxic AED, which decreased cell viability by leading to activation of caspase-3 and PARP cleavage in primary cultures of rat hippocampal neurons. Thus, it is plausible that decreased basal proliferation and neurogenesis may be due to a toxic effect induced by OXC. Indeed, some authors have associated AEDs with a widespread spur of apoptosis (Bittigau et al. 2003; Olney et al. 2004; Ikonomidou 2010). However, there is still lack of information regarding the effects of OXC, and an extra effort should be done to elucidate the molecular mechanisms underlying these effects on neurogenesis or on cell viability. Furthermore, CBZ exposure induced an increase in the number of mature neurons compared to control. CBZ had a positive effect on the maturation of new neurons, however again we need further information on survival and network activity of these new neurons, in order to understand how "normal" and functional they are, as well as which mechanisms are being affected by CBZ.

Regarding *in vitro* experiments, except for S-Lic, we observed that all AEDs decreased basal proliferation of SVZ-derived neural stem cell cultures at distinct concentrations. It is known that VPA is a histone deacetylase inhibitor (HDACi), thus VPA suppress HDAC activity, which in turn affects cell growth and proliferation, among other pathways (reviewed by Chateauvieux et al. 2010). Several studies have shown the anti-proliferative effect of VPA *in vitro* (Hsieh et al. 2004; Jung et al. 2008; Yu et al. 2009; Gotfryd et al. 2011). Our results are in line with the literature, since we observed a huge decreased in the cell proliferation due to exposure to VPA (1 and 3 mM). Besides VPA, CBZ, OXC and LTG are also HDACi (Beutler et al. 2005; Stettner et al. 2012). Thus, as in case of VPA, the effects of the other AEDs on proliferation may be due to their HDACi properties. To our knowledge, the present data is the first regarding the effects of ESL and its metabolites on the proliferation of SVZ cultures. Both ESL and R-Lic decreased proliferation at different concentrations, but the mechanisms underlying these effects are unknown at present.

In addition, in the experiments of this chapter we also analyzed cell cycle phases. EdU is incorporated during DNA division, which occurs in the course of S-phase. Differences in EdU incorporation may be explained by changes in S-phase, which in turn may be explained by alterations in both, G0/G1 and G2/M phases. However, this chain of actions is not linear in non-synchronized cell cultures as SVZ cultures, whose cells are at different phases of the cell-cycle. We observed that CBZ, LTG and VPA, at concentrations mentioned above, increased the number of cells in G0/G1, which means that these cells were arrested at this stage and did not enter into S-phase. Arrest in G0/G1 means that: a) cells may stop at this stage and lose cell-division features (e.g. cells that become differentiated, such as neurons), or b) enter a resting state (G0) when there are no environmental conditions to proceed into cell division. Our findings may be explained by the HDACi properties of some AEDs and consequent anti-proliferative effects of LTG, CBZ and VPA. However, further analysis is needed to understand the arrested cell´s fate, for instance to determine whether those cells have become differentiated or have adopted "resting state". As we have described before, we did not observe changes in the number of cells in S-phase, which means that our population of EdU-positive cells were EdU-positive "daughter" cells in G2/M or G0/G1 stages. Moreover, we observed that ESL and VPA, at concentrations mentioned above, decreased cells into G2/M, while OXC had the opposite effect. Regarding ESL, without supplementary analysis we can not speculate what underlying mechanism explains its effect.

We also measured apoptotic DNA in all the conditions studied. DNA content of apoptotic cells contains less DNA than a "healthy" cell, thus it is possible to quantify the number of apoptotic cells in the DNA histograms (Riccardi and Nicoletti 2006). We observed that exposure to VPA (3 mM) of neural stem cell cultures from SVZ had the biggest effect in inducing cell death by apoptosis. Indeed, VPA strongly increased the percentage of apoptotic cells, which may also explain the decrease of proliferation and the decrease of cells in G2/M phase. Although OXC (0.3 mM) did not significantly increase the number of apoptotic cells we observed a trend towards it. Moreover, cell arrest in G2/M may result from cells with DNA damage, which are not able to ensure mitosis. Since those cells were arrested they are eventually destroyed in order to prevent damaged daughter cells (reviewed by Sancar et al. 2004). Thus, OXC may damage cells to a lower extent than VPA and cells stay arrested in G2/M for a longer time until they are eliminated. It is plausible to propose that OXC is toxic to neural stem cell cultures from SVZ based on our results.

However, before concluding clearly that OXC is neurotoxic to SVZ cultures, further studies are needed.

In conclusion, we observed that ESL has potential advantages over other AEDs in AED therapy. In our study we showed that ESL exposure *in utero* and during nursing did not induce changes in proliferation and neurogenesis of SGZ in adult brain of progeny from treated females. Also, its main metabolite, S-Lic, did not change proliferation and distribution of cell-cycle phases of SVZ cultures. In addition, we showed that OXC exposure has a potential neurotoxic profile in adult brain, since decreased proliferation and neurogenesis in vivo. Moreover, OXC decreased proliferation in SVZ cultures and arrested neural stem cells in G2/M stage at high concentration (0.3 mM). However, to confirm its neurotoxic profile, other studies are needed.

Chapter 7

General Discussion

7.1. General Discussion – Major findings

Antiepileptic drug therapy is available for the treatment of epilepsy, among other neurologic disorders. Although AEDs have different mechanisms of action, all of them are widely used to treat epileptic seizures. However, AEDs have several side-effects, including toxicity to central nervous system, drug interaction and teratogenic effects (Loring et al. 2007; Pennell 2008; Johannessen Landmark and Patsalos 2010). Therefore, AEDs should be used cautiously in epileptic patients, with particular attention to special populations, such as pregnant women and children. In recent years, pharmaceutical companies have done an effort to develop new AEDs in order to overcome the adverse effects observed with the older AEDs. Eslicarbazepine acetate (ESL) is one of the third generation AEDs that entered the market in 2009 in Europe (Elger et al. 2009). ESL is a derivative of carbamazepine and acts by blocking voltage-gated sodium channels, and one advantage of ESL over other EDAs is that it does not cause enzyme-induction and autoinduction (Bialer 2006). In humans and mice after oral administration it is rapidly metabolized in the liver to its main metabolite, eslicarbazepine (S-Lic), which is responsible for approximately 95% of total systemic drug concentration. Then, a small percentage of S-Lic subsequently undergoes a minor chiral inversion to R-licarbazepine (R-Lic) through oxidation to oxcarbazepine (OXC). R-licarbazepine (4.5%) and oxcarbazepine (0.5%) are thus minor metabolites of ESL (Perucca et al. 2011). However, although ESL is already very well characterized, the neurotoxicity profile of its metabolites, namely S-Lic and R-Lic in had not been evaluated yet, neither in neural cell cultures (*in vitro* studies), nor in *in vivo* studies designed to test the effects of ESL exposure during prenatally period, gestation and nursing on female mice and its progeny.

The studies described in this thesis aimed at investigating the following main aspects regarding ESL and other AEDs: 1) The effects of ESL and its metabolites on activation of neurotoxicity and neuroprotection pathways in cultured hippocampal neurons, compared to other AEDs (chapter 3); 2) The impact of exposure to antiepileptic drugs during prenatal period, gestation and nursing on CD1 female mice (chapter 4); and 3) The effects of antiepileptic drug exposure *in utero* and during nursing on the behaviour and on neurogenesis in the hippocampus of CD1 mice during development (chapters 5 and 6).

Our main findings regarding these main points are discussed below:

1. The effects of ESL and its metabolites on activation of neurotoxicity and neuroprotection pathways in cultured hippocampal neurons, compared to other AEDs

In chapter 3 we compared the neurotoxicity profile of ESL and its metabolites, S-Lic and R-Lic, to the structurally-related AEDs CBZ and OXC, and to the unrelated AEDs LTG and VPA. We assessed cell death and cell viability parameters, as well as the activation of prosurvival intracellular signalling pathways in primary cultures of hippocampal neurons exposed to the AEDs. We found that ESL and its metabolites, as well as LTG, were not toxic to cultured hippocampal neurons. OXC stands out as the most toxic drug evaluated in this study, which is in agreement with previous studies (Ambrosio et al. 2000; Araujo et al. 2004), particularly for high concentrations. We found that cell death triggered by exposure to OXC in hippocampal neurons is caspase-dependent, since OXC exposure caused an increase in cleaved-caspase 3 and cleaved PARP, which are markers of programmed cell death. Furthermore, we did not observe release of the apoptosis-inducing factor (AIF) from mitochondria to the nuclei of dying hippocampal neurons, which is typical of caspase-independent apoptosis (Susin et al. 1999). VPA also induced the appearance of apoptotic markers in hippocampal cultures but did not decreased cell viability or induced nuclear condensation / fragmentation. In VPA exposed cultures, it is plausible that caspase-3 cleavage occurs earlier than nuclear condensation, and this AED may trigger activation of cell survival pathways. Actually, we observed that VPA increased phosphorylation of ERK1/2 (at 30 min of exposure), Akt (at 60 min of exposure) and SAPK/JNK (at 24 h of exposure), and these effects may trigger neuroprotective mechanisms. These findings are in agreement with other studies, which showed that treatment with VPA activated the aforementioned pathways and this may be responsible for its neuroprotective effects (Chuang 2005; Di Daniel et al. 2005). However, further studies should be performed to clarify these mechanisms. Interestingly, in chapter 6 we observed that VPA induced cell death in neural stem cell cultures from the rat SVZ. It is possible that the differences between the two *in vitro* models used may be responsible for the opposite effects of VPA obtained. In fact, other studies described that VPA may have pro- and anti-apoptotic effects depending on cells and tissue characteristics (Bittigau et al. 2002; Phillips et al. 2003). On the other hand, some authors argue that inactivation of MAPK/ERK pathway may be important during spontaneous seizures; thus, the observed effects of ESL and its

metabolites in inhibiting this pathway may constitute a compensatory response mechanism; however, further studies are needed to explore this hypothesis. Regarding LTG, we observed that LTG activated the JNK pathway, which is in agreement with the neuroprotective effect of LTG reported before (Willmore 2005). Thus, ESL and S-Lic showed a safer profile regarding neurotoxicity in cultured hippocampal cells. Since S-Lic is the main active metabolite of ESL in humans, our data sugests that ESL treatment may be a better alternative for the treatment of epilepsy than the other drugs studied here.

2. The impact of exposure to antiepileptic drugs during prenatal period, gestation and nursing on CD1 female mice

Pregnant women with epilepsy are not advised to stop AED therapy during gestation and nursing. However, it is known that AEDs have adverse effects on both the mother and the fetus, and although teratogenic effects on fetus deserve special attention, because of their impact and serious consequences, effects on mothers must not be overlooked. It has been shown that CBZ, OXC and VPA have pronounced and different effects on metabolism and serologic parameters in humans (Chuang et al. 2012). Biochemical parameters evaluated in blood serum can be used as indicators of healthy status of the organism, and they have been used as additional information to monitoring the health of a pregnant woman. Several studies have reported effects of AEDs in lipid profile, liver enzymes and indicators of kidney function, thus it is important to understand drug-specific effects of AED therapy in healthy pregnant subjects to predict its potential effects in epileptic pregnant women.

Therapy with VPA or CBZ in humans, has been associated with weight gain (Biton 2003), while in mice the same AEDs did not induce these effects (Christensen et al. 2004; Brown et al. 2008). Patients treated with VPA have showed weight gain due to lower basal metabolism associated with inhibition of lipid oxidation, thus the expenditure of energy is lesser than average (Gidal et al. 2003). In our studies we did not observe differences between CD1 pregnant female mice treated with AEDs and the control group regarding body weight, which was in agreement with the aforementioned studies in mice. In fact, since mice have a basal metabolic rate seven times greater than humans, weight variance during pregnancy may not be comparable between different species. These differences between

species, may also explain the lack of effects of AEDs on blood glucose levels. In the case of blood glucose levels, we did not observe significant differences between treated CD1 females and control group, but observed a trend to decreased blood glucose levels caused by VPA; VPA was previously shown to lower blood glucose levels in humans (Luef et al. 2003).

Regarding the possible effects of AEDs on lipid profiles, and according to what has been described in literature, we expected that CBZ, OXC and VPA would influence serum lipid profile via inhibiting or stimulating CYP450 iso-enzymes, since these are important in the metabolism of many drugs (Patsalos et al. 2002; Pylvanen et al. 2003; Mintzer et al. 2009), while ESL was shown as not affecting CYP450 enzymes (Almeida and Soares-da-Silva 2003). On the other hand, due to changes in hormonal profile that occur during gestation and lactation, other effects on lipid profiles may occur. Our data showed that total cholesterol levels, alanine and aspartate aminotransferase, and creatine kinase were not changed by exposure to AEDs during prenatal, gestation ant nursing periods. Although CBZ, OXC and VPA treated females did not show significant differences on TC as compared to controls, they showed a trend to an increase in TC as observed in humans (Chuang et al. 2012). Again, this lack of effect of AEDs in treated females compared to control may be due to species differences. Indeed, in mice, the fetus use maternal cholesterol to steroid synthesis; while in human, fetus is almost responsible for producing its own cholesterol; thus, it is plausible that TC levels are increased in pregnant women due to interchanges between mother and fetus (Yoshida and Wada 2005). Triglycerides were decreased by VPA and not changed by other AEDs. In the literature we found contradictory results regarding humans, for instance, in mice, long-term exposure to VPA was shown to increase TG (Lee et al. 2008), while we observed that VPA treatment decreased TG in CD1 females. As already mentioned, we do not have yet a convincing explanation for this effect, and further studies should be done. Creatinine has not been associated with changes induced by AEDs, but it is known that during pregnancy CREA clearance may be increased, which lowers serum CREA levels. OXC and VPA, but not ESL and CBZ, decreased CREA levels which means that during pregnancy OXC and VPA may increase renal function and hence CREA clearance.

3. The effects of antiepileptic drug exposure in utero and during nursing on behaviour and neurogenesis of CD1 mice during development.

Neurogenic zones are susceptible brain areas to any adjustment of its modulators. During development, brain undergoes a prolonged maturation process, which makes these areas further vulnerable to any changes in the surrounding environment (Ikonomidou and Turski 2010). It is known that during development there is a transient period of rapid growth which is known as "brain growth spurt", which is the period that brain weight increases in average 5% to 10% (reviewed by Ikonomidou et al. 2000). During this period occurs a significant growth of neural network arborization with elongation and branching of dendrites and neurons, which allows an enhancement in brain functioning (reviewed by Ikonomidou 2010). Brain growth spurt takes place at different times in human and rodents due to its evident differences on development, but both periods are comparable (Bayer et al. 1993). In mice and rats this period occurs postnatally, with peak growth velocity on P7-P10 until third week. In humans, this period starts prenatally during the third trimester and has its first peak of growth velocity at birth, (Ikonomidou and Turski 2010), while other peaks occur at different stages of brain development. However, formation of newly born cells beyond "brain growth spurt" occurs throughout life. In fact, neurogenesis plays an important role in postnatal developing brain; it proceeds at its greatest proliferative capability in neurogenic areas such as SGZ and SVZ. Furthermore, immature brain contains more neural progenitor cells than juvenile brain, and is more vulnerable to injury .When neurons in differentiation are disturbed, they won't be formed (reviewed by Kaindl et al. 2006), which means that hippocampus and lateral ventricles when negatively affected by intrinsic or extrinsic factors, such as antiepileptic drugs, may not provide new neurons to the respective networks. Consequently, different brain functions such as learning and memory, which are controlled by these networks, may be impaired. For instance, hippocampus is closely involved in cognitive functions, thus alterations on neurogenesis due to AEDs effects on neurotransmitters may affect any stage of this process (Chen et al. 2009). In our study, AED exposure coincided with the "brain growth spurt" of mice.

Antiepileptic drug therapy is used in pregnant women with epilepsy, thereby there is exposure of the fetus and breastfeeding babies to the AEDs. Some AEDs are teratogenic at clinical doses, such as CBZ, VPA, phenobarbital, phenytoin. Besides teratogenic effects, cognitive functions have also been studied in children exposed *in utero* to AEDs and during early-postnatally life in the nursing period, and it was reported that AED exposure *in utero* increases the risk of long-term effects of AEDs, including cognitive dysfunction that persists

until adulthood (Vinten et al. 2005; Meador et al. 2007); in rodents, VPA, at subteratogenic doses, caused microcephaly and behavioural changes (deficits in spatial learning and altered locomotor activity) (Ingram et al. 2000) and phenobarbital treatment, which enhances $GABA_A$-receptor activation, impaired cognitive function by decreasing proliferation and neurogenesis in the immature rat brain (Stefovska et al. 2008).

Effects of eslicarbazepine acetate (ESL) exposure *in utero* and during nursing, were studied for the first time in an animal model (CD1 mice), and our data suggests that ESL does not affect cognitive functions, namely learning and memory of mice progeny.

Furthermore, ESL did not induce anxious or depressive-behaviours. However, we observed that ESL had a gender and age-specific behaviour; it had a stimulatory effect on locomotor activity in juvenile males when compared to controls. Moreover, we did not observe changes in the proliferation, and neurogenesis in the DG of adult brains. Neither males nor females born to ESL treated females differ from respective controls. The absence of an effect on neurogenesis after long-term exposure to ESL may explain why this AED did not adversely affect cognitive and mood functions. Moreover, its main active metabolite, S-Licarbazepine (S-Lic) did not change proliferation and cell cycle distribution of cultured neural stem cells derived from the subventricular zone of P7-P8 Wistar rats. However, further studies are needed *in vivo* to confirm these effects. In summary, our data suggests that ESL may be considered a good AED therapy for pregnant women with epilepsy, since it has a better and safe profile, and better tolerability for progeny as well as for the mother, as compared to other AEDs.

Regarding the other AEDs tested, we observed that CBZ, OXC and VPA exposure *in utero* and during nursing were responsible for impairment of different cognitive tasks, as learning and memory in progeny namely in juvenile CD1 mice; OXC had a specific effect in depressive-behaviour. Most of the effects were transient, and were lost with age. We also observed that these AEDs affected proliferation and neurogenesis in the dentate gyrus of the hippocampus which may explain some of the impaired cognitive tasks.

Carbamazepine decreased memory performance in males and females in almost all the tests; however we did not observe a negative impact in proliferation (males and females) or in neurogenesis (females). However, males had a higher number of mature neurons ($BrdU^+/NeuN^+$ positive cells) than control animals. Since no negative effects were observed in adult neurogenesis, our results may suggest other mechanisms may be involved in the impairment of cognitive functions, during the long-term exposure to CBZ *in utero* and

during nursing. Deleterious effects of CBZ may have repercussions on other regulated and developmental processes on developing brain before neurogenesis starts. Hence, impairment of cognitive functions may be due to primarily neuronal damage and loss in the brain. However, an effort is need to elucidate these developmentally regulated processes in which CBZ may be involved.

Regarding OXC, it had a similar effect to ESL on motor activity of juvenile males, which was lost with age; while in females this effect was maintained with age. No further information exists that may explain how these AEDs induce higher levels of activity. However, OXC decreased performance of behavioural tests to a lower extent than CBZ, but, on the other hand, it had a higher effect on proliferation (males) and neurogenesis (males and females) than CBZ. In fact, OXC exposure decreased the number of newly-born cells in both genders and decreased the number of neurons (immature and mature) in females. It is plausible to associate the cognitive deficits with the reduction of hippocampal neurogenesis. In fact, it has been reported that newly formed neurons in the SGZ contribute to hippocampal function, while the progress of neurogenesis in the DG is directly associated with the hippocampus-dependent learning (Kitabatake et al. 2007). We should also take into account the neurotoxic effects of OXC, which were observed *in vitro* in previous studies (Ambrosio et al. 2000; Araujo et al. 2004), and in the present study (chapter 3). Further investigation should be done in order to understand whether OXC directly affects neurogenesis, or whether OXC is mainly inducing neuronal cell death, which in turn, decreases the number of newly neurons and hence, neurogenesis. It is known that physiological cell death is a normal process in the developing brain. After the "brain growth spurt", the initial excess of neurons is eliminated by apoptosis, which is strictly regulated by intrinsic factors such as neurotransmitters, growth factors, and executed by intracellular proteins. Thus, agents that hinder this regulation may elicit apoptotic death of neurons that should not be eliminated from the developing brain (reviewed by Meador et al. 2007; Ikonomidou and Turski 2010). Indeed, it was reported that the majority of AED exposure at therapeutic doses during gestation and nursing induce apoptotic neurodegeneration in the developing brain (Bittigau et al. 2003). In the literature there are few studies regarding the effects of OXC exposure in early-life, thus an effort is required to understand the underlying mechanism(s) that lead to the altered neurogenesis on adult brain of progeny exposed to OXC.

Valproate is one of the AEDs better characterized concerning its teratogenic and neurotoxic effects in humans and in animal models (Holmes et al. 2001; Morrow et al. 2006; Kim et al. 2007). Moreover, other studies showed that VPA enhanced neuronal differentiation in human fetal forebrain stem cell cultures (Laeng et al. 2004). In our study, we observed that VPA impaired performance of both, juvenile and adult male mice, on novel object recognition (which reflects the use of learning and recognition memory related to hippocampal functions). Regarding effects on proliferation and neurogenesis, our results showed that VPA decreased the number of newly born cells in SGZ and OGZ of males but did not change basal proliferation or the number of immature and mature neurons; in females VPA did not induce changes on these parameters. Our data are in line with the study of Umka and colleagues in rats (2010), showing that VPA decreases cell proliferation in the SGZ and is associated with impairment in their ability to perform a hippocampus-dependent spatial memory test (Umka et al. 2010). It is reasonable to associate the impairment of memory and learning task with the reduction of newly born cells in DG as some authors have been postulated, and as explained above. VPA has been considered an HDACi that induces hyperacetylation of DNA, which in turn may cause an increase in expression of pro-differentiation genes and induce a growth arrest (reviewed by Kostrouchova and Kostrouch 2007). However, in our animal model we did not assess yet these parameters. In our study, newly-born cells were detected by BrdU incorporation which was injected a long time after the animals were exposed to VPA. Thus, the observed effects in the reduction of newly-born cells may not due to the effects of VPA as HDACi at the time of BrdU injection. Moreover, VPA did not change basal proliferation of DG. However, HDACi properties of VPA may induce differentiation at the time of VPA exposure (gestation and during nursing) and its effects may subsequently be observed in adult brain. Whereas another mechanism is underlying reduction of newly born-cells, epigenetic effects of VPA (trend to differentiate) may mask the eventual loss of neurons, making this change undetectable in the adult brain. Otherwise, VPA may induce apoptotic neurodegeneration *in vivo* and *in vitro* (Bittigau et al. 2003), and as was the case of OXC, effects on the reduction of newly-born cells may be due to its neurotoxicity. However, our data suggests that VPA neurotoxic effects may not affect maturation and differentiation of neurons in the DG. Thus, VPA affected newly-born cells to a less extent than OXC.

Using cultured hippocampal neurons (chapter 3) we observed that after VPA exposure at high concentration, the levels of apoptotic markers were increased, such as

cleaved-caspase 3 and cleaved-PARP, which was also observed for OXC exposure. However, OXC increased the number of neurons with nuclear condensation while VPA did not induce any change in the nuclear morphology. Furthermore, in neural stem cell cultures (chapter 6), we observed that after VPA exposure (highest concentration) apoptosis was increased, while OXC (highest concentration) showed a trend to increase the amount of apoptotic cells and arrest cells in G2/M (a signal of DNA damage). However, it was described, that VPA may have pro- and anti-apoptotic effects depending on cells and tissues characteristics (Bittigau et al. 2002; Phillips et al. 2003), which may explain our results with VPA. Regarding OXC, we are presenting here for the first time data from OXC exposure, both in neural stem cell proliferation and cell cycle, and in neurogenesis of adult hippocampus. In this case, OXC has similar effects (neurodegeneration) on cell cultures and in the tissue. However, an effort should be done to explore these effects of OXC *in vitro* and *in vivo.*

Curiously, females treated with CBZ and VPA during pre-gestation period, gestation and nursing, had fewer newly-born cells compared to control females. These AEDs have been described as HDAC; thus, our data may be explained by HDACi properties of these AEDs, in fact treated females were exposed to AEDs for a long-period. Otherwise, as it was observed before, CBZ and VPA induce neurodegeneration *in vivo* (Bittigau et al. 2002), which may be reflected in the reduction of newly born cells in the SGZ and explain the decrease of newly born cells in DG.

In conclusion, based on the present data, it is advisable that AEDs that have negative impact on cognitive functions and on the formation of newly-born cells of progeny should be avoided during pregnancy and nursing, namely OXC, which is an AED for which less information is available than in the case of CBZ or VPA. Thus, in the present study ESL emerges as the most favourable AED to be used by women during pregnancy and nursing, since no negative effects on cognitive and non-cognitive functions of mice born to ESL treated females were detected; furthermore, its main metabolite, S-Lic, is not toxic to hippocampal neurons and neural stem cell cultures. Thus, ESL is a safe and efficacious anticonvulsive agent for the treatment of partial epilepsy (Elger et al. 2007).

Chapter 8

Main Conclusions

8. Main Conclusions

The work presented in this thesis allowed us to draw the following main conclusions:

- Eslicarbazepine acetate and its metabolites, S-Lic and R-Lic, up to 0.3 mM, do not induce neurotoxic effect in cultured hippocampal neurons;

- Inhibitory effects of ESL and its metabolites on MAPK/ERK pathways may be beneficial *in vivo* to face the overactivation of ERK1/2 that may occur during spontaneous seizures;

- Carbamazepine and LTG were not toxic to cultured hippocampal neurons at the concentration tested (0.03-0.3 mM) and LTG may exert neuroprotective effects through the activation of the JNK pathway;

- OXC and VPA exposure induced the appearance of markers related to cell death; OXC was the most toxic drug, and may induced neuronal cell death by caspase-dependent pathways;

- Eslicarbazepine acetate may be a better alternative for the treatment of epilepsy than the other drugs studied here, since it is endowed with an improved safety profile;

- Exposure to ESL, CBZ and VPA during the prenatal period did not affect fertility of CD1 females, while OXC exposure tend to decrease fertility; moreover, treatment with ESL, CBZ, OXC and VPA during prenatal period and gestation did not change litter size and duration of gestation in CD1 females;

- During whole treatment (prenatal period, gestation and during nursing) of CD1 females with ESL, CBZ, OXC or VPA, the body weight of the pregnant females increased in the normal range during the gestation and nursing periods, and a similar increase was observed in the experimental groups. Blood glucose levels of CD1 females were not affected by AED exposure;

- After long-term treatment (prenatal period, gestation and during nursing) with ESL and CBZ, CD1 females did not show changes in biochemical parameters measured in blood serum, such as TC, TG, ALT, AST, CREA and CK, compared to control females; however, TG levels were significantly decreased by VPA; CREA levels were decreased by VPA or OXC but the other biochemical parameters were not changed by these AEDs;

- After long-term treatment with ESL and OXC, the number of newly born cells that incorporated BrdU in SGZ, IGZ and OGZ of CD1 females DG, did not differ from control females; however, the number of newly born cells in SGZ of the DG of CD1 females was decreased after long-term treatment with CBZ and VPA, while in IGZ and OGZ no changes were observed;

- Eslicarbazepine acetate and CBZ exposure (*in utero* and during nursing) did not change body weight of CD1 mice born to treated females, while OXC and VPA increased body weight, compared to control animals; moreover, AEDs exposure did not induce changes in brain weight of CD1 mice born to treated females;

- Eslicarbazepine acetate exposure enhanced locomotor activity of juvenile males, but this effect was lost with age; locomotor activity was not affected in females by ESL exposure; OXC exposure also enhanced locomotor activity of juvenile males and of juvenile and adult females ;

- Eslicarbazepine acetate exposure did not affect cognitive functions (memory and learning), but CBZ exposure impaired cognitive performance in juvenile males (NOR and inhibitory avoidance test); CBZ also impaired cognitive performance in females (Y-Maze and inhibitory avoidance test); OXC impaired cognitive performance in juvenile males evaluated on NOR test (effect lost with age), as well as in females (on inhibitory avoidance test); VPA exposure also impaired cognitive performance in males (NOR test);

- Eslicarbazepne acetate or CBZ did not induce anxious or depressive behaviours in CD1 mice born to treated females; however, CBZ exposure tend to induce an

anxiolytic effect in adult males (EPM test); OXC exposure induced a depressive effect on juvenile males , but VPA did not induce anxious or depressive behaviours;

- AED exposure did not affect brain morphology of CD1 mice born to treated females, as evaluated by the cresyl violet staining.

- Eslicarbazepine acetate (*in vivo* studies) did not change basal proliferation and neurogenesis of SGZ in the adult hippocampus of CD1 mice born to treated females; however, CBZ slightly increased the number of mature neurons in SGZ of males DG; besides this effect, CBZ did not induce changes in proliferation and neurogenesis of males and females born to treated females; on the other hand, OXC exposure decreased the proliferation and formation of newly born cells in SGZ of males DG and decreased neurogenesis in SGZ of females DG ; VPA exposure also decreased the number of newly born cells in SGZ and OGZ of males DG, but did not change proliferation or neurogenesis in females;

- Eslicarbazepine, the main active metabolite of ESL (*in vitro* studies), did not alter basal neural stem cell proliferation and did not affect cell cycle phases of SVZ derived cultures form Wistar rats;

- Eslicarbazepine acetate (0.3 mM) and R-Lic (0.01 mM) (*in vitro* studies), decreased proliferation of SVZ cultures, and ESL (0.1 mM) decreased the percentage of cells in G2/M phase; however, R-Lic did not change cell cycle distribution;

- Carbamazepine, OXC, LTG and VPA (*in vitro* studies) decreased proliferation of SVZ cultures at different concentrations and extensions; noteworthy that VPA (1 or 3 mM) strongly decreased (85% or 95%) proliferation of SVZ cultures;

- Cell cycle phases, namely G1/G0 and G2/M phases were differently affected by CBZ, OXC, LTG and VPA, while S phase did not have changes; however its noteworthy that VPA (1 or 3 mM) highly decreased (75% or 70%) the percentage of cells in G2/M, while OXC (0.3 mM) increased (65%) the percentage of cells in G2/M, thus, OXC arrests cells in this cell cycle, which may be explained by damage on nuclear DNA;

- Oxcarbazepine and valproate at highest concentrations increased the percentage of apoptotic cells from SVZ, while none of the other AEDs induced apoptosis in these cultures;

- Eslicarbazepine acetate has potential advantages over the other AEDs in AED therapy; either in the *in vitro* or in the *in vivo* studies ESL had a better and safer profile – ESL did not induce cell death and was better tolerated by CD1 mice (pregnant females and its progeny) – ESL did not induce adverse-effects after long-term treatment on biochemical parameters of blood serum, neither on fertility rates, nor on cognitive or non-cognitive functions.

Chapter 9

References

9. References

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