Erythropoietin and enriched housing in Marlau™ cages protect neurons and cognitive function in epileptic rats

Raafat P. Fares

Université Claude Bernard Lyon 1

N° d'ordre : 343-2009 Année 2009

THÈSE présentée devant

L'UNIVERSITÉ CLAUDE BERNARD – LYON 1

pour l'obtention du DIPLOME DE DOCTORAT
(arrêté du 7 août 2006)

à soutenir publiquement le 22 décembre 2009 par

Raafat **P**. **FARES**

L'érythropoïétine et l'enrichissement du milieu en cage Marlau™ protègent les populations neuronales et la fonction cognitive chez le rat épileptique

Erythropoietin and enriched housing in Marlau™ cages protect neurons and cognitive function in epileptic rats

Directeur de thèse : **Laurent BEZIN**

JURY:

M. le Pr. Philippe RYVLIN,	Président
Mme le Dr. Mireille LERNER-NATOLI	Rapporteur
M. le Dr. Marcello SOLINAS	Rapporteur
M. le Dr. Didier LAGARDE	Examinateur
Mme le Dr. Claire RAMPON	Examinateur
M. le Dr. Jean-Jacques RISSO	Examinateur
M. le Dr. Laurent BEZIN	Directeur de thèse

UNIVERSITE CLAUDE BERNARD - LYON 1

Président de l'Université	**M. le Professeur L. Collet**
Vice-président du Conseil Scientifique	M. le Professeur J-F. Mornex
Vice-président du Conseil d'Administration	M. le Professeur G. Annat
Vice-président du Conseil des Etudes et de la Vie Universitaire	M. le Professeur D. Simon
Secrétaire Général	M. G. Gay

COMPOSANTES SANTE

Faculté de Médecine Lyon Est – Claude Bernard	Directeur : M. le Professeur J. Etienne
Faculté de Médecine Lyon Sud – Charles Mérieux	Directeur : M. le Professeur F-N. Gilly
UFR d'Odontologie	Directeur : M. le Professeur D. Bourgeois
Institut des Sciences Pharmaceutiques et Biologiques	Directeur : M. le Professeur F. Locher
Institut des Sciences et Techniques de Réadaptation	Directeur : M. le Professeur Y. Matillon
Département de Formation et Centre de Recherche en Biologie Humaine	Directeur : M. le Professeur P. Farge

COMPOSANTES SCIENCES ET TECHNOLOGIE

Faculté des Sciences et Technologies	Directeur : M. Le Professeur F. Gieres
UFR Sciences et Techniques des Activités Physiques et Sportives	Directeur : M. C. Collignon
Observatoire de Lyon	Directeur : M. B. Guiderdoni
Institut des Sciences et des Techniques de l'Ingénieur de Lyon	Directeur : M. le Professeur J. Lieto
Institut Universitaire de Technologie A	Directeur : M. le Professeur C. Coulet
Institut Universitaire de Technologie B	Directeur : M. le Professeur R. Lamartine
Institut de Science Financière et d'Assurance	Directeur : M. le Professeur J-C. Augros
Institut Universitaire de Formation des Maîtres	Directeur : M R. Bernard

I do feel the strong desire and the joy to acknowledge the people who have helped me to achieve this work.

To Doctor Laurent BEZIN

Some events in life have serendipitous consequences. For me, the occasion of meeting Dr Laurent BEZIN was one of the most flourishing and exciting experience I have ever lived. His teachings have reached not only my mind but also my soul. I will tell you about this man who has drastically changed the destiny of my life. With all my heart, I do really mean the destiny of my life.

I would like to express my sincere and deep gratitude to him. Without his faith, his generosity, his respect, his presence and his sacrifices it would have been impossible for me to finish my PhD, least of all, even start my Master's degree. I met him four years ago willing to join his research group. Coming from the Kinesiology's department of Lyon, I had only the motivation and nothing else regarding the neurobiology field. Despite the fact of my ignorance at that time, he made a very courageous decision by giving me the opportunity to become a student in his laboratory. He then taught me generously, respectfully with huge patience offering me a very secure and thriving environment. Not too later, I was delighted to discover that Dr Laurent BEZIN was transferring me not only his knowledge in science but also his deep values and peaceful principles in life, and that behind his great capacity and competence in work, he has a tender heart which constantly guides his personality.

I am very grateful to him for giving me the opportunity to participate in several international congresses in neurosciences and epilepsy, for associating me in the patent of his innovation of Marlau™ cage, for his considerable participation, help and support in my experiments especially those of enriched housing, for his very constructive criticism in preparing presentations, for his fascinating analysis of the data results and for his enormous investment in writing the articles.

I am extremely proud and delighted to have met and worked with Dr Laurent BEZIN.

I would like to thank

Professor Philippe RYVLIN for accepting to be the chairman of the jury, Dr. Mireille LERNER-NATOLI and Dr. Marcello SOLINAS for accepting to review this work in details, and Dr. Didier LAGARDE, Dr. Claire RAMPON and Dr. Jean-Jacques RISSO for accepting to be associate members of this jury.

Béatrice GEORGES and Colette MOULIN for their continuous assistance in gene expression experiments.

Chantal BONNET and Marion LE CAVORSIN MOREAU for their permanent support during *in vivo* experiments, in particular for the animals care in Marlau™ cages.

Hayet KOUCHI and Pascal SANCHEZ for accepting to get involved in my experiments and for their patience with me, especially when sharing the same office. Thank you for the nice moments spent together.

Sandrine BOUVARD for her help in collecting blood samples.

Frédérique COHEN ADAD for her continuous assistance in bibliography management.

Ahmed ATHAMEMA, Rachid ATTOF, Amor BELMEGUENAI, Jacques BODENNEC, Aurélien BOUSSOUAR, Emilie CARRE, Andréi CIVIDJIAN, Yvette DALMAZ, Rafik DEY, Thomas LIEUTAUD, Anne MORALES, Jérémie NADAM, Fabrice NAVARRO, Jean-Marc PEQUIGNOT, Luc QUINTIN and Emil TOADER for the time and discussions shared together.

All members of the UMR 5123 directed by Professor Bruno ALLRAD, and in particular Marie-Thérèse BOILLON, Christophe CHOUABE, Guy DALLEVET, Jean-Sébastien GAILLARD, Harry KOUBI, Sophie MOHAMED-SIDI, Gérard MOREL and Julie ULMANN.

The Kinesiology's department of Lyon especially Professor Christian COLLET, Doctor Cyril MARTIN, Professor Raphael MASSARELLI, Doctor Marcel SAGNOL and Professor Thierry TERRET.

My friends for their presence and their encouragements: Babeth, Florent, Guillaume, Jihad, Julie, Marie-Laure, Mario, Nadine, Paul, Peter, Sylva, Vanessa, and Yamina.

I dedicate this thesis to my family who supported me from Beyrouth

To my mother Fatmé,

To my father Abdel Rassoul,

To my brothers Ramzi, Rami, Rachad, Amir and to my uncle Youssef,

COMMUNICATIONS

Submitted Patent

Bezin L, Moreau M, Fares RP (2009). Cage d'hébergement pour rongeurs à milieu de vie enrichi /Cage allowing enriched housing of rodents. I.N.P.I., registration number: FR09/00544.

Published Articles

1. Sanchez PE*, Fares RP*, Risso JJ, Bonnet C, Bouvard S, Le-Cavorsin M, Georges B, Moulin C, Belmeguenai A, Bodennec J, Morales A, Pequignot JM, Baulieu EE, Levine RA, Bezin L. Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons. ***PNAS*** (2009) 106:9848-53 (* first co-authors).

2. Sanchez PE, Navarro FP, Fares RP, Nadam J, Georges B, Moulin C, Le Cavorsin M, Bonnet C, Ryvlin P, Belmeguenai A, Bodennec J, Morales A, Bezin L. Erythropoietin receptor expression is concordant with erythropoietin but not with common beta chain expression in the rat brain throughout the life span. ***J Comp Neurol*** (2009) 514:403-14.

3. Navarro FP, Fares RP*, Sanchez PE*, Nadam J, Georges B, Moulin C, Morales A, Pequignot JM, Bezin L. Brain heparanase expression is up-regulated during postnatal development and hypoxia-induced neovascularisation in adult rats. ***J Neurochem*** (2008) 105:34-45 (* second co-authors).

Submitted Article

1. Fares RP*, Sanchez PE*, Kouchi H, Bodennec J, Koubi H, Morales A, Le Cavorsin M, Georges B, Moulin C, Bonnet C, Bouvard S, Belmegenai A, Sloviter RS, Bezin L. Standardized enrichment procedures in the Marlau™ cage support enhanced brain plasticity in healthy rats and prevent cognitive impairment in peileptic rats (* first co-authors).

Article in Preparation

1. Fares RP*, Bonnet C, Belmeguenai A, Kouchi H, Georges B, Moulin C, Morales A, Ryvlin P, Bodennec J, Bezin L. Exogenous erythropoietin exerts opposite effects on cognitive deficits and anxiety in epileptic rats depending on the quality of the living environment (* first co-authors).

Presentations at International Meetings

Slide presentation in the 28th International Epilepsy Congress, Budapest 2009
Fares RP, Bonnet C, Sanchez PE, Le Cavorsin M, Belmeguenai A, Morales A, Bodennec J, Ryvlin P, Bezin L. Protective effects of environmental enrichment in Marlau™ cage on the development of spontaneous seizures and on cognition in rats subjected to status epilepticus at weaning.

Poster presentation in the 28th International Epilepsy Congress, Budapest 2009
Bonnet C, Fares RP, Sanchez PE, Le Cavorsin M, Belmeguenai A, Morales A, Bodennec J, Ryvlin P, Bezin L. Severe cognitive dysfunctions, but discrete neuronal lesions, in adult epileptic rats subjected to status epilepticus at weaning.

Slide presentation in the Neuroscience 2007 Congress, San Diego 2007
Bezin L, Fares RP, Le Cavorsin M, Georges B, Moulin C, Morales A,. Beneficial effects of environmental enrichment on brain plasticity are abolished by physical exercise.

TABLE CONTENTS

Commonly used abbreviations

AED:	Anti-epileptic drugs
AMPA:	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BBB:	Blood brain barrier
BDNF:	Brain derived neurotrophic factor
BSRS:	Behavioral spontaneous recurrent seizures
CA1:	Area 1 of the Ammon's horn
CA3:	Area 2 of the Ammon's horn
ChAT:	Choline acetyltransferase
CNS:	Central nervous system
DAT	Dopamine transporter
Elisa:	Enzyme-Linked ImmunoSorbent Assay
EPM:	Elevated plus maze
EpoR :	Erythropoeitin Receptor
GABA:	γ-aminobutyric acid
GAD:	glutamate decarboxylase
GC:	Granule cells
GDNF:	Glial cell line-derived neurotrophic factor
GluR:	Glutamate receptor
IAP:	Inhibitor of protein apoptosis
Icv:	Intracerebroventricular
IEG:	Immediate early genes
IGF-1:	Isuline-Like growth factor 1
Ip:	Intraperitoneal
IS-H:	In situ-Hybridization
Li/Pilo:	Lithium/pilocarpine
LTP:	Long term potentiation
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI:	Magnetic Resonance Imaging
MWM:	Morris Water Maze
mRNA:	messenger Ribonucleic acid
NA:	Noradrenaline
NGF:	Nerve growth factor
NR1:	N-methyl-D-aspartate (NMDA) receptor 1 subunit
NT-3:	Neurotrophin-3
Pilo:	Pilocarpine
r(h)Epo:	Recombinant (human) erythropoeitin
RT-PCR:	Reverse transcription polymerase chain reaction
SE:	Status epilepticus
TBI:	Traumatic brain injury
VEGF:	Vascular endothelial growth factor
VLR:	Ventral Limbic region
WB:	Western Blot
WET:	Water exploration test
5-HT:	Serotonin

Introduction

Epilepsy is one of the most serious neurological disorder. It is estimated that at least 50 million people have epilepsy worldwide with a huge burden not only on people with epilepsy, but also on their families and the society at large (Leonardi M, 2002). This disorder is characterised by i) the presence of at least one seizure, ii) an enduring alteration in the brain that increases the likelihood of future seizures, iii) and associated neurobiologic, cognitive, psychological, and social disturbances (Fisher RS, 2005) such as anxiety, psychoses, poor self-esteem, attention deficit and severe learning disabilities and memory impairment (de Boer HM, 2008). In the past 20 years, a large range of anti-epileptic drugs (AED) has been licensed for use. Unfortunately, AED administration failed to control seizures in a large number of patients and was associated with side effects such as cognitive and psychiatric disturbances (Jacobs MP, 2009). The anatomic analysis of epileptic brains or routine imaging revealed two groups of patients with epilepsy. One group displayed an extensive neuronal loss such as reported in mesial temporal lobe epilepsy while the second group exhibited no obvious neuronal loss (Margerison JH, 1966). Thus, protecting neurodegenerative processes occurring in some patients as well as cognitive impairment in others represent a major subject in epilepsy research. Today, while erythropoietin is considered as one of the most promising neuroprotective agents (Hasselblatt M, 2006; Maiese K, 2008), an increased body of studies provides evidence that enrichment (or complexity) of housing decreases the cerebral vulnerability and exerts a rehabilitative effect in the context of diverse brain insults (Nithianantharajah J 2006; Laviola G, 2008). In this thesis, we have tested 1) in a model of epilepsy with large neuronal lesions whether optimization of the neuronal expression of erythropoietin receptor extends neuroprotection to the most vulnerable neuronal populations to excitotoxic injury; 2) in a model of epilepsy associated with faint neuronal lesions i) whether erythropoietin prevents cognitive impairment; ii) whether enriched housing in Marlau™ cages is more efficient than erythropoietin, and iii) whether erythropoietin treatment may potentiate the effects of enriched housing.

Specific aims of the Thesis

1. Develop a new cage (Marlau™ cage) to standardize enriched housing for rodents and to demonstrate that it reproduces the main effects reported so far of environmental enrichment in rodents (Results Part I).
2. Find appropriate physiological conditions to optimize neuronal expression of erythropoietin receptor (EpoR) in the rat brain (Results Part II).
3. Establish whether rhEpo administration can protect vulnerable neuronal populations to excitotoxic injury following up-regulation of the expression of its receptor in neurons (Results Part II).
4. Ascertain, in animals prone to develop epilepsy following chemically-induced SE at weaning, whether: 1) administration of rhEpo after SE in rats housed in standard cages, 2) housing rats in Marlau™ cages just after SE, or 3) the combination of rhEpo administration and housing in Marlau™ cages after SE, can prevent cognitive decline usually observed in animals that have developed epilepsy (Results Part III).

Review of the Literature

Part I. Epilepsy

I.1. Definition, frequency, prognosis and causes

I.1.a Definition. Epilepsy is defined as a brain disorder characterized by recurrent epileptic seizures. An epileptic seizure is the clinical manifestation occurring after an episode of epileptic neuronal activity characterized by enhanced activation and synchronization of small or large neuronal populations. The clinical manifestations depend on the part of the brain involved in the epileptic neuronal discharge and the spread of the discharge (Shorvon S, 2009).

I.1.b Frequency and prognosis. Epilepsy is a common condition, affecting about 50 million people worldwide. In most studies, the incidence of epilepsy has been found to be about 65 cases per 100 000 persons per year. However, frequency is slighlty higher in lower social-economic classes, and is definitely greater in underdeveloped countries, likely due to poorer perinatal care, standards of nutrition and public hygiene and to greater risks of brain injury and cerebral infection.

Once patients are diagnosed as having epilepsy, approximately 60% of them will cease having seizures within the 5-year period after diagnosis has been established and half of them will have withdrawn therapy. Epilepsy will never remit for 20% of the patients, but reduction in frequency and severity of seizures may be achieved in most cases by appropriate therapy. Periods of remission and relapse are observed in the last 20% of patients (Shorvon S, 2009).

In patients with drug-refractory epilepsy, temporal lobe epilepsy is the most frequent type of epilepsy diagnosed, and may be associated with hippocampal sclerossis. In these patients, epilepsy surgery is carried out to control epileptic seizures and mainly consists in (i) temporal lobectomy for patients with hippocampal sclerosis or other lesions (vascular lesions, cortical dysplasia and tumors) in the mesial temporal lobe, (ii) corpus callosectomy, and (iii) implantation of a stimulator below the skin of the chest for stimulation of the left vagus nerve. Temporal lobectomy renders 60% of the patients seizure-free in the long term, while vagus nerve stimulation reduces by 50% seizure frequency in one-third of the patients implanted (Acharya MM, 2008; Shorvon S, 2009).

I.1.c Causes of epilepsy. Epilepsy can have many causes, which are categorized into 3 groups. When the cause is clearly identified: hippocampal sclerosis, prenatal or perinatal injury, post-vaccination encephalitic encephalomyelitis, traumatic brain injury, brain tumors, cerebral infections (meningitis and encephalitis), febrile seizures, cerebrovascular disease (cerebral haemorrhage, cerebral infarction, arteriovenous

malformations, cavernous haemangioma), status epilepticus, the epilepsy is categorized as symptomatic. When no cause is known, or when the cause is just suspected, the epilepsy is referred as cryptogenic. When the epilepsy is predominantly of genetic (or presumed genetic) origin, it is then categorized as idiopathic (Shorvon S, 2009).

I.2. Cognitive impairments and psychological disorders in people with epilepsy

I.2.a Psychopathology, anxiety and depression. The risk to develop psychopathology, anxiety, depression and to commit suicide is greater in people with epilepsy than in the general population. Anxiety and depression are the most prevalent psychiatric disorders in people with epilepsy (McCagh J, 2009). Anxiety is the most frequently reported psychiatric problem by people with epilepsy, and depression and suicide are 4-5 times more prevalent in people with epilepsy than the general population (Brodie MJ & Schachter SC, 2001). It has been suggested that anxiety may be a consequence of the unpredictability of seizures, restricted social activities, low self-esteem, stigma and rejection from society (Vasquez B & Devinsky 0, 2003). The feeling of social isolation might be reinforced by the fact that people with epilepsy develop agoraphobia and social phobia, due to the possibility that seizures may occur at any time (Thompson PJ, 2000). While most studies reported that anxiety was a consequence of epilepsy, a recent study revealed that 45% of children with epilepsy had a history of psychopathology (mostly anxiety, depression and attention deficit disorder) preceding the onset of seizures (Jones JE, 2007), psychopathology that might be in part facilitated by parental over protectiveness (Ferrari M, 1983). Depression is more common in patients with temporal lobe epilepsy with a focus in the dominant hemisphere, which is particularly apparent in people with mesial temporal sclerosis (Quiske A, 2000). As for anxiety, depression may not be a consequence of epilepsy only; the relationship may be bi-directional since people with major depression or, to a lesser extent, with a history of suicide attempt are at a greater risk of developing epilepsy (Hesdorffer DC, 2000, 2006).

I.2.b Cognitive dysfunction. Cognitive impairment is ranked as one of the biggest problems with having epilepsy. Common cognitive deficits in people with epilepsy are intellectual decline, reduced information processing speed, reduced reaction time, attention deficits and memory impairments (McCagh J, 2009). People with temporal lobe epilepsy, and especially mesial temporal lobe epilepsy, are commonly affected by impaired visuo-spatial functions, problem solving, learning and memory, compromising academic attainment (Hermann B & Seidenberg M, 2002). If the dominant hemisphere is affected, then language dedicits can also be apparent (Kent GP, 2006). For the majority

of seizure types, cognitive impairment arising from seizure activity can be reduced or reversed by effective seizure control (McCagh J, 2009). However, in seizures difficult to control, administration of a multiplicity of anti-epileptic drugs at high doses can be associated with or exacerbate cognitive impairments and psychological problems (see table 1). Finally, seizures or the side effects of anti-epileptic drugs may not be the only causes of cognitive dysfunction in people with epilepsy. Indeed, cognitive dysfunction has been demonstrated to precede the onset of diagnosed seizures in groups of children, who showed impaired attention, reaction time, location learning, executive function, language, psychomotor speed and academic skills. In addition, children with epilepsy who had academic difficulties prior to seizure onset were particularly at risk of cognitive impairment (Oostrom KJ, 2003; Hermann B, 2006).

Table 1: Side effects of Anti-epileptic drugs administration on cognition and behavior (Modified and adapated from the Book: Epilepsy by Simon Shorvon, ONL 2009).

Drug	Major putative mechanisms of action	Side effects on cognition
-Clobazam -lonazepamam	Enhances GABA action at the GABAergic receptors	Behavioral and cognitive disturbances, personality and behavior changes, hyperactivity, aggressiveness, withdrawal symptoms
-Phenobarbital -Primidone	Enhances GABA action at the benzodiazepine site at the $GABA_A$ receptor, inhibits glutamate excitability, weak effect on sodium, potassium, and calcium conductance	Aggressiveness, cognitive dysfunction (memory and attentional disturbances), paradoxial hyperkinesis and behavioral change in children
Tiagabine	Enhances GABAergic transmission at the $GABA_A$ receptor by inhibiting GABA re-uptake	Psychosis, depression, word-finding difficulties and other cognitive effects, emotional lability
Topiramate	Blocks conductance at the neuronal sodium channel, potentiates GABAergic inhibition at the $GABA_A$ receptor, reduces excitatory actions of glutamate at AMPA receptor, inhibits high-voltage calcium channels, inhibits carbonic anhydrase	Cognitive dysfunction especially difficulties with memory, attention, language and word finding, confusion, agitation, amnesia, depression, anxiety, psychosis, emotional lability
Vigabatrin	Enhances GABAergic transmission by inhibiting the catabolic enzyme gamma-aminobutyric acid transaminase (GABA-T), thereby increasing the synaptic concentration of GABA	Psychosis, depression, confusion
Valproate	Not fully understood. Action on GABA and glutaminergic activity, calcium (T) conductance, and potassium conductance	Cognitive disturbance, aggressiveness, tremor, ataxia
Zonisamide	Multiple actions including inhibition of sodium and T-type calcium currents, enhances GABAa receptor function, carbonic anhydrase inhibition, glutaminergic transmission inhibition	Attention and concentration difficulties, memory impairment, confusion, depression, mental slowing, speech disturbance, aggressiveness, tremor, ataxia
-Carbamazepine -Lamotrigine -Oxcarbazepine -Phenytoin	Blocks conductance at the neuronal sodium channel	Effects on behavior and mood, depression, psychosis, movement disorder, anxiety, memory and attention defects, other cognitive, neurological and psychiatric effects
Lacosamide	Enhances selectively slow inactivation at the neuronal sodium channel and modulates the action of the CMRP-2 protein	Depression, memory disturbance
Ethosuximide	Inhibits neuronal T-type calcium channels	Behavioral disturbances including aggression and irritability, hyperexcitability in children, acute psychotic reactions, depression
-Gabapentin -Pregabalin	Binds to the alpha2Theta subunit of the neuronal voltage-dependent calcium channel	Memory and attentional deficit, concentration difficulties, confusion and other cognitive effects, psychiatric disturbance, depression, anxiety
Levetiracetam	Binds to the synaptic vesicle protein SV2A	Behavioral change, irritability, aggression,mood change, depression, anxiety, psychosis, memory and attention disturbance

I.3. Neuroprotective strategies as potential therapeutic interventions in people with epilepsy

I.3.a Epilepsy and neuronal loss. Experimental data obtained from in vitro studies or in animal models of brain disorders indicate that excessive neuronal activation by the excitatory amino acid transmitter glutamate induces neuronal injury and death that has been referred to as excitotoxicity. Glutamate-mediated excitotoxicity leads to disturbances in the intracellular electrolyte metabolism, mitochondrial dysfunction, oxidative stress, growth factor depletion or withdrawal and increased concentration of cytokines (Ferriero DM, 2005). At cellular level, intense seizure activity initiates massive influx of calcium via voltage-gated and N-methyl-D-aspartate-dependent ion channels, which leads to biochemical cascades triggering acute neuronal cell death (Henshall DC & Simon RP, 2005; Acharya MM, 2008).

In people with epilepsy, it is difficult to determine whether seizures induce excitotoxicity in the long term. However, some indications arise from a detailed post-mortem anatomic analysis of the brains of 55 patients with epilepsy (Margerison JH & Corsellis JA, 1966). It was indeed reported that most patients did not exhibit widespread or severe brain damage, and the only common pathology present in all patients was neuronal loss in the hilus of the dentate gyrus. Only a minority of the brains examined exhibited classic hippocampal sclerosis (extensive hilar and pyramidal layer neuronal loss, with partial or majority survival of dentate granule cells and CA2 pyramidal cells). Such hippocampal sclerosis is associated with shrinkage of the hippocampus, which can be detected using routine Magnetic Resonance Imaging (MRI) (Briellmann RS, 2002). However, for over a century, there has been a controversy about hippocampal sclerosis as a cause or effect of the repeated, brief seizures.

A prospective MRI study of hippocampus volume determination in patients with focal epilepsy indicated that the average volume was identical between patients and controls at the time of diagnosis of epilepsy and after 5 years of treatment (Salmenpera T, 2005). However, cell death may have occurred, as already reported for patients with endfolium sclerosis (relatively selective loss of dentate gyrus neurons), which alone does not cause significant hippocampal shrinkage that can be detected by routine imaging (Sloviter RS, 2008). Interestingly, in the kindling model of epilepsy, it has been reported that long-term stimulations (150 provoked stage-5 seizures) caused no alteration in hippocampus volume, but led to a dramatic decrease in neuronal density in the hilus of dentate gyrus, area CA1 and CA3 of the hippocampus (Cavazos JE, 1994).

The above mentioned prospective study also revealed, when careful individual analysis were performed, that 13% of patients developed hippocampus volume decrease during 3 years of follow-up. It is noteworthy that these patients had longer duration of seizure

disorder and larger seizure number before the epilepsy was diagnosed compared with patients who did not show altered hippocampus volume (Salmenpera T, 2005). We cannot conclude from this observation that progressive morphological alteration of the hippocampus was accompanied with increased neuronal loss in the hippocampus. But, if confirmed, it will demonstrate that repeated seizures in people with epilepsy may result in increased neuronal death.

Finally, a minority (8%) of patients with temporal lobe epilepsy had a decreased hippocampus volume at the time of diagnosis (Salmenpera T, 2005), suggesting that the pathological hippocampus was damaged prior to epilepsy onset. Different reasons may account for this observation. Either anatomical malformations occurred in the hippocampus during development for unexplained reasons, or some initial-precipitating injury, such as a convulsive or non-convulsive status epilepticus, a history of febrile seizures, brain trauma or infection may have injured the hippocampus. This hypothesis is partly supported by experimental models raised in rodents. Indeed, convulsive status epilepticus triggered by systemic administration of chemoconvulsive agents (kainate or pilocarpine), or intrahippocampal injection of kainate, or non-convulsive status epilepticus triggered by 8 hour bilateral stimulation of the perforant pathway in awake rats induce neuronal damages that resemble those observed in human hippocampal sclerosis (Bouilleret V, 1999; Sloviter RS, 2007).

I.3.b Status epilepticus, epileptogenesis and epilepsy. As stated above, status epilepticus has been widely used to understand the mechanisms underlying hippocampal neurodegeneration and synaptic reorganization that occurs during epileptogenesis (Acharya MM, 2008). The term "epileptogenesis" refers to the process by which normal brain tissue is transformed into tissue capable of generating spontaneous seizures, a transition that can take as little as minutes or as long as years. After an initial precipitating injury such as status epilepticus, the immediate release of large concentration of glutamate leads to rapid activation of inflammatory processes and adaptative induction of neuroprotective genes. Then follows an intermediate period characterized by massive neuronal loss, with a lack of electrographic seizures as recorded by scalp electroencephalography. Finally, the latent period, also called the silent period, precedes the onset of recurrent and spontaneous seizures. This period is characterized by remodeling events that have all been found in combination or not in tissue of epileptic patients, including axonal sprouting, synaptogenesis, neurogenesis, gliosis, angiogenesis and circuit reorganization, increasing predisposition to synchronous activity (Jacobs MP, 2009).

While axonal sprouting has received great attention for many years (Acharya MM, 2008), it appears not to be a sufficient condition to support the onset of epilepsy (Vaydia

VA, 1999; Morimoto K, 2004). Increased neurogenesis observed after status epilepticus, and ectopic migration of newborn neurons in the hilus of dentate gyrus has been proposed as a mechanisms underlying pathological excitability in the epileptic brain (Acharya MM, 2008). Gliosis and angiogenesis have been among the first described characteristics accompanying neuronal loss in the hippocampus of patients with temporal lobe epilepsy (Thom M, 2008). Increased angiogenesis is receiving particular attention, since it has been positively correlated with the number of seizures (Rigau V, 2007). In addition, brain blood barrier permeability in the epileptic tissue, and in particular immunoglobulin G leakage, may play a critical role in neuronal excitability (Rigau V, 2007; van Vliet EA, 2007).

Considering neuroprotective strategies, most of the research has focused on preventing neurodegenerative processes occurring after status epilepticus (Acharya MM, 2008). However, as stated before, few studies have considered the value of increasing robustness of neurons vulnerable to excitotoxicity that may take place after each episode of recurrent seizure.

I.3.c Neuroprotective strategies for preventing epilepsy. When a patient is subjected to an initial precipitating injury leading to epilepsy, the administration of a neuroprotective compound that has demonstrated efficacy in models of epileptogenesis may be proposed prior to the onset of seizures. It is therefore essential to develop biomarkers of epileptogenesis in humans, and to develop drugs that may be anti-epileptic, neuroprotective or both (Jacobs MP, 2009). A battery of such drugs is already available, including lipids, neurotrophic factors, antioxidants, and hormones (Acharya MM, 2008). Erythropoietin is among the potent hormones that are well tolerated in humans and that may play a neuroprotective role during epileptogenesis, as detailed in the next chapter.

Part II. Erythropoietin

II.1. Neuroprotective effects of Erythropoietin (Epo). Epo, synthesized by the kidney, was originally described for its role in hematopoiesis, which consists of increasing red blood cells (Jelkmann W, 1992) by protecting erythroid progenitors against apoptosis (Ghezzi P, 2004). Besides the hematopoietic system, Epo and its receptor (EpoR) have been found to be expressed by diverse tissues (Maiese K, 2008). In the non pathological brain, Epo and EpoR are expressed throughout the life span, mainly by neurons, in humans (Juul SE, 1999; Siren AL, 2001) and rodents (Nadam J, 2007; Sanchez PE, 2009; 2009b). Epo and EpoR are also expressed in cultured neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells (Marti HH, 1996; Juul SE, 1999; Chin K, 2000; Siren AL, 2001; Hasselblatt M, 2006). This widespread expression of Epo and EpoR has led to studies of additional biological roles of Epo. It has been established that brain-derived Epo is an endogenous factor regulating physiological processes such as developmental and adult neurogenesis (Chen ZY, 2007; Shingo T, 2001; Tsai PT, 2006), angiogenesis (Wang L, 2004) and ventilatory acclimatization to hypoxia (Soliz J, 2007). The constitutive neuronal expression can counteract neuronal death in mild brain injuries (Sakanaka M, 1998), and its induced expression in astroyctes (Bernaudin M, 1999; Grasso G, 2005; Nadam J, 2007), microglia and endothelial cells (Bernaudin M, 1999) after severe brain damage is supposed to prolong neuroprotection and support tissue restructuring. In vitro studies have shown that rhEpo protects neuronal cells from noxious stimuli such as hypoxia (Lewczuk P, 2000), excess glutamate (Morishita E, 1997), serum deprivation or kainic acid exposure (Siren AL, 2001). To anticipate induced expression of Epo by brain cells in response to a damaging event, recombinant human Epo (rhEpo) can be used efficiently because its crosses the blood–brain barrier (BBB) through transcytosis when administered peripherally (Brines ML, 2000). To be neuroprotective, rhEpo must be used at much higher doses than those usually used to increase erythropoiesis. At 50 U/kg, rhEpo was erythropoietic but did not reduce infarct volume after an experimental stroke in mice (Wang Y, 2007). A dose-dependent effect of rhEpo has been shown against brain ischemic injury in gerbils (Calapai G, 2000). Among the doses of rhEpo tested (50, 500, 1150 and 5000 U/kg) after experimental stroke in mice, the higher dose (5000 U/kg) produced the greater brain protection (Wang Y, 2007). Beneficial effects of Epo have also been observed in animal models of subarachnoid hemorrhage (Grasso G, 2002; Alafaci G, 2000), intracerebral hemorrhage (Lee ST, 2006), traumatic brain injury (Brines ML, 2000; Yatsiv I, 2005; Lu D, 2005), spinal cord injury (Celik M, 2002; Gorio A, 2002) and in Parkinson disease (Kanaan NM, 2006; Xue YQ, 2007). In addition, encouraging results on the neuroprotective efficacy of rhEpo in humans have been obtained from clinical trials involving stroke patients

(Ehrenreich H, 2002), patients with chronic schizophrenia (Ehrenreich H, 2007b) and patients with chronic progressive multiple sclerosis (Ehrenreich H, 2007). Despite this wealth of information on neuroprotective effect of Epo in multiple models of brain injury, few data are available on its role in the development of epilepsy and/or prevention of cognitive impairment in epileptic animals. Indeed, some studies have shown its potential effect in models of SE.

II.2. rhEpo administration before SE. As shown in table 2, studies in rodent models of temporal lobe epilepsy (TLE) showed significant effects of rhEpo in antagonizing the development of chemically-induced SE (Brines ML, 2000; Uzum G, 2006; Nadam J, 2007), in decreasing the number of stage 4/5 seizures (Nadam J, 2007; Wen X, 2006; Yang J, 2007), the levels of pro-apototic proteins such as casapse-3, Bim, Bid and the total number of TUNEL positive cells in the hippocampus (Wen X, 2006; Yang J, 2007; Mikati M, 2007). In addition, pre-treatement with rhEpo before either kainate or pilocarpine-induced SE increased the levels of anti-apoptotic proteins like Bcl-w and Bcl2 (Yang J, 2007) and protected the pyramidal cells in CA1 area of the hippocampus (Mikati M, 2007).

Table 2: rhEpo increases brain resistance to seizures and SE models

rhEpo as a **prevention** tool	**SE & seizures models**	Effects
Female BALB/c mice Epo (5000 U/Kg; i.p, **24** hours prior to SE) ***Brines ML, 2000***	Kainate 20 mg/Kg; i.p	-Delayed onset of SE -Decreased mortality rate (~45%) -Increased survival time (~41%)
Male Wistar rats Epo (3000 U/Kg; i.p, **24** hours prior to SE) ***Uzum G, 2006***	Pentylentetrazol 80 mg/Kg; i.p	-Delayed onset of seizure -Decreased severity of seizures -Decreased total seizure time -Confined blood brain barrier leakage
Male Sprague Dawley rats Epo (10 000U/Kg; i.p, **4** hours prior to SE) ***Wen X, 2006; Yang J, 2007***	Li-Pilocarpine 30 mg/Kg; i.p/s.c	-Decreased seizure activity -Decreased TUNEL and levels of pro-apoptotic proteins (caspase-3, Bim and Bid) in the hippocampus -Increased levels of anti-apoptotic proteins (Bcl-w and Bcl-2) in the hippocampus
Male Sprague Dawley rats Epo (5000 U/Kg; i.p, **24** hours and **30 min** prior to SE) ***Nadam J, 2007***	Pilocarpine 300 mg/Kg; i.p	-Decreased number (-40%) of rats developing SE -Decreased number of stage 4/5 seizures

II.3. rhEpo administration after SE. As shown in table 3, in pilocarpine-induced SE, the therapeutic administration of rhEpo 30 minutes, 1 and 3 days after the end of SE showed neuroprotective effects only in the hippocampus and not in the ventral limbic region (VLR). The pyramidal neurons in the CA1 area of the hippocampus, hilar interneurons and the glutamatergic mossy cells were partially protected, while complete protection was observed in the SLMo and in the CA3 area of the hippocampus (Nadam J, 2007). Recently, our group showed that neuroprotective effects of rhEpo following pilocarpine-induced SE could be extended to VLR, when the expression of its receptor EpoR was preliminary up-regulated in neurons using physiological tools, such as repeated (3 times, 4 days apart) exposures to hypoxia (6h at 8% O2) (Sanchez PE, 2009). In Lithium/Pilocarpine-induced SE, it has been shown by Chu et al 2008 that administartion of rhEpo immediately after the cessation of Lithium/Pilo-induced SE and once daily during the 7 following days prevented the BBB leakage, and reduced neuronal death, microglial activation, and the generation of ectopic granule cells in the hippocampus. Interestingly, this study showed a reduction not only in the number of rats that developed spontaneous generalized convulsive (stage4/5) seizures, but also in the frequency and duration of the recorded spontaneous seizures in the hippocampus (Chu K, 2008).

Table 3: Beneficial effects of therapeutic administartion of rhEpo in SE models

rhEpo as a **therapeutic** tool	SE model	Effects
Male Sprague Dawley rats Epo (5000 U/g; i.p, 30min, 1 and 3 days post-SE) ***Nadam J, 2007***	Pilocarpine 300 mg/Kg; i.p	-Full protection of CA3 and SLMo -Partial protection of Hilar interneurons, mossy cells and CA1pyramidal neurons -No protection of ventral limbic region (VLR)
Male Sprague Dawley rats 3 repeated hypoxia exposures Epo (5000 U/g; i.p, 30min, 1 and 3 days post-SE) ***Sanchez PE, 2009***	Pilocarpine 300 mg/Kg; i.p	-As above plus protection of VLR
Male Sprague Dawley rats Epo (5000 U/g; i.p, Immediately after SE cessation and once daily for 7 days) ***Chu K, 2008***	Li-Pilocarpine repeated doses of 30 mg/Kg; i.p	-Cell protection in CA1,CA3 and the Hilus -Decreased blood brain barrier leakage -Decreased number of rats developing convulsive seizures -Decreased frequency and duration of hippocampal seizures

II.4. Effect of rhEpo on cognition in normal animals. Potential behavioral or cognitive effect of the administration of Epo in nonlesioned animals has rarely been studied. An early report in C57BL/6 mice showed that a low dose of Epo (1,5U ~ 50U/Kg) administered subcutaneously every other day for 19 weeks, but not for 8 weeks, was able to improve the water maze performance (Hengemihle JM, 1996). In a recent study using 100-fold greater dose of Epo (5000U/kg i.p., the neuroprotective dose), treatment of 28 day old mice every other day during 22 days significantly had no effect on anxiety, spontaneous activity, exploratory behavior and motor performance established using the different tests as the elevated plus maze, open field tests, hole board and rota-rod, but improved the contextual memory in fear conditioning. This latter result was supported by the ex vivo observation that rhEpo improved LTP within the hippocampus (Adamcio B, 2008).

II.5. Effect of rhEpo on cognition in lesioned animals. The effect of rhEpo on behavior and cognition has been tested in various models of brain injury as ischemia (Sadamoto Y, 1998; Sakanaka M, 1998; Catania MA, 2002; Wang L, 2004; Belayev L, 2005; Yu YP, 2005), hypoxia-ischemia (Kumral A, 2004; Demers EJ, 2005; Spandou E, 2005), traumatic brain injury (Lu D, 2005; Yatsiv I, 2005), transection of fimbria-fornix (Mogensen J, 2004; Mala H, 2005) and glutamate injection in the lateral nucleus of amygdala (Miu AC, 2004). Details on the dose and the mode of administration are given in table 4. The administration of rhEpo was usually associated with positive effects. Indeed, continuous icv infusion of rhEpo at the dose of 5U/day in the lateral ventricle following middle cerebral artery occlusion for 28d in rats has been shown to ameliorate the short and long-term spatial memory deficit in the Morris Water Maze (MWM) test (Sadomoto Y, 1998). This positive effect on the MWM performance was also observed in Hypoxia-ischemia (Kumral A, 2004), TBI (Lu D, 2005) and transaction of fimbria-fornix models (Mogensen J, 2004) when rhEpo wash administered intraperitoneally (i.p) in young and adult Wistar rats. Beneficial effects of rhEpo on behavior have also been observed in the passive avoidance task, another tool to measure learning, in the ischemic brain of adult animals (Sakanaka M, 1998; Catania MA, 2002). In addition rhEpo protected fear conditioning performances following excitotoxic lesions in the lateral nucleus of the amygdala (Miu AC, 2004), enhanced functional recovery in the corner and foot-fault tests (Wang L, 2004) and improved functional outcomes by reducing long-term sensorimotor deficits after ischemia (Demers EJ, 2005; Spandou E, 2005).

Table 4: rhEpo effects on cognition/behavior has been investigated in various models of brain injury but not in models of epilepsy

Brain injury model	Study details
Hypoxia-Ischemia	*Epo*: 1000U/Kg administered i.p after oxygenation in Wistar rats at P7 Test: MWM ***Kurmal A, 2004***
	Epo: 2500U/Kg administered s.c after oxygenation for 3d in Sprague-Dawley rats at P7 Test: sensorimotor response ***Demers EJ, 2005***
	Epo: 2000U/Kg administered intraperitoneally in Wistar rats at P7 Test: Rota-Rod treadmill-Grip traction-Foot fault-Postural reflex and Limb placing ***Spandou E, 2005***
Ischemia	*Epo*: 5U/day continuously infused in the lateral ventricle for 28d of 12w SH-SP rats Test: MWM ***Sadamoto Y, 1998***
	Epo: 2,5 to 25U/day infused for 7d in the lateral ventricle of gerbils Test: Passive avoidance task ***Sakanaka M, 1998***
	Epo: 0,5 to 25U immediately injected after restoration of blood flow in the lateral ventricle of adult gerbils Test: Passive avoidance task ***Catania MA, 2002***
	Epo: 5000 or 10 000U/Kg administered i.p for 7d in Wistar rats starting 24h after artery occlusion Test: Corner and foot-fault ***Wang L, 2004***
	Darbepoetin Alfa: 10µg/Kg administered i.p 2h after onset artery occlusion in adult Sprague-Dawley (SD) rats Test: postural reflex and forelimb placing ***Belayev L, 2005***
	Epo: 5000U/Kg ip or 4,8 to 24U intranasaly 10 min after artery occlusion and 24h after reperfusion in SD rats Test: neurological score ***Yu YP, 2005***
Controlled cortical injury	*Epo*: 5000U/Kg administered i.p daily for 14d in adult Wistar rats Test: MWM ***Lu D, 2005***
Closed head injury	*Epo*: 5000 U/Kg administered i.p 1h and 24h after injury in Sabra mice Test: Neurological severity score and Object recognition ***Yatsiv I, 2005***
Transection of fimbria-fornix	*Epo*: 5000U/Kg administered i.p during the injury in adult Wistar rats Test: MWM and 8 arm-radial maze ***Mogensen J, 2004; Mala H, 2005***
Glutamate excitotoxic injury	*Epo*: 2,5U injected consecutively with glutamate in the lateral nucleus of Amygdala in adult Wistar rats Test: Open field and Trace fear conditioning ***Miu AC, 2004***

II.6. Mechanisms of Epo-mediated neuroprotection. The neuroprotective effects of Epo involve its binding to receptors expressed at high levels in the CNS, in particular within the limbic system (Digicaylioglu M, 1995). The binding sites for Epo in the CNS have been proposed to be different from those present at the surface of the peripheral cells, based on studies using Epo analogs that are neuroprotective but lack erythropoietic effects (Leist M, 2004). Based on studies showing that Epo analogs were devoid of neuroprotective effects in transgenic mice lacking the common beta chain (a transmembrane protein expressed by the receptors of interleukin-3 (IL-3), IL-5 and the granulocyte-macrophage/colony-stimulating-factor) (Brines M, 2004), it has been suggested that the heteroceptor mediating the cytoprotective effects of Epo might be composed of one subunit of the classical Epo receptor "EpoR" and two subunits of the common beta chain (Brines M, 2005). We have recently shown that the common beta chain is expressed at trace levels in the brain of rats throughout the life span, and cannot be detected in differentiated PC12 cells, known to be protected by Epo under cytotoxic conditions. These results indicate that most of the neuroprotective effects of Epo may involve a receptor complex that does not contain the common beta chain (Sanchez PE, 2009b). From our knowledge from cells of the immune system and cultured cell lines, the effects of Epo are initiated by its binding to the classical Epo receptor "EpoR". Upon this

binding, dimerization of EpoR enables autophosphorylation of the receptor associated Janus-tyrosine Kinase-2 and leads to the activation of several downstream signalling cascades. These signalling cascades counteract the mechanisms of cell death and apoptosis (Kawakami M, 2001). Most significant signaling events for the neuronal functions seem to be the activation of phosphatidylinositol-3-kinase (PI3-K)-AKt/protein Kinase B pathway, the Ras-mitogen-activated protein Kinases (MAP kinases), signal transducers and activators of transcription-5 (STAT-5) (Siren Al, 2001), and nuclear factor-kB-dependent transcription (Nf-KB) which controls the expression of "inhibitor of apoptosis proteins" (IAP) (Digicaylioglu M, 2001). These molecular pathways are depicted in figure 1. In vivo studies showed that inhibition of JAK2 or PI3K abolished the neuroprotective effects of EPO (Zhang F, 2006; 2007). The activation of PI3K induces the translocation and subsequent activation of NFκB and/or stimulates STAT5 homodimerization thereby initiating a number of downstream molecular cascades (Sola A, 2005).

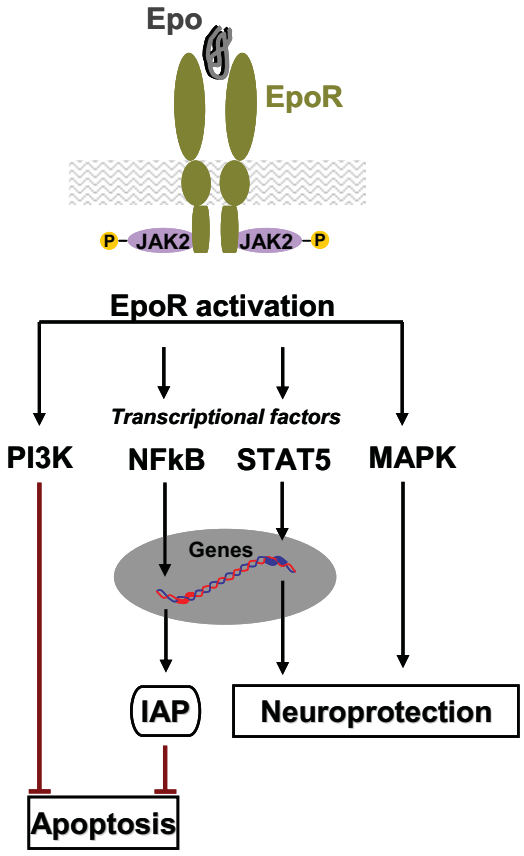


Figure 1: *Signal pathways of EpoR*. Epo binds to EpoR dimer and stimulates JAK2 kinase activity resulting in phosphorylation (P) of JAK2 and EpoR. Activated JAK2 leads to the activation of several downstream signalling cascades. These downstream signalling messengers include the phosphatidylinositol-3-kinase (PI3-K)-AKt/protein Kinase B pathway, the Ras-mitogen-activated protein Kinases (MAP kinases), signal transducers and activators of transcription-5 (STAT-5), and nuclear factor-kB-dependent transcription (Nf-KB) which controls the expression of "inhibitor of apoptosis proteins" (IAP).

II.7. Constitutive expression of EpoR in the brain. After providing evidence that numerous Epo binding sites are present in the limbic system (Digicaylioglu M, 1995), it has been shown that EpoR is constitutively expressed in the mammalian brain, both in humans and rodents (Hasselblatt M, 2006). EpoR gene is expressed at very high levels in brain tissue during the fetal life, the levels of both transcript and protein decreasing dramatically before birth. After birth, EpoR transcript and protein levels are also varying throughout the life span, but to an extent that never reaches that observed during the prenatal period (Sanchez PE, 2009b). In both human and rodent tissue, EpoR protein is primarily detected in neurons, and scarcely in astrocytes (Bernaudin M, 1999; Siren AL, 2001; Nadam J, 2007). The presence of EpoR has also been reported in vivo in endothelial and ependymal cells in the rat brain (Yamaji R, 1996; Grasso G, 2005).

II.8. Induced brain expression of EpoR following brain insult and in brain disease. In humans, hypoxic events caused by cardio-respiratory dysfunctions are associated with increased immunolabeling of EpoR in both neurons and astrocytes, and detection of EpoR in endothelial cells (Siren AL, 2001). In resected tissue from patients with temporal lobe epilepsy, EpoR immunolabeling has been shown to be overexpressed in endothelial cells and astrocyte foot processes (Eid T, 2004). Finally, schizophrenic patients have increased expression of EpoR in both neurons and astrocytes (Ehrenreich H, 2007b), and overexpression of neuronal EpoR in brain tissue from Alzheimer's disease patients are correlated with greater severity of the disease (Assaraf MI, 2007). It may thus be possible that increased expression of EpoR in injured brain tissue is an adaptative mechanism to better cope with the nocivity of the microenvironment, particularly in the context of excitotoxicity. In rodents, increased expression of EpoR in models of stroke has been well documented, and would involve at different times following the insult, microglial cells, endothelial cells and astrocytes (Bernaudin M, 1999). After spinal cord injury, increased expression of EpoR has been shown in neurons, astroyctes and endothelial cells (Grasso G, 2005). In the acute/silent phase following pilocarpine-induced status epilepticus in adult rats, EpoR was transiently increased in astrocytes (Nadam J, 2007).

Part III. Environmental enrichment

III.1. A starter for enrichment

III.1.a The birth of enrichment concept. The story stems from the work of the Canadian psychologist Donald Hebb in 1940s. He noticed that when pet rats were allowed to roam freely around the house, they performed in a maze-running test better than the locked-up lab rats. He then hypothesized that raising animals in a stimulating environment would render their brains better at solving problems than animals raised in laboratory cages. The concept of "environmental enrichment" emerged later in the late 1950s at Berkeley, California, as reported by Marian Diamond in the book "Magic trees of the Mind" published in 1999. Based on the observation of Hebb's work, David Krech, Mark Rosenzweig, and Edward Bennett decided to house pet rats in two kinds of cages which they named: "enrichment cage", a large cage filled with toys and housing twelve rats; and "impoverished cage", a small cage devoid of toys and housing one rat only. Then, they found that rats housed in the "enrichment cage" run better mazes than those housed in the impoverished condition, in agreement with the results obtained by Hebb. Furthermore, they defined the "enriched environment" as the combination of complex inanimate and social stimulations (Rosenzweig MR, 1978).

III.1.b The craze for enrichment. There are two main reasons to explain the craze for enrichment. The first one is due to the fact that enriched environment induces brain plasticity. The second one is related to the well-being of animals. In fact, until the early 1960s, a firm dogma considered that brain, once developed, never changed. When Diamond first discovered that growing rats in an enriched environment increased the thickness of their cerebral cortex (Diamond MC, 1964), the scientific thought changed. The enriched housing became a condition highly sought (a tool), and numerous studies assessed how life experience induced by enriched environment could modify brain development, anatomy, chemistry, robustness, rehabilitation after brain insults, and the improvement of disease states, and behavior. Abnormal or stereotypic behaviors were observed not only in zoo enclosures, but also in laboratory, testifying that housing environment was unchallenging and boredom. Thus, in the 1980s, the enrichment was introduced as a concept in laboratory animal care in order to stimulate essential species-specific behavior and fulfill the needs of animals, which participate to the wellbeing of animals. Then, huge efforts were and are still being made to define the principles and the procedures aiming at developing a standardized program for enriched environment.

III.1.c The current constituent of enrichment. Since the pioneer work of Rosenzweig and colleagues, several attempts have been made to define the concept of environmental enrichment (Chamove AS, 1989; Newberry RC, 1995). In each case, the major goal to achieve was, and still, to guarantee the stimulation of sensory, cognitive and motor systems of the brain. Thus, innumerable cages of different shapes and sizes were conceived to reach this goal (see figure 2). The analysis of the constituents of these enriched cages showed frequently:

-An increased exploration area (larger cages or two-interconnected cages)

-An increased number of animals housed together (starting from 3 to 12 per cage)

-The addition of running wheels

-The addition of a variety of objects: tunnels and toys of different colors and shapes, wooden objects of different textures, cardboard boxes, small houses, rubber balls, maze, chain, swing, climbing ladders, and nesting material.

One of the major principles of environmental enrichment is to provide novelty. In these enriched cages used so far, the most used or unique method described to introduce novelty and complexity is to move or remove objects within the experiment.

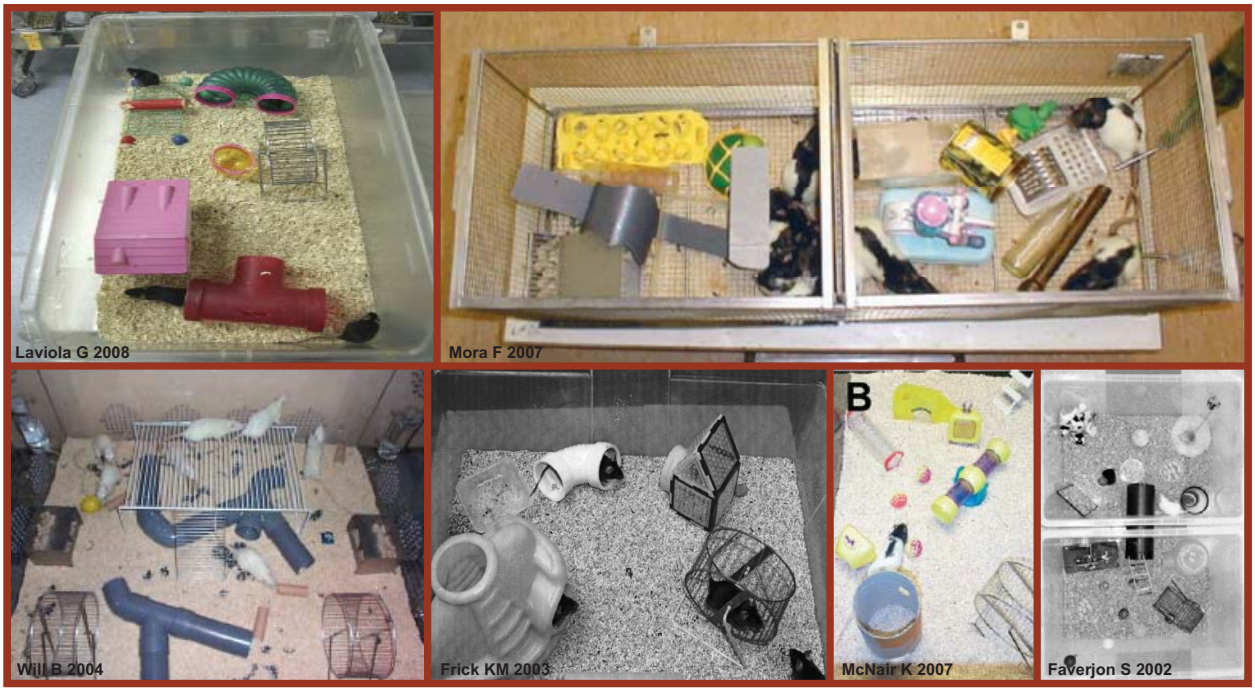


Figure 2: Some of the non-standardized enriched cages reported in the literature.

III.1.d Relevance to standardize enriched housing. Standardization is a fundamental concept in the animal science aiming to increase the reproducibility of the results and consequently the comparison between studies; it is designed to reduce individual differences (intra-experimental variation) within the groups and as well differences between studies (inter-experiment variation). A remarkable study by Crabbe and colleagues has revealed that current standard housing conditions are completely ineffective to offer reproducibility between studies. Indeed, despite equating rigorously the environmental variables of mice in three laboratories, large significant effects for the same behavioral tests were observed between the different sites (Crabbe JC, 1999). Moreover, it has been shown that rearing mice in bare cages did not remove individual differences in behavioral tests used in drug screening and in behavioral phenotyping of transgenic and "knockout" mice (Wolfer DP, 2004). Thus, current system of standardization, based on economic criteria and ergonomic factors for the personnel caring for the animals, is not as optimal for scientific research. Housing animals in complex environment was shown not to increase neither the intra-experimental variation (Wolfer DP, 2004; Van de weerd HA, 2002) nor the risk of obtaining conflicting results between laboratories that have precisely used the same enrichment protocol (Wolfer DP, 2004). However, screening the literature in which different protocols of enrichment were used has showed conflicting results between studies (see tables 5, 6 and 7). Thus, standardization of enrichment is highly required not only for good laboratory practices but also because it offers wellbeing for animals.

III.1.e Potential recommendations for an optimal enrichment program. Considerable progress was made by the FELASA Working group in order to identify the principles of enrichment to be used for enhancing animal welfare. These principles, aiming at satisfying animal's physiological and behavioral needs, are considered to be the starting point for standardization when developing an enrichment program. Evolving animals in a complex environment should take into account the complexity, control, and predictability features (FELASA 2006). The cage size is an important aspect but the third spatial dimension within it and the interaction between individuals and responsiveness to the environment seems to be more important than just the space provided per individual (Chamove AS, 1989; Ward GE, 1991). In addition, the appropriate structure of the cage with physical, visual and space barriers should offer the possibility to hide or escape from a subordinate individual, the opportunity to have a choice, to make a decision, to be an active participant, and to feel a certain level of control on the environment (Sambrook TD, 1997). In addition, animals' life in the wild highlights an important aspect that should strongly be regarded: the cognitive mechanism for solving complex problem. Animals use navigational and cooperative social skills in order to procure their food. At the same time,

they face problems regularly and are able to overcome them, survive and thrive. This solving problem ability requires animal to process, store, retrieve and act upon information from the environment (Meehan CL, 2007). This type of cognition is known by "higher-level" or "off-line" controls in which the representation of information can be used flexibly in the control of behavior (Toates F, 2004). Thus, in addition to features described below, it seems that challenging animals with a problem is a very important, even a necessary feature of an enrichment program as long as they possess the skills and resources to effectively solve the problems with which they are presented (Meehan CL, 2007). These recommendations have been developed to some extent in Marlau™ cage (see figure 3).

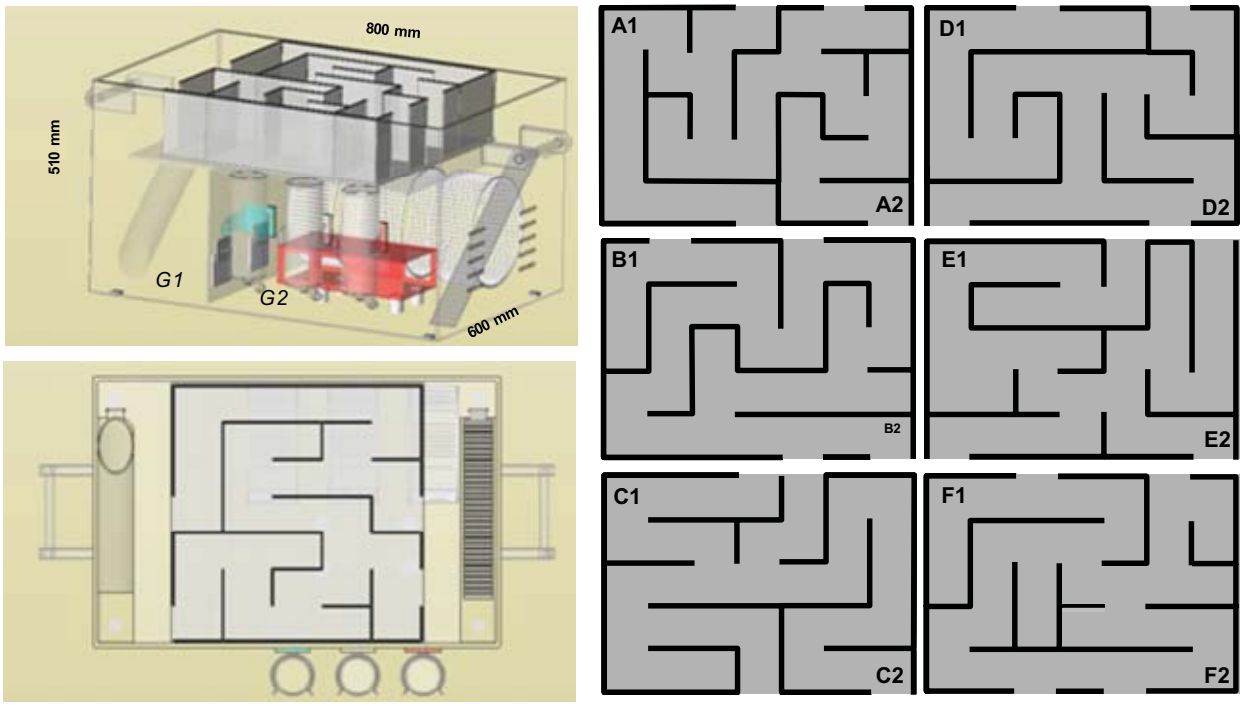


Figure 3: Marlau™ cage (patent FR09/00544) is developed to standardize the enriched environments for rodents. For more details see Results Part II.

Table 5	Animals				Program			Enrichment design							MWM
Author	Strain	Sex	En	Ctrl	Begin at	Length	Type	RW	TNL	RMP	TYS	CHE	LBH	CPT	Result
Wolf M, 2008	Rat	F	12	3-4	42w	40d	C	-	-	-	+	daily	-	-	+
Holguin S, 2008	Rat	M	2	1	4w	?	C	-	-	-	+	2/w	-	-	+
Zanardi A, 2007	Mice	F	10	2	3w	2m	C	+	-	+	+	2/w	+	+	+
Fan Y, 2007	Gerbil	M	?	3	2m	2m	C	+	+	-	+	2/w	-	+	+
Toscano CD, 2006	Mice	M-F	6-8	1	3w	6m	C	+	+	-	+	1/w	-	-	+
Meshi D, 2006	Mice	F	8	4	12w	6w	C	+	+	-	+	-	-	+	+
Huang FL, 2006	Mice	?	?	?	3-5m	3w	C	+	-	-	+	2/w	-	-	+
Benett JC, 2006	Mice	?	5	5	23m	6w	C	+	+	-	+	daily	-	-	+
Jankowski JL, 2005	Mice	F	16	3-4	2m	6m	C	+	+	-	+	-	-	-	+
Giza CC, 2005	Rat	M	16-18	?	3w	17d	C	-	+	+	+	daily	-	+	+
Foley AG, 2005	Rat	?	30	15	25d	55d	C	+	+	+	+	daily	-	+	+
Hellemans KG, 2005	Rat	M	12	2	3w	12w	C	+	-	+	+	2/w	-	+	+
Schrijver NC, 2004	Rat	M	3	3	3w	80d	C	-	-	+	+	1/w	-	-	+
Guilarte TR, 2003	Rat	?	8	1	3w	30d	C	+	+	+	+	1/w	-	+	+
Frick KM, 2003	Mice	M-F	4	4	?	23d	3h/d	+	-	-	+	-	-	+	+
Bredy TW, 2003	Rat	?	8	2	22d	50d	3h/d	-	-	-	+	regularly	-	+	+
Schrijver NC, 2002	Rat	M	3	3	3w	80d	C	-	-	+	+	1/w	-	-	+
Faverjon S, 2002	Rat	M	9	?	3w	30d	4h/d	+	+	+	+	2/w	-	+	+
Pham TM, 1999	Rat	?	8	1	22d	2m	4h/d	+	+	+	+	daily	-	-	+
Nilsson M, 1999	Rat	F	6	1	10w	4-8m	4h/d	-	-	+	+	2/w	-	-	+
Kempermann G, 1997	Mice	F	12	4	3w	40d	C	+	+	-	+	-	-	-	+
Galani R, 1997	Rat	M	12	1	?	30d	4h/d	-	-	+	+	daily	-	+	+
Falkenberg T, 1992	Rat	M	6	1	50d	34d	4h/d	?	?	?	?	?	?	?	+
Paylor R, 1992	Rat	?	?	?	15d	12d	4h/d	?	?	?	?	?	?	?	+

Table 5: Studies reporting positive effect of enriched environment on the MWM test

Animals: En/Ctrl, The number of animals per cage**. Program: Begin at,** the age at which enrichment started**; length,** the duration of housing**; Type,** continuous (**C**) or intermittent**; Enrichment design: RW,** Running wheel**; TNL,** Tunnel; **RMP**, Ramp; **TYS**, Toys; **CHE**, change toys; **LBH**, Labyrinth; **CPT**, compartment. **MWM: Morris Water Maze result;** (+)**,** positif result Abbreviations: **M**, Male; **F**, Female; **w**, week; **d**, day; **m**, month; **C**, continuous housing; (**+**), presence;(**-**), absence;(?), not known.

Table 6	Animals				Program			Enrichment design							MWM
Author	Strain	Sex	En	Ctrl	Begin at	Length	Type	RW	TNL	RMP	TYS	CHE	LBH	CPT	Result
Mandolesi L, 2008	**Rat**	**M**	**10**	**2**	**3w**	**3m**	**C**	**+**	**-**	**+**	**+**	**2/w**	**-**	**+**	-
Comeau W, 2008	Rat	M-F	4-6	3-4	3w	3m	C	-	-	-	+	1/w	-	-	-
Cao X, 2008	Rat	M	8	2	3w	8w	C	+	+	+	+	1/w	-	+	-
Wurm F, 2007	Rat	M	6-8	4	10-12w	5-8w	C	-	+	-	+	1/w	-	-	-
Wang CA, 2007	Rat	M	8-10	2-3	25d	2w	C	+	-	+	+	3/w	-	-	-
Miu AC, 2006	Rat	M	5-7	3-4	adult	10w	C	-	+	-	+	2/w	-	-	-
Ciu M, 2006	Rat	?	?	?	3w	30d	C	+	+	+	+	1/w	-	+	-
Briones TL, 2006	Rat	M	9	2	3-4m	2w	C	+	+	-	+	daily	-	-	-
Briones TL, 2006b	Rat	M	9	2	3-4m	2w	C	+	+	-	+	daily	-	-	-
Ueda S, 2005	Rat	M	5	2	4w	4w	C	+	-	-	+	daily	-	-	-
Martinez-Cue C, 2005	Mice	M	8-10	2-3	22d	7w	C	+	-	+	+	-	-	+	-
Leggio MG, 2005	Rat	?	10	2	3w	3w	C	+	-	+	+	1/w	-	+	-
Gobbo OL, 2005	Rat	M	4	3-4	adult	10w	C	+	+	-	+	1/w	-	+	-
Gobbo OL, 2004	Rat	M	4	3	adult	10w	C	+	+	-	+	1/w	-	+	-
Hicks RR, 2002	Rat	M	6	1	3w	2w	C	-	-	-	+	3/w	-	-	-
Schneider JS, 2001	Rat	M	8	1	25d	75d	C	-	+	-	+	3/w	-	-	-
Passineau MJ, 2001	Rat	M	?	1	adult	11d	C	+	+	+	+	-	-	+	-
Pursky GT, 2000	Mice	F	?	4-5	21d	40d	C	-	+	+	+	daily	-	-	-
Caston J, 1999	Mice	?	?	5	1d	3m	C	+	+	+	-	-	-	+	-
Van Rijzingen IM, 1997	Rat	M	6-7	2	2m	6w	C	-	+	+	+	1/w	-	-	-
Puurunen K, 1997	Rat	?	6-7	1	?	1w	C	+	+	-	+	1/w	+	+	-
Boehm GW, 1996	Mice	F	4-6	4-6	3w	9w	C	+	-	+	-	-	-	-	-
Wainwright PE, 1993	Mice	?	12	1	3w	7w	C	-	-	+	+	3/w	?	?	-

Table 6: Studies reporting no effect of enriched environment on the MWM test

Animals: En/Ctrl, The number of animals per cage**. Program: Begin at,** the age at which enrichment started**; length,** the duration of housing**; Type,** continuous (**C**) or intermittent**; Enrichment design: RW,** Running wheel**; TNL,** Tunnel; **RMP**, Ramp; **TYS**, Toys; **CHE**, change toys; **LBH**, Labyrinth; **CPT**, compartment. **MWM: Morris Water Maze result;** (-)**,** negatif result Abbreviations: **M**, Male; **F**, Female; **w**, week; **d**, day; **m**, month; **C**, continuous housing; (**+**), presence;(**-**), absence;(?), not known.

Table 7	Animals				Program			Enrichment design							BDNF data			
Author	Strain	Sex	En	Ctrl	Begin at	Length	Type	RW	TNL	RMP	TYS	CHE	LBH	CPT	LOC	State	TCN	Result
Kondo M, 2008	**Rat**	**M-F**	**5-6**	**5-6**	**4w**	**10-26w**	**C**	**+**	**-**	**-**	**+**	**3/w**	**-**	**-**	**Hi**	**Prot**	**Elisa**	**-**
Segovia G, 2008	Rat	M	10-12	1	3m	19m	C	+	+	-	+	1/w	-	-	Hi	Prot	Elisa	-
Bindu B, 2007	Rat	M	8-10	2-3	2m	10d	6h/d	-	+	+	+	daily	-	-	Hi	Prot	WB	-
Rossi C, 2006	Mice		5-6	2-3	10w	8w	C	+	+	+	+	1/w	-	-	Hi	Prot	WB	+
Zhu SW, 2006	Mice	M	6	6	3w	4m	C	?	?	?	?	?	-	-	Hi (D-V)	Prot	WB	+
Gobbo OL, 2004;5	Rat	M	4	3-4	adult	10w	C	+	+	-	+	1/w	-	+	Hi (DG)	Prot	Elisa	+
Gresack JE, 2004	Mice	F	?	?	3w	7m	3h/d	+	-	-	+	daily	-	-	Hi	Prot	Elisa	-
Spires TL, 2004	Mice	?	?	?	4w	4m	C	-	+	-	+	3/w	-	-	Hi	Prot	WB	-
Guilarte TR, 2003	Rat	?	8	1	3w	4w	C	+	+	+	+	1/w	-	+	Hi	mRNA	IS-H	-
Hicks RR, 2002	Rat	M	6	1	3w	2w	C	-	-	-	+	3/w	-	-	Hi	mRNA	IS-H	-
Schneider JS, 2001	Rat	M	6	1	25d	10w	C	-	+	-	+	3/w	-	-	Hi (D)	mRNA	RT-PCR	+
Zhao L, 2000	Rat	M	8-10	4	3m	12-20d	C	-	-	-	+	3/w	-	-	CA2	mRNA	IS-H	-
Ickes B, 2000	Rat	M	6	1	8w	12m	C	+	+	+	+	2/w	-	-	Hi (V)	Prot	Elisa	+
Young D, 1999	Rat	M	6	1	3w	3w	C	+	+	-	+	-	-	-	Hi (MF)	Prot	Immuno	+
Falkenberg T, 1992	Rat	M	?	7w	7w	34d	C	?	?	?	?	?	?	?	Hi (D)	mRNA	IS-H	-

Table 7: Studies reporting conflicting results regarding BDNF gene expression following enriched environment

Animals: En/Ctrl, The number of animals per cage**. Program: Begin at,** the age at which enrichment started**; length,** the duration of housing**; Type,** continuous (**C**) or intermittent**; Enrichment design: RW,** Running wheel**; TNL,** Tunnel; **RMP**, Ramp; **TYS**, Toys; **CHE**, change toys; **LBH**, Labyrinth; **CPT**, compartment. **BDNF data: LOC,** Location or structure in which BDNF was measured**; State,** mRNA or Protein**; TCN,** technique used to detect the BDNF. Abbreviations: **M**, Male; **F**, Female; **w**, week; **d**, day; **m**, month; **C**, continuous housing; (**+**), presence;(**-**), absence; (D-V), dorsal and venral; (DG), Dentate Gyrus; (D), dorsal; (V), ventral; (MF), mossy fibers; (?), not known.

III.2. Enrichment induced brain plasticity

III.2.a Morphology and anatomy. This variable was the first to be observed on brain slides after housing in enriched environment by Diamond. She discovered a 6% increase in cortical thickness and precisely in the visual cortex of rats reared in enriched environment compared to impoverished environment (Diamond MC, 1964). Later, her group has noticed that the nerve cell bodies were farther apart in the thicker cortex of enriched environment rats than in the thinner cortex of isolated rats. It has been shown that more dendritic branches were the result of enhanced environmental stimulation (Holloway RL, 1966). Moreover, Greenough et al 1973 have found that more higher-order branches were present in the brain of rats reared in enriched environment and more lower branches in the brains of isolated rats (Greenough WT, 1973). A difference in the morphology of the dendritic spines was also observed in these higher order branches as has been reported by Diamond's group (Connor JR, 1982). While it has originally been observed that the increase in cortical thickness was restricted to caudal brain regions precisely to occipital region, Walsh has reported an increase in the hippocampal thickness of enriched rats (Walsh RN, 1969). This alteration of hippocampal thickness has not been confirmed, neither by Diamond's group on a large colony of rats (Diamond MC, 1976), nor more recently (Pereira LO, 2008). In 1982, it has been reported that environmental enrichment increased the brain weight and the number of granule cells in the dorsal hippocampus (Susser ER, 1982). Recently, several studies have validated that enriched environment increased the dendritic length, nodes, and the spines of pyramidal neurons either in the hippocampus or in the layer III of the cortex (Bindu B, 2007; leggio MG, 2005), the brain weight (kolb B 1991), the granule cell (GC) number and the volume of the GC layer (Kemperman G, 1997; Young D, 1999). For more details on enrichment induces structural plasticity see the review of Rampon (Rampon C, 2000c).

III.2.b Neurogenesis. The increase in neurogenesis after enriched environment exposure was seen whatever the age, the sex of animals and the duration of exposure to enrichment. ***Enrichment protocol starting at weaning.*** At postnatal day 21, C57BL/6 mice were housed in number of 12 in an enriched cage during 68 days. The detection of BrdU positive cells was realized either 1 or 28 days post BrdU injection for assessing proliferation and survival rate, respectively. While no difference was observed in the proliferation rate, the survival of BrdU positive cells was increased in the hippocampal granule cell layer (GCL). These cells co-localized with the mature neuronal marker calbindin D28k (Kempermann G, 1997). However, the same protocol realized this time on 129Svj mice has shown that proliferation rate is increased following enriched environment exposure (Kempermann G, 1998). In addition, by using the nuclear marker

of proliferating cells (PCNA), the increased proliferation was confirmed in Wistar rats housed in an enriched environment, in a number of 6 per cage during only 3 weeks (Young D, 1999). ***Enrichment protocol starting at 6 weeks or more.*** Many studies have provided evidence that housing mice at 10 weeks (Hattori S, 2007), 2 (Rossi C, 2006), 3 (Meshi D, 2006; van Praag H, 1999), 6, (Wolf SA, 2006; Kempermann G, 1998), 10 (Kempermann G, 2002), and 18 months (Kempermann G, 1998b) in enriched environment increased neurogenesis. This effect of increased neurogenesis was also observed in rats, when enriched environment started at 6 weeks (Auvergne R, 2002), 2 (Segovia G, 2006; Nilsson M, 1999; Bruel-Jungermann E, 2005) or 3 months (Leal-Galicia P, 2007). However, enriched environment had no effect on neurogenesis when it started at very old age (25 months) and lasted 8 weeks (Segovia G, 2006).

III.2.c Learning and memory. ***Morris water Maze (MWM).*** This spatial learning test is a powerful tool allowing the evaluation of cognitive function in rodents in particularly learning and memory, two mechanisms highly associated to the hippocampal function. Analysing 47 studies that have primarily used this test to assess whether EE exposure could alter the cognitive function has shown conflicting results (see fig 4).

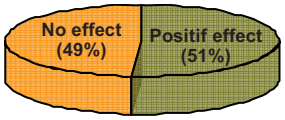


Figure 4: Effect of enriched environment on the latency to platform in the **Morris Water Maze** test reported in the literature (47 articles)

Some have provided evidence that enriched environment enhanced cognitive function of animals allowing them to find the hidden platform faster than animals housed either in social or isolated environments (see table 5). However, others failed to demonstrate any significant effect between animals reared in enriched environment and those reared in standard or isolated cages (see table 6).

III.2.d Anxiety-like behavior. ***Elevated Plus Maze (EPM).*** Analysing 17 articles that have used the EPM as a test to measure anxiety-like behavior has demonstrated in 12 studies a positive effect (dercreased anxiety) of enriched environment (Imanaka A, 2008; Abramov U, 2008; Leal-Galicia P, 2007; Iwata E, 2007; Pena Y, 2006; Imanaka A, 2006; Schneider T, 2006; Friske JE, 2005; Benaroya-Milshtein N, 2005; Hellemans KG, 2004; Chapillon P, 1999; Caston J, 1999). However in the 5 remaining studies, no effect has been observed (Gortz N, 2008; Zambrana C, 2007; Kobayashi K, 2006; Zhu SW, 2006; Martinez-cue C, 2005).

III.2.e Long Term Potentiation (LTP). Enriched environment has shown to enhance learning and memory in hippocampus-dependent task such as the Morris Water Maze and in other tasks such as the fear conditioning and the object recognition. The changes in neural function responsible for this improvement are poorly understood. It is believed that LTP is the mechanism underlying certain types of learning and memory in the hippocampus. It has thus been tested whether the beneficial effects of enriched environment exposure could be mediated through the modulation of LTP. The electrophysiological recordings to measure the LTP varied between studies. In hippocampal slices, some recorded the synaptic transmission either in perforant path-dentate gyrus synapses (Feng R, 2001; Foster TC, 2000; Cao X, 2008) or in the stratum radiatum of CA1 region (Huang FL, 2006; Yang JL, 2007; Li S, 2006; Cui MH, 2006; Artola A, 2006; Duffy SN, 2001). In freely moving animals, recordings were taken only from the dentate gyrus of the hippocampus (Irvine G, 2006; Bredy TW, 2003). In addition, one study was interested by the anterior cingulate cortex (Shum FW, 2007) because of its implication in higher order emotional responses and several forms of memory. Among all studies investigating the LTP in hippocampal slices, those recording from dentate gyrus showed no difference between enriched environment and controls groups (Feng R, 2001; Cao X, 2008; Foster TC, 1996; Foster TC, 2000). Nevertheless, those measuring from the CA1 region showed a significant increase in the LTP after enriched environment exposure compared to controls (Huang F, 2006; Cui MH, 2006; Artola A, 2006; Duffy S, 2001; Li S, 2006). Data recorded from layer II-III neurons in the ACC showed for the first time an induction of LTP in mice housed in enriched environment compared to controls (Shum FW, 2007). In freely moving animals, seven weeks of enriched environment exposure did not modify the LTP in the dentate gyrus of rats (Bredy TW, 2003). However, it has been shown that the nature of home cages plays a key role in determining the effect of enriched environment exposure on LTP induction: enrichment in plastic containers but not in metal cages, the former facilitating the natural range of exploratory behavior compared to the latter, increased the LTP in the dentate gyrus versus controls (Irvine GI, 2006).

III.2.f Neurotransmitters. Numbers of studies were interested to explore the enriched environment induced neurotransmitter alterations in different brain regions (Bownling SL, 1993; Naka F, 2002; Bezard E, 2003; Frick KM, 2003; Melendez RI, 2004; Neugebauer NM, 2004; Zhu J, 2004; Faherty CJ, 2005; Wagner AK, 2005; Zhu J, 2005; Segovia G, 2006; Del Arco A, 2007; 2007b; Dhanushkodi A, 2007; Galani R, 2007; Rahman S, 2008; Segovia G, 2008). ***GABA and its synthesis enzyme glutamate decarboxylase (GAD).*** The activity of GAD enzyme is increased in the hippocampus but not in the neocortex of male middle-aged (18 m) mice following exposure to enriched environment

compared to controls (Frick KM, 2003). However, the extracellular concentration of GABA in CA3 area of the hippocampus of young (2 m) and old (25 m) male Wistar rats remained unchanged after exposure to enriched environment (Segovia G, 2006). ***Glutamate.*** Basal extracellular concentration of glutamate following enriched environment exposure is age and region specific. While it only increases in the CA3 area of the hippocampus of old (25 m) male Wistar rats, it remains stable in the young animals (Segovia G, 2006). In the nucleus accumbens (Rahman S, 2008) and in the prefrontal cortex (Melendez RI, 2004) of immature Sprague-Dawley rats (21 d), basal extracellular concentration of glutamate remains unchanged. ***Acetylcholine ACh and its synthesis enzyme "choline acetyltransferase" (ChAT).*** This neurotransmitter was only investigated in the prefrontal cortex by microdialysis. No alteration was found in its basal concentration following enriched environment (Del Arco A, 2007; 2007b; Segovia G, 2008). In addition, protein level of ChAT examined in the hippocampus of Wistar rats using Western Blot technique revealed an insignificant increase compared to controls (Dhanushkodi A, 2007). ***Dopamine and its transporter DAT.*** Dopaminergic system has received the lion's share of attention. It plays a major role in mediating multiple aspects of cognition and is implicated in a wide variety of neurological and psychiatric disorders (Jaber M, 1996). In addition, DAT is primary in determining the extraneuronal concentration of dopamine and an important therapeutic target for neurostimulants. Thus, several researchers explored the modification of this system to enriched environments (Bownling SL, 1993; Naka F, 2002; Bezard E, 2003; Neugebauer NM, 2004; Zhu J, 2004; Faherty CJ, 2004; Wagner AK, 2005; Zhu J, 2005; Del Arco A, 2007b; Galani R, 2007; Segovia G, 2008; El Rawas R, 2009). While most of these studies did not show an alteration of extracellular or tissue dopamine content in different brain regions such as the prefrontal cortex (Neugebauer NM, 2004; Zhu J, 2004; Del Arco A, 2007; Segovia G, 2008), the frontal cortex (Naka F, 2002; Wagner AK, 2005), the frontoparietal cortex (Galani R, 2007), the parieto-temporal-occipital cortex, the pons/medulla oblongata (Naka F, 2002), the hippocampus (Galani R, 2007), the striatum (Bownling SL, 1993; Zhu J, 2004; Zhu J, 2005), and the nucleus accumbens (Bownling SL, 1993; Zhu J, 2004; Zhu J, 2005; El Rawas R, 2009), others noted a significant decrease in mRNA concentration of DAT in the striatum (Bezard E, 2003) and the substantia nigra pars compacta (Faherty CJ, 2004) of animals housed in enriched environment compared to controls. Moreover, enriched environment alters the function of DAT by decreasing the maximal velocity for [3H] DA uptake in medial prefrontal cortex compared to controls and by reducing its cell surface expression (Zhu J, 2004; Zhu J, 2005). ***Noradrenaline (NA) and serotonin (5-HT).*** While basal concentration of NA has been reported to increase in the dorsal hippocampus of rats housed in enriched environment, the basal concentration of 5-HT decreased in the ventral hippocampus.

Basal concentration of both NA and 5-HT remained stable in the frontoparietal cortex of rats raised in enriched environment (Galani R, 2007). While NA increased in the parieto-temporal-occipital cortex, the cerebellum and in the pons/medulla oblongata, the concentration of 5-HT remained unchanged in these structures (Naka F, 2002).

III.2.g Neurotransmitters receptors. ***5-HT receptors.*** Though few studies investigated the 5-HT receptor, it has been reported that the transcript (using HIS) and protein levels (using Western Blot) of the 5-HT2A receptor were not altered following enriched environment exposure in the hippocampus and the striatum area (Rasmuson S, 1998; Adriani W, 2006). However, the gene expression of 5-HT1A receptor was increased in the hippocampus of rats raised in enriched environment compared to controls (Rasmuson S, 1998). ***Dopamine receptors.*** Binding studies showed that Dopamine 1 (D1) and Dopamine 2 receptors were not altered neither in the striatum of mice after long period of enrichment (Glass M, 2004) nor in the nucleus accumbens after short period of housing in the enriched environment (Bardo MT, 1991). However, protein level assessed by immunolabeling revealed a decrease in D1 receptor in the prefrontal cortex of adult rats raised in enriched environment (Del Arco A, 2007b). ***GABA receptor.*** To our knowledge, the only study that explored the GABAA receptor reported no change in its transcript level in the striatum of mice reared in enriched environment (Glass M, 2004). ***Ionic glutamate receptors (AMPA).*** Whatever the method used to determine whether AMPA receptor was modified after housing in enriched environment, transcript and protein levels of GluR1, GluR2, GluR3 subunits were not changed in the hippocampus (Nithianantharajah J, 2008; Andin J, 2007; Gagne J, 1998), except one study reporting that mRNA of the subunits GluR2 and GluR4 were increased in the hippocampus of mice housed in enriched environment (Naka F, 2005). In addition GluR1 protein in the nucleus accumbens NAcc (Wood DA, 2005), in the forebrain (Tang YP, 2001), in the visual cortex (Restivo L, 2005), in the striatum and the frontal cortex (Nithianantharajah J, 2008) was not changed following enriched environment exposure. ***Ionic glutamate receptors (NMDA).*** No change in the gene expression of NR1 subunit in the brain (Guilarte TR, 2003; Toscano CD, 2006) or in the protein level in the nucleus accumbens (Toscano CD, 2006; Wood DA, 2005) and in the forebrain (Tang YP, 2001) was observed following enriched environment exposure. Concerning the NR2 subunit, the mRNA of the NR2A was increased in the right hemisphere (Bredy TW 2004) and in the hippocampus (Andin J 2007) after enriched environment exposure. While protein level of NR2A and NR2B subunits was increased in the forebrain (Tang YP, 2001), it did not change in the anterior cingulate cortex of mice raised in enriched environment (Shum FW, 2007). ***Metabotropic glutamate receptors.*** Protein levels of mGluR1/R5 and mGluR2/3 remained stable in the striatum of rats after enriched environment exposure. Conversely, alterations were seen

in the prefrontal cortex with an increase in the dorsal prefrontal cortex and no change in the ventral prefrontal cortex (Melendez RI, 2004).

III.2.h Cellular and molecular basis of enrichment-induced neuroplasticity.

Immediate Early Gene (IEG). IEG can alter cellular properties in response to extracellular stimuli. Some studies (Toscano CD, 2006; Ronnback A, 2005; Koh S, 2005; Pinaud R, 2001; Wallace CS, 1995; Olsson T, 1994) have investigated the influence of enriched environment exposure on some of these IEGs such as the neuronal activity-dependent transcription factors: Zif268 (also known as Egr-1, Krox-24, TIS8, NGFI-A or ZENK), Krox-20 (also known as egr-2), NGFI-B (also known as Nurr77, N10, TIS1, or TR3) and the activity-regulated cytoskeletal protein (arc).

Zif268. In general, mRNA of Zif268 is increased in the hippocampus (Toscano CD, 2006; Ronnback A 2005; Olsson T 1994), and in the visual cortex (Wallace CS, 1995) of animals reared in enriched environment. Using the in situ hybridization technique, the induction of Zif268 was region and study dependent. For example, Olsson and colleagues (Olsson T, 1994) have shown an increase in CA2 area, while Toscano and colleagues (Toscano CD, 2006) have reported an increase in CA3-CA4 and Ronnback and colleagues (Ronnback A, 2005) only in CA1 and not in CA3 area compared to controls. ***Krox-20.*** Concerning this IEG, an induction of its gene expression has been shown in the cortex (Ronnback A, 2005; Pinaud R, 2001), in the hippocampus using RT-PCR technique (koh S 2005) and an increase in CA3 area only when detecting its mRNA by in situ hybridization (Ronnback A, 2005). ***NGFI-B.*** One study has reported an increase in the transcript level of NGFI-B in CA1-CA2 (Olsson T, 1994) whereas another did not show any change in CA1-CA3 areas of the hippocampus (Ronnback A, 2005). ***Arc.*** One study has reported no change in the gene expression of arc in the hippocampus of animals housed in enriched environment (Toscano CD, 2006), while other studies have shown an increase in the hippocampus (koh S, 2005; Pinaud R, 2001; Olsson T, 1994), in the striatum and the cortex (Pinaud R, 2001).

Large scale analysis of transcriptomic and proteomic alterations. In a pioneer study using a gene chip array realized on the cortex of mice, it has been reported that the expression of genes whose products are involved in DNA/RNA synthesis, neuronal growth/structure, protease/cell death, protein processing and neuronal signaling are changed after short (3h) and long (14d) period of enriched environment exposure (Rampon C, 2000). Analysis of transcript levels realized this time on the striatum of mice has shown that the expression of 48 genes implicated in signal transduction, cell proliferation/differentiation, transcription and translation, structural rearrangements and metabolism are differently regulated, with 34 genes which are up-regulated and 14 down-regulated (Thiriet N, 2008). Finally, proteomic analysis assessed in the

hippocampus of rats has shown that proteins involved in the cytoplasmic organization and energy metabolism were the dominant classes to be altered (McNair K, 2007).

III.2.i Neurotrophic and growth factors. ***BDNF in the hippocampus.*** It is accepted that up-regulation of neurotrophic factors such as BDNF is a signature of the enrichment-induced plasticity in different brain regions (Ickes B, 2000) and notably in the hippocampus (Young D, 1999). Many studies have tried to correlate the induction of BDNF in the hippocampus with other plasticity markers as neurogenesis (Rossi C, 2006). Analysing very carefully the literature, we have observed that most of studies investigating the neurogenesis have focused on the dorsal hippocampus. However, the detection of BDNF in the dorsal hippocampus using in situ hybridization technique has not revealed any difference between animals reared either in enriched or standard/impoverished environments (Guilarte TR, 2003; Hicks RR, 2002; Zhao LR, 2000; Falkenberg T, 1992). A single study using a competitive RT-PCR technique has reported that exposure to enriched environment up-regulated the transcript level of BDNF in the dorsal hippocampus, notably in the Dentate Gyrus (see table 7). It is noteworthy that this result was obtained when rats were subjected to Morris water Maze test during the housing period (Schneider JS, 2001). Protein level of BDNF has also been assessed in the hippocampus using different methods like immunolabeling, Elisa and Western blot (Bindu B, 2007; Rossi C, 2006, Zhu SW, 2006, Gobbo OL, 2004; 2005; Ickes B, 2000; Young D, 1999). An increase in protein level of BDNF has been observed in the hippocampus using Elisa (Zhu SW, 2006; Gobbo OL, 2004; Gobbo Ol, 2005) western blot (Rossi C, 2006) and in the mossy fibers using density measurements of BDNF immunoreactivity (Young D, 1999). Finally, raising rats during 1 year in enriched environment increased BDNF level in the ventral hippocampus (Ickes B, 2000). ***BDNF in other brain regions.*** Transcript level of BDNF has been shown to increase in the striatum of mice housed in enriched environment using in situ hybridization technique (Bezard E, 2003), to remain unchanged using RT-PCR technique in the striatum and the cortex (Faherty CJ, 2005) and to decrease in the substantia nigra pars compacta (Faherty CJ, 2005). Protein level has been reported to increase in the cortex, basal forebrain, hindbrain (Ickes B, 2000), the cerebellum, prefrontal cortex, striatum (Dobrossy MD, 2006), occipital cortex (Sale A 2004), superior colliculus, visual cortex (Franklin TB, 2006) and retina (Franklin TB, 2006; Landi S, 2007). On the other hand, some studies have shown that BDNF level was not altered in the amygdala (Segovia G, 2008), the anteromedial cortex (Spires TL, 2004), the cerebellum, cortex (Kondo M, 2008), the frontal cortex (Chen X, 2005), the prefrontal cortex (Segovia G, 2008) and the striatum (Kondo M, 2008; Spires TL, 2004) following exposure to enriched environment.

Other factors. The gene expression of ***NGF*** (Torasdotter M, 1998; Schneider JS, 2001) and its protein levels (Pham TM, 1999; 1999b; Ickes B, 2000) has been reported to increase in the hippocampus after exposure to enriched environment. In addition, the protein level in the basal forebrain, the hindbrain (Ickes B, 2000), the visual cortex (Pham TM, 1999b; Torasdotter M, 1998) and the entorhinal cortex (Pham TM, 1999b) has been shown to increase and not to change in the cortex (Ickes B, 2000) of animals housed in enriched environment compared to controls. ***GDNF.*** After enriched environment exposure, tissue level of GDNF mRNA remained stable in the striatum (Faherty CJ, 2005), but protein level in the hippocampus (Young D, 1999) and mRNA level in the substantia nigra pars compacta (Faherty CJ, 2005) were up-regulated. ***NT-3***. Quantification of protein level by Elisa has shown an increase in NT-3 in the cortex, but not in the hippocampus (Ickes B, 2000). Transcript level of NT-3 in the visual cortex and the hippocampus increased or decreased in rats housed in enriched environment compared to isolated rats (Torasdotter M, 1996). ***IGF-1.*** Transcript level of IGF-1 has been reported to decrease in the substantia nigra pars compacta and the striatum (Faherty CJ, 2005). Protein level was up-regulated in the visual cortex (Ciucci F, 2007). ***VEGF.*** Its transcript level was induced in the hippocampus following enriched environment compared to standard housing condition (Cao L, 2004; Navarro FP, 2008).

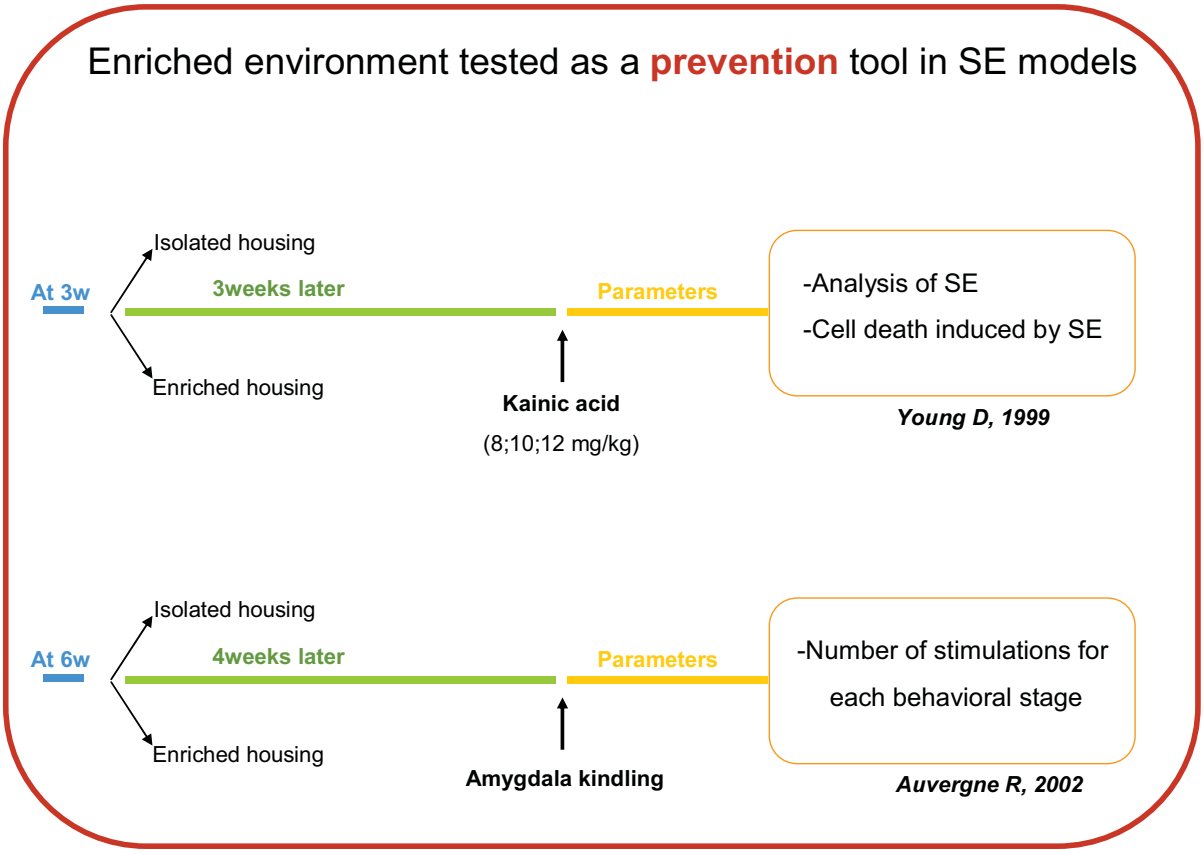
III.3. Enrichment and seizures.

Although a wealth of information is available on the effect of enriched environment in conditions such as stroke, Parkinson's, Alzheimer's and Huntington's diseases, only few studies have reported the effects of different housing conditions on seizure development and its consequences. Some were interested to know whether enriched environment could modulate the response to brain insults consecutive to neurotoxin-induced seizures (Young D, 1999), or kindling development (Auvergne R, 2002). Other studies were conducted in order to discover the therapeutic effect of enrichment on cognition and behavior following the neurotoxin-induced brain damage and status epilpeticus (Faverjon R, 2002; Rutten A, 2002; Koh S, 2005, Koh S, 2007, Wang CA, 2007).

III.3.a Enrichment as a prevention tool against epileptogenesis. Study by Young et al 1999 using Kainic Acid. Male wistar rats were reared at weaning either in impoverished (isolated) or enriched condition for 3 weeks. Enriched environment consisted of housing 6 rats in a large cage containing a running wheel, tunnels, toys, a maze, a bar-pressing food administration station and access to corn snacks. After a 3 week period of either impoverished or enriched housing, rats received an injection of kainic acid at the dose of 8mg/kg. Fifty percent of rats reared in the impoverished condition developed "wet-dog shakes" (WDS), compared to 17% only for the rats raised

in the enriched condition. In addition, while none of the rats of the enriched condition showed motor seizures, those housed in the impoverished condition and which exhibited WDS developed motor seizures, and one of these rats progressed toward SE (i.e. 17% of the rats housed in the impoverished condition). When a suprathreshold dose of kainate (12mg/kg) was used, 83% and 60% of rats reared in the impoverished and enriched condition, respectively, developed a sustained SE. Histological markers showed less cell death in area CA3 and the hilus of the hippocampus in rats reared in the enriched condition compared to those reared in the impoverished condition, suggesting that EE produces resistance not only to seizures but also to excitotoxic injury.

Study by Auvergne et al 2002 using amygdala kindling. Male wistar rats were reared at 6-week-old either in impoverished (isolated) or enriched condition for 4 weeks before implanting stimulating electrodes in the right amygdala. Kindling development was delayed in rats housed in enriched condition compared to rats raised in isolated conditions, suggesting that enriched housing may inhibit epileptogenesis.



III.3.b Enrichment as a rehabilitation tool after chemically-induced SE. Two studies by Holmes' group (Rutten et al 2002; Faverjon et al 2002) using pilocarpine. In these 2 studies, the authors used a Lithium-pilocarpine to induce SE. The protocol consisted of injecting rats with 3 mEq/kg lithium chloride (i.p.) at 19 days after birth, followed by 60 mg/kg pilocarpine (s.c.) the day after. No drug was administered to stop SE at a precise time. At P55, substantial cell loss was evident in the hilus and areas CA1 and CA3 of the hippocampus. In addition, rats demonstrated a cognitive impairment in the Morris Water Maze (MWM) task, compared to naïve rats.

This model was then used in order to evaluate the effect of enriched housing on the recovery of cognitive function and the protection of cell loss. Rats were subjected to an intermittent enrichment protocol for 30 days: they were housed in groups of 9 for 4 hours a day in a large cage including toys, auditory stimuli (Mozart's piano concerts), mirror and a running treadmill. No details are provided regarding the non-enriched condition. Although this enrichment protocol failed to protect cell loss, it improved the learning and memory performances in the MWM test. However, we should mention that this improvement of cognitive function was not complete since naive enriched rats performed better in the MWM test than enriched rats subjected to SE.

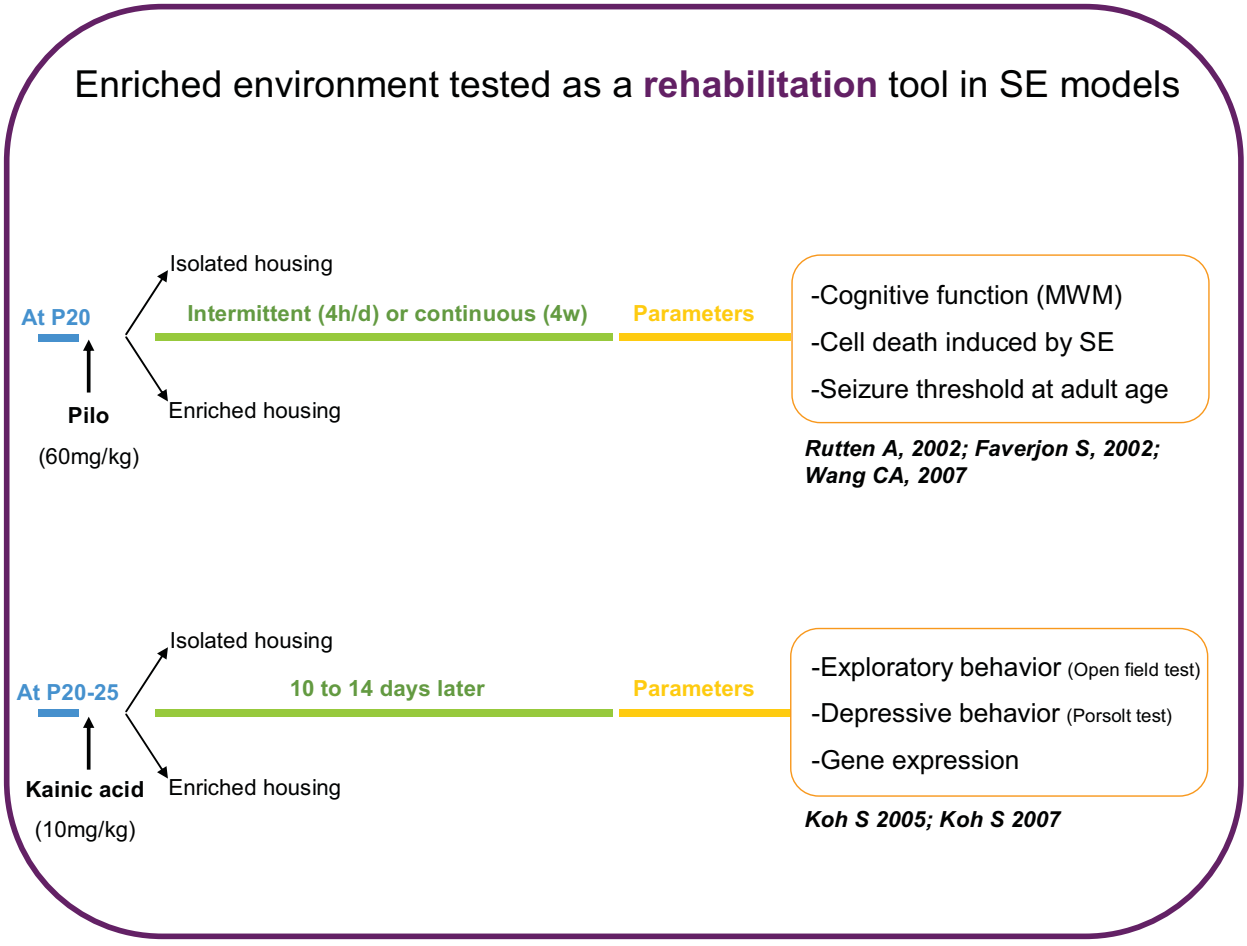
Two studies by Koh et al 2005/2007 using the Kainic Acid. Male Long Evans rats were injected with kainic Acid (10mg/kg) at days 20-25 after birth and housed either in group of 8 to 10 in an enriched environment or singly (isolated) in standard cages. The enriched cage is similar to that of Holmes' group. Seven days after housing, rats were tested in the open field during 5 minutes. Exploratory behavior of isolated rats that experienced SE was decreased compared to rats housed in EE. Moreover, 14 days after exposure to enriched conditions, rats subjected to SE exhibited less depression-like trait than their counterparts raised in isolated conditions, as assessed in the Porsolt test. Finally, genes involved in synaptic plasticity and memory consolidation (Arc, Homer1a, Egr1) were increased in rats subjected to Kainate-induced SE and exposed to enriched housing, compared to their counterparts raised in isolated conditions.

Study by Wang et al 2007 using pilocarpine. This study followed the same protocol as the one reported by Holmes and colleagues, and confirmed that cognitive function, assessed in the MWM, is protected when rats are housed in enriched conditions. However, no difference in seizure threshold was found between rats housed in enriched and isolated groups, determined at the adult stage after pentylenetetrazol injection.

III. 4. Enrichment and other models of brain impairment.

The beneficial effects of housing animals in enriched environment has been tested and well documented in different syndromes and diseases as reported in the review of Laviola (Laviola G, 2008). In models of Alzheimer's disease, enriched environment prevented the

onset of memory deficits and restrained the progression of neurodegeneration with the association of increasing neurotrophic factors (Berardi N, 2007; Wolf SA, 2006). In model of Parkinson's disease, mice raised in an enriched environment were shown to be more resistant to MPTP compared to mice housed in standard condition and to have reduced cell death of dopaminergic neurons in the substantia nigra pars compacta (Faherty CJ, 2005; Bezard E, 2003). Positive effects were also observed in the Huntington's disease by rescuing protein deficits and improvement of motor symptoms (Spires TL, 2005), in brain trauma by attenuating learning deficits (Hicks RR, 2002), in Rett's syndrom by improving the motor coordination (Kondo M, 2008), in lead toxicity by protecting against the behavioral and neurochemical alterations induced by lead exposure (Schneider JS, 2001), after cranial irradiation by ameliorating the functional recovery on the rotarod test (Fan Y, 2007), in the cocaine model of addiction by reversing addiction (Solinas M, 2008) and reducing reinstatement induced by cues and stress (Chauvet C, 2009), by reducing the behavioral, neurochemical, and molecular effects of cocaine (Solinas M 2009) and by enhancing the recovery of the locomotor activity in the open field test (Bezard E, 2003).



Results

RESULTS – PART I

TITLE :

STANDARDIZED ENRICHMENT PROCEDURES IN THE MARLAU™ CAGE SUPPORT ENHANCED BRAIN PLASTICITY IN HEALTHY RATS AND PREVENT COGNITIVE IMPAIRMENT IN EPILEPTIC RATS

PUBLICATION STATE :

Submitted

SPECIFIC AIMS :

1. Develop a new cage (Marlau™ cage) to standardize enriched housing for rodents.
2. Demonstrate that it reproduces the main effects reported so far of environmental enrichment in rodents.

MAIN RESULTS :

- Rats housed in Marlau™ cages had increased : cortical thickness (primary motor cortex and primary somato-sensory cortex), hippocampal neurogenesis, and levels of transcripts encoding TrkB.FL, IGF-1, Epo and EpoR in the dorsal hippocampus, and BDNF, TrkB.FL, TrkB.T1, TrkB.T2, IGF-1, VEGF, Epo and EpoR in the ventral hippocampus.
- Rats housed in Marlau™ cages exhibited better performances in spatial learning, decreased anxiety, and better recovery of basal plasma corticosterone level after acute restraint stress, likely due to increased expression of glucocorticoid receptors in the hippocampus.
- Rats housed in Marlau™ cages after severe *status epilepticus* at weaning had no learning impairment compared to rats subjected to the same insult and housed in conventional cages once they developed behavioral spontaneous recurrent seizures.
- All rats with behavioral spontaneous recurrent seizures exhibited an increased anxiety-traited compared to their respective controls, but anxiety was less severe in rats raised in Marlau™ cages compared to those raised in conventional cages.

➔ All of the beneficial effects of environmental enrichment reported so far in the literature have been found to be reproduced in rats housed in the Marlau ™ cage. Thus, this cage, by providing standardized enrichment procedures for rodents during housing, should facilitate transfer of reproducible programs of environmental enrichment across laboratories.

Standardized enrichment procedures in the Marlau™ cage support enhanced brain plasticity in healthy rats and prevent cognitive impairment in epileptic rats

Raafat P. Fares[1-4]*, Pascal E. Sanchez[1-4]*, Hayet Kouchi[1-4], Jacques Bodennec[1-4], Harry Koubi[1-3], Marion Le Cavorsin[1-4], Béatrice Georges[1-4], Colette Moulin[1-4], Chantal Bonnet[1-4], Sandrine Bouvard[1,2,4], Anne Morales[1-4], Amor Belmeguenai[1-4], Robert S. Sloviter[5], Laurent Bezin[1-4]

1. University of Lyon, F-69003 Lyon, France;
2. University Lyon 1, F-69003 Lyon, France;
3. Centre National de la Recherche Scientifique, UMR 5123, Integrative Cellular and Molecular Physiology Lab., 43 bd du 11 novembre 1918, F-69622 Villeurbanne cedex, France;
4. CTRS-IDEE, F-69003 Lyon, France;
5. Department of Pharmacology, University of Arizona, Tucson, AZ 85724 USA.

* These authors contributed equally to the study.

Abstract

Environmental enrichment (EE) during housing of laboratory animals is known to induce structural brain plasticity, stimulate neurogenesis, increase neurotrophic factor expression and protect against brain insults. However, these positive effects are not constantly observed, likely because standardized procedures of EE are lacking. Therefore, we engineered an enriched cage (the so-called Marlau™ cage) offering (i) reduced stressful social interactions, (ii) increased voluntary exercise, (iii) diverse entertaining activities, (iv) cognitive stimulation (maze exploration), and (v) novelty (maze configuration changed three times a week). The maze, which separates food pellet and water bottle compartments, guarantees cognitive stimulation for all animals. Compared to rats raised in groups in conventional cages, we report that rats housed in Marlau™ cages have increased cortical thickness, hippocampal neurogenesis and hippocampal levels of transcripts encoding various genes involved in tissue plasticity and remodelling. In addition, rats housed in Marlau™ cages exhibit better performances in spatial learning, decreased anxiety-trait, and better recovery of basal plasma corticosterone level after acute restraint stress. We also show that Marlau™ cage ensures inter-experiment reproducibility in spatial learning and brain gene expression assays. Finally, housing rats in Marlau™ cages after severe *status epilepticus* at weaning prevents the cognitive impairment observed in rats subjected to the same insult and then housed in conventional cages. In conclusion, Marlau™ cage, by providing standardized enrichment procedures for rodents during housing, should facilitate transfer of reproducible programs of EE across laboratories.

INTRODUCTION

Experimental models of human diseases using living animals are the main resources available so far to approach what might be the etiology and the progression of a disease, and to test the effect of potential therapeutics. In joint research programs, multidisciplinary approaches may be required, involving diverse laboratories and research groups sometimes located at long distances from each other. In that case, distinct animal facilities are needed to perform research in each location. In this frame, standardization of housing procedures is a fundamental requirement, aimed at producing, in distinct places, "equivalent" animals, i.e. with similar steady states, symptom development and treatment response, allowing consequently the compilation of all results for further integrative analysis. Unfortunately, a remarkable study has revealed that current standard housing conditions in animal facilities are not satisfactory to produce animals with similar behaviors in three different sites despite rigorous efforts to equate test apparatus, testing protocols and all possible features of animal husbandry (1). Similarity of results between three different sites could be attained when animals were housed in enriched cages (2), offering a combination of complex inanimate and social stimulation (3). This type of housing conditions defines what is usually termed "enriched environment" or "environmental enrichment" (EE) (4, 5).

While encouraging at reducing inter-experiment variability (2), important inconsistency has been observed by different research groups using EE paradigms also, as exemplified by performance measurement in the Morris Water Maze used to test spatial learning and memory (see table 1). At the present time, these discrepancies are difficult to explain. They may be caused by the use of variant controls, which can consist of animals living either in individual or group cages. A lack of accurate description of the enrichment protocols in publications (4), a pronounced variability in the enrichment protocols used by different investigators (6) and the possibility that all animals housed together are not exposed equally to the features characterizing the EE protocol used may be other sources of variability across studies.

Therefore, in order to circumvent some of the interpretational problems inherent in the use of various enrichment protocols, we developed the "Marlau™" cage to standardize the procedures of EE for rodents. The enrichment program offered by this cage is bereft of toys and has been inspired from the training program in the original "environmental complexity and training" (ECT) paradigm. Training in ECT consisted of daily exploration in mazes with pattern of barriers changed frequently (7). Marlau™ cage includes a series of mazes, the configuration of which is changed regularly, thus supporting complexity and novelty. In addition, we ensured that all animals were exposed to maze training by placing mazes between food pellet and water bottles compartments. We provide evidence that housing in Marlau™ cages meets the criteria of enrichment-induced beneficial effects on brain function in physiological and pathological conditions.

MWM test	References during the 1993-2008 period	Total : 49
No effect of environmental enrichment on the different variables analyzed	Boehm G.W. 1996. Brain Res, 726: 11-22.; Briones T.L. 2006. Experimental Neurology, 198: 530-8.; Briones T.L. 2006. Behavioural Brain Research, 168: 261-71.; Cao X. 2008. Dev Psychobiol, 50: 307-13.; Caston J. 1999. Dev Psychobiol, 35: 291-303.; Comeau W. 2008. Dev Psychobiol, 50: 134-46.; Costa D.A. 2007. Neurobiol Aging, 28: 831-44.; Cui M. 2006. Neurosci Lett, 404: 208-12.; Gobbo O.L. 2004. Behav Brain Res, 152: 231-41.; Gobbo O.L. 2005. Behav Brain Res, 159: 21-6.; Hamm R.J. 1996. J Neurotrauma, 13: 41-7.; Hicks R.R. 2002. Neuroscience, 112: 631-7.; Leggio M.G. 2005. Behav Brain Res, 163: 78-90.; Mandolesi L. 2008. J Alzheimers Dis, 15: 11-28.; Martinez-Cue C. 2005. Behav Brain Res, 163: 174-85.; Miu A.C. 2006. Behav Brain Res, 172: 135-44.; Passineau M.J. 2001. Exp Neurol, 168: 373-84.; Prusky G.T. 2000. Behav Brain Res, 114: 11-5.; Puurunen K. 1997. Stroke, 28: 623-31.; Schneider J.S. 2001. Brain Res, 896: 48-55.; Ueda S. 2005. Neuroscience, 135: 395-402.; van Rijzingen I.M. 1997. Neurobiol Learn Mem, 67: 21-8.; Wainwright P.E. 1993. Neurotoxicol Teratol, 15: 11-20.; Wang C.A. 2007. Epilepsy Behav, 11: 303-9.; Wurm F. 2007. Stroke, 38: 2833-40.	25 / 49 (51%)
Decreased latency and/or distance to platform in animals raised in enriched environments	Bennett J.C. 2006. Neurobiol Learn Mem, 85: 139-52.; Bredy T.W. 2003. Neuroscience, 118: 571-6.; Falkenberg T. 1992. Neurosci Lett, 138: 153-6.; Fan Y. 2007. Eur J Neurosci, 25: 38-46.; Faverjon S. 2002. Neurology, 59: 1356-64.; Foley A.G. 2005. Journal of Neuroscience Research, 82: 245-54.; Frick K.M. 2003. Neurobiol Aging, 24: 615-26.; Galani R. 1997. Neurobiol Learn Mem, 67: 43-56.; Giza C.C. 2005. Behav Brain Res, 157: 11-22.; Guilarte T.R. 2003. Ann Neurol, 53: 50-6.; Hellemans K.G. 2004. Brain Res Dev Brain Res, 150: 103-15.; Holguin S. 2008. Behav Brain Res, 191: 11-6.; Huang F.L. 2006. J Neurosci, 26: 6230-7.; Jankowsky J.L. 2005. J Neurosci, 25: 5217-24.; Kempermann G. 1997. Nature, 386: 493-5.; Meshi D. 2006. Nat Neurosci, 9: 729-31.; Nilsson M. 1999. J Neurobiol, 39: 569-78.; Paylor R. 1992. Behav Brain Res, 52: 49-59.; Pham T.M. 1999. Behav Brain Res, 103: 63-70.; Schrijver N.C. 2002. Pharmacol Biochem Behav, 73: 209-24.; Schrijver N.C. 2004. Behav Brain Res, 152: 307-14.; Toscano C.D. 2006. Exp Neurol, 200: 209-15.; Wolff M. 2008. Hippocampus, 18: 996-1007.; Zanardi A. 2007. Faseb J, 21: 4028-37.	24 / 49 (49%)

Table 1 : Discrepancies in the effect of environmental enrichment on navigational/spatial-temporal memory test (Morris water Maze test) in articles published during the last 15 years (1993-2008 period).

MATERIALS AND METHODS

All animal procedures were in compliance with the guidelines of the European Union (directive 86/609), taken in the French law (decree 87/848) regulating animal experimentation. All efforts were made to minimize the number of animals used.

Animals

Male Sprague-Dawley rats (Harlan, Gannat, France) were used throughout the experiments. Pups arrived at 15 day-old with foster dams, maintained in groups of 10 in plastic cages and had free access to food and water. At day (d) 21, rats were weaned and housed either in standard (S) cages (type "E", length 405 mm, depth 255 mm, height 197 mm, total exploration surface = 800 cm^2; Charles River, France) or in enriched (En) cages (Marlau™ cage, described below) for up to 13 weeks. Animals were housed in groups of 6 in "S" cages and in groups of 12 in "En" cages in approved facilities, at 21°C under diurnal lighting conditions (lights on from 06:00 to 18:00). In both "S" and "En" cages, bedding material was changed once a week on Fridays at 16:00 and was made of aspen wood: half of Litaspen Premium 6 and half of Litaspen Premium 8/20. All rats were fed using the same pellets (type A04, Safe). All animals were weighed twice each week, and removed from their cage when bedding material was changed.

Marlau™ cage

This cage was engineered in the laboratory to standardize procedures of enrichment for rodents (Bezin et al., patent # FR09/0054). Two cage dimensions exist, a large one for rats, described below and used in this study (Fig. 1), and a smaller one designed for mice. Marlau™ cage provides welfare by introducing crucial features of the wild, such as novelty, complexity, and physical activity. The internal dimensions (height, width, depth) of the cage for rats are: 510 mm, 800 mm, 600 mm. The cage is divided into 2 floors. The ground floor (G) is 300 mm high and is divided into 2 sections including one section (G1: 296 mm x 600 mm) with food pellets, and another (G2: 496 mm x 600 mm) with 3 water bottles. G2 also contains a red rectangular house (height, width, depth: 100 mm, 400 mm, 200 mm) with 4 lateral windows and 2 opened extremities) and 3 running wheels. G2 is connected to the upper floor via a ramp. The upper floor, which contains a maze, is connected to G1 via a tunnel. When rats are in G1, they can access to G2 using 2 one-way opening doors. Exposure to complexity is assured by imposing all rats to go through the maze once they are in G2 to reach food. Physical activity is encouraged by the enlarged exploration area (9-fold greater than in "E" cages) and free access to running wheels. Finally, increased social interactions are promoted by the possibility to house together a larger number of animals (12 rats / cage). At each change of the bedding material, paper wool and aspen wood wool were added in G1 and G2, respectively. Standardization of novelty is assured by changing the maze configuration 3 times a week (Monday, Wednesday and Friday) at 16:30, using 6 mazes (A-F), each offering 2 different configurations (1 and 2; e.g. A1 and A2 for maze A). Maze changes are made in a defined order to offer reproducibility across experiments (starting with the series of A1, B1, C1, and ending with the series of D2, E2, F2). After F2 configuration has been used, another series is restarted from A1. When the terms "A1" or "D2" are employed, it

signifies that the side 1 of the maze A, or the side 2 of the maze D, is positioned facing the ramp, respectively. To avoid territorial dominance, rats can enter and exit the maze using 2 gates on each side.

Experimental design of animal studies

Experiment 1. *Overall physiological status: body weight, food intake and stress coping.* In this experiment, 12 rats were housed in one Marlau™ cage and 12 rats in two standard cages (2 x 6) for a period of six weeks. All rats were weighed twice each week, and food pellets were weighted once a week in each cage at bedding material change. In order to investigate the impact of housing condition on stress coping ability, plasma corticosterone (CORT) level was measured in basal conditions and in response to restraint stress in 10 randomly chosen rats (with 5 in each S and En groups). The 7 remaining rats in each group were used to assess hippocampal glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) transcript level, and total lipids in the whole body (without brain tissue).

Experiment 2. *Histological and biochemical characterization of the enrichment procedures using the Marlau™ cage.* Four weeks after weaning, 4 of the "En" rats, and 2 "S" rats in each of the 2 standard cages were injected with BrdU, as described below. The other rats received vehicle instead. Two weeks later, rats injected with BrdU (8 in total) were prepared for histological analysis (hippocampal neurogenesis, cortical thickness). The other rats (n=8 in each S and En groups) were used to assess gene expression in the hippocampus (Hi) at transcript level using RT-real time PCR (RT-qPCR).

Experiment 3. *Testing the repeatability of the results obtained in Marlau™ cage.* The experiment has been repeated twice: Exp.3a and Exp.3b, with 12 rats of each S and En conditions in each experiment (n=48 in total). Rats were subjected at 6 weeks to the Morris Water Maze test (MWM), and then sacrificed two weeks later to assess gene expression in the ventral hippocampus at transcript level using RT-qPCR.

Experiment 4. *Longitudinal analysis of exploratory behavior.* After weaning, rats (n=24, with 12 in each S and En groups) were subjected to three different tests, as described below, performed at a different developmental ages: Water Exploration Test (WET), Black/White/Black Box test (BWB), and finally Elevated Plus Maze test (EPM) at 4, 6 and 15 week-old, respectively.

Experiment 5. *Testing the protective effect of Marlau™ cage against cognitive impairment in epileptic rats*. At weaning, rats were subjected to Pilocarpine-induced *status epilepticus* (Pilo-SE) and were housed, 1 day later, in standard or Marlau™ cages. Control and rats subjected to Pilo-SE were housed in separate cages. Cognitive performance was assessed at 15 and 16 weeks using the WET and the MWM, respectively, in rats that developed behavioral spontaneous recurrent seizures (BSRS) and controls.

In vivo Procedures

BrdU administration. At 7 weeks (4 weeks after weaning), rats received 5 consecutive injections of 5'-bromodeoxyuridine (BrdU; Sigma B5002, 50 mg/kg, i.p.), at 17:00 the first day, and at 12:00 and 17:00 the two following days, as previously described (8). Rats were sacrificed two weeks later.

Acute restraint stress protocol. After 6 weeks of housing conditions, rats (n=5 in each S and En groups) were placed in well-ventilated plexiglas restraint tubes for 30 min. Animals were anesthetized with

halothane and blood samples (250-300µL) were collected retro-orbitally in EDTA tubes (BD Microtainer K_2E) to determine CORT level. Samples were collected at basal level, at the end of restraint stress, and after 2 hours of recovery. Blood samples were centrifuged for 5 min at 10 000 rpm and plasma was kept at -20°C.

Morris Water Maze (MWM). A circular tank (180 cm diameter, 60 cm high) was filled with water maintained at 25°C to a depth of 40 cm. The water was made opaque by addition of black gouache, which prevented visualization of the platform by the rat. All visual clues around the room were kept in a constant location from day to day. The pool was divided into 4 virtual quadrants defined as North (N), East (E), South (S), and West (W). A circular platform (10 cm diameter) was hidden 1 cm below the surface of the water, and was kept at a constant position within the northern quadrant, close to the NW border. Rats were tested for a total of 16 trials performed during 4 consecutive days. Each day, rats were subjected to 4 trials, at 2-hour intervals, each trial lasting 90 sec. By a camera positioned on the ceiling just above the center of the tank, rats were videotracked using a software, which provided the distance and the time to find the platform. Animals were introduced into the water facing the wall of the pool, at a position that was changed at each trial as follows: 1^{rst} day, ESWE; 2nd day, SWES; 3rd day, WESW; 4th day, ESWE. When rats found the platform, they were allowed to stay onto it for 15 sec. When they did not find the platform, they were placed onto it for 15 sec. Investigators were not allowed to stay in the room during the tests.

Water Exploration Test (WET). The tank used for the MWM was divided in four identical quadrants (6,358 cm^2 each) using two 180 cm long opaque plastic separators. Four rats were tested for 5 min in the same time, but in different quadrants. The VideoTrack system provided the time, the distance, and the velocity of exploration within the full quadrant, and within a virtual central zone of 2,921 cm^2 (Fig. 8.A.a).

Black/White/Black box test (BWB). This test was inspired of the Dark/Light box test (9). The box (Fig. 8.B.a) consisted of two black compartments of different size, 800 cm^2 and 600 cm^2, separated by a larger white compartment (1,000 cm^2). Rats were always introduced in a same corner of the large black compartment. Animals had free-access to the white compartment. A gate, easily removable by the investigator, prevented access to the small black compartment from the white compartment. On the first day of the test, rats had only access to the large black and the white compartments. On the second day of the test, rats had access to the three compartments. Test duration was 90 sec on both days. The VideoTrack system provided the time spent and the distance spent in each compartment.

Elevated Plus Maze test (EPM). The maze (Fig. 8.C.a) consisted of two open arms (OA) (50x15 cm) perpendicularly positioned to two arms (50x15 cm) closed by 40-cm high walls. The maze was elevated 60 cm above the floor. Rats were isolated 30 min in a standard cage (type "E") before the test. Each animal was placed for 5 min in the central platform (15×15 cm), facing the same OA. The maze was cleaned with tap water and dried after each trial to eliminate possible odour cues left by previous rats. All rats were video monitored and the following variables were measured: the number of entries, the time spent and the distance travelled in each arm.

Entries were considered each time rats positioned their four paws into a new arm.

Pilocarpine-induced *status epilepticus*. On day 21, pups (n=24) were injected with lithium chloride (Sigma, 3 mEq/kg, i.p.) freshly dissolved in saline. Eighteen hours after, rats were given scopolamine (Sigma, 1mg/kg s.c.) followed, 40 min later, by pilocarpine hydrochloride (Sigma, 25 mg/kg, i.p.). After 30 min of continuous behavioral SE, a single injection of diazepam (Valium®, 10 mg/kg, i.p; Roche) was administered to terminate behavioural seizures. Controls received 4 saline injections instead of pharmacological compounds. Sixteen rats survived to Pilo-SE, and were randomly distributed in two standard cages (n=4 in each) and one Marlau™ cage (n=8). Equal numbers of control rats (rats not subjected to Pilo-SE) were then housed similarly.

Ex vivo procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (250 mg/kg) before being sacrificed. For biochemical analysis, brain structures were rapidly microdissected, frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry analysis, animals were transcardially perfused (30 mL/min) with chilled 4% paraformaldehyde in 0.1M phosphate buffer. After cryoprotection in 25% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

Total body fat determination. After brain removal, the rest of the body was weighted and digested in hot 30% KOH. Aliquots (5g) of the saponified samples were neutralized with concentrated HCl; lipids were then extracted and washed as previously described (10). Briefly, the lipids were extracted overnight at 4°C in chloroform-methanol (2:1, v/v) and washed twice by addition of 0.25 % (w/v) aqueous KCl solution. The chloroform lower phase was evaporated to dryness until the mass of lipids remained constant. Total lipid content was expressed as the percentage of lipid mass / rat body weight.

Quantitative determination of CORT using Elisa. CORT was extracted from plasma samples and measured using an Elisa kit (Neogen Corporation) following manufacturer's instruction.

Quantitation of transcript level variations by RT-qPCR. Variations in transcript levels were determined by real time PCR amplification of cDNAs of interest after reverse transcription (RT) of total mRNAs, as previously detailed (8, 11-13). A synthetic external and non-homologous poly(A) Standard RNA (SmRNA) was used to normalize the reverse transcription of mRNAs of biological samples (Morales and Bezin, patent WO2004.092414). Sequences of the different primer pairs used are: ***BDNF*** (GenBank accession no. X67108) forward 5' AAA TTA CCT GGA TGC CGC AA 3', reverse 5' CGC CAG CCA ATT CTC TTT TT 3' (345 bp); ***Epo*** (GenBank accession no. NM_017001) forward 5' GCT CCA ATC TTT GTG GCA TC 3', reverse 5' ATC CAT GTC TTG CCC CCT A 3' (66 bp); ***EpoR*** (GenBank accession no. D13566) forward 5' CCA GCT CTA AGC TCC TGT GC 3', reverse 5' CTT CAG GTG AGG TGG AGT GG 3' (68 bp); ***GR*** (GenBank accession no. AY293740) forward 5' CAC AAG CAA TGT GCA G 3', reverse 5' AAG TGA AAC GGC TTT GGA TAA G 3' (103 bp); ***IGF-1*** (GenBank accession no. NM_178866.2) forward 5'ATG CCC AAG ACT CAG AAG GA 3', reverse 5' CGT GGC ATT TTC TGT TCC TC 3' (110 bp); ***MAP-2*** (GenBank accession no. NM_013066) forward 5' GTG TTA AGC GGA AAA CCA CAG 3', reverse 5' GAC TTT GTC CTT CGC CTG TT 3' (80 bp); ***MR*** (GenBank

accession no. M36074.1) forward 5′ TGA AGG TTT TGC TGC TAC TAA GC 3′, reverse 5′ TGT AAT TTG TCC TCA TCT CCT CAA 3′ (84 bp); ***TrkB.FL*** (GenBank accession no.M55291) forward 5′ TGA AGA CGC TGA AGG ACG CCA 3′, reverse 5′ CAG GTT CTC TCC TAC CAA GCA 3′ (353 bp); ***TrkB.T1*** (GenBank accession no. M55292) forward 5′ CTG GAT GGC TAG CTG AGA TAA AGG A 3′, reverse 5′ AGT CAC AGC TCA CAA CAA GCA GGC T 3′ (187 bp); ***TrkB.T2*** (GenBank accession no. M55293) forward 5′ TAC TCA GCC TTG CCC ACT TT 3′, reverse 5′ GCC ATA ACA TAT CCT TGC CC 3′ (162 bp); ***VEGF*** (GenBank accession no. NM_031836) forward 5′ CCT GTG TGC CCC TAA TGC 3′, reverse 5′ AGG TTT GAT CCG CAT GAT CT 3′ (107 bp).

Fluorescent detection of BrdU-positive cells in the hippocampus. Free-floating coronal cryostat-cut sections (40 µm thick) were mounted on SuperFrost®Plus slides and air dried. After DNA denaturation for 30 min in 2M HCl at 65°C and neutralization in borate buffer pH 8.5, sections were incubated overnight at 4°C with a rat monoclonal antibody raised against BrdU (OBT-0030; Oxford Biotechnology) diluted at 1/25, and then with an Alexa-488-conjugated donkey anti-rat IgG antibody (A21208; Molecular Probes) diluted at 1/1000. In dual immunolabelings, astroglial marker GFAP or neuronal marker NeuN was detected together with BrdU, using mouse monoclonal antibodies raised against GFAP (G3893, Sigma) and NeuN (MAB377, Chemicon) diluted at 1/1000, respectively. In this case, anti-BrdU antibody was detected as above, and mouse monoclonal antibodies raised against GFAP or NeuN were recognized using an Alexa-633-conjugated goat anti-mouse IgG antibody (A21052; Molecular Probes) diluted at 1/2000. Sections were then analyzed at the same conditions of photomultiplier gain, offset and pinhole aperture using a TCS SP2 confocal microscopy system (Leica), allowing the comparison of fluorescence intensity between regions of interest. Images were finally imported into Adobe Photoshop 8.0.1 (Adobe Systems) for further editing.

Morphological analysis

Measurement of cortical thickness and hippocampus volume. After sections were Nissl-stained, images were captured with a video camera 3CCD (DXC-9300; Sony) coupled to an image analysis system (Visilog 6.3; Noesis). Cortical thickness was measured as shown (Fig. 3A) at IA 9.70 mm and 5.40 mm (14). The surface area of the whole dorsal hippocampus was measured from IA 5.86 mm to 4.70 mm.

BrdU-positive cell counting. For each brain, we analyzed three sections containing the dorsal hippocampus, selected at IA 5.70 mm, 5.40 mm and 5.10 mm (14). The granule cell area was traced using an image analysis system (Visilog 6.3; Noesis). The number of BrdU-labeled cells was counted in this delimited area for all sections by an investigator blind to the group (S and En).

Statistical Analysis

Data are expressed as mean ± SEM of the different variables analyzed and are compared among groups by using either Student's t test, two-way ANOVA (ANOVA 2), and ANOVA 2 with repeated measures. ANOVA 2 were followed by Fisher's protected Least Significance Differences (LSD) test.

RESULTS

General behavioral observation

Rats were observed during the one-hour period after each maze change. They moved with a high level of activity throughout the maze until they found the exit, and then returned back to it for ~30 min to explore all alleys with both horizontal and vertical movements. After that time, rats ate food pellets within the maze, after carrying them from the G1 ground compartment. The day when both bedding material and maze configuration were changed, rats climbed first to the maze until they found the exit, and then explored intensively the ground floor for ~20 min. Most rats started to visit the maze more in details once general activity decreased in the ground floor. During ground exploration, social activity, including play-fighting behavior, was enhanced.

Body weight, food intake and total lipids

Because enriched housing in Marlau™ cages started at weaning in our paradigm, we first verified whether housing conditions (enriched compared to standard) altered body growth. We show that body weight gain in rats raised in Marlau™ cage (enriched group, En) was greater than that of rats raised in standard cages (standard group, S): +12.9% ($p<0.001$; ANOVA 2 with repeated measures) from day 28 to day 63 (Fig. 2A). The body weight gain in En was associated with a greater food intake (+7.4% from weeks 1 to 6 in Marlau™ cages, data not shown), with no modification of total body lipid proportion (Fig. 2B). The body weight gain in En rats was confirmed in the other experiments: +11.5%, +9.8%, +10.8 and +11.2% in experiments 2, 3a, 3b and 4, respectively.

Restraint stress coping

Basal plasma level of CORT was similar in rats raised in Marlau™ cages and standard cages (Fig. 3A). Peak plasma level of CORT after 30 min restraint stress was also similar in rats raised in the two housing conditions, but recovery of basal plasma level of CORT was faster in rats raised in Marlau™ cages than in rats raised in standard cages, as measured 2 hours after stress cessation (Fig. 3A).

One of the most important brain areas involved in the recovery of baseline level of plasma CORT after a stressful condition is the hippocampus. CORT can bind to MR and GR, and both receptors are largely present in the hippocampus (15, 16). MR have a high affinity for CORT and are thought to regulate the basal activity of the hypothalamic-pituitary-adrenal (HPA) system. Conversely, GR have a low affinity for CORT and hippocampal GR are thought to play an important role in the recovery of baseline plasma level of CORT after high release of CORT as seen during acute stress (15, 16). Here, we show that MR transcript level in the ventral hippocampus of enriched rats is greater than that of standard rats, and that GR transcript level is greater in both the dorsal and ventral hippocampus of enriched rats compared to standard rats (Fig. 3B). Similar results were previously obtained for GR in the dorsal hippocampus (17). These results indicate that hippocampal upregulation of MR and GR gene expression in rats raised in Marlau™ cages might participate to faster recovery of basal plasma CORT level after exposure to an acute stress.

Cortical thickness and hippocampal surface area

Marlau™ cage has been designed so that it enhances voluntary exercise (large surface to explore, presence of running wheels) and motivated exercise (exploratory behavior in the maze to find food at each maze change). Exercise has been shown to increase the thickness of primary motor cortex (M1) and primary somatosensory cortex (S1) (18). We thus verified whether thickness of M1 and S1 was increased in rats raised in Marlau™ cage. From Nissl-stained coronal brain sections taken at 120 µm intervals, two sections of each brain were selected at coronal planes corresponding to IA +9.70 and +5.40 according to Paxinos and Watson (14). We measured cortical thickness, as illustrated (Fig. 4) in three different subregions on each sections: cingular cortex 1 and 2 and secondary motor cortex (Cg1/2;M2), primary motor cortex (M1) and primary somatosensory cortex, upper lip region (S1ULp) at IA +9.70 mm, and retrosplenial granular b cortex (RSGb), primary somatosensory cortex, barrel field (S1BF) and secondary motor cortex (M2) at IA +5.40 mm. While there was a tendency for an overall increase in cortical thickness, M1 and S1 (S1ULp and S1BF) were the subregions where the increase was statistically different (Fig. 4).

Surface area measurements of the hippocampus were performed at 240 µm intervals from IA +5.86 mm to IA +4.90 mm in the dorsal anterior hippocampus (daHi), and from IA +4.20 mm to IA +3.72 mm in the dorso-ventral hippocampus (dvHi). We did not measure any difference in the surface area of the hippocampus between rats raised in standard and enriched cages (daHi, S: 30.29 ± 2.05 mm^2, En vs. S: 100 ± 1 % ; dvHi, S: 50.07 ± 2.30 mm^2, En vs. S: 88 ± 10 %).

Neurogenesis

As reported by others in many studies, EE has been shown to increase neurogenesis in rodents (19, 20). Here, we show fourteen days after BrdU administration that rats raised in Marlau™ cages had a greater number of BrdU-positive cells in the granule cell layer of the dentate gyrus (Fig. 5A). Double immunolabeling of BrdU together with either NeuN (specific neuronal marker) or GFAP (specific astroglial marker) revealed that BrdU-positive cells were primarily neurons (Fig. 5B).

Transcript level of genes involved in brain plasticity

Effects of EE on brain plasticity are likely sustained by modulation of the expression patterns of many different genes (21). Among these genes, Vascular Endothelial Growth Factor *"vegf"* and Brain-Derived Neurotrophic Factor *"bdnf"* have been shown to play major roles in enrichment-induced neurogenesis (22, 23), and VEGF and BDNF transcript levels are induced in the hippocampus following EE (8, 22, 24). Here, we show that BDNF and VEGF transcript levels were significantly induced in the ventral hippocampus, but not in the dorsal hippocampus of enriched rats compared to standard rats (Fig. 6). BDNF exerts its function via full-length tyrosine kinase receptor TrkB.FL (25), and the truncated receptors TrkB.T1 and TrkB.T2 may regulate the local concentration of BDNF for extended periods of time following its endocytosis and release by astrocytes (26). We show that transcript levels of TrkB.FL, TrkB.T1 and TrkB.T2 were significantly higher in the

ventral hippocampus of enriched rats, compared to standard rats. By contrast, only TrkB.FL transcript level was significantly higher in the dorsal hippocampus of enriched rats (Fig. 6).

Erythropoietin (Epo) is recognized as a potent endogenous neuroprotective molecule (27, 28), and is involved in the regulation of a variety of physiological processes, including neurogenesis (29, 30) and dendritic and axonal outgrowth (31-33). Interestingly, exogenous Epo induces BDNF transcript level in the neocortex (34). Here, we show that rats raised in Marlau™ cages had greater transcript levels of Epo and Epo receptor (EpoR) in the dorsal and ventral hippocampus (Fig. 6).

Compelling studies have provided evidence that Insulin-like Growth Factor 1 "IGF-1" works in concert with VEGF and BDNF to produce complementary effects of exercise on brain plasticity (35). Hippocampal upregulation of IGF-1 transcript level has been reported only after voluntary (36) but not after forced (37) exercise. Because voluntary exercise is one of the behaviors promoted by the Marlau™ cage, we hypothesized that IGF-1 transcript level might be elevated in the hippocampus of rats raised in Marlau™ cages. In addition, exogenous IGF-1 induces transcript levels of Epo (38) and BDNF (37) in the hippocampus and the neocortex, respectively. Here, we show for the first time that IGF-1 transcript level was significantly increased in the dorsal and the ventral hippocampus of rats housed in enriched environment compared to rats housed in standard cages (Fig. 6).

Finally, we show that transcript level of Microtubule-Associated Protein MAP-2 was also significantly induced in rats raised in Marlau™ cages compared to rats raised in standard cages, by +24 ± 3.7 % ($p<0.05$) and +60.8 ± 15.0 % ($p<0.01$) in the dorsal and ventral hippocampus, respectively, as previously reported by others for MAP-2 protein in the mouse hippocampus (39).

	Dorsal Hippocampus	**Ventral Hippocampus**
BDNF	1,109,200 ± 63,320	428,2800 ± 18,000
Epo	13,640 ± 116	14,320 ± 2,480
EpoR	55,120 ± 4,760	20,720 ± 680
GR	7,504,040 ± 382,560	2,303,800 ± 323,080
IGF-1	412,240 ± 18,800	227,080 ± 4,840
MAP-2	1,157,800 ± 50,960	464,720 ± 81,760
MR	2,109,520 ± 163,560	609,960 ± 34,680
TrkB.FL	983,080 ± 43,200	426,920 ± 47,520
TrkB.T1	399,960 ± 25,120	179,240 ± 2,640
TrkB.T2	2,040 ± 764	600 ± 76
VEGF	375,760 ± 30,812	217,320 ± 12,387

Table 2 : Number of cDNA copies (mean ± SEM) after reverse transcription of 1 µg of total RNAs contained in the ventral and dorsal hippocampus dissected from the brains of 9 week-old rats housed in standard cages (n=8).

Repeatability of the results obtained in Marlau™ cages

The dentate gyrus in the hippocampal formation plays an important role in learning and memory, in particular during navigational/spatial learning in the Morris Water Maze (MWM) test (40). The improved performances in the MWM promoted by EE (41) have been confirmed by 51% of the 47 studies that we have referenced (table 2) so far. We thus investigated in a first series of experiments (Exp.3a) whether performances in the MWM were enhanced in rats raised in Marlau™ cages, and tested in a second series of experiments (Exp.3b) whether it could be replicated. We first expressed the latency to find the platform as usually reported in the literature, i.e. maximum time (90 sec) was assigned to rats that did not find the platform. We report that similar results were obtained between experiments 3a and 3b, and that rats housed in Marlau™ cages performed better during the first day trial (Fig. 7A). However, such a difference between the two groups of rats (enriched vs. standard) was not the result of greater individual performances in enriched rats (see insert panel in Fig. 7A). Indeed, when rats found the platform on the first day trial, they performed similarly whether they were raised in Marlau™ cages or standard cages. The only difference between enriched and standard housing was the bigger proportion of rats that found the platform when raised in Marlau™ cages (Fig. 7A). Similar results were obtained when the distance traveled, and not the escape latency, was chosen as a variable (data not shown).

The transcript level of EpoR in the ventral hippocampus was chosen as another variable to test the repeatability of the results obtained in Marlau™ cages, since we recently demonstrated that the efficacy of exogenous Epo to protect neurons against excitotoxic injury was determined by the expression of EpoR in brain tissue (42).Here, we report that the difference in EpoR transcript level measured in the ventral hippocampus of rats raised in Marlau™ cages and standard cages was similar between experiments 3a and 3b (Fig. 7B).

Longitudinal analysis of exploratory behavior

One week after weaning and housing in Marlau™ cages or standard cages, the behavior of the rats was tested in the Water Exploration Test (WET), as described (Fig. 8A.a). Rats were allowed to explore for 5 min a quadrant of a circular pool; the central zone of the quadrant was referred as the anxiogenic zone. The path (Fig. 8A.b), the swimming distance in the full quadrant (Fig. 8A.c1), the time spent (Fig. 8A.c2) and the swimming distance (Fig. 8A.c3) in the central zone of the quadrant, and the velocity in the central zone (Fig. 8A.d1-3) were measured. We show that enriched and standard rats had different exploration patterns: while rats in both groups swam the same distance (Fig. 8A.c1), enriched rats, compared to standard rats, explored thoroughly the central zone (Fig. 8A.b,c2,c3). In addition, when present in the central zone, enriched rats adopted a floating behavior (null velocity, Fig. 8A.d3), while standard rats just crossed it rapidly (rapid velocity, Fig. 8A.d1).

After 3 weeks of housing in Marlau™ cages or standard cages, rat behavior was tested for 90 sec during two consecutive days in the three-compartment Black/White/Black (BWB) box, as described (Fig. 8B.a). Visiting the white zone was indicative of less anxiety. On the first day, when rats had access to 2 compartments only and were introduced in the large black zone, the adjacent white zone

was much more visited by rats of the enriched group than by rats of the standard group (Fig. 8B.b). O n the second day, when rats were introduced in the large black zone, and were allowed to access a small black zone after crossing the white zone, less rats of the standard group that visited the white zone on the first day revisited it on the second day. By contrast, all rats of the enriched group revisited the white zone on the second day (Fig. 8B.c), and the majority of them explored the small black zone (Fig. 8B.d).

Finally, after 12 weeks of housing in standard or Marlau™cages, rat behavior was tested for 5 min in the elevated plus maze (EPM), as described (Fig. 8C.a). The proportion of rats visiting the open arms was three-fold greater in the enriched group than in the standard group, and the number of entries and the time spent in the open arms were significantly greater in the enriched group (Fig. 8C.b). In addition, analysis of the exploratory behavior in the closed arms revealed that rats in the standard group spent most of the time in one arm (the so-called "preferred closed arm") than in the other, while rats in the enriched group spent about half of the time in each closed arm (Fig. 8C.c).

Cognitive performances in epileptic rats

To our knowledge, no study explored at the adult stage the cognitive function of epileptic animals subjected to Pilo-SE at weaning, whether they were housed in enriched or standard environments after the initial injury. In our study, 11 weeks after Pilo-SE, we ascertain that 7/8 rats raised in standard cages and 4/8 rats raised in Marlau cages exhibited BSRS. In the groups of rats subjected to Pilo-SE, we only monitored exploratory behavior of rats with BSRS.

Anxiety has been shown to increase in rats raised in standard housing conditions after kainic acid-induced SE, this effect being reversed by housing in non-standardized enriched environments (43). In our study, all rats with BSRS displayed increased anxiety-like behavior compared to respective controls (standard or Marlau™ cages). However, anxiety-trait in rats with BSRS raised in Marlau™ cages was far below that measured in rats with BSRS raised in standard cages (Fig. 9A).

Non-standardized EE has already been shown to protect navigational/spatial memory in the Morris Water Maze of rats subjected to Pilo-SE either partially (44) or fully (45). However, in the latter study, no difference was observed in the latency to find the immerged platform between standard and enriched control groups (45). Here, we show that performances in the Morris Water Maze test are highly protected in epileptic rats raised in Marlau™ cages. Indeed, while none of the epileptic rats raised in standard cages found the platform during the first two trial days, most of the rats (>75%) that were raised in Marlau™ found the platform on the first trial day. In general, epileptic rats raised in Marlau™ cages displayed memory performances similar to control enriched rats (Fig. 9B).

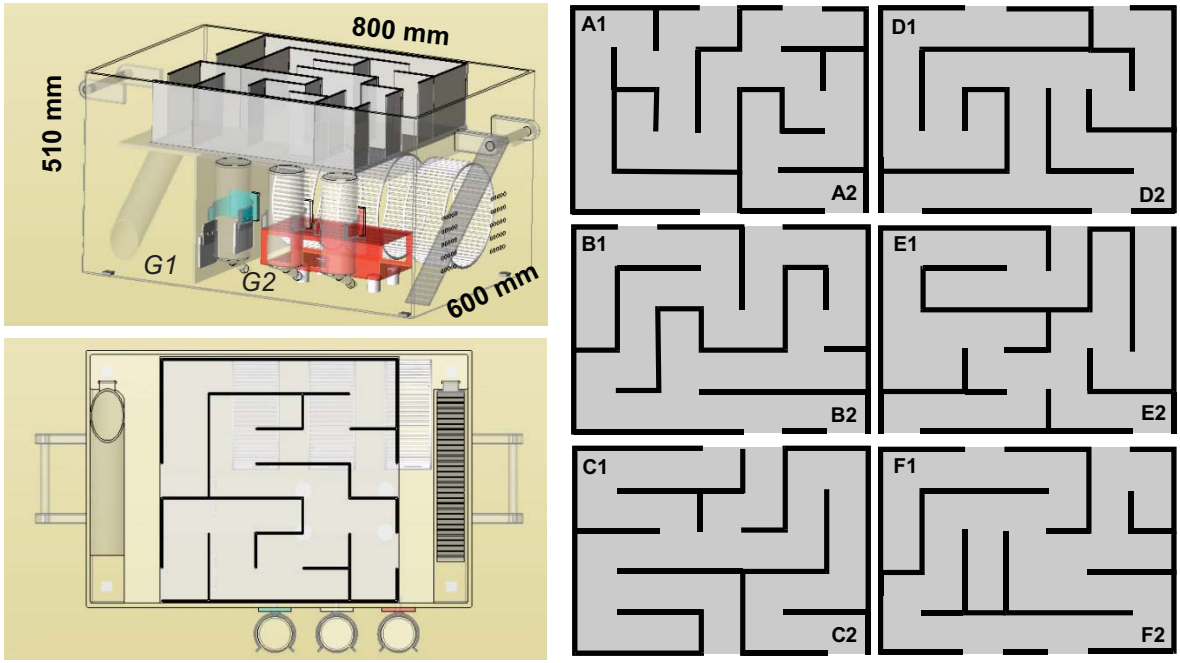


Figure 1. Graphic design of Marlau™ cage showing its complex structure. The cage displayed (Marlau™-Rat) allows housing of 12 rats in a large exploration area. The ground floor is divided into 2 sections including one section with food pellets (G1) and another (G2) with 3 water bottles, a red house and 3 running wheels. In order to reach food, rodents need to climb to the upper floor via a ramp, cross a labyrinth, and then go down to G1 via a tunnel. Access back to G2 is made possible by 2 one-way opening doors located in the wall separating the ground floor. Six labyrinths (A-F) are permanently used, offering a total of 12 different configurations. The labyrinth is changed 3 times a week (thus guaranteeing novelty and sustained cognitive stimulation) in a defined order to offer reproducibility across experiments (starting with the series of A1, B1, C1, and ending with the series of D2, E2, and F2). Territorial dominance is avoided by the presence of the 2 one-way opening doors and 2 gates on each side of the labyrinths. This complex well-organized structure of the Marlau™ cage thus introduces crucial features of the wild, including novelty, complexity, cognitive stimulation and physical activity.

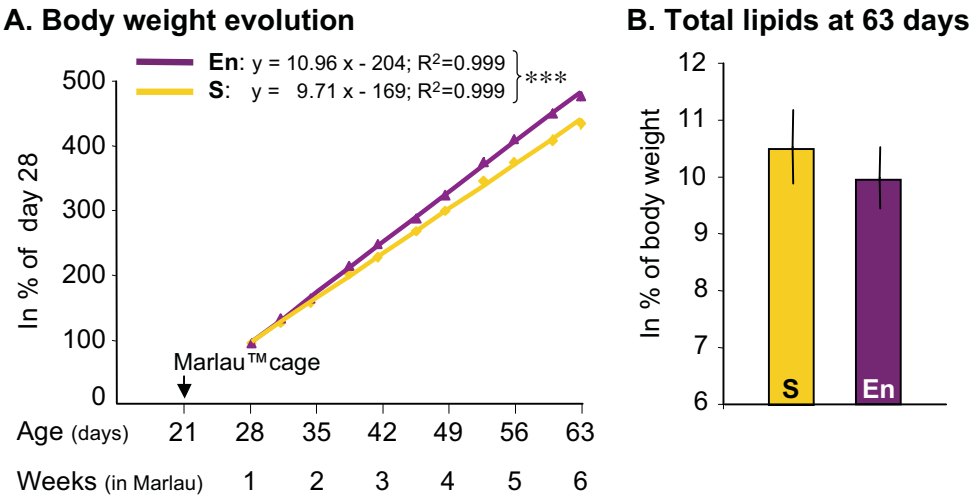


Figure 2. Rat weight, but not total body fat, is increased after housing in the Marlau™ cage. A. After 4 weeks in the Marlau™ cage, enriched rats had an increase in body weight that was significantly greater than that of rats raised in standard conditions (n=12 in each group). **B.** Total body fat percentage measured at termination time was not altered by housing conditions (n=8 in each group). En vs. S: *** $p<0.001$, ANOVA 2 with repeated measures. All results are expressed as the mean ± SEM. Abbrebiations: S, rats in standard cages; En, rats in Marlau™ cages.

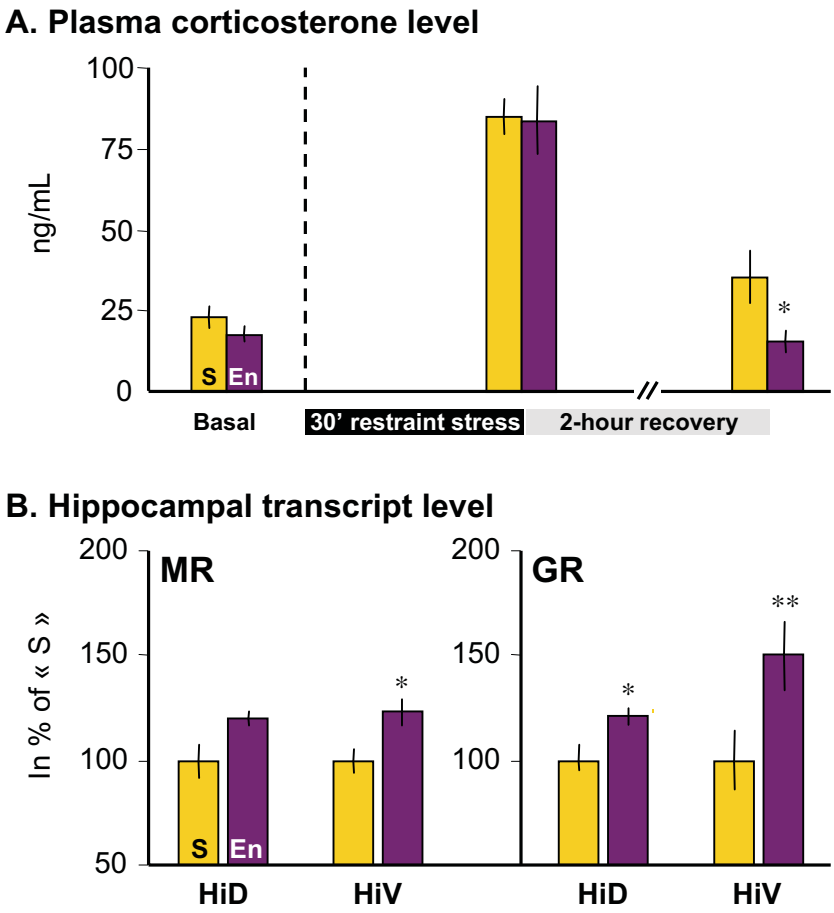


Figure 3. Rats raised in Marlau™ cages display better capacity to cope with restraint stress. A. Plasma corticosterone level was measured 30 min prior to (basal), at the end of a 30 min-restraint stress and after 2-hour recovery period (n=5 in each group). Note that increased hippocampal transcript level of GR, and to a lesser extent of MR, was associated to a faster recovery of basal CORT level after restraint stress. En vs. S: * $p<0.05$, ** $p<0.01$, following Student's *t* test (A) and ANOVA 2 with repeated measures (B). Abbreviations: S and En as in Fig. 2. **B.** Transcript levels for corticosteroid receptors (MR and GR) were measured in the dorsal and ventral hippocampus of naïve rats raised in standard or Marlau™ cages (n=7 in each group).

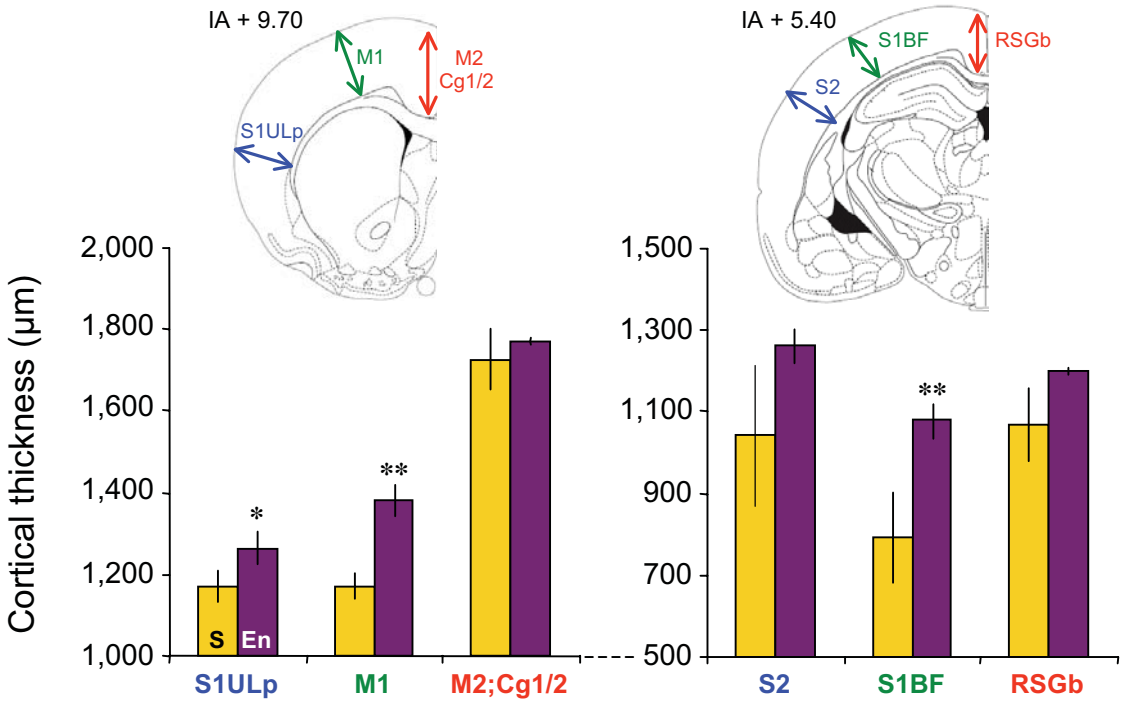


Figure 4. Marlau™ cages meet the criterion of enrichment-induced cortical thickness. Six weeks after housing in Marlau™ cages, averaged cortical thickness measured as shown by vertical arrows on Nissl-stained sections was increased compared to standard conditions, especially in M1 and S1 subregions. En vs. S: * $p<0.05$, ** $p<0.01$, ANOVA 2, factor 1: rat group, factor 2: subregion. Each bar represents the mean ± SEM ($n=4$ in each group). Abbreviations: S and En as in Fig. 2; Cg1/2, cingular cortex 1 and 2; M1, primary motor cortex; M2, secondary motor cortex; RSGb, retrosplenial granular b cortex; S1BF, primary somatosensory cortex, barrel field; S1ULp, primary somatosensory cortex, upper lip region.

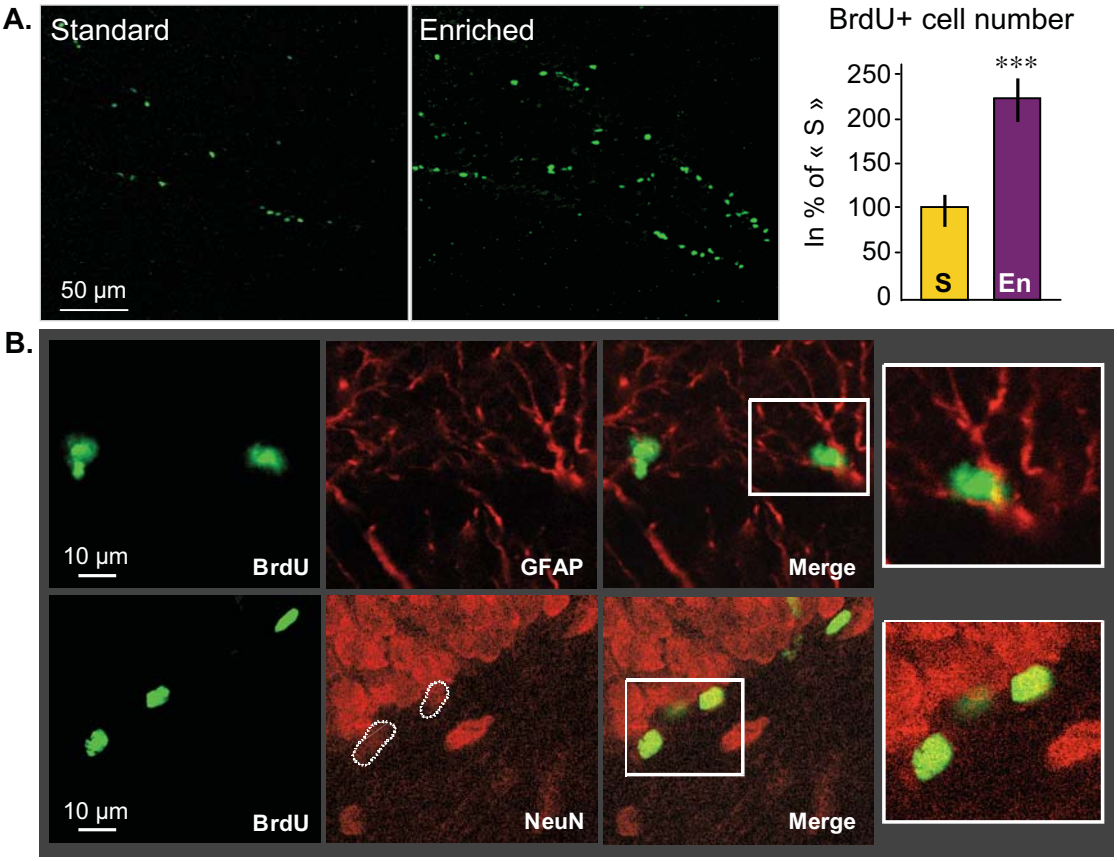


Figure 5. Marlau™ cages meet the criterion of enrichment-induced hippocampal neurogenesis. Fourteen days after BrdU injections, the averaged number of cells having incorporated BrdU (counted in 3 sections, 300 µm apart) nearly doubled in the hippocampus of rats raised in Marlau™ cages. While BrdU could be detected in both neurons and astrocytes, respectively identified by NeuN and GFAP, BrdU was primarily found in neurons. En vs. S: *** $p<0.001$, ANOVA 2, factor 1: rat group, factor 2: anatomical plane. Each bar represents the mean ± SEM ($n=4$ in each group). Abbreviations: S and En as in Fig. 2.

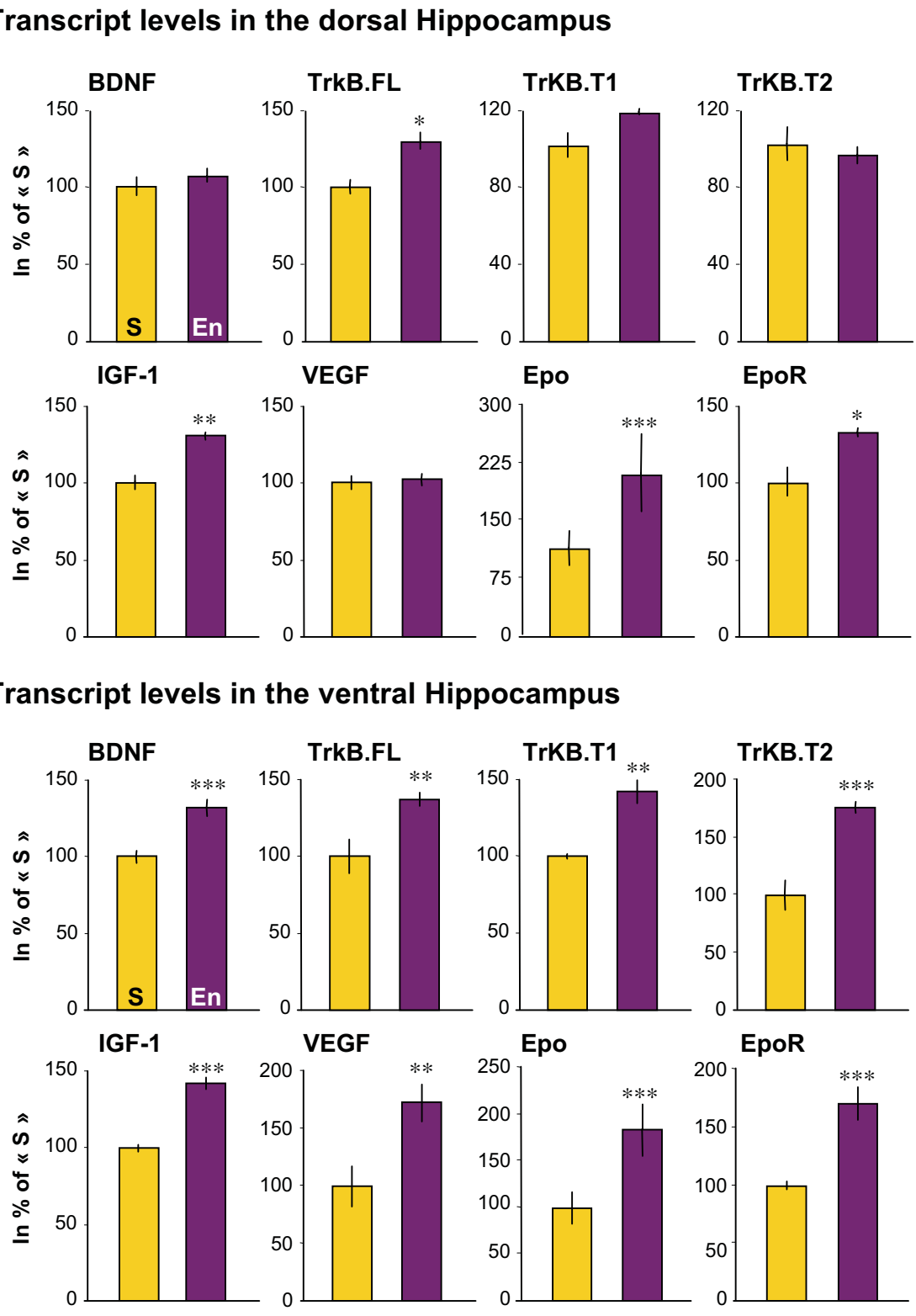
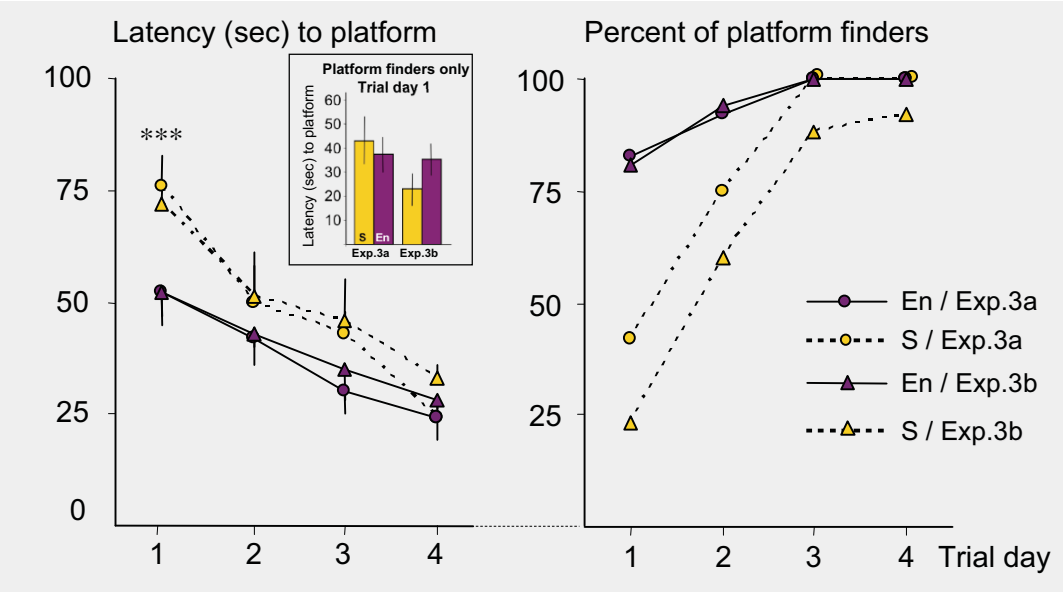


Figure 6. Marlau™ cages meet the criterion of enrichment-induced increase in transcript levels in the hippocampus. Enrichment-induced alterations in transcript levels were greater in the ventral hippocampus than in the dorsal hippocampus, for all genes measured after 6 weeks of housing in Marlau™ cages. En vs. S: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's *t* test. Each bar represents the mean ± SEM ($n=8$ in each group). Abbreviations: S and En as in Fig. 2.

A. Morris Water Maze performance



B. EpoR transcript level

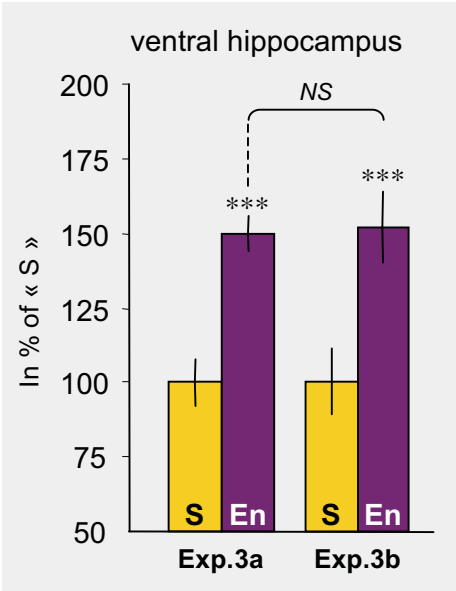
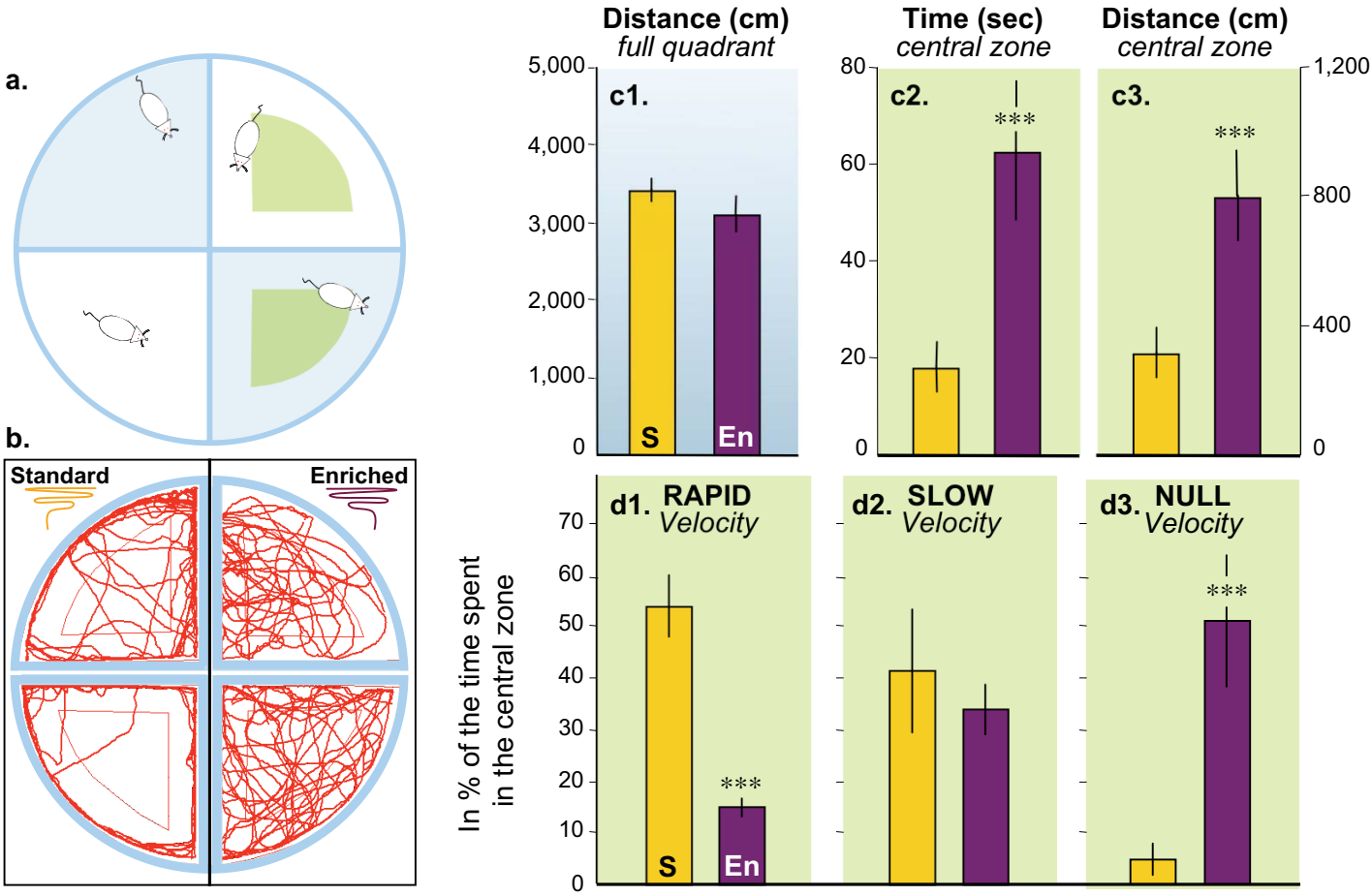


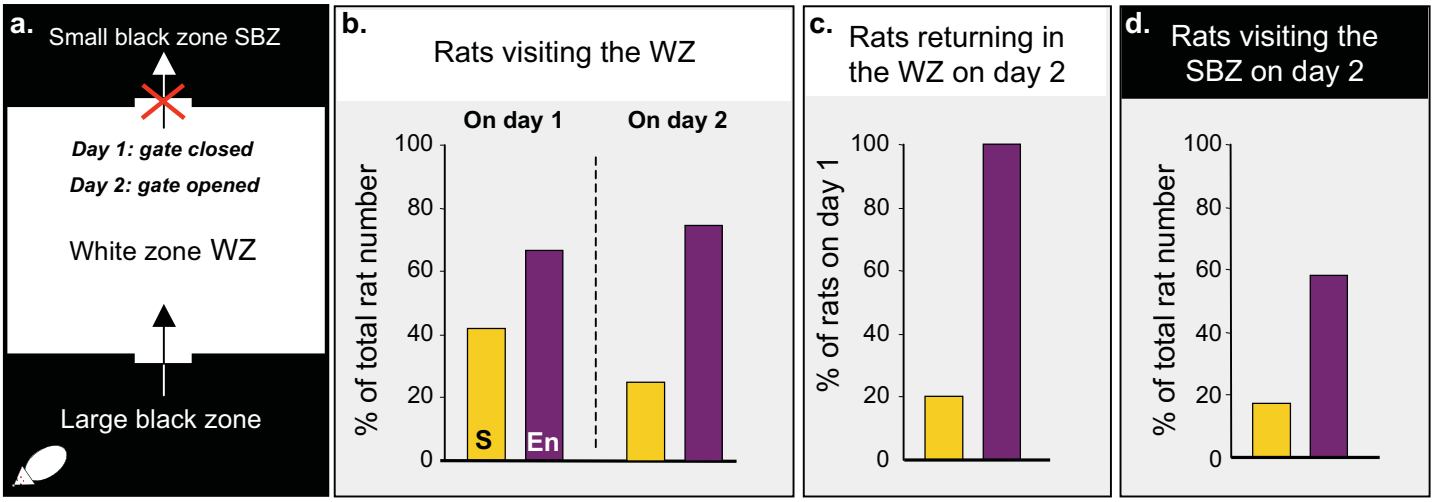
Figure 7. Marlau™ cages provide highly reproducible activation of hippocampal plasticity-related variables. Experiments were repeated twice (a and b), with 12 rats in each group. **A.** Six weeks after housing in Marlau™ or standard cages, rats were tested four times a day for spatio-temporal learning in the Morris Water Maze. Insert represents data obtained for the latency to platform on trial day 1, considering only rats that found the platform. En vs. S: *** $p<0.001$, ANOVA 2 with repeated measures. **B.** Two weeks later, EpoR transcript level was measured in the ventral hippocampus. En vs. S: *** $p<0.001$, ANOVA 2. Abbreviations: S and En as in Fig. 2.

Figure 8. Longitudinal analysis of exploratory behavior during housing in Marlau™ cages is in favor of decreased anxiety in enriched rats. Rats were consecutively tested in the WET (A), in the BWB (B), and in the EPM (C), respectively after one, three and twelve weeks of housing in Marlau™ or standard cages. **A.** WET. *a,* rats are placed in a pool divided into 4 quadrants for 5 min; visiting the virtual "central zone" (CZ, green) was indicative of reduced anxiety; *b,* typical trajectories of S and En rats; *c,* time spent and distance traveled in the full quadrant and the CZ; *d,* time spent in the CZ swimming with different velocity profiles: rapid, slow, and null (floating behavior). Each bar represents the mean ± SEM (*n=12* in each group). **B.** BWB. *a,* rats were tested during 2 consecutive days (with access to the SBZ on day 2 only); visiting the WZ on day 1 and the SBZ on day 2 was indicative of reduced anxiety; *b-d,* we measured the number of rats visiting the WZ on days 1 and 2 (*b*), the percentage of those returning to the WZ on day 2 (*c*), and the number of rats visiting the SBZ on day 2 (*d*). Each bar represents the mean of the group (n=12 in each group). **C.** EPM. a, exploratory behavior of rats was tested for 5 min once placed in an open arm (OA), as shown; exploration of the OA was indicative of reduced anxiety; *b,* we measured the number of rats visiting the OA, the number of entries in the OA, and the time spent in the OA; *c,* while the time spent to explore the closed arms (CA) was mostly restricted to one arm in standard rats, the enriched rats explored nearly equally both CA. En vs. S: ** $p<0.01$, *** $p<0.001$, Student's *t* test. Abbreviations: S and En as in Fig. 2.

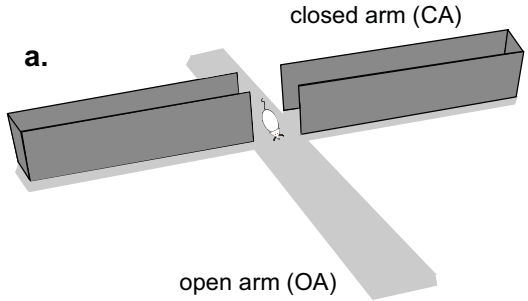
A. Water Exploration Test (WET) - 1 week post-weaning



B. Three-compartment Black/White/Black (BWB) Box - 3 weeks post-weaning

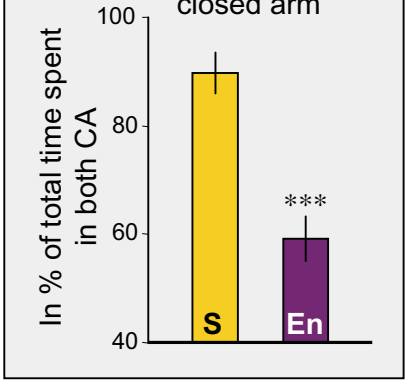


C. Elevated plus maze (EPM) - 12 weeks post-weaning



b. Exploration of both open arms

	S	En
Rats visiting the OA	25%	75%
Nb of entries in the OA	8 ± 2	19 ± 3 **
Time (sec) in the OA	24 ± 4	41 ± 7 **



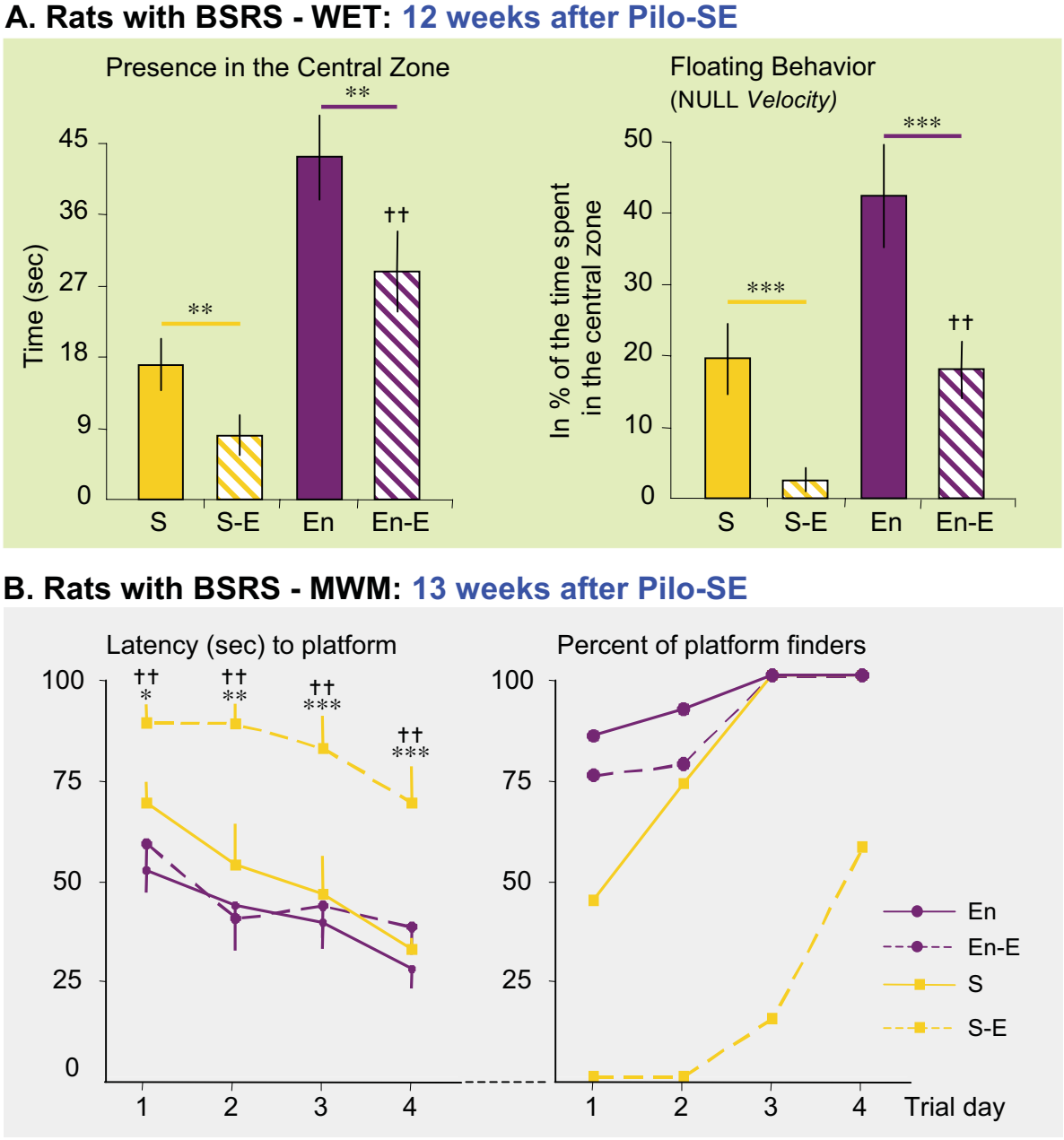


Figure 9. Rats raised in Marlau™ cages after excitotoxic brain injury displayed decreased cognitive impairments. Rats underwent pilocarpine-induced *status epilepticus* (Pilo-SE) at 3 week-old, and were raised, on the following day, either in standard or Marlau™ cages. Rats developing behavioral spontaneous recurrent seizures (BSRS, or "epileptic") were then subjected at 15- and 16-week old to the WET and to the MWM, respectively. In these results, 8 rats not subjected to Pilo-SE were included in each S and En groups. The number of rats subjected to Pilo-SE that developed BSRS was not identical in each S (n=7/8) and En (n=4/8) groups. **A.** WET. While rats undergoing BSRS displayed marked anxiety-like behavior, the positive effect of enrichment in control rats was observed in epileptic rats, as demonstrated by the increased time spent floating in the central zone. S-E vs. S and En-E vs. En: ** $p<0.01$, *** $p<0.01$; En-E vs. S-E: †† $p<0.01$, ANOVA 2. **B.** MWM. Enriched housing prevented the deterioration of learning and memory observed in standard housing, as indicated by the latency to find the platform and the proportion of rats finding the platform. S-E vs. S: * $p<0.05$, ** $p<0.01$, *** $p<0.01$; En-E vs. S-E: †† $p<0.01$, ANOVA 2 with repeated measures. Abbreviations: S and En as in Fig. 2; En-E, enriched epileptic rat; S-E, standard epileptic rat.

DISCUSSION

Until now, enrichment procedures during housing of rodents in preclinical research studies were lacking standardization. We engineered the Marlau™ cage, offering reduced stressful social interactions, increased voluntary exercise, diverse entertaining activities, and standardized provision of cognitive stimulation and novelty. Compared to rats raised in conventional cages, rats raised in Marlau™ cages exhibited a large number of phenotypic characteristics reported for animals housed in non-standardized EE, i.e. increased (i) cortical thickness, (ii) hippocampal neurogenesis, (iii) hippocampal levels of BDNF and VEGF transcripts, better performances in spatial learning, and decreased anxiety-trait. In addition, housing rats in Marlau™ cages after severe *status epilepticus* at weaning prevents cognitive impairments.

Development of the Water Exploration Test to measure anxiety.

Most of the tools available so far to measure anxiety in laboratory animals, in particular rodents, are mainly composed of branching mazes. Rats raised in Marlau™ cages are living in an environment that includes a series of branching mazes. Therefore, to circumvent any potential bias when comparing their level of anxiety with that of rats unacquainted with branching mazes, we developed the Water Exploration Test (WET) as a new tool to measure anxiety. When measured one week or twelve weeks after weaning, we found that the time spent in the central zone of the quadrant, and the percentage of time spent with a floating behavior in this central zone, are always greater in rats housed in Marlau™ cages compared to rats housed in standard cages. This finding indicates that Sprague-Dawley rats raised in enriched Marlau™ cages exhibit lower anxiety compared to rats raised in standard cages.

The Elevated Plus Maze (EPM) belongs to the family of the branching mazes. It comprises four arms and is widely used to measure anxiety in rodents. However, interpretation bias may occur when it is used to compare anxiety between rats raised either in Marlau™ or standard cages, as mentioned above. Anyway, it is noteworthy that, twelve weeks after weaning, two independent studies using either EPM or WET reach to the conclusion that anxiety-trait in rats raised in Marlau™ cages is lower than that of rats raised in standard cages.

We noticed that WET presents an important advantage over the EPM when testing anxiety in rodents with unaltered locomotion activity. In the WET, rats have to swim continuously, allowing uninterrupted exploration of the quadrant. By contrast, in the EPM, absence of motivation to explore the maze can lead to an ambiguous interpretation of the time spent in the open arms.

Marlau™ cage fulfills neuroscience research and animal welfare objectives.

A distinction has been made between enrichment protocols used in neuroscience research and in animal welfare research (46). In neuroscience research, EE includes increased social and sensory stimulations; it has been used for a long time to evoke brain plasticity or to solicit greater recovery after brain insults or resistance in drug addiction (4, 5, 47). In this case, animals are housed in large cages full of toys that are changed frequently to induce changes in brain and

behavior. By contrast, the main focus in animal welfare research is to improve the wellbeing of animals by allowing the development of species-specific behavior. To meet the principles of the two research fields, we developed the enriched Marlau™ cage.

In the wild, rats are constantly facing problems that they must overcome in order to survive and thrive, using their complex range of cognitive skills (48). We introduced this characteristic in the Marlau™ cage by making food acquisition a critical factor in survival. Food and water are not easily within reach in the Marlau™ cage. When rodents are in the compartment comprising water bottles, they are separated from the food pellet compartment by a maze that increases the difficulty to acquire food. Complexity is reinforced at every maze configuration change, since animals have to find a new path through the maze three times a week, i.e. at every maze change, engaging each time cognitive processes such as learning and memory.

Change in maze configuration has been inspired of the training program in the original ECT paradigm (7). In ECT training, rodents were subjected to daily exploration of a maze with frequent changes in the pattern of barriers. The major advantage of the Marlau™ cage compared to the ECT program is that the maze is a permanent constituent of the Marlau™ cage, while daily trainings in the ECT program were performed in cages distinct from the housing cages.

The design of the Marlau™ cage makes it possible for animals to feel a certain level of control on the environment, by making decision and by facilitating escape behavior from a dominant animal. These features, which all contribute to increased wellbeing (48) may explain our data showing that rats raised in Marlau™ cages had a greater capacity to cope with stress, as demonstrated by the faster recovery of basal plasma CORT levels in response to restraint stress, compared to rats raised in standard cages. These results, which are concordant with prior results obtained by others in mice (49), may be partly explained by the increased expression of glucocorticoid receptors in the hippocampus of rats raised in Marlau™ cages, known to contribute to the rapid recovery of basal plasma CORT levels following acute stress (16).

Regular maze change configuration in the Marlau™ cage generates complexity and novelty, two crucial features of EE in neuroscience research. Maze change configuration also offers to rodents the opportunity to develop species-specific behaviors, especially when challenged by critical factors in survival. This feature may primarily contribute to wellbeing when animals possess the skills to overcome the challenge (48). The Marlau™ cage thus fulfills neuroscience research and animal welfare objectives.

Marlau™ cage provides equal exposure to challenge and homogenized results.

Due to the location of the maze separating water and food compartments, the Marlau™ cage presents two main advantages. First, the challenge that consists in navigating through the maze to acquire food is affecting all animals similarly. Second, the challenge is such that all rats possess the skills to overcome it. Equal exposure to the food-searching challenge is certified by body-weigh measurements. Indeed, while contrasting with a recent study performed on Sprague-Dawley rats housed in non-standardized EE (50), we did not find any decrease but instead an increase in the body weight of all rats housed in Marlau™ cages,

as already reported for diverse mouse strains (51). This weight gain was associated with stable total body fat percentage, indicating that development of rats raised in the Marlau™ cage was not better, but instead was as proportionate as the development of rats raised in standard cages.

We found that rats raised in Marlau™ cages performed better in the Morris Water Maze than rats raised in standard cages, a result consistent with ~50% of the studies analyzed (see table 1 for references). However, when looking at individual performances, we noticed that a minority of rats raised in standard cages (1 to 2 in each cage of 6) was doing as well as rats raised in Marlau™ cages. Such good performers in standard cages might correspond to dominant rats, as previously observed in CD-1 mice (52). One of the most prevalent challenges for dominant rodents is to maintain social competition over subordinates. Such a competition might also exist in the Marlau™ cage. However, due to the large space areas and the presence of shelters in Marlau™ cages, social dominance might not impact animals as it does occur in standard cages. If dominant rats in standard cages really do perform as well as the majority of rats in the Marlau™ cage, we might have to associate good scores in the Morris Water Maze (MWM) with the ability of the rats to overcome challenges in their environment. When rats are housed in boring cages, the only challenge to overcome is social competition. Housing rats in the Marlau™ cage might thus optimize homogeneity between animals, because they all overcome one of the most challenging issues in survival, which consists of food acquisition.

Gene expression and neurogenesis in EE

BDNF has been considered a hallmark variable induced by exposure to EE (53). As BDNF protein level has been reported to increase in the ventral hippocampus (53), we provide evidence here that housing in Marlau™ cages up-regulates BDNF-mRNA level in the ventral hippocampus, and confirm that BDNF-mRNA level remains stable in the dorsal hippocampus (41, 54). Up-regulation of BDNF-mRNA level has been reported in the dorsal hippocampus, only when animals were subjected both to EE and to spatial learning task and when results were compared to those obtained in animals housed in isolated conditions (24, 41).

Up-regulation of BDNF has been suggested to mediate enhanced neurogenesis in the hippocampus of animals raised in EE (23). However, as mentioned above, BDNF gene expression remains stable in the dorsal hippocampus, where enhanced neurogenesis has been reported in animals raised in EE (19, 20).

Other genes, such as IGF-1 and Epo, up-regulated in the dorsal hippocampus of rats raised in the Marlau™ cage might mediate enhanced hippocampal neurogenesis (29, 30, 55, 56). Concerning VEGF, while its enhanced hippocampal expression has been shown to be crucial for the induced neurogenesis and performance in learning and memory tasks following EE (22), and despite our previous study showing increased VEGF-mRNA level in the whole hippocampus of rats raised in non-standardized EE (8), we show here that the level of VEGF-mRNA increased in the ventral, but not the dorsal, hippocampus of rats raised in Marlau™ cages, compared to rats raised in standard cages.

Housing in Marlau™ cages protects cognitive function in epileptic rats.

EE has been shown to have beneficial effects in animal models of neurodegenerative diseases, psychiatric disorders (4, 47, 57-59) and in different models of brain impairment (60, 61). EE has also been shown to delay the development of kindling (62), inhibit kainite-induced SE (63), and to prevent cognitive decline during epileptogenesis in rats subjected to Pilo-SE at weaning (44, 45, 64). However, the question of whether EE can prevent or restrain the development of chronic epilepsy has never been addressed (65). For the first time, we provide evidence that housing in enriched cages (Marlau™ cages) after Pilo-SE induced at weaning reduces the number of rats developing behavioral spontaneous recurrent seizures (BSRS) later in life. In addition, we show that rats with BSRS housed in Marlau™ cages do not develop the cognitive decline (evaluated in the MWM) observed in rats with BSRS housed in standard cages. This effect is likely due to continuous solicitation of the hippocampus function in Marlau™ cages. Indeed, place navigation in the MWM depends on the functionality of "place cells", that are neurons in the hippocampus which identify or represent points in space in an environment (66). In rats housed in standard cages, seizures induce a decline in the precision and the stability of "place cells" (67). By contrast, in rats housed in Marlau™ cages, the functionality of "place cells" may be protected, despite the presence of BSRS, likely through the solicitation of "place cell" activity each time rats had to learn new spatial representation forms (68), that occurred at every change in maze configuration. This result is of particular importance for translational research, since place- and view-specific hippocampal activity has been reported in both humans and non-human primates (69-71).

Persistance of anxiety in rats with BSRS raised in Marlau™ cages.

In the WET, healthy rats raised in Marlau™ cages exhibited a reduced level of anxiety compared to their counterparts raised in standard cages, as indicated by the increased time spent floating in the center of the quadrant. At first glance, this may suggest that rats housed in Marlau™ cages developed an inadequate behavior with respect to predator threat in the wild. But this may also suggest that these rats felt safe, because they overcame the challenge of finding new routes through the maze each time its configuration was changed.

Rats with BSRS raised in Marlau™ cages exhibited reduced anxiety compared to their counterparts raised in standard cages. However, increase in anxiety was the same between the two housing conditions when rats with BSRS were compared with their respective controls. This result may correspond to the development of an adaptative behavior in all rats with BSRS, since seizures, with loss of consciousness, may represent a great source of danger. Indeed, anxiety has been proposed as a protective mechanism preventing the organism from engaging into potentially harmful behaviors (72, 73).

In conclusion, Marlau™ cage, by providing standardized enrichment procedures for rodents during housing, should facilitate transfer of reproducible programs of EE across laboratories. This implies that the changes in maze configuration and in bedding material must strictly occur across studies as described in this study. Standardization of enrichment

procedures appears now to be crucial, since interest in gene / environment interactions extends far beyond the field of neuroscience research (74).

REFERENCES

1. Crabbe JC, Wahlsten D, & Dudek BC (1999) Genetics of mouse behavior: interactions with laboratory environment. *Science* 284(5420):1670-1672.
2. Wolfer DP, Litvin O, Morf S, Nitsch RM, Lipp HP, & Wurbel H (2004) Laboratory animal welfare: cage enrichment and mouse behaviour. *Nature* 432(7019):821-822.
3. Rosenzweig MR, Bennett EL, Hebert M, & Morimoto H (1978) Social grouping cannot account for cerebral effects of enriched environments. *Brain Res.* 153(3):563-576.
4. Nithianantharajah J & Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat Rev Neurosci* 7(9):697-709.
5. van Praag H, Kempermann G, & Gage FH (2000) Neural consequences of environmental enrichment. *Nat Rev Neurosci* 1(3):191-198.
6. Fox C, Merali Z, & Harrison C (2006) Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. *Behav. Brain Res.* 175(1):1-8.
7. Diamond MC, Krech D, & Rosenzweig MR (1964) The Effects of an Enriched Environment on the Histology of the Rat Cerebral Cortex. *J. Comp. Neurol.* 123:111-120.
8. Navarro FP, Fares RP, Sanchez PE, Nadam J, Georges B, Moulin C, Morales A, Pequignot JM, & Bezin L (2008) Brain heparanase expression is up-regulated during postnatal development and hypoxia-induced neovascularization in adult rats. *J. Neurochem.* 105(1):34-45.
9. Hellemans KG, Benge LC, & Olmstead MC (2004) Adolescent enrichment partially reverses the social isolation syndrome. *Brain Res. Dev. Brain Res.* 150(2):103-115.
10. Bodennec J, Koul O, Aguado I, Brichon G, Zwingelstein G, & Portoukalian J (2000) A procedure for fractionation of sphingolipid classes by solid-phase extraction on aminopropyl cartridges. *J. Lipid Res.* 41(9):1524-1531.
11. Morales A, Bonnet C, Bourgoin N, Touvier T, Nadam J, Laglaine A, Navarro F, Moulin C, Georges B, Pequignot JM, & Bezin L (2006) Unexpected expression of orexin-B in basal conditions and increased levels in the adult rat hippocampus during pilocarpine-induced epileptogenesis. *Brain Res.* 1109(1):164-175.
12. Nadam J, Navarro F, Sanchez P, Moulin C, Georges B, Laglaine A, Pequignot JM, Morales A, Ryvlin P, & Bezin L (2007) Neuroprotective effects of erythropoietin in the rat hippocampus after pilocarpine-induced status epilepticus. *Neurobiol. Dis.* 25(2):412-426.
13. Sanchez PE, Navarro FP, Fares RP, Nadam J, Georges B, Moulin C, Le Cavorsin M, Bonnet C, Ryvlin P, Belmeguenai A, Bodennec J, Morales A, & Bezin L (2009) Erythropoietin receptor expression is concordant with erythropoietin but not with common beta chain expression in the rat brain throughout the life span. *J. Comp. Neurol.* 514(4):403-414.
14. Paxinos G & Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic Press) 4th Ed p 256.
15. Phillips LJ, McGorry PD, Garner B, Thompson KN, Pantelis C, Wood SJ, & Berger G (2006) Stress, the hippocampus and the hypothalamic-pituitary-adrenal axis: implications for the development of psychotic disorders. *Aust. N. Z. J. Psychiatry* 40(9):725-741.
16. Sapolsky RM, Krey LC, & McEwen BS (1984) Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. *Proc. Natl. Acad. Sci. U. S. A.* 81(19):6174-6177.

17. Mohammed AH, Henriksson BG, Soderstrom S, Ebendal T, Olsson T, & Seckl JR (1993) Environmental influences on the central nervous system and their implications for the aging rat. *Behav. Brain Res.* 57(2):183-191.
18. Anderson BJ, Eckburg PB, & Relucio KI (2002) Alterations in the thickness of motor cortical subregions after motor-skill learning and exercise. *Learn. Mem.* 9(1):1-9.
19. Kempermann G, Kuhn HG, & Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature* 386(6624):493-495.
20. van Praag H, Kempermann G, & Gage F (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* 2(3):266-270.
21. Rampon C, Jiang CH, Dong H, Tang YP, Lockhart DJ, Schultz PG, Tsien JZ, & Hu Y (2000) Effects of environmental enrichment on gene expression in the brain. *Proc. Natl. Acad. Sci. U. S. A.* 97(23):12880-12884.
22. Cao L, Jiao X, Zuzga DS, Liu Y, Fong DM, Young D, & During MJ (2004) VEGF links hippocampal activity with neurogenesis, learning and memory. *Nat. Genet.* 36(8):827-835.
23. Rossi C, Angelucci A, Costantin L, Braschi C, Mazzantini M, Babbini F, Fabbri ME, Tessarollo L, Maffei L, Berardi N, & Caleo M (2006) Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *Eur. J. Neurosci.* 24(7):1850-1856.
24. Schneider JS, Lee MH, Anderson DW, Zuck L, & Lidsky TI (2001) Enriched environment during development is protective against lead-induced neurotoxicity. *Brain Res.* 896(1-2):48-55.
25. Hennigan A, O'Callaghan RM, & Kelly AM (2007) Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. *Biochem. Soc. Trans.* 35(Pt 2):424-427.
26. Alderson RF, Curtis R, Alterman AL, Lindsay RM, & DiStefano PS (2000) Truncated TrkB mediates the endocytosis and release of BDNF and neurotrophin-4/5 by rat astrocytes and schwann cells in vitro. *Brain Res.* 871(2):210-222.
27. Hasselblatt M, Ehrenreich H, & Siren AL (2006) The brain erythropoietin system and its potential for therapeutic exploitation in brain disease. *J. Neurosurg. Anesthesiol.* 18(2):132-138.
28. Maiese K, Chong ZZ, Li F, & Shang YC (2008) Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies. *Prog. Neurobiol.* 85(2):194-213.
29. Shingo T, Sorokan ST, Shimazaki T, & Weiss S (2001) Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J. Neurosci.* 21(24):9733-9743.
30. Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, Liu X, Wu H, & Carmichael ST (2006) A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. *J. Neurosci.* 26(4):1269-1274.
31. Byts N, Samoylenko A, Fasshauer T, Ivanisevic M, Hennighausen L, Ehrenreich H, & Siren AL (2008) Essential role for Stat5 in the neurotrophic but not in the neuroprotective effect of erythropoietin. *Cell Death Differ.*
32. Kretz A, Happold CJ, Marticke JK, & Isenmann S (2005) Erythropoietin promotes regeneration of adult CNS neurons via Jak2/Stat3 and PI3K/AKT pathway activation. *Mol. Cell. Neurosci.* 29(4):569-579.
33. Ransome MI & Turnley AM (2008) Erythropoietin promotes axonal growth in a model of neuronal polarization. *Mol. Cell. Neurosci.* 38(4):537-547.
34. Viviani B, Bartesaghi S, Corsini E, Villa P, Ghezzi P, Garau A, Galli CL, & Marinovich M (2005) Erythropoietin protects primary hippocampal neurons increasing the expression of brain-derived neurotrophic factor. *J. Neurochem.* 93(2):412-421.
35. Cotman CW & Berchtold NC (2007) Physical activity and the maintenance of cognition: Learning from animal models. *Alzheimers & Dementia* 3(2):S30-S37.

36. Ding Q, Vaynman S, Akhavan M, Ying Z, & Gomez-Pinilla F (2006) Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. *Neuroscience* 140(3):823-833.
37. Carro E, Nunez A, Busiguina S, & Torres-Aleman I (2000) Circulating insulin-like growth factor I mediates effects of exercise on the brain. *J. Neurosci.* 20(8):2926-2933.
38. Chavez JC & LaManna JC (2002) Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: potential role of insulin-like growth factor-1. *J. Neurosci.* 22(20):8922-8931.
39. Fischer A, Sananbenesi F, Wang X, Dobbin M, & Tsai LH (2007) Recovery of learning and memory is associated with chromatin remodelling. *Nature* 447(7141):178-182.
40. McNamara RK & Skelton RW (1993) The neuropharmacological and neurochemical basis of place learning in the Morris water maze. *Brain Res. Brain Res. Rev.* 18(1):33-49.
41. Falkenberg T, Mohammed AK, Henriksson B, Persson H, Winblad B, & Lindefors N (1992) Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. *Neurosci. Lett.* 138(1):153-156.
42. Sanchez PE, Fares RP, Risso JJ, Bonnet C, Bouvard S, Le-Cavorsin M, Georges B, Moulin C, Belmeguenai A, Bodennec J, Morales A, Pequignot JM, Baulieu EE, Levine RA, & Bezin L (2009) Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons. *Proc. Natl. Acad. Sci. U. S. A.* 106(24):9848-9853.
43. Koh S, Chung H, Xia H, Mahadevia A, & Song Y (2005) Environmental enrichment reverses the impaired exploratory behavior and altered gene expression induced by early-life seizures. *J. Child Neurol.* 20(10):796-802.
44. Faverjon S, Silveira DC, Fu DD, Cha BH, Akman C, Hu Y, & Holmes GL (2002) Beneficial effects of enriched environment following status epilepticus in immature rats. *Neurology* 59(9):1356-1364.
45. Wang CA, Lai MC, Lui CC, Yang SN, Tiao MM, Hsieh CS, Lin HH, & Huang LT (2007) An enriched environment improves cognitive performance after early-life status epilepticus accompanied by an increase in phosphorylation of extracellular signal-regulated kinase 2. *Epilepsy Behav* 11(3):303-309.
46. Baumans V (2004) Use of animals in experimental research: an ethical dilemma? *Gene Ther.* 11 Suppl 1:S64-66.
47. Laviola G, Hannan AJ, Macri S, Solinas M, & Jaber M (2008) Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiol. Dis.* 31(2):159-168.
48. Meehan CL & Mench JA (2007) The challenge of challenge: Can problem solving opportunities enhance animal welfare? *Applied Animal Behaviour Science* 102(3-4):246-261.
49. Meijer MK, Sommer R, Spruijt BM, van Zutphen LF, & Baumans V (2007) Influence of environmental enrichment and handling on the acute stress response in individually housed mice. *Lab. Anim.* 41(2):161-173.
50. Pena Y, Prunell M, Rotllant D, Armario A, & Escorihuela RM (2009) Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology* 34(9):1390-1404.
51. Augustsson H, van de Weerd HA, Kruitwagen CL, & Baumans V (2003) Effect of enrichment on variation and results in the light/dark test. *Lab. Anim.* 37(4):328-340.
52. Francia N, Cirulli F, Chiarotti F, Antonelli A, Aloe L, & Alleva E (2006) Spatial memory deficits in middle-aged mice correlate with lower exploratory activity and a subordinate status: role of hippocampal neurotrophins. *Eur. J. Neurosci.* 23(3):711-728.
53. Ickes B, Pham T, Sanders L, Albeck D, Mohammed A, & Granholm A (2000) Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. *Exp. Neurol.* 164(1):45-52.
54. Zhao L, Mattsson B, & Johansson B (2000) Environmental influence on brain-derived neurotrophic factor messenger RNA expression after middle cerebral artery occlusion in spontaneously hypertensive rats. *Neuroscience* 97(1):177-184.

55. Chen ZY, Asavaritikrai P, Prchal JT, & Noguchi CT (2007) Endogenous erythropoietin signaling is required for normal neural progenitor cell proliferation. *J. Biol. Chem.* 282(35):25875-25883.
56. O'Kusky JR, Ye P, & D'Ercole AJ (2000) Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J. Neurosci.* 20(22):8435-8442.
57. Bezard E, Dovero S, Belin D, Duconger S, Jackson-Lewis V, Przedborski S, Piazza PV, Gross CE, & Jaber M (2003) Enriched environment confers resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and cocaine: involvement of dopamine transporter and trophic factors. *J. Neurosci.* 23(35):10999-11007.
58. Faherty CJ, Shepherd KR, Herasimtschuk A, & Smeyne RJ (2005) Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. *Molecular Brain Research* 134(1):170-179.
59. Spires TL, Grote HE, Varshney NK, Cordery PM, van Dellen A, Blakemore C, & Hannan AJ (2004) Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. *J. Neurosci.* 24(9):2270-2276.
60. Hicks RR, Zhang L, Atkinson A, Stevenon M, Veneracion M, & Seroogy KB (2002) Environmental enrichment attenuates cognitive deficits, but does not alter neurotrophin gene expression in the hippocampus following lateral fluid percussion brain injury. *Neuroscience* 112(3):631-637.
61. Kondo M, Gray LJ, Pelka GJ, Christodoulou J, Tam PP, & Hannan AJ (2008) Environmental enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome--Mecp2 gene dosage effects and BDNF expression. *Eur. J. Neurosci.* 27(12):3342-3350.
62. Auvergne R, Lere C, El Bahh B, Arthaud S, Lespinet V, Rougier A, & Le Gal La Salle G (2002) Delayed kindling epileptogenesis and increased neurogenesis in adult rats housed in an enriched environment. *Brain Res.* 954(2):277-285.
63. Young D, Lawlor PA, Leone P, Dragunow M, & During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat. Med.* 5(4):448-453.
64. Rutten A, van Albada M, Silveira DC, Cha BH, Liu X, Hu YN, Cilio MR, & Holmes GL (2002) Memory impairment following status epilepticus in immature rats: time-course and environmental effects. *Eur. J. Neurosci.* 16(3):501-513.
65. Dhanushkodi A & Shetty AK (2008) Is exposure to enriched environment beneficial for functional post-lesional recovery in temporal lobe epilepsy? *Neurosci. Biobehav. Rev.* 32(4):657-674.
66. Brun VH, Otnass MK, Molden S, Steffenach HA, Witter MP, Moser MB, & Moser EI (2002) Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. *Science* 296(5576):2243-2246.
67. Liu X, Muller RU, Huang LT, Kubie JL, Rotenberg A, Rivard B, Cilio MR, & Holmes GL (2003) Seizure-induced changes in place cell physiology: relationship to spatial memory. *J. Neurosci.* 23(37):11505-11515.
68. Frank LM, Stanley GB, & Brown EN (2004) Hippocampal plasticity across multiple days of exposure to novel environments. *J. Neurosci.* 24(35):7681-7689.
69. Ekstrom AD, Kahana MJ, Caplan JB, Fields TA, Isham EA, Newman EL, & Fried I (2003) Cellular networks underlying human spatial navigation. *Nature* 425(6954):184-188.
70. Hori E, Tabuchi E, Matsumura N, Tamura R, Eifuku S, Endo S, Nishijo H, & Ono T (2003) Representation of place by monkey hippocampal neurons in real and virtual translocation. *Hippocampus* 13(2):190-196.
71. Rolls ET (1999) Spatial view cells and the representation of place in the primate hippocampus. *Hippocampus* 9(4):467-480.
72. Zald DH, Hagen MC, & Pardo JV (2002) Neural correlates of tasting concentrated quinine and sugar solutions. *J. Neurophysiol.* 87(2):1068-1075.

73. Zald DH & Pardo JV (1997) Emotion, olfaction, and the human amygdala: amygdala activation during aversive olfactory stimulation. *Proc. Natl. Acad. Sci. U. S. A.* 94(8):4119-4124.

74. Williams JB, Pang D, Delgado B, Kocherginsky M, Tretiakova M, Krausz T, Pan D, He J, McClintock MK, & Conzen SD (2009) A model of gene-environment interaction reveals altered mammary gland gene expression and increased tumor growth following social isolation. *Cancer Prev Res (Phila Pa)* 2(10):850-861.

RESULTS – PART II

TITLE :

OPTIMAL NEUROPROTECTION BY ERYTHROPOIETIN REQUIRES ELEVATED EXPRESSION OF ITS RECEPTOR IN NEURONS

PUBLICATION STATE :

Published in the Proceedings of the National Academy of Sciences, USA.

PNAS | 2009 | vol. 106 | pages 9848-9853

SPECIFIC AIMS :

1. Find appropriate physiological conditions to optimize neuronal expression of erythropoietin receptor (EpoR) in the rat brain.
2. Establish whether rhEpo administration can protect vulnerable neuronal populations to excitotoxic injury following up-regulation of the expression of its receptor in neurons.

MAIN RESULTS :

- Neurons in the ventral limbic region (VLR), which includes the amygdala, the piriform cortex and the insular agranular cortex, are highly vulnerable to excitotoxic injury and express low levels of EpoR transcript and protein, compared to the dorsal hippocampus, the neocortex and the spinal cord.
- Repeated, but not single, hypoxia sessions (6h at 8% O_2) induce EpoR level in the VLR to that measured in the dorsal hippocampus, in association with a super-induction of *Epo* gene expression.
- Environmental enrichment in Marlau™ cages also up-regulates brain *EpoR* gene expression in the VLR, and makes it possible to induce EpoR in the dorsal hippocampus and the neocortex following repeated hypoxia.
- In the condition of increased EpoR levels in the VLR following repeated hypoxia sessions, exogenous administration of erythropoietin can protect VLR neurons after pilocarpine-induced *status epilepticus*.

➔ This study provides evidence that optimization of the neuroprotective effect of a molecule such as erythropoietin requires that the expression of its receptor is sufficiently elevated in the brain area to protect at the time of its administration.

Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons

Pascal E. Sanchez [1-4]*, Raafat P. Fares [1-4]*, Jean-Jacques Risso [5], Chantal Bonnet [1-4], Sandrine Bouvard [1,2,4], Marion Le-Cavorsin [1-4], Béatrice Georges [1-4], Colette Moulin [1-4], Amor Belmeguenai [1-4], Jacques Bodennec [1-4], Anne Morales [1-4], Jean-Marc Pequignot[1-4], Etienne-Emile Baulieu [6,7], Robert A. Levine [8] , Laurent Bezin[1-4]

1. University of Lyon, F-69003 Lyon, France;
2. University Lyon 1, F-69003 Lyon, France;
3. Centre National de la Recherche Scientifique, UMR 5123, Integrative Cellular and Molecular Physiology Lab., 43 bd du 11 novembre 1918, F-69622 Villeurbanne cedex, France;
4. CTRS-IDEE and Hospices Civils de Lyon;
5. Institut de Médecine Navale du Service de Santé des Armées, BP 610, F-83800 Toulon Armées, France;
6. Institut National de la Santé et de la Recherche Médicale, UMR 788, 80 rue du Général Leclerc, F-94276 Le Kremlin-Bicêtre cedex, France;
7. MAPREG Company, Centre Hospitalier Universitaire de Bicêtre, 78 rue du Général Leclerc, F-94276 Le Kremlin-Bicêtre cedex, France
8. Center for Integrative Wellness, Henry Ford Health System, Southfield Medical Center, 22777 West Eleven Mile Road, Southfield, MI 48033, USA
* These authors contributed equally to the study.

Abstract

Epo receptor (EpoR) binding mediates neuroprotection by endogenous erythropoietin (Epo) or by exogenous recombinant human Epo (rhEpo). The level of *EpoR* gene expression may determine tissue responsiveness to Epo. Thus, harnessing the neuroprotective power of Epo requires an understanding of the Epo-EpoR system and its regulation. We tested the hypothesis that neuronal expression of EpoR is required to achieve optimal neuroprotection by Epo. The ventral limbic region (VLR) in the rat brain was used because we determined that its neurons express minimal EpoR under basal conditions and they are highly sensitive to excitotoxic damage, such as occurs with pilocarpine-induced *status epilepticus* (Pilo-SE). We report that: (1) EpoR expression is significantly elevated in nearly all VLR neurons when rats are subjected to 3 moderate hypoxic exposures, with each separated by a 4-day interval, (2) synergistic induction of EpoR expression is achieved in the dorsal hippocampus and neocortex by the combination of hypoxia and exposure to an enriched environment, with minimal increased expression by either treatment alone, (3) rhEpo administered after Pilo-SE cannot rescue neurons in the VLR unless neuronal induction of EpoR is elicited by hypoxia prior to Pilo-SE. This is the first demonstration using environmental manipulations in normal rodents of the strict requirement for induction of EpoR expression in brain neurons to achieve optimal neuroprotection. Our results indicate that regulation of *EpoR* gene expression may facilitate the neuroprotective potential of rhEpo.

INTRODUCTION

Recombinant human erythropoietin (rhEpo) is now recognized as a promising molecule to prevent or protect against neurodegeneration in a wide variety of experimental neurological disorders (1-3). In addition, encouraging results on the neuroprotective efficacy of rhEpo in humans have been obtained from clinical trials involving stroke patients (4), patients with chronic schizophrenia (5), and patients with chronic progressive multiple sclerosis (6).

The level of Epo receptor (*EpoR*) expression in brain tissue has been proposed to determine the cytoprotective effects of Epo (7). *In vivo*, all neurons may not be prone to the protective effects of Epo, based on previous results showing that constitutive *EpoR* gene expression is heterogeneous in the rat central nervous system (8). Furthermore, all brain areas do not exhibit the same neuronal vulnerability to excitotoxic injury; in comparison to the dorsal regions of the brain, the ventral limbic region (VLR) is subjected to intense neuronal death in response to a pilocarpine-induced *status epilepticus* (Pilo-SE) (9). The present study was thus aimed at finding physiological conditions making it possible to increase expression of *EpoR* in neurons of the VLR, in order to test the hypothesis that increased neuronal expression of *EpoR* is required to achieve optimal neuroprotection by rhEpo following excitotoxicity induced by Pilo-SE.

In vitro studies showed that hypoxic exposure increases *EpoR* gene expression in cultured neurons (10-12). However, in the adult mouse brain, a single hypoxic exposure *in vivo* failed to increase *EpoR* gene expression (13, 14). We thus hypothesized that in rats, *EpoR* gene induction in neurons may require repetitive hypoxic challenges. Here, we show that three hypoxic exposures significantly increase neuronal expression of *EpoR*, and secondly, that *EpoR* induction is required for rhEpo to counteract neurodegenerative processes in the VLR following Pilo-SE.

MATERIAL AND METHODS

In vivo procedures

All animal experiments were in compliance with the guidelines of the European Union (directive 86/609), taken into the French law (decree 87/848), regulating animal experimentation. All efforts were made to minimize animal suffering and to reduce the number of animals used. Sprague-Dawley male rats were used throughout the study. For more detailed information see *supporting materials and methods*.

Hypoxic exposure. Hypoxia was realized by introducing rats within a chamber (Biospherix), the oxygen (O_2) proportion of which decreased progressively from 21% to 8% in 1h. Each hypoxia exposure was maintained at 8% O_2 during 6h. O_2 proportion was automatically regulated by the Pro-Ox system (Biospherix). The 3 hypoxia exposures were carried out 4 days apart.

Administration of rhEpo. Recombinant human Epo (rhEpo; Eprex®, generously provided by Janssen-Cilag) was administered at 5,000 IU/kg (i.p.). For more detailed information see *supporting materials and methods*.

Environmental enrichment. We engineered a cage (Marlau™ cage, patent # FR09/00544) promoting standardization of the procedures of enrichment. This cage (supplemental Fig. 3) allows increased social interactions (12 rats per cage), increased voluntary exercise (large surface area and presence of 3 running wheels), "diverting" activities (red tunnel, ladder, toboggan slide), and cognitive stimulations using labyrinths, the configuration of which is changed

3 times a week. Standard rats were housed in groups of 6 from weaning to adulthood in type "E" cages (Charles River, France).

Pilo-SE. Scopolamine methylnitrate (1 mg/kg, s.c.; Sigma) was administered 30 min prior to pilocarpine hydrochloride (350 mg/kg, i.p.; Sigma). SE was stopped 2 h after its onset by i.p. injection of 20 mg/kg diazepam (Valium®, Roche), as previously described (8, 39).

Ex vivo procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (250 mg/kg) before being sacrificed. For biochemical analysis, brain structures were rapidly microdissected, frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry analysis, animals were transcardially perfused with chilled 4% paraformaldehyde in 0.1M phosphate buffer. After cryoprotection in 25% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C. For Elisa measurement, rats were intracardially perfused for 2 min with chilled 0.9% NaCl. After brain removal, the VLR was dissected, weighted, frozen in liquid nitrogen, and stored at -80°C.

Reverse Transcriptase real time Polymerase Chain Reaction (RT-real time PCR). Variations in transcript levels were determined by real time PCR amplification of cDNAs of interest after reverse transcription (RT) of total mRNAs, as previously detailed (8). For more detailed information on primers used for PCR, see *supporting materials and methods*.

Quantitative determination of rhEpo using Elisa. RhEpo was measured using an Elisa kit (R&D Systems), as previously described (8).

Immunohistochemistry. Free floating sections of fixed tissue were used for colorimetric or fluorescent labelling of Epo and *EpoR*, in combination or not with either labeling of NeuN or GFAP. Images were captured by a TCS SP2 confocal microscopy system (Leica). For more detailed information about antibody characterization, see (40) and *supporting materials and methods*.

Labeling of neuronal degeneration. Fluoro-Jade B (Chemicon) was used to identify degenerating neurons after Pilo-SE in rats (41). Cell death occurring with DNA breaks was detected using terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling of DNA breaks (Roche).

Image analysis. Measurements of (i) neuronal density and (ii) fluorescent intensity were performed using an image analysis system (Visilog, Noesis). For more detailed information see *supporting materials and methods*.

Statistical analysis

Data are expressed as mean ± SEM of the different variables analyzed (mRNA level, neuronal density, brain uptake of rhEpo) and were compared among groups by using one- or two-way ANOVA followed by Fisher's protected Least Significance Differences (LSD) test.

RESULTS

Constitutive expression of *EpoR* is low in VLR neurons

The *EpoR* gene is expressed at different levels in the adult rat hippocampus (Hi), neocortex (NC) and spinal cord (8). Here, we have refined the analysis of *EpoR* gene expression by examining the VLR, which includes the insular agranular cortex (IAC), the amygdala (AMG) and the piriform cortex (PC). *EpoR* gene expression has been analyzed at both the transcript and protein level, by targeting the full length-*EpoR* isoform involved in intracellular signaling (15-17). Therefore, the PCR primers and the antibody used in this study are specific for the C-terminal cytoplasmic domain of *EpoR* cDNA and protein,

respectively. We provide evidence that (*i*) *EpoR* transcript level in the VLR is lower than that measured in the dorsal hippocampus (HiD) (Fig. 1A), (*ii*) *EpoR* protein is detected with ease using colorimetric immunohistochemistry in the pyramidal layer of the PC, while it is expressed at a barely detectable level in the other areas of the VLR (Fig. 1B), and (*iii*) *EpoR* protein is exclusively detected in neurons when using dual immunofluorescent labeling of *EpoR* with NeuN (Fig. 1B).

Repeated hypoxic exposures activate neuronal expression of *EpoR* in the VLR

In regard to the faint expression of *EpoR* in most of the neurons of the VLR, we explored the possibility of activating *EpoR* gene expression above detection threshold in these neurons. Hypoxia had already been shown to induce *EpoR* gene expression in cultured neurons (10, 11, 18), but not *in vivo*. Here we show that a single hypoxic exposure (1H) has no effect on the *EpoR* transcript level in HiD or the VLR immediately after hypoxia (Fig. 1A) or 1, 2 or 8 days after reoxygenation (data not shown). However, by repeating hypoxic exposure on three occasions (3H) with each separated by 4 days, we demonstrate for the first time a significant increase (+85%) in *EpoR* transcript level in the VLR, which was observed at the time of reoxygenation after the last hypoxic exposure (Fig. 1A); the basal level recovered 1 day later (data not shown). The increased *EpoR* transcript level was associated 3 days after 3H with an increased number of cells expressing *EpoR* protein above detection threshold in the piriform cortex, the amygdala and the insular agranular cortex. All *EpoR*-positive cells appeared to be neurons (Fig. 1B). Qualitative analysis of immunohistochemical detection of *EpoR* revealed that the number of cells detected 1 day after 3H was intermediate between the number determined in controls and the number obtained 3 days after 3H (data not shown). Quantitative analysis of *EpoR* immunofluorescent labeling over all NeuN-positive neurons revealed that: (*i*) fluorescent labeling was detected in all neurons, with the lowest values ranging from 25 to 43 arbitrary units (A.U); this represented 5% of total neurons both in controls and after 3H, (*ii*) neurons with a concentration greater than 250 A.U. represented 11% of total neurons in controls and 48% after 3H, and (*iii*) the average cellular concentration of *EpoR* increased by 74% in VLR neurons after 3H (Fig. 1C).

Hypoxia-induced expression of *EpoR* in VLR neurons is associated with induction of Epo

In vivo, a single hypoxic exposure (1H) is well-known to increase *Epo* gene expression in the brain of rodents (19). Here we show that, after 1H, Epo transcript level was increased at the time of reoxygenation to the same extent in the two brain regions studied (HiD and VLR), and was further increased after 3H in the VLR only (Fig. 2A). After either 1H or 3H, the apparent peak of Epo mRNA was observed at the time of reoxygenation only, basal level being recovered 1 day later (data not shown).

The greater increase in Epo mRNA observed after 3H was, 3 days after reoxygenation, associated with an increased number of cells expressing Epo protein above detection threshold in the piriform cortex, the amygdala and the insular agranular cortex, and all Epo+ cells appeared to be neurons (Fig. 2B). Qualitative analysis of immunohistochemical detection of Epo revealed that the number of cells detected 1 day after 3H was intermediate between the number determined in controls and the number obtained 3 days after 3H (data not shown). Quantitative analysis of Epo immunofluorescent labeling over all NeuN-positive neurons revealed that: (*i*) 100% of VLR neurons expressed an Epo concentration greater than 100 A.U. after 3 H, compared to 26% in

controls, and (*ii*) the average cellular concentration of Epo increased by 349% in VLR neurons after 3H (Fig. 2C). Our results demonstrate that repeated hypoxic exposures elevate both *Epo* and *EpoR* gene expression in neurons in the VLR.

Hypoxia-induced expression of *EpoR* is not associated with degenerative processes

Increased *EpoR* expression within the central nervous system was reported in pathological conditions in humans (20-23), and in rodent models of neurodegeneration (8, 24-26). Here, after 3H, we did not detect any degenerating neurons, either by Fluorojade B staining performed 1 day (data not shown) and 3 days after reoxygenation (supplemental Fig. 1A), or using terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling of DNA breaks (supplemental Fig. 1B). These results indicate that induced expression of *EpoR* following repeated hypoxic exposures was unrelated to neurodegenerative events in our system.

Environmental enrichment extends hypoxia-induced *EpoR* gene expression beyond the VLR

Environmental enrichment refers to housing conditions with enhanced sensory, cognitive and motor stimulation. It has a variety of what are considered beneficial effects on structural brain plasticity and behavior (27, 28). These effects in brain are likely sustained by modulation of the expression patterns of many different genes (29). We report here for the first time that rats housed in Marlau™ enriched cages had greater *EpoR* transcript level in the dorsal (HiD) and ventral (HiV) parts of the hippocampus, and the VLR, compared to rats housed in "standard" cages (Fig. 3A). Maximal *EpoR* transcript levels in the NC, the HiD, and the VLR were reached in rats reared in enriched cages and subjected to 3H (Fig. 3A). In the VLR of rats raised in enriched cages, the maximal level of *EpoR* transcript was associated with an increased number of cells detected for *EpoR* at 3 days after 3H (Fig. 3B); we also verified that all *EpoR* expressing cells were neurons (data not shown). In the ventral hippocampus (HiV), the lowest *EpoR* transcript level was observed in rats raised in standard cages, and the maximal level was attained in rats raised in enriched cages or subjected to 3H, independently of the rearing condition (Fig. 3A). Rats raised in enriched cages displayed increased Epo transcript level in the hippocampus (HiD and HiV) and the VLR (Fig. 3C), compared to rats housed in standard cages; however, responsiveness of Epo transcript level to 3H in all brain areas studied was similar in the two housing conditions (Fig. 3D).

Induced-*EpoR* gene expression in the VLR determines the neuroprotective effect of rhEpo after Pilo-SE

High dose (5,000 IU/kg) of rhEpo (administered immediately, 1 day and 3 days after Pilo-SE), which is known to induce neuroprotective effects in the HiD (8) (supplemental Fig. 2), failed to protect neurons in the VLR (Fig. 4A-C). This lack of rhEpo effect in the VLR cannot be attributed to weak passage of rhEpo across the brain barrier, since parenchymal rhEpo concentration was greater in the VLR than in the HiD (Fig. 4D).

We then tested whether neuroprotective effects of rhEpo could be achieved in the VLR after 3H-induced neuronal expression of *EpoR*. We first verified that the way rats entered into sustained SE following pilocarpine administration was not altered by 3H: continuous convulsions were attained 24.6 ± 2.9 min and 21.6 ± 1.0 min after pilocarpine administration in control rats and in rats subjected to 3H, respectively. We observed that rhEpo exerted neuroprotective effects in the VLR of rats subjected to 3H (Fig. 4A-C). However, the intense induction in endogenous Epo measured after 3H alone (Fig.

2A) was not sufficient to protect vulnerable neurons in the VLR following Pilo-SE (Fig. 4A-C). Interestingly, in the HiD, where 3H did not induce *EpoR* gene expression, we observed identical neuroprotective effects of rhEpo between rats subjected or not to 3H, (supplemental Fig.2), suggesting that lack of *EpoR* gene induction after 3H prevented rhEpo from exhibiting optimal neuroprotective effects.

Repeated hypoxic exposures do not alter IGF-1, and Tpo/TpoR transcript levels in the VLR

Endogenous factors can either act in synergy with Epo, as is the case with Insulin-like Growth Factor 1 (IGF-1) (30), or interfere with Epo, as is the case with thrombopoietin (Tpo) (10). Interestingly, hypoxia was shown to decrease Tpo and TpoR expression in cultured neurons at both the transcript and protein level (10). We expected that 3H would elevate the expression of IGF-1 and/or downregulate that of Tpo and its receptor TpoR. We show that Tpo and TpoR transcript levels tended to be decreased up to 1 day after reoxygenation at the end of 3H, while IGF-1 mRNA level remained stable (Fig. 5).

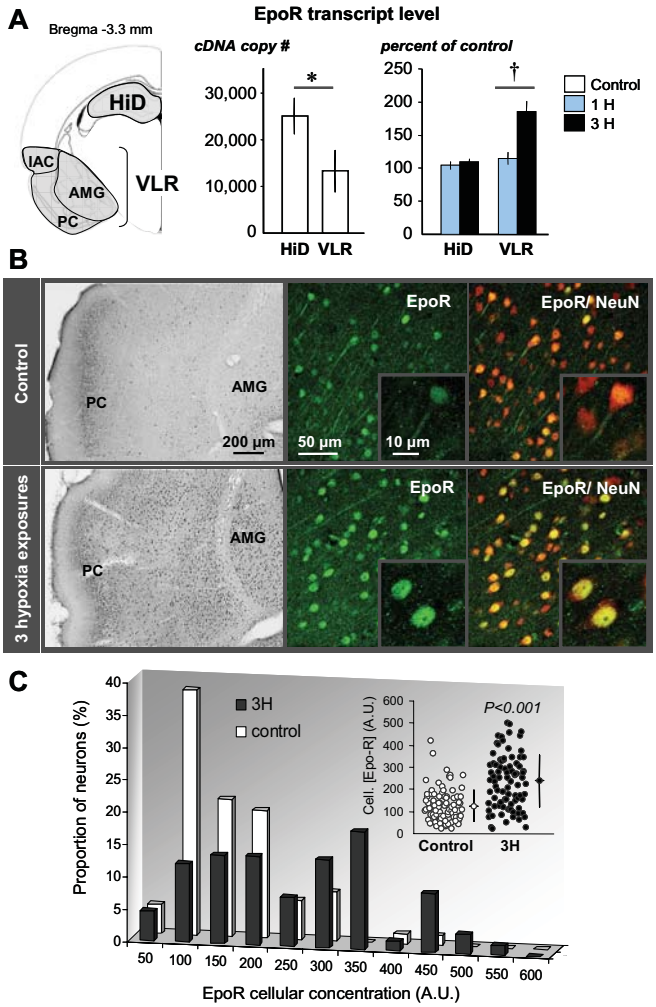


Fig. 1. Repetition of hypoxia exposures activates *EpoR* gene expression in the VLR. (A) EpoR transcript level measured by RT-qPCR in the HiD and the VLR of control rats revealed that EpoR-mRNA level was lower in the VLR than that measured in the HiD (* *P<0.05*). When measured at re-oxygenation time in rats subjected to either 1 (1H) or 3 (3H) hypoxic episodes, a significant increase in EpoR-mRNA level was found in the VLR after 3H only († *P<0.05* compared to 1H). All bars represent mean ± SEM (*n=4* in each group). (B) Three days after re-oxygenation time in rats subjected to 3H, the number of cells detected for EpoR remarkably increased compared to controls as shown after colorimetric detection; all EpoR-detected cells appeared to be neurons (NeuN+), as illustrated in the IAC: EpoR is in green and NeuN in red. (C) In the IAC, the increased number of cells detected after 3H was associated with an increased intensity of EpoR-immunofluorescent labeling (in A.U.), considered as the EpoR cellular concentration (*n=132* neurons in controls, *n=130* neurons after 3H). Graph in the panel represents all neurons measured and the mean ± SD for each group. Abbreviations: AMG, amygdala; HiD, dorsal hippocampus; IAC, insular agranular cortex; PC, piriform cortex; VLR, ventral limbic region.

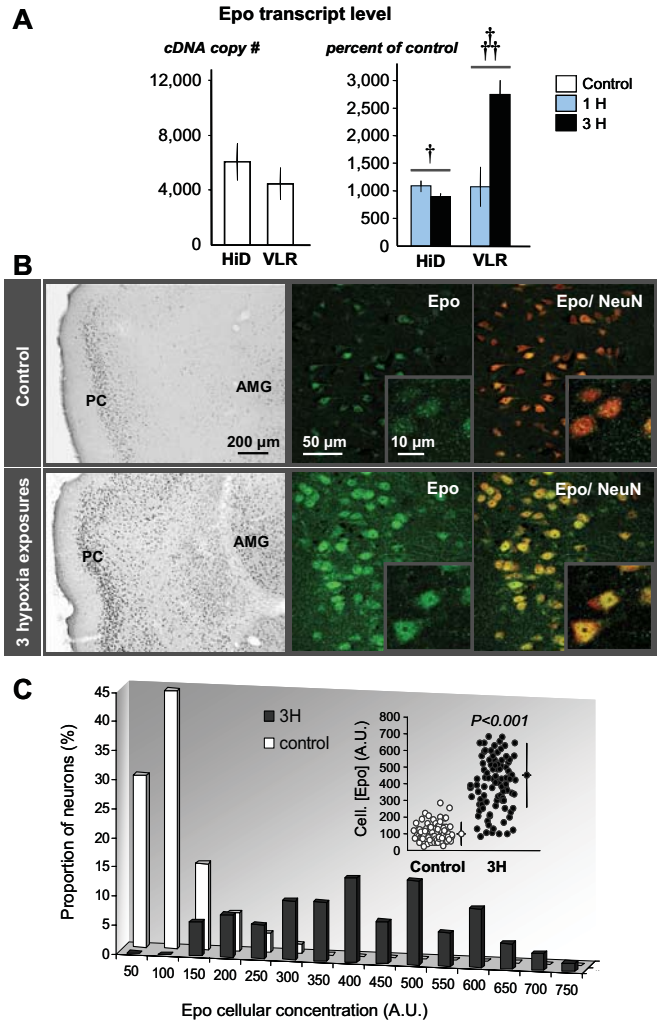


Fig. 2. Repetition of hypoxia exposures superinduces *Epo* gene expression in the VLR. (A) Constitutive level of Epo transcript measured by RT-qPCR was similar in the HiD and the VLR. At re-oxygenation time after 1H, Epo-mRNA level was significantly increased to the same extent in the three brain areas (*P<0.001* between control and 1H). However, at re-oxygenation time after 3H, Epo-mRNA level was superinduced in the VLR only († P<0.05, ††† *P<0.001* between 1H and 3H). All bars represent mean ± SEM (*n=4* in each group). (B) Three days after re-oxygenation time in rats subjected to 3H, the number of cells detected for Epo remarkably increased compared to controls, as shown after colorimetric detection; all Epo-detected cells appeared to be neurons (NeuN+), as illustrated in the IAC: Epo is in green and NeuN in red. (C) In the IAC, the increased number of detected cells after 3H was associated with an increased intensity of Epo-immunofluorescent labeling (in A.U.), considered as the Epo cellular concentration (*n=141* neurons in controls, *n=153* neurons after 3H). Graph in the panel represents all neurons measured and the mean ± SD for each group. Abbreviations: as in Fig. 1.

Figure 2

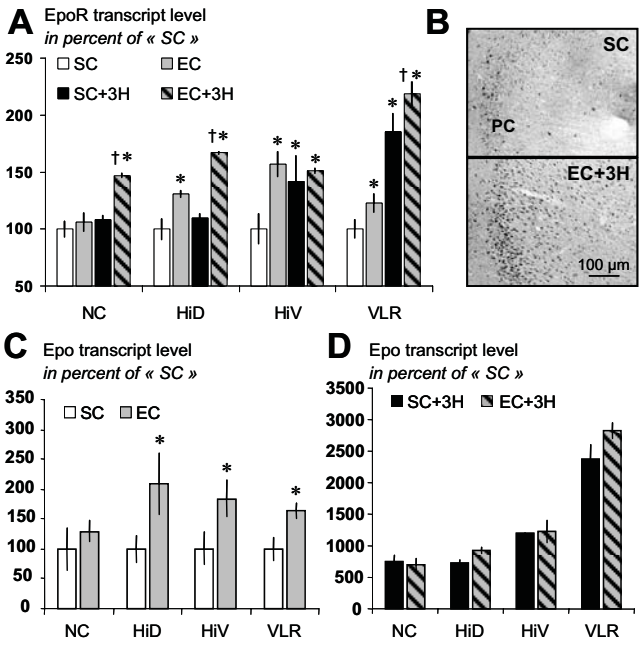


Fig. 3. Environmental enrichment increases brain *EpoR and Epo* gene expression. (A) Level of Epo-R trancript was measured in the NC, the HiD, the HiV and the VLR of rats raised in standard cages (SC) or enriched cages (EC), and subjected or not to 3H. In 3H groups, tissues were collected at re-oxygenation time of the last hypoxic exposure (* *P<0.01* compared to SC, † *P<0.01* compared to EC). **(B)** Three days after re-oxygenation time in rats subjected to EC+3H, the number of cells detected for EpoR increased compared to controls. **(C)** Constitutive expression of Epo was increased in the HiD, the HiV and the VLR in rats raised in EC compared to SC (* *P<0.05*). **(D)** Brain reactivity of *Epo* gene expression to 3H, measured at transcript level, was not affected by housing conditions. All bars represent mean ± SEM (*n=4* in each group). Abbreviations: as in Fig. 1; HiV, ventral hippocampus.

Figure 3

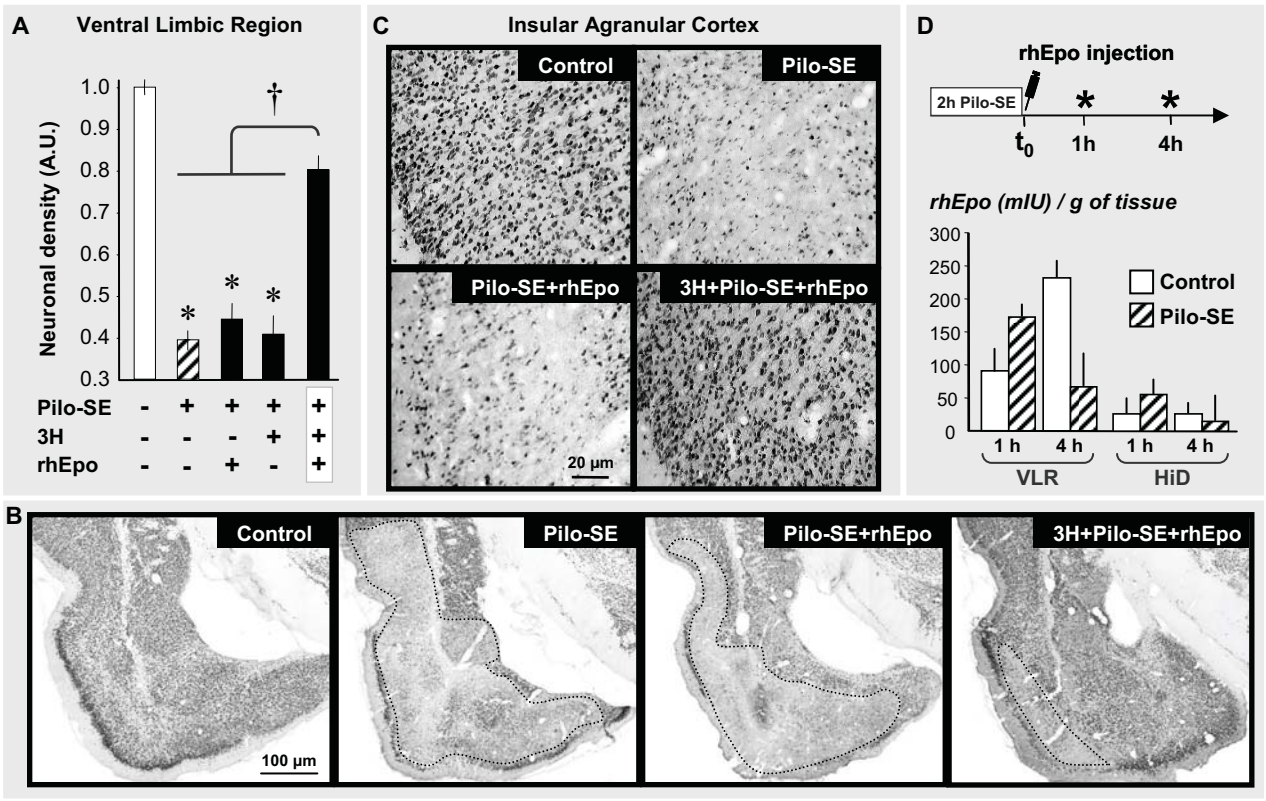


Fig. 4. Neuroprotective effects of rhEpo in the VLR are only observed in rats subjected to 3H following Pilo-SE. (A) Neuronal density in the VLR was measured at anatomical planes corresponding to IA +6.44 mm and + 5.40 mm according to (Paxinos and Watson, 1998). Because the anatomical plane itself had no effect, and no significant interaction was found between "anatomical plane" and "treatment condition", results for neuronal density were collapsed over the "anatomical plane" factor. Neither 3H alone, nor rhEpo alone, induced neuroprotective effects in the VLR after Pilo-SE. However, rhEpo administered in rats subjected to 3H significantly protected VLR neurons against neurodegeneration after Pilo-SE. * *P<0.001* compared to controls, † *P<0.05* (*n=6* in each group). **(B)** Immunohistochemical detection of NeuN six days after Pilo-SE in the lAC and the AMG: rhEpo had neuroprotective effects only when rats were subjected to 3H prior to Pilo-SE. **(C)** Parenchymal uptake of rhEpo was greater in the VLR than that measured in the HiD (*P<0.001*, ANOVA 2 in both control and Pilo-SE groups; factor 1 is "brain area", factor 2 is "time after rhEpo injection"), both in controls and in rats subjected to Pilo-SE. Each bar represents the mean ± SEM (*n=3* in each group). *: time of termination.

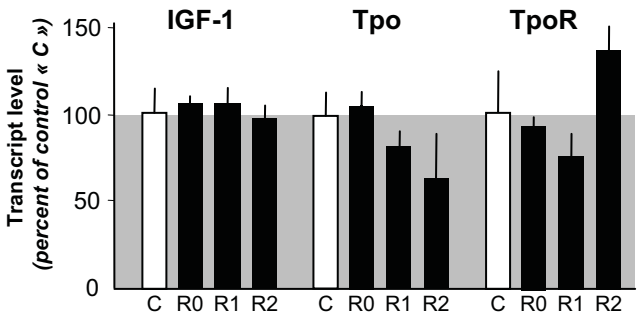


Fig. 5. Repetition of hypoxia exposures does not alter IGF-1, Tpo and TpoR transcript levels in the VLR. Controls and rats subjected to 3H were sacrificed immediately after (R0), 1 (R1) and 2 (R2) days after the last hypoxic exposure. Levels of transcripts were measured by RT-qPCR. Each bar represents the mean ± SEM (*n=4* in each group).

DISCUSSION

While advances have been made in the understanding of the mechanisms that contribute to premature brain cell death, efforts to discover and implement effective neuroprotection strategies have lagged behind. Our study provides evidence that *EpoR* gene expression is up-regulated in the adult rat brain under physiological conditions in which the brain attempts to decrease vulnerability. This up-regulation that occurs predominantly in neurons is required for rhEpo to exert any neuroprotective effect. Thus, EpoR upregulation appears to be an effective way to increase the neuroprotective efficacy of rhEpo.

We have also refined previous findings (8) that not all brain areas similarly express *EpoR* gene by showing, for example, that the level of EpoR is greater in the hippocampus than in the VLR. Consistent with the hypothesis that the tissue level of EpoR determines the tissue response to Epo (31), our data indicate that not all brain areas respond similarly to rhEpo. Indeed, we show in the Pilo-SE model that rhEpo significantly protects hippocampal neurons from degeneration, but is ineffective in protecting neurons of the VLR. This observation led us to search for physiological conditions that might enhance *EpoR* gene expression within the VLR.

Induction of *EpoR* has been proposed as a tissue-protective response to injury (2, 8, 25), and the only physiological response reported so far to enhance brain *EpoR* protein has been acclimation to ambient heat (32). Sublethal exposures to extreme environmental conditions, known to increase brain tolerance to a subsequent damaging event (33, 34), may also increase brain *EpoR* gene expression. Here, we show that non-deleterious repetition of hypoxic exposures dramatically increases *EpoR* gene expression at both the transcript and protein level, primarily in neurons of the VLR. We confirmed that *EpoR* gene expression remains unchanged in the brain of adult rodents after a single hypoxic exposure (13, 14). Hypoxia has been shown in the adult rat spleen to elevate the expression of *EpoR* (16), and the reason why repetition of hypoxic exposures induces *EpoR* in the VLR is not known. The superinduction of Epo, occurring exclusively in the VLR after hypoxia repetition, may play a role, involving the transcription factor GATA-3, previously linked with induced *EpoR* transcript level in cultured neurons in an Epo-dependent fashion (12). Intriguingly, we demonstrate for the first time that extreme environmental manipulations are not the only condition inducing *EpoR* gene expression in the brain. Indeed, environmental enrichment significantly elevated *EpoR* transcript level in the HiD and the VLR, and rendered dorsal brain areas (HiD and NCx) sensitive to repeated hypoxia. By contrast, maximal *EpoR* gene expression in the ventral brain areas (HiV and VLR) was attained after repeated hypoxia exposures, independently of whether rats were raised in standard or enriched housing conditions. There are likely to be other physiological interventions that will selectively raise *EpoR* in specific brain areas, but remain unidentified.

Consistent with the notion that enhanced *EpoR* gene expression confers increased tissue response to rhEpo, we ascertained that rhEpo protects VLR neurons following excitotoxic injury in rats subjected to repeated hypoxic exposure only. This effect is mainly explained by the increased expression of *EpoR* gene in VLR neurons, since neither rhEpo nor repeated hypoxic exposures were sufficient to induce neuroprotective effects. In addition, this effect is very likely independent of other adaptive

mechanisms activated by the repetition of hypoxic exposures, since: (*i*) in the dorsal hippocampus of rats subjected to 3H which showed no induction of *EpoR*, rhEpo had no additional protective effects, (*ii*) the expression of IGF-1, known to potentiate rhEpo effects *in vitro* (30), was not modified by 3H. Altogether, our results indicate that induction of *EpoR* gene expression in vulnerable brain areas by 3H is a prerequisite to optimize neuroprotective effects of rhEpo. Unfortunately, this concept could not be tested in the dorsal hippocampus, where *EpoR* gene expression is enhanced in rats raised in enriched cages and subjected to 3H, due to the inhibitory effect of environmental enrichment on the development of brain excitability and *status epilepticus* (35, 36).

Epo, which is a molecule induced by hypoxia, is considered to play a key role in the enhancement of brain robustness by hypoxia (19). Hence, rhEpo can be considered as an 'enviromimetic', defined as any exogenous molecule that mimics the beneficial effects of environmental changes (27). Here we show that repeated hypoxic exposures rendered rhEpo effective in the VLR by induced *EpoR*. These results are in line with the concept that optimization of the effect of neuroprotective agent may require the preliminary induction of its targeted receptor (37). Concerning rhEpo, future studies should elucidate mechanisms promoting trafficking of *EpoR* toward the cell surface (38) and the mechanisms selectively involved in the induction of EpoR after environmental manipulations, in order to develop drugs capable of inducing EpoR.

Acknowledgements

This work was supported by grants from the CNRS and the University of Lyon 1. P.E. Sanchez is a fellow from the Délégation Générale pour l'Armement, Ministère français de la défense. We thank D. Ressnikoff and Y. Tourneur from the Centre Commun de Quantimétrie of the University of Lyon 1 for their assistance in the use of the confocal microscope.

REFERENCES

1. Brines M, Cerami A (2005) Emerging biological roles for erythropoietin in the nervous system. *Nature Reviews Neuroscience* 6:484-494.
2. Maiese K, Chong ZZ, Li F, Shang YC (2008) Erythropoietin: Elucidating new cellular targets that broaden therapeutic strategies. *Prog Neurobiol* 85:194-213.
3. Noguchi CT, Asavaritikrai P, Teng R, Jia Y (2007) Role of erythropoietin in the brain. *Crit Rev Oncol Hematol* 64:159-171.
4. Ehrenreich H*, et al.* (2002) Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* 8:495-505.
5. Ehrenreich H*, et al.* (2007) Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. *Mol Psychiatry* 12:206-220.
6. Ehrenreich H*, et al.* (2007) Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis. *Brain* 130:2577-2588.
7. Chen ZY, Warin R, Noguchi CT (2006) Erythropoietin and normal brain development: receptor expression determines multi-tissue response. *Neurodegener Dis* 3:68-75.

8. Nadam J*, et al.* (2007) Neuroprotective effects of erythropoietin in the rat hippocampus after pilocarpine-induced status epilepticus. *Neurobiol Dis* 25:412-426.
9. Covolan L, Mello LE (2000) Temporal profile of neuronal injury following pilocarpine or kainic acid-induced status epilepticus. *Epilepsy Res* 39:133-152.
10. Ehrenreich H*, et al.* (2005) A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. *Proc Natl Acad Sci U S A* 102:862-867.
11. Liu R, Suzuki A, Guo Z, Mizuno Y, Urabe T (2006) Intrinsic and extrinsic erythropoietin enhances neuroprotection against ischemia and reperfusion injury in vitro. *J Neurochem* 96:1101-1110.
12. Yu X*, et al.* (2002) Erythropoietin receptor signalling is required for normal brain development. *Development* 129:505-516.
13. Bernaudin M*, et al.* (2000) Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia* 30:271-278.
14. Soliz J, Gassmann M, Joseph V (2007) Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. *J Physiol (Lond)* 583:329-336.
15. Liu C, Shen K, Liu Z, Noguchi CT (1997) Regulated human erythropoietin receptor expression in mouse brain. *J Biol Chem* 272:32395-32400.
16. Shams I, Nevo E, Avivi A (2005) Erythropoietin receptor spliced forms differentially expressed in blind subterranean mole rats. *FASEB J* 19:1749-1751.
17. Yamaji R*, et al.* (1998) The intron 5-inserted form of rat erythropoietin receptor is expressed as a membrane-bound form. *Biochim Biophys Acta* 1403:169-178.
18. Chin K*, et al.* (2000) Production and processing of erythropoietin receptor transcripts in brain. *Molecular Brain Research* 81:29-42.
19. Sharp FR, Bernaudin M (2004) HIF1 and oxygen sensing in the brain. *Nature Reviews Neuroscience* 5:437-448.
20. Assaraf MI*, et al.* (2007) Brain erythropoietin receptor expression in Alzheimer disease and mild cognitive impairment. *J Neuropathol Exp Neurol* 66:389-398.
21. Ehrenreich H*, et al.* (2004) Erythropoietin: a candidate compound for neuroprotection in schizophrenia. *Mol Psychiatry* 9:42-54.
22. Eid T*, et al.* (2004) Increased expression of erythropoietin receptor on blood vessels in the human epileptogenic hippocampus with sclerosis. *J Neuropathol Exp Neurol* 63:73-83.
23. Siren AL*, et al.* (2001) Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol (Berl)* 101:271-276.
24. Bernaudin M*, et al.* (1999) A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J Cereb Blood Flow Metab* 19:643-651.
25. Grasso G*, et al.* (2005) Erythropoietin and erythropoietin receptor expression after experimental spinal cord injury encourages therapy by exogenous erythropoietin. *Neurosurgery* 56:821-827; discussion 821-827.
26. Malhotra S, Savitz SI, Ocava L, Rosenbaum DM (2006) Ischemic preconditioning is mediated by erythropoietin through PI-3 kinase signaling in an animal model of transient ischemic attack. *J Neurosci Res* 83:19-27.
27. Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews. Neuroscience* 7:697-709.
28. van Praag H, Kempermann G, Gage FH (2000) Neural consequences of environmental enrichment. *Nature Reviews. Neuroscience* 1:191-198.
29. Rampon C*, et al.* (2000) Effects of environmental enrichment on gene expression in the brain. *Proc Natl Acad Sci U S A* 97:12880-12884.
30. Digicaylioglu M, Garden G, Timberlake S, Fletcher L, Lipton SA (2004) Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. *Proc Natl Acad Sci U S A* 101:9855-9860.

31. Chen ZY, Asavaritikrai P, Prchal JT, Noguchi CT (2007) Endogenous erythropoietin signaling is required for normal neural progenitor cell proliferation. *J Biol Chem* 282:25875-25883.
32. Shein NA, Horowitz M, Alexandrovich AG, Tsenter J, Shohami E (2005) Heat acclimation increases hypoxia-inducible factor 1alpha and erythropoietin receptor expression: implication for neuroprotection after closed head injury in mice. *J Cereb Blood Flow Metab* 25:1456-1465.
33. Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance. *Nature Reviews. Neuroscience* 7:437-448.
34. Ruscher K*, et al.* (2002) Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. *J Neurosci* 22:10291-10301.
35. Auvergne R*, et al.* (2002) Delayed kindling epileptogenesis and increased neurogenesis in adult rats housed in an enriched environment. *Brain Res* 954:277-285.
36. Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med* 5:448-453.
37. Lipton SA (2007) Pathologically activated therapeutics for neuroprotection. *Nature Reviews. Neuroscience* 8:803-808.
38. Ravid O*, et al.* (2007) An extracellular region of the erythropoietin receptor of the subterranean blind mole rat Spalax enhances receptor maturation. *Proc Natl Acad Sci U S A* 104:14360-14365.
39. Morales A*, et al.* (2006) Unexpected expression of orexin-B in basal conditions and increased levels in the adult rat hippocampus during pilocarpine-induced epileptogenesis. *Brain Res* 1109:164-175.
40. Sanchez P*, et al.* (2009) Erythropoietin receptor expression is concordant with erythropoietin but not with common beta chain expression in the rat brain throughout the life span. *J Comp Neurol* in press.
41. Schmued LC, Hopkins KJ (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res* 874:123-130.
42. Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic Press) 4th Ed p 256.

SUPPORTING MATERIALS AND METHODS

In vivo experimental design

Animals. Male Sprague-Dawley rats (Harlan, Gannat, France) were used throughout the experiments. They arrived at 5 weeks old (experiments, 1, 3 and 4) or at 2 weeks old with a dam (exeriment 2) in approved facilities, and housed at 21°C under diurnal lighting conditions (lights on from 06:00 am to 06:00 pm). They were maintained in groups of 6 in plastic cages (type "E" cages, Charles River, France) with free access to food and water. After 2 weeks acclimatization, rats underwent 3 weeks of experimental procedure. Following Pilo-SE, rats were housed individually to support recovery until being sacrificed. Control rats were housed in groups of 6 throughout all the experiment to avoid deleterious effects related to stress isolation. Control rats received systematically corresponding injections of saline solution.

Experiment 1. Brain expression of *Epo* and *EpoR* genes was determined in the Hi and the VLR of control rats (n=10) and rats subjected to either 1 (n=13) or 3 (n=22) hypoxic (H) exposure(s). Expression profiles of Epo and EpoR mRNAs were measured by RT-real time PCR (RT-qPCR) in control rats (n=6) and at various re-oxygenation times following either 1H (0h, n=4, 1 day, n=3; 2 days, n=3; 8 days, n=3) or 3H (0h, n=4; 1 day, n=4; 2 days, n=4; 8 days, n=4). In addition, immunostaining on brain sections of Epo and EpoR was realized in combination or not with neuronal (NeuN) and astrocytic (GFAP) markers at 1 (n=3) and 3 (n=3) days following 3H (n=6). Degenerative processes were also assessed after reoxygenation in all groups. Control rats (n=4) were included in this analysis.

Experiment 2. Rats were housed in MARLAU™ enriched cages (EC), developed in our group to standardize environmental enrichment, or two standard cages (SC, type "E", Charles River, France). Six and twelve rats were grouped together in SC and EC, respectively, from weaning until completion of the experiment. For more detailed information see *supporting materials and methods*. Six weeks after weaning, half of the rats of each SC and EC conditions (6/12 rats in each condition) were subjected to 3 hypoxic exposures. Levels of EpoR and Epo transcripts were measured by RT-qPCR in the HiD, the HiV, the NC and the VLR of 4 rats in each group. The remaining rats (n=2 per group) were used for EpoR immunostaining.

Experiment 3. To determine whether 3 hypoxic exposures could enhance rhEpo neuroprotective effects after Pilo-SE, rats were subjected to 3H (n=12) or not (n=12) prior to Pilo-SE. In each group, half of the rats that underwent Pilo-SE were treated or not with rhEpo at 5,000 IU/kg i.p. immediately after cessation of SE, and then 1 day and 3 days later. This dose of rhEpo corresponds to the optimal neuroprotective dose with this route of administration (1). Neuronal protection was evaluated 6 days after Pilo-SE by measuring the density of NeuN+ cells in the VLR. Control rats (n=6) received the same injections as rats subjected to SE, except that saline was substituted for pilocarpine.

Experiment 4. Tissue concentration of rhEpo in the VLR and the HiD was determined at different times (1 h and 4 h) following its peripheral administration in control rats (n=6) and in rats subjected to Pilo-SE (n=6). In these latter, rhEpo was administered just after cessation of SE.

Methods

RT-qPCR. Sequences of the different primer pairs used are: ***Epo*** (GenBank NM_017001) forward 5' GCT CCA ATC TTT GTG GCA TC 3', reverse 5' ATC CAT GTC TTG CCC CCT A 3' (66 bp); ***EpoR*** (GenBank D13566) forward 5' CCA GCT CTA AGC TCC TGT GC 3', reverse 5' CTT

CAG GTG AGG TGG AGT GG 3′ (68 bp), ***IGF-1*** (GenBank NM_178866.2) forward 5′ ATG CCC AAG ACT CAG AAG GA 3′, reverse 5′ CGT GGC ATT TTC TGT TTC TC 3′ (110 bp), ***Tpo*** (GenBank D32207) forward 5′ CCC AAG CAG AAC CTC TCA AC 3′, reverse 5′ TCA GGT ATC CAG GGA TTT GG 3′ (200 bp), ***TpoR*** (2) forward 5′ AGA ACC CAC AGA GTG GTG TG 3′, reverse 5′ TCA CAC CAT CCA GGA GCA AGA AT 3′ (220 bp). All primer pairs were designed using "Primer 3" software (NIH; www.basic.nwu.edu).

Immunohistochemistry. Free floating sections (40 μm thick) from paraformaldehyde-fixed tissue were incubated either with a rabbit polyclonal anti-Epo antibody (sc-7956; Santa Cruz), or with a rabbit polyclonal anti-EpoR antibody (sc-697; Santa Cruz), or with a mouse monoclonal anti-NeuN antibody (MAB-377; Chemicon) or a GFAP antibody (G3893; Sigma). For colorimetric immunolabeling, sections were then incubated with a biotinylated donkey antibody raised against mouse IgG (715-065-151; Jackson ImmunoResearch). Sections were incubated with avidin biotin peroxydase (1:500; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3′,3-diaminobenzidine (DAB, Vector). For fluorescent dual-immunolabeling, sections were exposed to an Alexa-488-conjugated donkey anti-rabbit IgG antibody (A-21206; Molecular Probes) and to an Alexa-633-conjugated goat anti-mouse IgG antibody (A21052; Molecular Probes). Sections were then mounted on Superfrost®Plus slides and coverglassed with Prolong Gold Antifade reagent (Molecular Probes). Images were captured by a TCS SP2 confocal microscopy system (Leica). Images were then imported into Adobe Photoshop 8.0.1 (Adobe Systems) for further editing.

Antibody characterization.

1. For Epo-R, a polyclonal rabbit antibody (Santa-Cruz # sc-697 (M-20)) raised against the C-terminal cytoplasmic domain of the precursor form of mouse EpoR was used. This antibody reveals in rat brain homogenates a single band slightly above the expected 66 kDa size(3, 4). Brain mapping of Epo-R protein using this antibody has been a matter of intensive debate due to the production of diverging results (4). Its specificity has then been questioned in a report showing immunolabeling in *Epo-R*-knockout mice (*Epo-R*$^{-/-}$) (5). However, conclusions raised from these *Epo-R*$^{-/-}$ mice must be taken with caution, since *Epo-R* gene, which is composed of eight exons, has been silenced by deletion of exons 1-6 only (3, 6). In the eventuality of a chimeric protein, partly encoded by the remaining exons 7-8, a signal may be obtained with the M-20 antibody, which is directed against an epitope encoded by exons 7-8 of the *Epo-R* gene. A complete abolition of Epo-R immunohistofluorescent labeling in rat brain tissue sections has been reported, when this antibody was pre-incubated with a 5-fold excess of the M-20-immunizing peptide (sc-697P) (7, 8).

2. For Epo, a polyclonal rabbit antibody (Santa-Cruz # sc-7956 (H-162)) raised against amino acids 28-189 from mature human Epo was used. This antibody stains a single 33 kDa band in western blot analysis of Epo expression in Normal Rat Kidney cell line transformed by the Kirsten murine sarcoma virus (KRNK cells) (manufacturer's technical information).

3. For the detection of neuronal cell type in brain slices, we used the monoclonal mouse antibody (Chemicon # MAB-377) raised against undetermined nuclear proteins of neurons (NeuN). This antibody recognizes 2-3 bands in the 46-48 kDa range and possibly another band at approximately 66 kDa (manufacturer's technical information). Even if the sequence of the immunizing antigen(s) has not been established yet, this antibody has been shown to stain neurons exclusively, both *in vivo* and *in*

vitro, recognizing most of neuronal cell types throughout the central nervous system of numerous vertebrates (9).

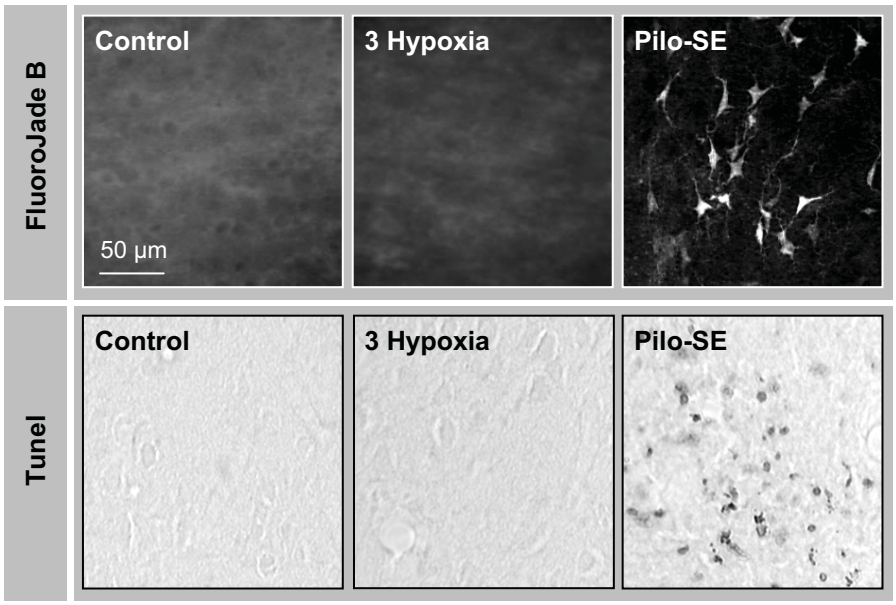
4. Mouse anti-GFAP (Sigma # G3893, monoclonal, immunogen: whole GFAP protein from pig spinal cord) reacts specifically with GFAP in immunoblotting assays and labels astrocytes in brain tissue sections (manufacturer's technical information).

Neuronal density measurement. Sections immunostained with NeuN were observed under a light microscope (Diaplan; Leitz) by two independent investigators who were blind to the groups from which the sections were taken. Images were captured with a video camera 3CCD (DXC-930P; Sony) coupled to an image analysis system (Visilog® 6.3; Noesis). The system allows to scan at magnification 10X adjacent fields throughout the whole ventral region and to reconstruct a single image composed of a mosaic of the digitized adjacent fields. Mosaics of the ventral region, composed by the amygdala, the piriform cortex and the insular agranular cortex were captured at both interaural +6.44 mm and +5.40 mm. To obtain an index of the neuronal density, the surface area occupied by NeuN+ cells within the VLR or within areas CA1 and CA3 of the HiD was divided by the total surface area.

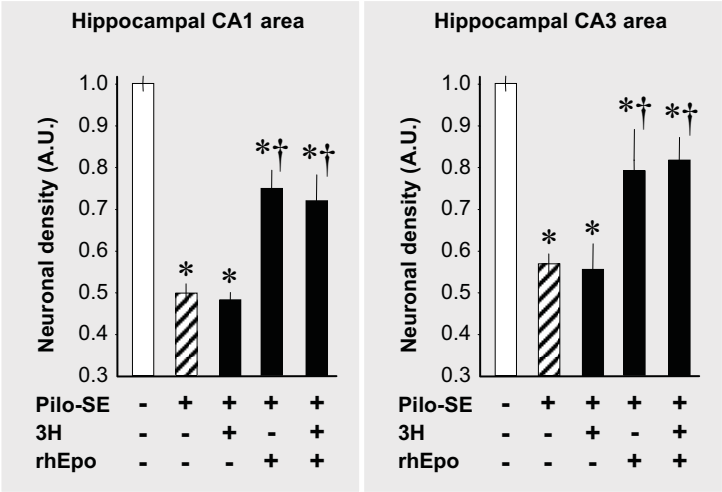
Measurement of the intensity of Epo- or EpoR-immunofluorescent labeling in neurons. Sections used were all processed together for the dual immunofluorescent labeling of Epo or EpoR with NeuN. They were then analyzed at the same conditions of photomultiplier gain, offset and pinhole aperture using a TCS SP2 confocal microscopy system (Leica), allowing the comparison of fluorescence intensity in all neurons contained in the image using an image analysis system (Visilog 6.3, Noesis).

REFERENCES OF THE SUPPORTING INFORMATION

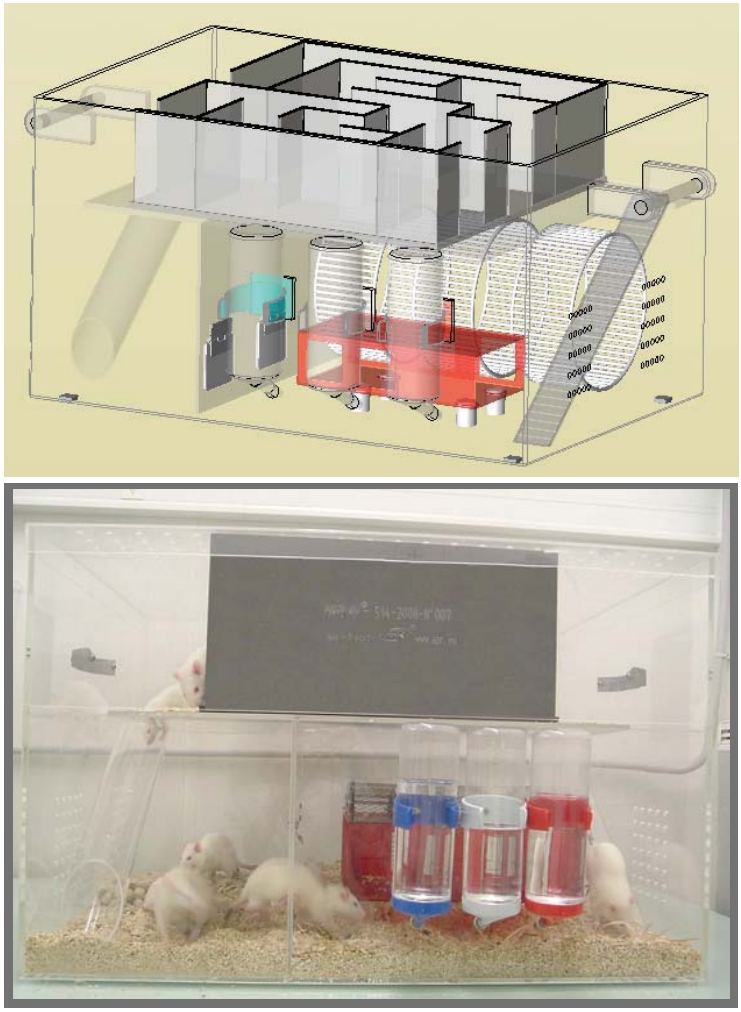
1. Calapai G*, et al.* (2000) Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *Eur J Pharmacol* 401:349-356.
2. Ehrenreich H*, et al.* (2005) A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. *Proc Natl Acad Sci U S A* 102:862-867.
3. Fu QL*, et al.* (2008) Up-regulated endogenous erythropoietin/erythropoietin receptor system and exogenous erythropoietin rescue retinal ganglion cells after chronic ocular hypertension. *Cell Mol Neurobiol* 28:317-329.
4. Kirkeby A, van Beek J, Nielsen J, Leist M, Helboe L (2007) Functional and immunochemical characterisation of different antibodies against the erythropoietin receptor. *J Neurosci Methods* 164:50-58.
5. Elliott S*, et al.* (2006) Anti-Epo receptor antibodies do not predict Epo receptor expression. *Blood* 107:1892-1895.
6. Wu H, Liu X, Jaenisch R, Lodish HF (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 83:59-67.
7. Grimm C*, et al.* (2002) HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nat Med* 8:718-724.
8. Sanchez P*, et al.* (2009) Erythropoietin receptor expression is concordant with erythropoietin but not with common beta chain expression in the rat brain throughout the life span. *J Comp Neurol* in press.
9. Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116:201-211.
10. Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic Press) 4th Ed p 256.



Supplemental Fig. 6. Repetition of hypoxia exposures does not induce detectable degenerative processes. Fluorojade B staining or Tunel were used to detect neurodegeneration or cell death associated with DNA breaks, respectively. No signal was obtained in controls and 3H rats with both methods, as shown here in the dorsal hippocampus three days after reoxygenation. By contrast, both Fluorojade B and Tunel+ cells were detected in the hippocampus 24h after Pilo-SE.



Supplemental Fig. 7: Neuroprotective effects of rhEpo in the dorsal hippocampus six days after Pilo-SE. Neuronal density in CA1 and CA3 areas was measured at anatomical planes corresponding to IA +6.44 mm and + 5.40 mm according to (Paxinos and Watson, 1998). Because the anatomical plane itself had no effect, and no significant interaction was found between "anatomical plane" and "treatment condition", results for neuronal density were collapsed over the "anatomical plane" factor. 3H alone did not induce any neuroprotective effects in the two hippocampal areas after Pilo-SE. By contrast, rhEpo administered alone or in rats subjected to 3H significantly protected pyramidal neurons of CA1 and CA3 areas against neurodegeneration after Pilo-SE. However, no greater neuroprotective effect of rhEpo was observed in rats subjected to 3H. * *P<0.001* compared to controls, † *P<0.001* compared to "Pilo-SE" or "Pilo-SE + 3H" (*n=6* in each group).



Supplemental Fig. 8. Marlau^{TM}: a new cage to calibrate environmental enrichment in rodents. This cage allows increased social interactions (12 rats per cage), increased voluntary exercise (large surface area and presence of 3 running wheels), "diverting" activities (red tunnel, ladder, toboggan slide), and cognitive stimulations using labyrinths, the configuration of which is changed 3 times a week.

RESULTS – PART III

TITLE :

EXOGENOUS ERYTHROPOIETIN EXERTS OPPOSITE EFFECTS ON COGNITIVE DEFICITS AND ANXIETY IN EPILEPTIC RATS DEPENDING ON THE QUALITY OF THE LIVING ENVIRONMENT

PUBLICATION STATE :

In preparation

SPECIFIC AIMS :

Ascertain in rats subjected to lithium/pilocarpine-induced *status epilepticus* (Li/Pilo-SE) at weaning whether:

1. administration of rEpo after SE in rats housed in standard cages,
2. housing rats in enriched Marlau™ cages just after SE,
3. the combination of rEpo administration and housing in enriched Marlau™ cages after SE,

can prevent cognitive decline and anxiety usually observed in epileptic rats.

MAIN RESULTS :

- Rats subjected to Li/Pilo-SE at weaning developed behavioral spontaneous recurrent seizures (BSRS) 4 weeks later without massive neurodegenerative processes, and exhibited increased anxiety and deficits in spatial learning and memory.
- Brain *epo* gene expression was dramatically decreased following Li/Pilo-SE, while brain *epor* gene expression remained unchanged, justifying the supplementation with exogenous recombinant Epo (rEpo) following Li/Pilo-SE.
- Treatment with rEpo exerted positive effects on anxiety and on learning and memory deficits in rats with BSRS reared in standard cages, while it exacerbated anxiety and generated memory deficits in rats with BSRS reared in enriched Marlau™ cages.

➔ Exogenous rEpo can exert positive effects on anxiety and can prevent the impairment in spatial learning and memory in rats prone to develop BSRS following Li/Pilo-SE, but only when rats are reared in standard (impoverished) environments. When determining the potential therapeutic effect of a molecule, one must take into consideration the quality of the living environment.

Exogenous erythropoietin exerts opposite effects on cognitive deficits and anxiety in epileptic rats depending on the quality of the living environment

Raafat P. Fares[1-4]*, Chantal Bonnet[1-4]*, Amor Belmeguenai[1-4], Hayet Kouchi[1-4], Béatrice Georges[1-4], Colette Moulin[1-4], Anne Morales[1-4], Philippe Ryvlin[1,2,4,5], Jacques Bodennec[1-4], Laurent Bezin[1-4]

1. University of Lyon, F-69003 Lyon, France;
2. University Lyon 1, F-69003 Lyon, France;
3. Centre National de la Recherche Scientifique, UMR 5123, Integrative Cellular and Molecular Physiology Lab., F-69622 Villeurbanne cedex, France;
4. Institute for children and adolescent with epilepsy, CTRS-IDEE, F-69003 Lyon, France;
5. Hospices Civils de Lyon, Neurological Hospital, Functional Neurology and Epileptology Department, F-69677 Bron, France.

* These authors contributed equally to the study.

INTRODUCTION

Anxiety represents a major problem for people with epilepsy (1). It is mostly associated with a past history and a current history of depression (2). Anxiety may be mediated by epilepsy-related factors such as increased seizure severity, earlier age of onset and poor seizure control (2-4). Anxiety in people with epilepsy may also be the consequence of other psychosocial factors, such as low self-esteem, restricted social activities and rejection from society (5).

Cognitive impairment is one of the biggest problems reported by people with epilepsy, and memory deficits are frequently reported, especially in patients with mesial temporal lobe epilepsy (1). While number of seizure-related variables, such as seizure onset, seizure frequency, and duration of epilepsy can impact on cognitive function (1), cognitive impairments can precede the first recognized seizure, as shown in subsets of children with new-onset epilepsy (6-8). Behavioral and cognitive impairments can also be aggravated by psychosocial factors (1).

In an animal model of epilepsy developed after lithium/pilocarpine-induced *status epilepticus* (Li/Pilo-SE) at weaning, enrichment of the living environment by provisions of cognitive stimulations and diverting activities, the so-called enriched housing conditions (EH), has been shown to protect against spatial learning deficits usually observed in animals reared in standard housing conditions (SH). Behavioral spontaneous recurrent seizures (BSRS) in this model were reported to appear more than 3 months after SE (9). It is noteworthy

that the protective effect against learning deficits was reported not only during the silent phase after SE (10, 11), but also once BSRS were installed, with no effect on pathological anxiety (see Results – Part I).

In an adult animal model of temporal lobe epilepsy, exogenous administration of recombinant human erythropoietin (rEpo) has been shown to protect neurons from excitotoxic damages occurring in the hippocampus, the amygdala and the piriform cortex (12, 13). However, the effect of rEpo treatment on anxiety and cognitive impairments has not been tested in animals with BSRS.

In this study, we determined whether: 1) rEpo treatment after Li/Pilo-SE at weaning could interfere with the development of anxiety and deficits in spatial learning and memory, 2) combined enriched housing and rEpo treatment could have synergetic effects on anxiety and deficits in cognition, and 3) anxiety-like disorders occur before the onset of BSRS, and may be counteracted either by rEpo, or EH or both.

MATERIALS AND METHODS

All animal procedures were in compliance with the guidelines of the European Union (directive 86/609), taken in the French law (decree 87/848) regulating animal experimentation. All efforts were made to minimize the number of animals used.

Animals

Male Sprague-Dawley rats (Harlan, Gannat, France) were used throughout the experiments. Pups arrived at 15 day-old with foster dams, maintained in groups of 10 in plastic cages and had free access to food and water. For experiments 1 and 2 described below, rats were weaned at day 21 and reared in standard housing conditions (SH) using type "E" cages (length 405 mm, depth 255 mm, height 197 mm, total exploration surface = 800 cm^2; Charles River, France). For experiment 3, rats were weaned as above and reared, from day 23 and throughout the end of the experiment (week 29), either in SH conditions or in enriched housing conditions (EH) using Marlau™ cages (see Results – Part I). Animals were housed in groups of 5 in SH and in groups of 8-12 in EH in approved facilities, at 21°C under diurnal lighting conditions (lights on from 06:00 to 18:00). In both SH and EH, bedding material was changed once a week on Fridays at 16:00 and was made of aspen wood: half of Litaspen Premium 6 and half of Litaspen Premium 8/20. All rats were fed using the same pellets (type A04, Safe). All animals were weighed twice each week, and removed from their cage when bedding material was changed.

Experimental design of animal studies

Experiment 1. Characterization of the optimal dose of pilocarpine inducing SE in young rats. In order to determine an optimal dose guaranteeing the development of SE in high number of rats at day 22 with minimal death rate**,** five doses of pilocarpine were tested 18h following lithium (Li) administration. Accordingly, 15 (n=7), 20 (n=7), 25 (n=7), 30 (n=7), and 40 (n=7) mg/kg of pilocarpine were administered in 5 groups of rats. After pilocarpine injections, the latency to SE, the number of rats developing SE, and the survival rate to 45 min SE were measured. Surviving rats were sacrificed two weeks post-SE for further immunostaining of neuronal markers NeuN,

Somatostatin (SOM) and Calcitonin gene-related peptide (CGRP).

Experiment 2. Hippocampal Epo/EpoR system expression in normal young rats and following Li/Pilo-SE. In this study, rats (n=25) at day 22 experienced 45 min SE induced by 25mg/kg pilocarpine, 18 hrs after Li administration. To determine the levels of mRNAs encoding for Epo and EpoR proteins, animals were sacrificed under deep anesthesia. The dorsal hippocampus (HiD), the dorsal thalamus (ThD), the Neocortex (NCx) and the Ventral Limbic Region (VLR) comprising the amygdala, the insular agranular cortex and the piriform cortex (13) were rapidly removed and frozen in liquid nitrogen at different times (t) after the onset of SE: t=10h (n=5), t=24h (n=5) and t=72h (n=5). Naïve rats were sacrificed at day 24 (n=5)

Experiment 3. Longitudinal assessment of cognitive function and anxiety in rats with BSRS following Li/Pilo-SE induced at day 22: effect of housing conditions and rEpo administration. In order to determine whether rEpo administration following Li/Pilo-SE could prevent the development of anxiety and cognitive impairments and whether this effect was dependent on housing conditions, 50 rats were subjected to Li/Pilo-SE as described in experiment 2. Of the 34 rats that survived SE, 18 received a daily administration of rEpo (5000 IU/kg, i.p.) on 5 consecutive days, starting immediately after cessation of SE. Naïve control rats (n=22) received saline injections instead of rEpo. One day later, at day 23, rats subjected to SE and control naïve rats where either reared in SH or EH, as indicated (Fig. 1). Starting two weeks post-SE, anxiety and cognitive performances were assessed in all rats using three different tests: the Water Exploration Test (WET), the Morris Water Maze (MWM) and the Elevated Plus Maze (EPM), as indicated (Fig. 1). Based on observations in our pilot studies conducted on a 4-month period following Li/Pilo-SE, we noted that behavioral spontaneous recurrent seizures (BSRS) occurred within the 4th week post-SE. Electrode implantation for continuous EEG monitoring was not possible in our social housing conditions, in particular in Marlau™ cages. Thus, to identify the rats that indeed developed BSRS by the end of the 5th week post-SE, rats were observed 8 hours a day by two persons, who watched for BSRS occurrence, 5 days a week during a 5 week-period (from 1 to 5 weeks post-SE). After completion of all tests, 6 rats of each group were sacrificed for further immunostaining of neuronal markers NeuN, SOM and CGRP.

In vivo Procedures

Li/Pilo-SE. On day 21, pups were injected with lithium chloride (Sigma; 3 mEq/kg, i.p.) freshly dissolved in saline. Eighteen hours after, rats were given scopolamine (Sigma; 1 mg/kg s.c.) followed, 30 min later, by pilocarpine hydrochloride (Sigma; 25 mg/kg, i.p for experiment 2 and 3). Rats developing a sustained SE showed forelimb clonus leading to frequent rearing and falling, as well as occasional running seizures, which culminated sometimes in tonic extension and death. After 45 min of continuous SE, diazepam (Valium®, Roche) was first administered at the dose of 10 mg/kg, and then, 2 hrs later, at the dose of 5 mg/kg. Naïve controls received 5 saline injections instead of pharmacological compounds.

Treatment with rEpo. Injected solution of rEpo (Eprex®, generous gift from Janssen-Cilag, France) was prepared at the concentration of 1 IU/µL by diluting the

source solution (40000 IU/mL; i.e. 336 µg) with saline. Rats were then treated i.p. with 5000 IU/kg (i.e. 42 µg/kg), previously determined as the optimal neuroprotective dose with this route of administration (14). Rats received 5 rEpo injections on 5 consecutive days. The first dose was given ~10 min after diazepam administration.

Morris Water Maze (MWM). Spatial learning was tested in the MWM as previously described (see Results – Part I), in control rats and in rats that developed BSRS by the end of the 5th week post-SE.

Retention Test. This study was performed only on rats that did find the platform in the MWM task 11 weeks earlier. During retention test, rats had to remember the position of the hidden platform, the position of which remained unchanged compared to the initial MWM task. The test consisted of a single session comprising 3 trials at 2 hr intervals. At each trial, rats were given 90 sec to find the platform and 15 sec to rest on the platform. When rats did not find the platform, they were placed onto it for 15 sec.

Water Exploration Test (WET). Anxiety was measured in the WET as previously described (see Results – Part I), in control rats and in rats that developed BSRS by the end of the 5th week post-SE.

Elevated Plus Maze test (EPM). Anxiety in the EPM was measured as previously described (see Results- Part I), in control rats and in rats that developed BSRS by the end of the 5th week post-SE.

Ex vivo procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (250 mg/kg) before being sacrificed. For biochemical analysis, brain structures were rapidly microdissected, frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry analysis, animals were transcardially perfused (30 mL/min) with chilled 4% paraformaldehyde in 0.1M phosphate buffer. After cryoprotection in 25% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

Quantitation of transcript level variations by RT-qPCR. Variations in levels of transcripts encoding Epo and EpoR has been determined as previously described (see Results – Part I).

Neuronal degeneration. Fluoro-Jade B (Chemicon) was used to stain degenerating neurons after Pilo-SE in rats, as previously described (see Results – Part II).

Colorimetric immunohistochemistry. Free floating sections (40 µm thick), selected precisely at IA +5.40 mm (15) from PF-fixed tissue, were incubated either with a mouse monoclonal anti-NeuN antibody (MAB-377; Chemicon), or rabbit polyclonal anti-SOM (RGG-8004), or rabbit polyclonal anti-CGRP (chemicon AB5920). Sections were then incubated with either a biotinylated donkey antibody raised against mouse IgG (715-065-151; Jackson ImmunoResearch) or biotinylated goat antibody raised against rabbit IgG (chemicon AP187B). Sections were incubated with avidin biotin peroxydase (1:500; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3',3-diaminobenzidine (DAB, Vector). Sections were then mounted on Superfrost®Plus slides. Images were captured with a video camera 3CCD (DXC-9300, Sony) coupled to an image analysis system (Visilog 6.3, Noesis). SOM-immunoreactive (SOM-IR) and CGRP-IR neurons were counted manually. Results are expressed as the mean ± SEM number of SOM-IR and CGRP-IR neurons detected in a

40 µm-thick section selected at IA +5.40 mm.

Specificity of the rabbit primary antibodies

Polyclonal anti-somatostatin-28 IgG (RGG-8004, Peninsula Laboratories, San Carlos, CA) raised in rabbit with the peptide SANSNPAMAPRERKAGCKNFFWKTFTSC (disulfide bond) has 100% cross-reactivity with somatostatin-28 and 25, less than 0.01% cross-reactivity with somatostatin-28 (1-12), and no cross-reactivity with somatostatin-14, prosomatostatin-32, Substance P, amylin amide (human), CCK 26-33 (nonsulfated), VIP (human, rat, porcine), and insulin (human). Preabsorption with 10 nM somatostatin-28 resulted in complete loss of immunoreactivity (16). The antigen distribution was similar to the distribution of mRNA detected by *in situ* hybridization (16-18).

Polyclonal anti-CGRP IgG (Chemicon, No. AB5920) raised in rabbit with a synthetic rat CGRP conjugated to keyhole limpet hemocyanin [KLH] reacts with CGRP in dot blot (manufacturer's information). Immunoblot of rat DRG reveals a band at the expected 75-kDa size and also a band at around 120 kDa. Specific reactivity of the antiserum is eliminated by preincubation of the antiserum with 10 µM of rat CGRP (19).

Statistical Analysis

Data are expressed as mean ± SEM of the different variables analyzed and are compared among groups by using either one-way ANOVA (ANOVA 1), ANOVA 2, and ANOVA 2 with repeated measures. ANOVAs were followed by Fisher's protected Least Significance Differences (LSD) test.

Results and Discussion

Optimal dose of pilocarpine to induce SE in rats at weaning. In the majority of the studies published so far (9-11, 20, 21), 60 mg/kg is the dose of pilocarpine used to induce SE at weaning, when administered the day after Li (3 mEq/kg). In our hands, this dose of pilocarpine caused 100% death, which occurred within the first 20 min of SE. We determined that the 25 mg/kg pilocarpine provided the greater number of rats developing SE and the best survival rate (Fig. 2). At 15, 20, 25, 30 and 40 mg/kg, SE onset was observed >60 min, 27.6 ± 4.2 min, 27.7 ± 2.1 min, 29.4 ± 4.7 min, and 25.0 ± 0.9 min after pilocarpine administration, respectively. We used the dose of 25 mg/kg pilocarpine in all further experiments, diazepam being administered at the doses of 10 mg/kg after 45 min of sustained SE, and 5 mg/kg 2 hrs after the first dose.

Transient and discrete neuronal degeneration following Li/Pilo-SE at weaning. Prior studies established that neurodegenerative processes occurred in the hippocampus following Li/Pilo-SE induced at weaning, in particular within areas CA1 and CA3, (10, 11, 20) and at the hilar border of the dentate granule cells (9). Here, we observed 24 hrs after Li/Pilo-SE an intense Fluoro-Jade B labeling in scarce neurons of the neocortex, the hilus and the dorsal nuclei of the thalamus, but in numerous neurons of the area CA1 of the hippocampus and the basolateral nucleus of the amygdala (Fig. 3A). Two weeks later, we did not notice any obvious neuronal loss in the brain areas where Fluoro-Jade B labeling was observed, as depicted qualitatively in the hilus and the basolateral nucleus of the amygdala (Fig.

3B). In support of these observations, we did not quantify any decrease in the number of the hilar neurons that are known to be vulnerable to excitotoxic insults (Fig. 3C), i.e. SOM-IR GABAergic interneurons (22, 23) and CGRP-IR glutamatergic mossy cells (12, 24).

Reactivity of Epo and EpoR gene expression following Li/Pilo-SE at weaning. In experiment 2, onset of SE was observed 27.3 ± 0.4 min (n=24) after pilocarpine administration. Variations in Epo- and EpoR-mRNA levels were determined 10 hrs, 1 day and 3 days post-SE and compared to basal levels (naïve controls at day 24) (Fig. 4) in the dorsal hippocampus (HiD), the neocortex (NCx), the dorsal nuclei of the thalamus (ThD) and the ventral limbic region (VLR). While Epo-mRNA increased by 3 days post-SE in the HiD (+167 ± 46%, $p<0.01$) and the NCx (+106 ± 14%, $p<0.01$), it dramatically decreased throughout the time period examined in the ThD, reaching -76 ± 5% ($p<0.001$) 1 day post-SE, and decreased transiently in the VLR (-35 ± 12%, $p<0.05$). No decrease in EpoR-mRNA was measured in the four brain areas examined. Instead, a significant increase was observed, transiently in the acute phase (10 hrs) after SE in the ThD (+77 ± 20%, $p<0.05$), but lastingly in the NCx (+101 ± 50% at 3 days post-SE, $p<0.01$). The dramatic decrease in brain *epo* gene expression, while brain *epor* gene expression was maintained, justified the administration of exogenous rEpo post-SE to compensate for a potentially decreased Epo/EpoR signaling in vulnerable brain areas of rats that underwent severe SE.

Development of BSRS. In experiment 3, onset of SE in rats that survived was observed 26.3 ± 0.2 min (n=44) after pilocarpine administration. Five weeks after Li/Pilo-SE, the percentage of rats with BSRS was lower in rats reared in EH compared to SH (Fig. 5). The percentage of rats that were seen with BSRS during the hours of observation during the 5 week-period post-SE is provided on Figure 5. From that age, only rats that indeed exhibited BSRS were included in the study to determine: i) retrospectively, whether anxiety-trait was altered before the occurrence of BSRS, ii) whether rEpo and quality of housing could improve anxiety and cognitive outcome once BSRS developed.

Increased anxiety-like behavior is not altered by rEpo during the silent phase after Li/Pilo-SE induced at weaning. In the WET, the more time the animals spend in the central zone of a quadrant, the less anxious they are considered (see Results – Part I). After only 2 weeks of rearing in EH, control rats are less anxious than their counterparts reared in SH. We previously reported that rats subjected to Li/Pilo-SE at weaning developed anxiety-trait at the adult stage (see Fig. 9A of the Results – Part I). Here, during the week that preceded the WET, no BSRS was observed in all groups of rats. All rats that experienced SE at weaning, and that developed BSRS during the 4^{th} week post-SE, showed an increased anxiety-trait, independently of the housing condition, that was not altered by the administration of rEpo (Fig. 6). While we cannot fully exclude the possibility that BSRS may have occurred out of the observation periods during the week that preceded the WET, these results strongly suggest that anxiety develops during the silent phase after Li/Pilo-SE and cannot be prevented by EH and/or rEpo.

rEpo improves anxiety in rats with BSRS when reared in SH, but produces negative effects when reared in EH. Anxiety has been recognized as the most common psychiatric comorbidity in patients with epilepsy (1-3, 25). Here, we determined in rats with BSRS whether the quality of the living environment (SH and EH) and rEpo may improve anxiety-trait. Therefore, control rats (C/SH and C/EH) and rats with BSRS reared in SH or EH, and treated or not with rEpo were subjected to the WET and to the EPM, 12 and 15 weeks post-SE (i.e. 8 and 11 weeks after the occurrence of BSRS), respectively.

In the WET, the less time rats explore the central zone of the quadrant, the more anxious they are considered. Rats with BSRS reared in SH exhibited an increased anxiety-trait, which was no more significantly different from controls (C/SH) when rats were treated with rEpo after SE (Fig. 7). By contrast, rEpo exacerbated anxiety-trait in rats with BSRS reared in EH, as shown by the percentage of time spent in the central zone, and the swimming profile: an anxious rat navigates at rapid velocity in the central zone, reducing the time spent at null velocity (floating behavior) in this zone (Fig. 7).

Results obtained in the EPM confirmed those obtained in the WET for rats reared in EH: rEpo exacerbated the reduction in time spent by rats with BSRS in the open arm (Fig. 8), indicating that rats with BSRS are more anxious when treated with rEpo after SE. Results obtained for rats reared in SH are totally different, and confirm the tendency obtained in the WET. Indeed, pathological anxiety-trait was significantly reduced - not to say almost abolished - in rats treated with rEpo before BSRS, compared to those not treated with rEpo before BSRS (Fig. 8).

Thus, EH is not sufficient to prevent the development of anxiety in rats with BSRS, and rEpo has opposite effects on anxiety, depending on the quality of the living environment. Indeed, rEpo treatment reduces anxiety in rats reared in SH, while it exacerbates anxiety in rats reared in EH.

Effect of rEpo on learning and memory in rats with BSRS depends on housing conditions. We previously established that spatial learning in the MWM was fully protected in rats with BSRS reared in EH immediately after Li/Pilo-SE at weaning, while rats with BSRS reared in SH were barely able to learn the position of the platform by the end of task (see Fig. 9B, Results – Part I). Here, we confirmed this result (Fig. 9A). In addition, we show that, 11 weeks after the MWM task, the platform was found by all rats with BSRS reared in EH, and by 50% only of the rats with BSRS reared in SH (Fig. 10).

In rats reared in EH, treatment with rEpo did not improve learning in the MWM of rats with BSRS; rearing in EH was sufficient to completely protect this function in rats with BSRS (see Fig. 9A, Results – Part I and actual Fig. 9). However, performance in the memory retention test was dramatically impaired in rats that were treated with rEpo. Indeed, ~30% only of the rats that found the platform in the MWM task remembered its position (Fig. 10). Thus, rEpo treatment after Li/Pilo-SE has negative effects on memory in rats with BSRS reared in EH.

In rats reared in SH, treatment with rEpo fully protected spatial learning in the MWM in rats with BSRS (Fig. 9A), by increasing the percentage of platform finders in the last day trial (Fig. 9B). In addition, it substantially increased the percentage of rats that did remember the position of the

platform in the memory retention test (Fig. 10).

Hypervascularity in rats with BSRS treated with rEpo and reared in EH cannot explain aggravated cognitive impairments. The increased anxiety and decreased cognitive performances in rats with BSRS treated with rEpo and reared in EH cannot be explained by massive degenerative processes, as revealed by NeuN-immunostaining in the hippocampus and the neocortex (Fig. 11A), and by detection of hilar vulnerable neurons (Fig. 11B) expressing SOM (GABAergic interneurons) or CGRP (glutamatergic mossy cells). However, it is noteworthy that 50% of the rats that exhibited aggravated anxiety and cognitive impairments displayed an unquestionable increase in brain capillary (arrows in Fig. 11A) density in the hippocampus and the neocortex (compare with similar rats but not treated with rEpo, Fig. 11A). Because this hypervascularity was not observed in all rats treated with rEpo and reared in EH, it cannot explain the aggravated behavioral impairments.

CONCLUSION

In this study, we provide for the first time evidence that anxiety-like disorders may occur before BSRS in rats subjected to Li/Pilo-SE at weaning. These results complement the prior observation that learning deficits in the MWM also occur during the silent phase after Li/Pilo-SE (10, 11), and support the clinical observation that cognitive impairments can precede onset of epilepsy in children (6-8). Neither rEpo, nor housing in enriched conditions (21) has a protective effect on the occurrence of this anxiety-trait.

In rats with BSRS reared in standard housing conditions (SH), treatment with rEpo after SE fully reverses the development of pathological anxiety-trait as measured in the EPM, prevents spatial learning impairment as measured in the MWM, and reduces memory retention deficits in the long term.

By contrast, in rats with BSRS reared in EH, rEpo treatment after SE exacerbates the anxiety-trait and profoundly altered retention memory performance, that was fully preserved in rats with BSRS reared in EH but not treated with rEpo.

Altogether, these data indicate that rEpo treatment given after SE at immature stages may counteract the development of anxiety and cognitive deficits once BSRS occurred and when living in an impoverished environment. Such positive effects may thus be expected in patients with epilepsy, whose majority is reporting poor quality of live, with increased feelings of social isolation (1). However, our results also reveal that rEpo may be a double-edged sword in the treatment of individuals prone to BSRS. Indeed, while producing positive effects in rats reared in impoverished environments, rEpo turned out to exacerbate anxiety-like disorders and memory in rats reared in enriched/complex environments.

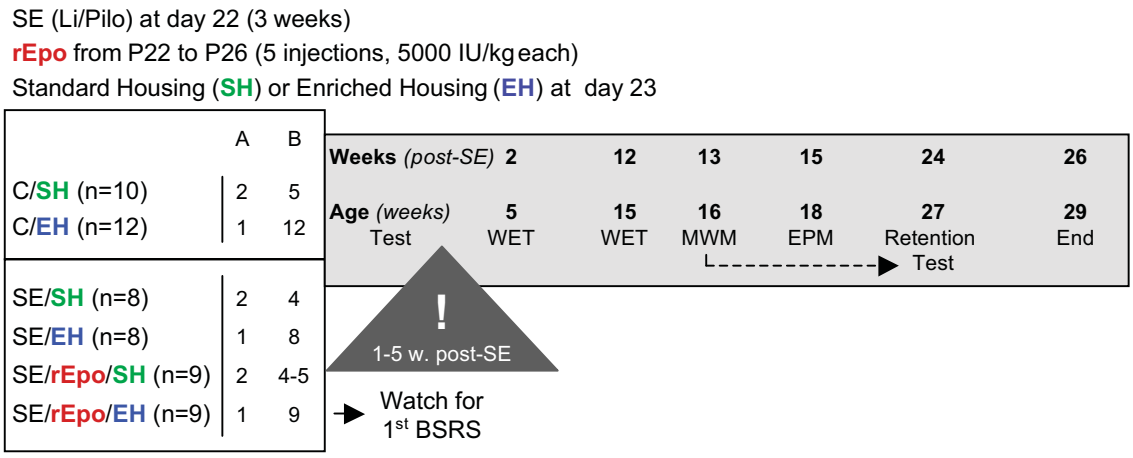


Figure 1. Experimental design of behavioral studies. Anxiety and cognitive performances were assessed longitudinally in rats from 5 to 27 weeks of age. Rats were distributed in 6 groups, reared in standard (SH) or enriched (EH) housing from day 23 and throughout the experiment. The number of cages in which the "n" rats were distributed in each group (A), and the number of animals per cage (B) is provided. After Li/Pilo- SE at P22, half of rats received daily rEpo injections for 5 consecutive days, and were reared either in standard (SE/rEpo/SH) or in enriched (SE/rEpo/EH) cages, while the other half received saline injections instead of rEpo (SE/SH and SE/EH). Control rats receiving saline only were also added to the experiment (C/SH and C/EH). Abbreviations: BSRS, behavioral spontaneous recurrent seizures; C/EH, control rats in enriched Marlau™ cages ; C/SH, control rats in standard cages; EPM, Elevated Plus Maze; MWM, Morris Water Maze; SE/EH, rats subjected to SE and reared in enriched Marlau™ cages; SE/rEpo/EH, rats subjected to SE, treated with rEpo and reared in enriched Marlau™ cages; SE/SH, rats subjected to SE and reared in standard cages; SE/rEpo/SH, rats subjected to SE, treated with rEpo and reared in standard cages; WET, Water Exploration Test.

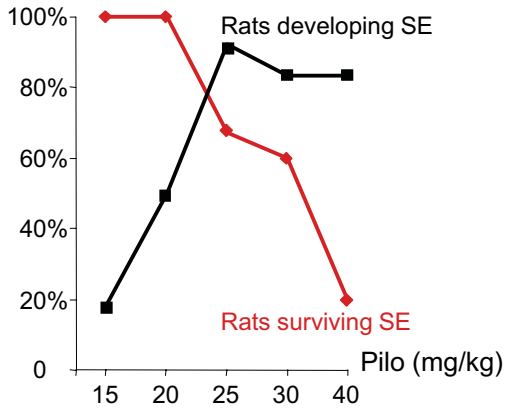


Figure 2. Effect of pilocarpine dose on *status epilepticus* development and survival in young rats. Lithium (3 mEq/kg, i.p.) was administered at day 21, 18 hours prior to pilocarpine. Among the five doses of pilocarpine tested at day 22, 25mg/kg was the optimal dose to induce a sustained SE in high number of rats with minimal death rate compared to other doses.

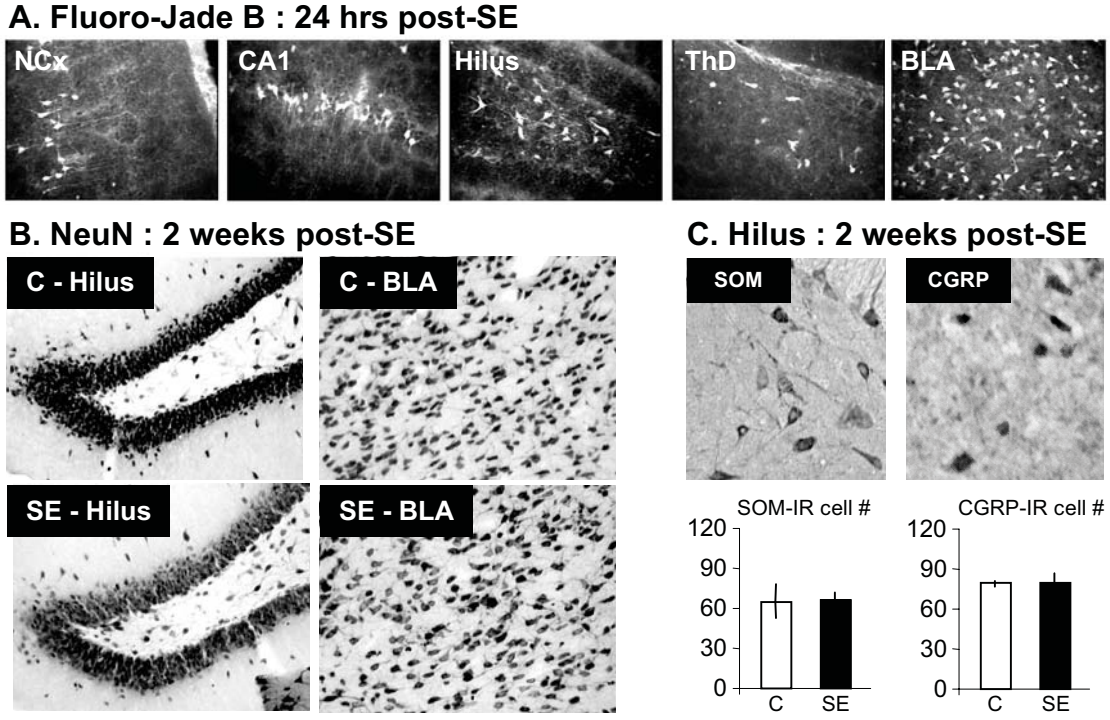


Figure 3. Lack of evidence of massive neurodegenerative processes following pilocarpine induced SE in young rats. A. Fluora-Jade B staining, which is a marker of neuronal degeneration, was observed 24hours post-SE (at day 23) in several brain regions. **B.** Two weeks post-SE, no obvious neuronal loss was observed in the brain, notably in the Hilus and the BLA using the neuronal marker NeuN. **C.** Counting hilar neurons using SOM and CGRP markers showed no difference between controls and rats subjected to SE two weeks earlier. Abbreviations: BLA, basolateral nucleus of the amygdala; C, controls; CGRP, calcitonin gene-related peptide; IR, immunoreactive; NCx, Neocortex; ThD, dorsal part of the thalamus; SE, *status epilepticus*; SOM, somatostatin.

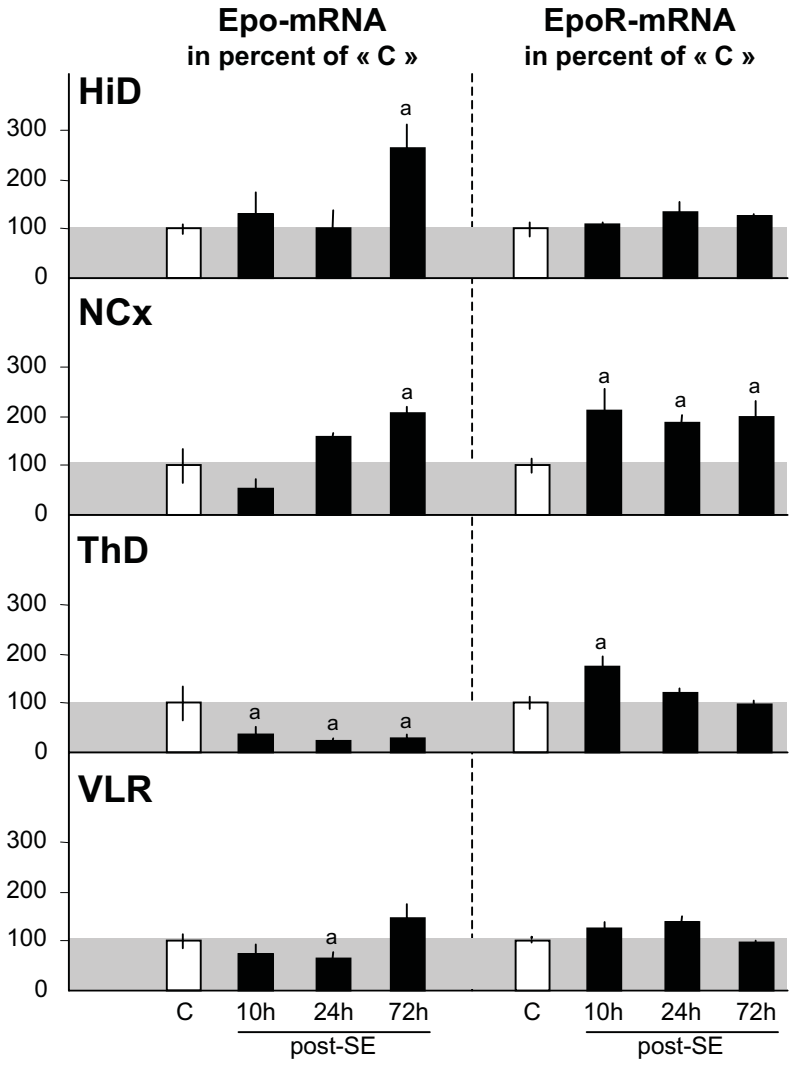


Figure 4. Brain expression profile of Epo/EpoR system following SE. Transcripts encoding Epo and EpoR were quantified using RT-qPCR in the dosal hippocampus (HiD), the neocortex (NCx), the dorsal part of the thalamus (ThD) and the basolateral nucleus of the amygdala (BLA) following SE induced at P22 by pilocarpine. All values were normalized against the SmRNA, as explained in Materials and Methods. Abbreviations: C, controls; HiD, dorsal hippocampus; NCx, Neocortex; ThD, dorsal part of the thalamus; VLR, ventral limbic region; SE, *status epilepticus*. a: $p<0.05$ compared to C, Fisher's LSD test following ANONA1.

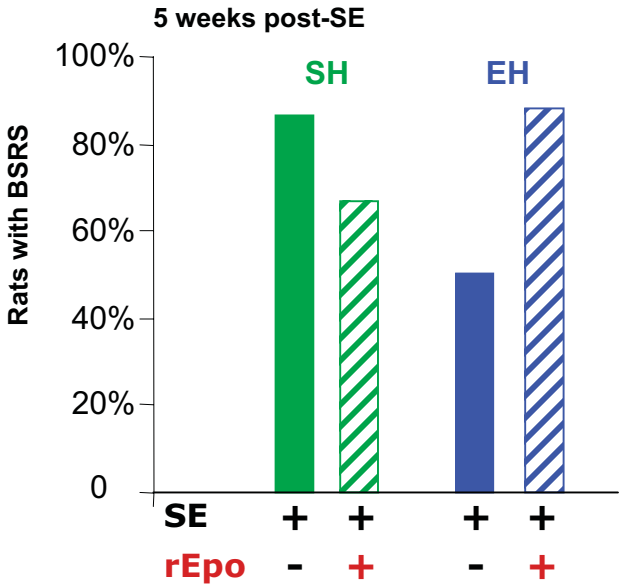


Figure 5. Percentage of rats with BSRS monitored during the 5 weeks following SE. BSRS, when occurred, appeared within the 4th week after Li/Pilo-SE. Abbreviations: SH, standard housing; EH, enriched housing.

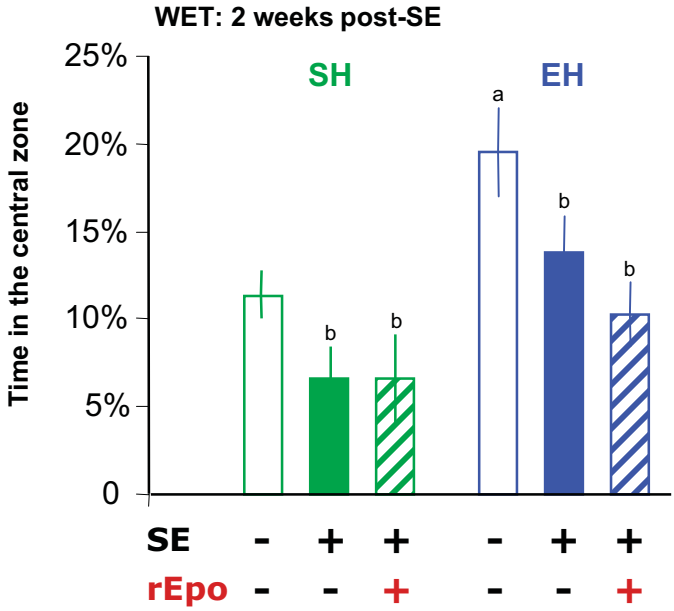


Figure 6. Anxiety increases in all rats prior to BSRS, independently of the housing conditions and rEpo treatment. Performances of all rats were measured in the WET two weeks after SE. In this test, the more time rats spend in the central zone, the less anxious they are considered. Group explanations are given in figure 1. Control rats reared in EH had a less anxious profile than those reared in SH. SE increased anxiety-trait in both housing conditions, and rEpo had no effect. a: $p<0.05$, control EH vs. control SH; b: $p<0.05$, vs. respective SH or EH controls; Fisher's LSD test following ANOVA2. Abbreviations: SE, *status epilepticus*; SH, standard housing; EH, enriched housing.

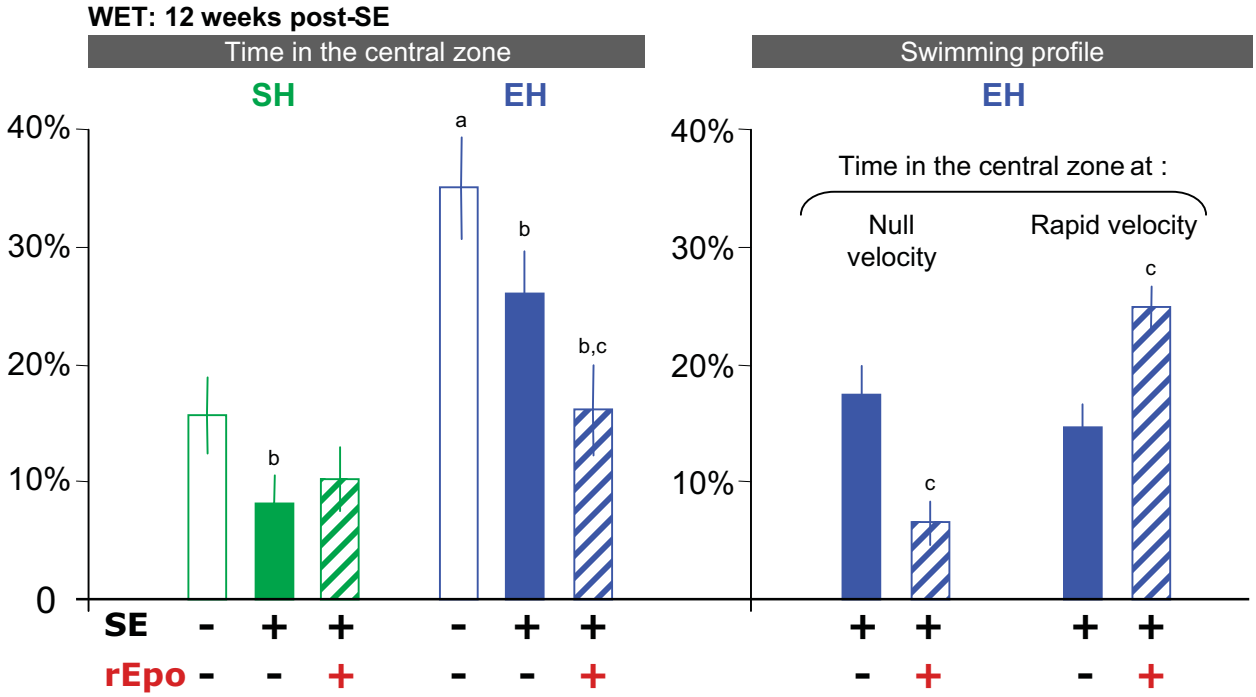


Figure 7. At 12 weeks post-SE, rEpo increased anxiety-like behavior in rats with BSRS reared in enriched but not in standard housing

At 15 weeks of age (12 weeks post-SE), anxiety-like behavior was assessed in control rats and rats with BSRS using the WET. In this test, the more time rats spend in the central zone, the less anxious they are considered. Rats with BSRS in SH and EH groups were more anxious than their respective controls. In addition, rEpo administered after SE exacerbated anxiety in rats with BSRS, as shown by the decreased time spent in the central zone and the significant alteration of the swimming profile (decreased floating behavior, i.e. null velocity, and increased rapid velocity). a: $p<0.05$, control EH vs. control SH; b: $p<0.05$, vs. respective SH or EH controls; c: $p<0.05$, vs. SE without rEpo; Fisher's LSD test following ANOVA2. Abbreviations: EH, enriched housing; SE, *status epilepticus*; SH, standard housing.

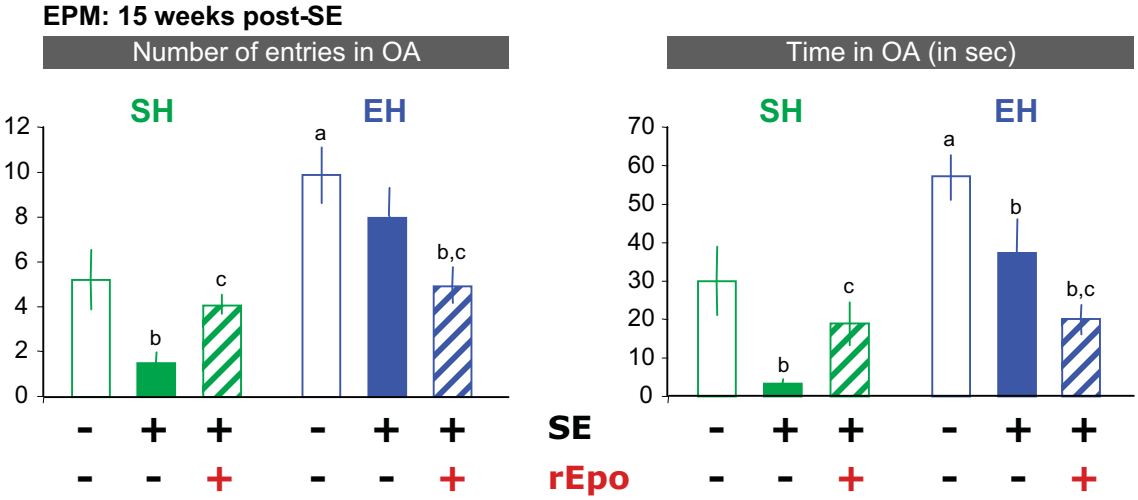


Figure 8. At 15 weeks post-SE, rEpo exerts opposite effects on anxiety in the EPM in rats reared in SH compared to EH. At 18 weeks of age (15 weeks post-SE), anxiety was assessed in rats using the EPM. In this test, the more rats enter and spend time in the open arm (OA), the less anxious they are considered. All rats with BSRS exhibited a highly anxious profile, which was almost completely recovered and exacerbated by rEpo administration when rats were reared in SH and EH, respectively. a: $p<0.05$, control EH vs. control SH; b: $p<0.05$, vs. respective SH or EH controls; c: $p<0.05$, vs. SE without rEpo; Fisher's LSD test following ANOVA2. Abbreviations: EH, enriched housing; SE, *status epilepticus*; SH, standard housing.

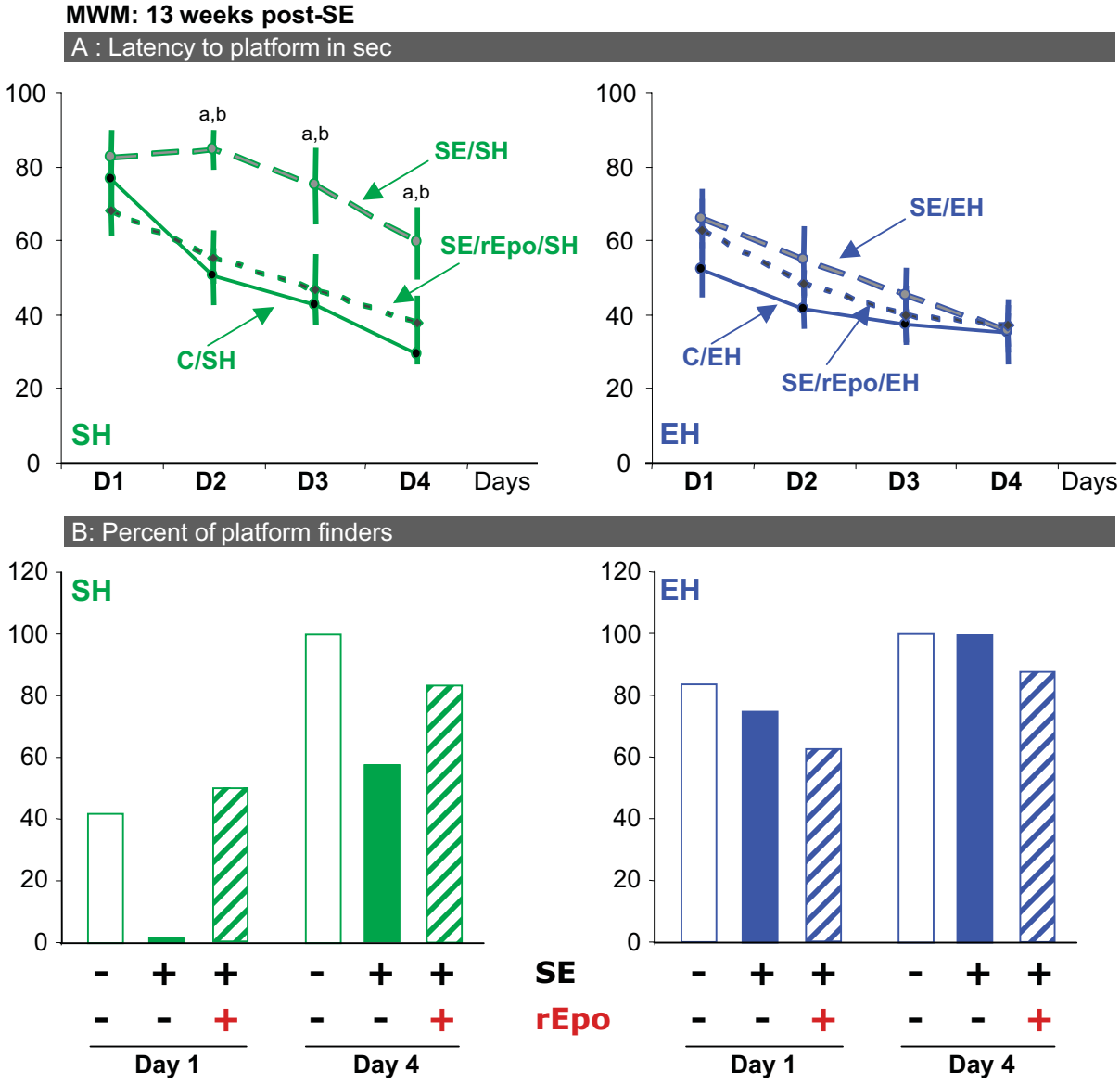


Figure 9. At 13 weeks post-SE, rEpo protects rats with BSRS from learning impairment in MWM when reared in SH. At 16 weeks of age (13 weeks post-SE), learning was assessed in the MWM in control rats and rats with BSRS developed after SE. The upper graph represents the latency to find the platform from days 1 to 4, while the lower graph shows the percent of rats that found the platform on days 1 and 4. Only rats with BSRS reared in SH exhibited a severe cognitive impairment compared to controls. rEpo administration completely abolished the development of this cognitive decline. Note that rats with BSRS reared in EH were completely protected with no additional effect of rEpo. a: $p<0.05$, C/SH vs. SE/SH; b: $p<0.05$, SE/rEpo/SH vs. SE/SH; Fisher's LSD test following ANOVA2 with repeated measures. Abbreviations: EH, enriched housing; SE, *status epilepticus*; SH, standard housing.

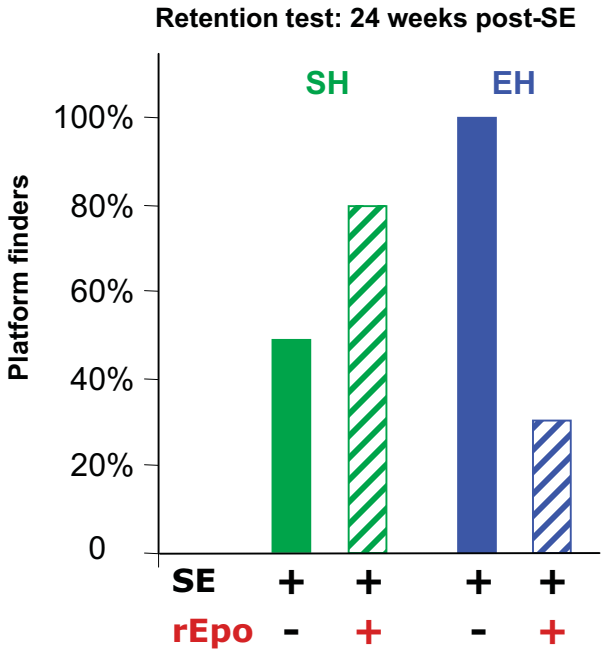


Figure 10. At 24 weeks post-SE, rEpo exerts opposite effects on spatial memory retention in rats reared in SH compared to EH. Eleven weeks after the achievement of the MWM test, we tested whether rats with BSRS that did find the platform at the 4th trial day (see fig. 8B) could find it again in a three-trial session. Rats were considered as "platform finders" when they found the platform in 2 consecutive trials. When reared in EH, all rats with BSRS not treated with rEpo remembered the position of the platform. However, rEpo treatment abolished this effect. By contrast, rEpo administered to rats with BSRS reared in SH improved memory retention. Abbreviations: EH, enriched housing; SE, *status epilepticus*; SH, standard housing.

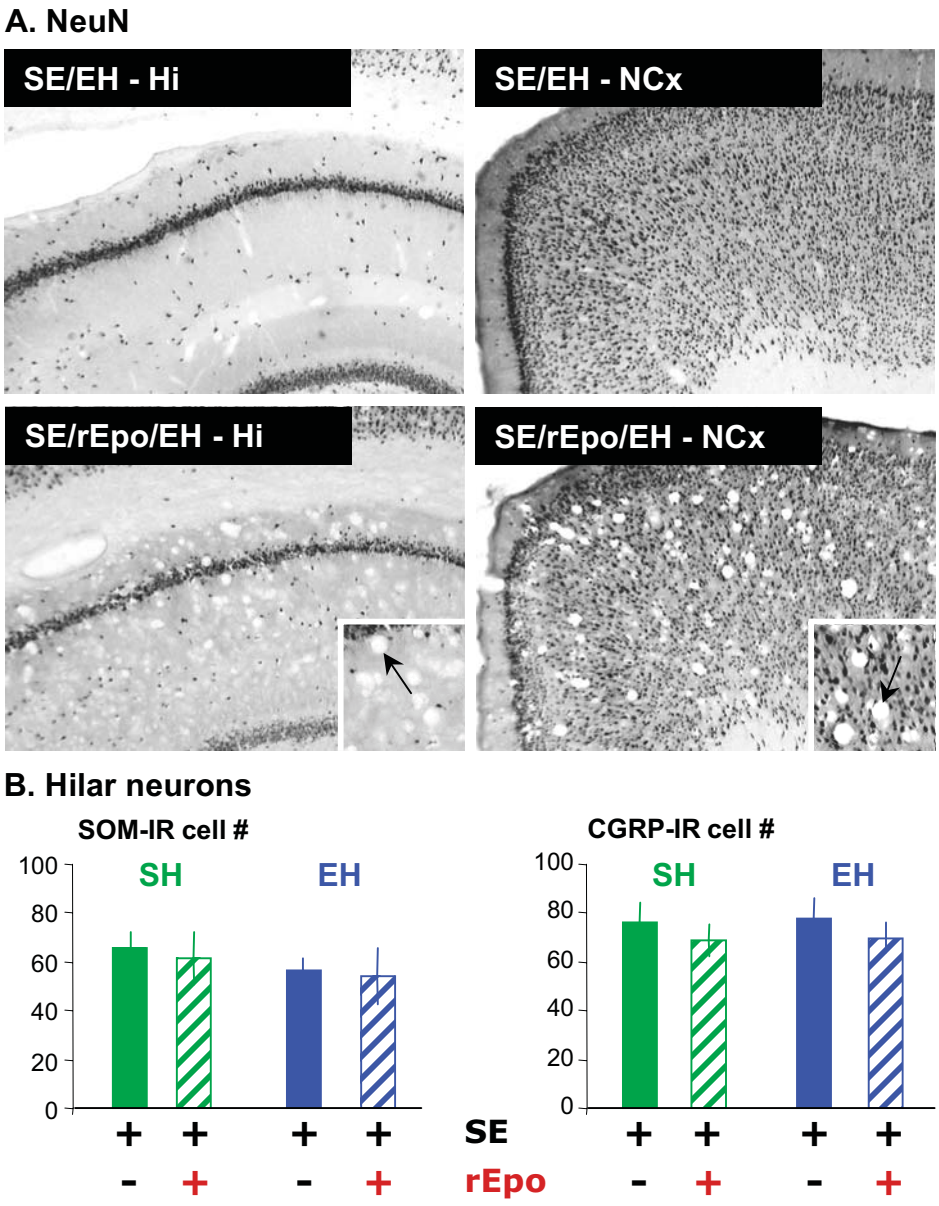


Figure 11. Administration of rEpo post-SE increased cerebral vascularity in rats with BSRS, only when reared in EH. At 29 weeks of age, all rats were sacrificed for histological analysis. **A.** While no neuron loss was noted following NeuN staining at this age in rats with BSRS, an important increase in blood vessel (see arrows) density was observed in rats (n=3/6) treated with rEpo and reared in EH compared to rats not treated with rEpo. **B.** No difference in the number of SOM-IR and CGRP-IR neurons was found in the hilus of rats raised either in SH or EH conditions, and treated or not with rEpo. Abbreviations: CGRP, calcitonin gene-related peptide; EH, enriched housing; Hi, hippocampus; IR, immunoreactive; NCx, neocortex; SE, *status epilepticus*; SH, standard housing; SOM, somatostatin.

REFERENCES

1. McCagh J, Fisk JE, & Baker GA (2009) Epilepsy, psychosocial and cognitive functioning. *Epilepsy research* 86(1):1-14.
2. Mensah SA, Beavis JM, Thapar AK, & Kerr MP (2007) A community study of the presence of anxiety disorder in people with epilepsy. *Epilepsy Behav* 11(1):118-124.
3. Jacoby A, Baker GA, Steen N, Potts P, & Chadwick DW (1996) The clinical course of epilepsy and its psychosocial correlates: findings from a U.K. Community study. *Epilepsia* 37(2):148-161.
4. Smith DF, Baker GA, Dewey M, Jacoby A, & Chadwick DW (1991) Seizure frequency, patient-perceived seizure severity and the psychosocial consequences of intractable epilepsy. *Epilepsy research* 9(3):231-241.
5. Vazquez B & Devinsky O (2003) Epilepsy and anxiety. *Epilepsy Behav* 4 Suppl 4:S20-25.
6. Hermann BP & Jones JE (2006) Intractable epilepsy and patterns of psychiatric comorbidity. *Advances in neurology* 97:367-374.
7. McNelis AM, Johnson CS, Huberty TJ, & Austin JK (2005) Factors associated with academic achievement in children with recent-onset seizures. *Seizure* 14(5):331-339.
8. Oostrom KJ, Schouten A, Kruitwagen CL, Peters AC, & Jennekens-Schinkel A (2003) Behavioral problems in children with newly diagnosed idiopathic or cryptogenic epilepsy attending normal schools are in majority not persistent. *Epilepsia* 44(1):97-106.
9. Sankar R, Shin DH, Liu H, Mazarati A, Pereira de Vasconcelos A, & Wasterlain CG (1998) Patterns of status epilepticus-induced neuronal injury during development and long-term consequences. *J Neurosci* 18(20):8382-8393.
10. Faverjon S, Silveira DC, Fu DD, Cha BH, Akman C, Hu Y, & Holmes GL (2002) Beneficial effects of enriched environment following status epilepticus in immature rats. *Neurology* 59(9):1356-1364.
11. Rutten A, van Albada M, Silveira DC, Cha BH, Liu X, Hu YN, Cilio MR, & Holmes GL (2002) Memory impairment following status epilepticus in immature rats: time-course and environmental effects. *The European journal of neuroscience* 16(3):501-513.
12. Nadam J, Navarro F, Sanchez P, Moulin C, Georges B, Laglaine A, Pequignot JM, Morales A, Ryvlin P, & Bezin L (2007) Neuroprotective effects of erythropoietin in the rat hippocampus after pilocarpine-induced status epilepticus. *Neurobiology of disease* 25(2):412-426.
13. Sanchez PE, Fares RP, Risso JJ, Bonnet C, Bouvard S, Le-Cavorsin M, Georges B, Moulin C, Belmeguenai A, Bodennec J, Morales A, Pequignot JM, Baulieu EE, Levine RA, & Bezin L (2009) Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons. *Proceedings of the National Academy of Sciences of the United States of America* 106(24):9848-9853.
14. Calapai G, Marciano MC, Corica F, Allegra A, Parisi A, Frisina N, Caputi AP, & Buemi M (2000) Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *European Journal of Pharmacology* 401(3):349-356.
15. Paxinos G & Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic Press) 4th Ed p 256.
16. Naus CC, Miller FD, Morrison JH, & Bloom FE (1988) Immunohistochemical and in situ hybridization analysis of the development of the rat somatostatin-containing neocortical neuronal system. *The Journal of comparative neurology* 269(3):448-463.
17. Naus CC & Bloom FE (1988) Immunohistochemical analysis of the development of somatostatin in the reeler neocortex. *Brain research* 471(1):61-68.
18. Priestley JV, Rethelyi M, & Lund PK (1991) Semi-quantitative analysis of somatostatin mRNA distribution in the rat central nervous system using in situ hybridization. *Journal of chemical neuroanatomy* 4(2):131-153.
19. Chao T, Pham K, Steward O, & Gupta R (2008) Chronic nerve compression injury induces a phenotypic switch of neurons within the dorsal root ganglia. *The Journal of comparative neurology* 506(2):180-193.
20. Cilio MR, Sogawa Y, Cha BH, Liu X, Huang LT, & Holmes GL (2003) Long-term effects of status epilepticus in the immature brain are specific for age and model. *Epilepsia* 44(4):518-528.
21. Wang CA, Lai MC, Lui CC, Yang SN, Tiao MM, Hsieh CS, Lin HH, & Huang LT (2007) An enriched environment improves cognitive performance after early-life status epilepticus accompanied by an increase in phosphorylation of extracellular signal-regulated kinase 2. *Epilepsy Behav* 11(3):303-309.
22. Sloviter RS (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science (New York, N.Y* 235(4784):73-76.
23. Sun C, Mtchedlishvili Z, Bertram EH, Erisir A, & Kapur J (2007) Selective loss of dentate hilar interneurons contributes to reduced synaptic inhibition of granule cells in an electrical stimulation-based animal model of temporal lobe epilepsy. *The Journal of comparative neurology* 500(5):876-893.
24. Freund TF, Hajos N, Acsady L, Gorcs TJ, & Katona I (1997) Mossy cells of the rat dentate gyrus are immunoreactive for calcitonin gene-related peptide (CGRP). *The European journal of neuroscience* 9(9):1815-1830.
25. Beyenburg S & Damsa C (2005) [Psychiatric comorbidity in epilepsy]. *Bulletin de la Societe des sciences medicales du Grand-Duche de Luxembourg* (3):283-292.

General Discussion

One of the main objectives of our laboratory is to enhance brain robustness to better cope with deleterious processes associated with insults of different etiology, such as increased and uncontrolled release of excitatory neurotransmitters occurring in the context of stroke, epilepsy and trauma. Enhanced brain robustness encompasses not only the prevention of cell death, but also preservation of neuronal network function. Here, we discuss 1) the relevance of the development of the Marlau™ cage and the Water Exploration Test for anxiety, 2) some points related to the protection of i) the neuronal entity and ii) the network function. Finally, we discuss the influence of the environment and rhEpo on the development of seizures and related cognitive disorders.

Part I. Development of behavioral tools to improve standardization

I.1. Development of the Marlau™ cage. One of the major contributions of this thesis has been the development and validation of the Marlau™ cage, as a simple way to standardize enriched housing (environmental enrichment) of rodents in experimental paradigms. Most of the setups used so far in the scientific literature to enrich the living environment of rodents are insufficiently described to allow accurate repetition in other laboratories (Nithianantharajah J, 2006). In addition, all setups that we have found cannot guarantee that all rats raised together in an enriched environment were equally exposed to the positive elements constituting the enriched environment. Indeed, if getting familiar to a new object added in the environment is an important point of enrichment in most setups used, it is difficult to anticipate whether all animals of the group will explore the new object, either because of motivational differences among animals, or because of the possibility that an animal, more aggressive than the others, prevents the free access to this new object. Finally, most of the studies modified repeatedly the arrangements of different objects in the cage as a tool to introduce novelty (a crucial point in enriched programs); however, such a procedure is difficult to reproduce within and between experiment(s). In the Marlau™ cage, the novelty is provided by the change in maze configuration that occurs three times a week. By this way, we are sure that all animals are exposed to novelty since they are obliged to explore the maze to seek food. Indeed, food pellets and bottles of water are located in two different compartments in the cage, and to go from the compartment containing water to that of pellets, rats must go through the maze. Enrichment in the Marlau™ cage also consists in providing to the animals: 1) increased voluntary exercise due to the large surface exploration areas and the presence of three running wheels, 2) ways to rest in secure areas such as in the red tunnel, and then 3) ways to escape from the aggression of a dominant male through the presence of multiple compartments, including all cells

within the mazes. Moreover, by changing the configuration of the maze three times a week, hippocampal function, in particular place cell functionality, is highly stimulated. Using Marlau™ cages, we reproduced most of the characteristics of brain plasticity reported by others in response to enriched environments: increased cortical volume (Diamond MC, 1964), increased hippocampal neurogenesis (Kempermann G, 1997), increased expression of genes involved in hippocampal plasticity (Rampon C, 2000), including BDNF (Young D, 1999) and VEGF (Cao L, 2004). We also found that learning and memory in the Morris Water Maze was more efficient in rats raised in Marlau™ cages than in rats raised in standard cages, a result that was found by only ~50% of the studies so far. Last but not least, a significant number of the studies that have analyzed the positive effects of enriched housing on different variables compared the results obtained to those of rats raised in isolated conditions, likely to accentuate the contrast between the two housing conditions to obtain statistically different results (53% of the studies reported in table 7). By contrast, in this thesis, results obtained after housing in the Marlau™ cage have been compared to those of rats raised in groups of 6 in standard cages, and the robustness of the differences observed between the two conditions underscores that enrichment tools provided by the Marlau™ cage are highly efficient in increasing brain plasticity.

I.2. Development of the Water Exploration Test. This test was developed to test anxiety-like disorders in rats, and to force rats to provide quantitative data, even when they are highly anxious. Indeed, performance in the Elevated Plus Maze, which is one of the most used test to evaluate anxiety, depends on whether rodents are willing to explore the anxiogenic area (open arms). If a rodent remain in the closed arms for different reasons (including increased anxiety, but also fatigue, and, why not, laziness) no value will be measured in the open arms, and the value of zero second (or zero entry, or zero centimer) will be assigned to this rat. As exemplified in this thesis (Results Part II), the percentage of rats that visited the open arms was 25% and 75% when housed in standard or Marlau™ cages respectively, this percentage difference influencing largely the quantitative evaluation of the time spent in the open arms (24 sec and 41 sec, respectively). We thus developed a test avoiding immobility: rats have to swim for 5 min in a quadrant from which they cannot exit, and what is measured is the velocity and the time spent in the central zone of this quadrant, considered as "anxiogenic". The less anxious is a rat, the more time it will spend "floating" (null velocity) in the central zone. Using this test, so-called Water Exploration Test (WET), we found that rats housed in standard and Marlau™ cages explored the same distance, but with different profiles: rats housed in Marlau™ cages explored extensively the central zone, or even, remained in this

zone with a floating behavior. In this case, it was easier to compare the results obtained in the central zone between the two groups, since all rats explored exactly the same distance in the total quadrant (Results Part I).

Part II. Decreased vulnerability of the neuronal entity

Recombinant human erythropoietin (rhEpo) has been recognized as a promising molecule to prevent or protect against neurodegenration in a wide variety of experimental neurological disorders (Brines M, 2005; Maiese K, 2008; Nogouchi CT, 2007). Pioneer studies by our group have shown that therapeutic administration of rhEpo following pilocarpine-induced status epilepticus (Pilo-SE) provided a robust protection of vulnerable hippocampal neurons (Nadam J, 2007). Nevertheless, rhEpo did not rescue vulnerable neurons of the Ventral Limbic Region (VLR) (Results Part I). Indeed, we have demonstrated that constitutive EpoR gene expression is heterogeneous in the rat central nervous system with higher levels in the hippocampus than in the VLR (Nadam J, 2007; Results Part I). In brain tissue, the level of EpoR expression has been proposed to determine the cytoprotective effects of Epo (Chen ZY, 2006). We have thus hypothesized that increased neuronal expression of EpoR in the VLR is required for rhEpo to exert its neuroprotective effects after severe excitotoxic injury induced by Pilo-SE. Thus, our first objective was to find appropriate physiological conditions making it possible to increase expression of EpoR in neurons of the VLR.

II.1. Activation of EpoR gene expression in the VLR by repeated hypoxic exposures. A single hypoxic exposure has been shown to increase EpoR gene expression in cultured neurons (Yu X, 2002; Liu R, 2006; Ehrenreich H, 2005) but not in the adult mouse brain (Bernaudin M, 2000; Soliz J, 2007). We hypothesized that upregulation of EpoR gene expression in the VLR might be triggered by repeated hypoxic exposures. In this thesis, we have shown that 3 sessions of hypoxia (8% O_2 for 6 hours) at 4-day intervals in rats raised in standard cages activated neuronal expression of EpoR in the VLR and in the ventral hippocampus (HiV), but not in the dorsal part of the brain, notably in the neocortex (NCx) and the dorsal hippocampus (HiD) (Results Part II). The unequal response and the absence of reactivity in other brain structures led us to presume that repeated hypoxic exposures is not sufficiently challenging.

II.2. Activation of EPO and EpoR gene expression in the VLR by environmental enrichment. Enrichment provided in the cage where animals are living, so-called environmental enrichment or enriched housing, has been shown to modulate the expression pattern of different genes (Rampon C, 2000). Among these genes, Insulin-

Like Growth factor 1 "IGF-1" was reported to be a crucial candidate for enriched housing to exert its beneficial effects. Koopmans and colleagues have demonstrated that neutralizing circulating IGF-1 in rats significantly blocked the effectiveness of enriched housing on functional recovery from spinal cord injury (Koopmans GC, 2006). In addition, pioneer studies by Torres-Aleman's group in Spain have reported that beneficial effects of physical exercise on the brain are mediated by circulating IGF-1 (Carro E, 2001; Trejo JL, 2001), knowing that physical exercise can also up-regulate the expression of IGF-1 in the hippocampus (Ding Q, 2006). The literature reports two major studies providing evidence of a tight link between IGF-1 and Epo: 1) exogenous IGF-1 induced transcript levels of Epo (Chavez JC, 2002), and 2) there is a synergetic neuroprotective effect of Epo and IGF-1 (Digicaylioglu M, 2004). Therefore, because voluntary exercise (through the presence of running wheels and large exploration areas) is one of the behaviors promoted by Marlau™ cages, we have hypothesized that rearing rats in Marlau™ cages may induce IGF-1, which in turn, may induce Epo expression. In this thesis, we report for the first time that both IGF-1 and Epo were significantly increased in the HiD and the HiV of rats housed in Marlau™ cages compared to rats housed in standard cages (Results Parts I and II). This is also verified in the VLR, since greater expression of Epo (Results Part II) and IGF-1 (+40%, $p<0.05$, Fares et al., personal communication) were found in rats raised in Marlau™ cages compared to rats housed in standard cages. Interestingly, when enriched environment failed to increase IGF-1 in the NCx (+9%, $p>0.05$, Fares et al., personal communication), it also failed to induce that of Epo (Results Part II). Intriguingly, each time brain Epo was upregulated in rats reared in Marlau™ cages, EpoR expression was also activated. These results are in line with prior studies showing that: 1) EpoR expression in cultured neurons is dependent on that of Epo via the transcription factor GATA-3 (Yu X, 2002); 2) constitutive expression of Epo is concordant with EpoR throughout the life span (Sanchez PE, 2009b); and 3) exogenous administration of Epo induces EpoR expression 5 and 29 hours later (Sanchez PE Thesis 2008). It may thus be possible that in response to enriched housing, IGF-1 induction precedes and governs that of Epo, which, in turn, determines EpoR upregulation via GATA-3 signaling.

II.3. Ubiquitous upregultion of EpoR gene expression after repeated hypoxic exposures when housed in Marlau™ cages. In the HiV, we observed that upregulation of EpoR was maximal each time rats were in a condition different from just being housed in standard environments under normoxic conditions. Indeed, housing in Marlau™ cages, or repeated hypoxia exposures independently of the housing condition, elevated EpoR gene expression in the HiV to the same extent. In the VLR, while rats housed in Marlau™ cages exhibited greater level of EpoR gene expression compared to

rats raised in standard cages, it appeared that repeated hypoxia exposures was the most efficient stimulus to elevate EpoR gene expression at its greatest value, independently of the housing condition also. In the HiD and the NCx, it appeared that the greatest expression of EpoR was observed after repeated hypoxia exposures when rats were housed in Marlau™ cages. A last point deserves to be highlighted: while neither enriched environment alone nor repeated hypoxic exposures in rats raised in standard cages increased EpoR expression in the NCx, we showed that rearing rats in Marlau™ cages rendered this structure sensitive to hypoxia. The fact that two factors need to be combined to enhance expression of a crucial gene involved in brain plasticity/repair has already been reported by Falkenberg and coll. when they demonstrated that neither enriched housing, nor training rats reared in standard cages in the Morris Water Maze (MWM) task, increased BDNF in the hippocampus by themselves; however, induction of BDNF could be achieved by combining MWM task and enriched housing (Falkenberg T, 1992).

II.4. Neuroprotection by rhEpo in the VLR following elevated EpoR gene expression. While maximal EpoR expression was observed under the combination "housing in Marlau™ cages plus repeated hypoxic exposures", we could not test whether rhEpo could exert its maximal neuroprotective effect following *status epilepticus* (SE) under this combination, since enriched housing by itself exerts inhibitory effects on the development of brain excitability and SE and produces resistance to excitotoxic injury (Young D, 1999; Auvergne R, 2002). Thus, to circumvent any interpretational issue inherent to the enrichment paradigm, we have decided to conduct the experiment on rats raised in standard cages and exposed to repeated hypoxia. This decision did not cause any problem to test the neuroprotective efficacy of rhEpo in the VLR, which is the most devastated brain area following SE, since EpoR gene expression reached similar levels after repeated hypoxia, whether rats were housed in standard or Marlau™ cages (Results Part II). We provide evidence that: 1) upregulated Epo/EpoR system is not sufficient by itself to counteract deleterious processes activated following SE; 2) high dose of rhEpo by itself was not efficient to counteract neurodegenerative processes; 3) high dose of rhEpo in rats with elevated EpoR gene expression displayed significant neuroprotection in the VLR after SE (Results Part II).

Part III. Functional neuroprotection

Several studies have reported that both rhEpo and enriched environment can exert beneficial effects on cognition and behavior in different models of brain injury (see Review of the literature, Part III.4). Since all experiments in our laboratory on enriched

environment were designed to start at weaning, we have adjusted a model of severe SE at this stage, using lithium (Li) and pilocarpine (Pilo), so-called Li-Pilo. The interesting finding was to discover that SE at weaning, in contrast to SE at the adult stage, did not cause any obvious neuronal loss (Results Part III). However, rats exhibited severe cognitive decline with age. Using this model, we have addressed the following questions: 1) can therapeutic administration of rhEpo rescue cognitive function in rats raised in standard cages and subjected to Li-Pilo SE at weaning; 2) can enriched housing in Marlau™ cages be as efficient as rhEpo; and 3) is it possible to potentiate the effects of rhEpo and enriched housing by combining them ?

III.1. Preserved spatial learning performances in epileptic rats housed in Marlau™ cages: towards an adaptative process. We have noticed an important cognitive decline in "epileptic" rats housed in standard cages, as evaluated in the Morris Water Maze task. By contrast, cognitive performances were greater in healthy ("non-epileptic") rats raised in Marlau™ cages compared to those raised in standard cages (Results Part I), and they were preserved in "epileptic" rats housed in Marlau™ cages. Pioneer studies, conducted during the latent period following Li-Pilo SE at weaning, had already provided evidence that housing rats in enriched environment protects against cognitive decline (Rutten A, 2002; Faverjon S, 2002; Wang CA, 2007). Here, we refined those earlier reports by measuring cognitive performances, not only during the latent period, but also once behavioral spontaneous recurrent seizures occurred. We show that housing rats in Marlau™ cages from the day after Li-Pilo SE is enough to protect the cognitive performance in the Morris Water Maze task (Results Part I). We cannot provide any information at the present time on whether seizures were of the same type and at the same frequency between rats raised in standard and Marlau™ cages. Indeed, it is currently not feasible to monitor EEG in rats housed in social groups, especially in cages as complex as the Marlau™ cages. This observation reveals that wireless EEG recordings must be developed to circumvent this issue. Assuming that dysfunction of neural networks underlying "epilepsy" is the same in rats housed in the two different conditions, we can thus propose that seizures *per se* do not play a crucial role in cognitive impairment. Indeed, if that were the case, "epileptic" rats housed in Marlau™ cages should have displayed cognitive impairments following Pilo-SE. Instead, we propose that cognitive preservation is mainly the outcome of environmental context. When the housing environment is stimulating the spatial orientation (as discussed below), then navigational learning and memory in the Morris Water Maze is preserved, despite the presence of seizures. However, seizures may precipitate cognitive decline in rats whose hippocampal function is not sufficiently stimulated, as occurs during housing in standard cages.

Place navigation in the Morris Water Maze, which consists for the rat of finding a hidden platform in a circular tank full of water using visual (spatial) cues located outside of the pool (Morris R, 1984), depends on the functionality of "place cells", that are neurons in the hippocampus which identify or represent points in space in an environment (Brun VH, 2002). Interestingly, it has been reported that seizures induce changes in "place cell" physiology by rendering them less precise and less stable (Liu X, 2003). This may thus explain cognitive impairment in place navigation in the Morris Water Maze, when "epileptic" rats are housed in standard cages.

The observation that place navigation is preserved when "epileptic" rats are housed in Marlau™ cages may rely on the intrinsic properties of these cages. Indeed, this cage is an efficient ways to standardize reinforcement of hippocampal place cell functionality. By changing three times a week the configuration of the maze that rats had to explore to find their food, we engaged rats in learning repeatedly new spatial representation forms (Frank LM, 2004), and hence, in maintaining high plasticity of place cell activity. We propose that steady reinforcement of place navigation, which is an essential function of the hippocampus, is protective against cognitive impairment in "epileptic" rats. This result is of particular importance, since place- and view-specific hippocampal activity has been reported in both humans and non-human primates (Rolls ET, 1999; Ekstrom AD, 2003; Hori E, 2003).

III.2. Persistence of anxiety-trait in epileptic rats housed in Marlau™ cages: can it be considered as an adaptive process? Our study has shown that rats subjected to Pilo-SE and reared in standard cages presented a severe cognitive decline notably in the navigational learning and memory task (Morris Water Maze). In addition, these rats exhibited severe anxiety-like disorders, as measured in the Elevated Plus Maze and in the Water Exploration Test that we have developed. While housing rats in Marlau™ cages following Pilo-SE fully preserved the cognitive function in the Morris Water Maze task, it did not protect against the development of anxiety-like disorders. At first, we considered this result as disappointing and disconcerting. But, on an adaptive point of view, it may be of good value that "epileptic" rats raised in Marlau™ cages maintain a high level of anxiety.

Anxiety has to be distinguished from fear. The first is a future-oriented mood state, preparing to cope with potentially upcoming negative events (Barlow DH, 2000). The latter is related to the specific behaviors of escape and avoidance in context of present and real dangers. Anxiety has been proposed as a protective mechanism preventing the organism from engaging in potentially harmful behaviors (Zald DH, 1997; 2002). Thus, when the animal is in good health, exploring the unknown might be a positive adaptive behavior. The more secure is the living environment, the greater might be the chance to

be willing at exploring the unknown. By contrast, in a context of animal suffering in pathological conditions, getting out of the secure environment might be a source of anxiety: seizures can occur in the new environment, like the open arms of the Elevated Plus Maze or the center of the quadrant in the Water Exploration Test. Translated into the wild, the risk of being attacked by a potential predator is particularly high in these areas, in particular when rats might lose consciousness during seizures. There is no reason for this danger to be different whether animals are living in standard or Marlau™ cages. This means that rodents might be aware of their "epileptic" state, and that they adapt their behavior constantly to avoid potentially dangerous situations.

III.3. rhEpo provided functional protection in epileptic rats reared in standard cages but not in Marlau™ cages. To our knowledge no one has yet investigated the role of rhEpo on cognition in rats subjected to Pilo-SE either at adult age or at weaning. Here, we report that rhEpo administered daily for 5 consecutive days to rats housed in standard cages following Li-Pilo SE at weaning has little beneficial effects on anxiety, as measured in the long term (18 weeks after Li-Pilo SE) using the Elevated Plus Maze test. We also confirm that its administration protects against learning and memory alterations in the Morris Water Maze task, as reported in other models of brain injury (see table 4). All these positive effects of rhEpo were observed in rats that developed "epilepsy", i.e. behavioral spontaneous recurrent seizures (BSRS), and may be associated to the following mechanisms:

1. Recently, Epo has been shown to enhance long term potentiation in CA1 and hippocampus-dependent memory via profound changes in the balance between excitatory and inhibitory transmission: without modifying the number of synapses, or vesicle fusion and dynamics, Epo increased the efficiency of the bursting activity of selected synapses/neuronal networks, while increasing the proportion of silence synapses in the hippocampus (Adamcio B, 2008). Our results thus suggest that rhEpo administered after Li-Pilo SE may have prevented the development of learning and memory deficits by maintaining the balance between excitatory and inhibitory transmission in neuronal networks involved in long-term memory processes, at learning, consolidation, storage and/or retrieval stages (Wang H, 2006).
2. NMDA receptor-dependent synaptic reinforcement in hippocampus CA1 area is a crucial process for memory consolidation (Shimizu E, 2000). We do not know whether alteration in NMDA receptor expression and/or NMDA receptor-dependent synaptic reinforcement is altered in the CA1 area of rats subjected to Li-Pilo SE at weaning. If that is the case, then preventive effects of rhEpo treatment may have counteracted the development of learning and retrieval of remote spatial memory deficits in "epileptic" rats by maintaining NMDA receptor expression in area CA1.

3. Finally, a recent study provided evidence that newly generated neurons in the dentate gyrus are recruited in neuronal networks that support retrieval of remote spatial memory (Trouche S, 2009). While enhanced neurogenesis occurring in the hippocampus has been proposed as a mechanism that may support dysfunctional excitability in the epileptic brain of rats subjected to Pilo-SE at the adult stage (Parent JM, 1997), it is thought to preserve cognitive functions when rats are subjected to Li-Pilo SE at weaning (Faverjon S, 2002). Since Epo is involved in regulating developmental and adult neurogenesis (Chen ZY, 2007, Shingo T, 2001, Tsai PT 2006), it may be possible that rhEpo administered in rats subjected to Li-Pilo SE at weaning and housed in standard cages preserved: 1) the functionality of neuronal progenitor cells in the subgranular zone of the dentate gyrus, and/or 2) the mechanisms involved in the recruitment of newly-generated neurons in neuronal networks that support retrieval of remote spatial memory.

Since we observed beneficial effects of enriched housing regarding cognitive performances in the Morris Water Maze, while anxiety-like disorders were not prevented, we questioned the possibility of improving the effects of enriched housing, notably on anxiety, using rhEpo therapy. We report for the first time an intriguing result when rats were reared in Marlau™ cages and treated with rhEpo. Indeed, rhEpo exacerbated anxiety and completely abolished the positive effects of enriched housing in the retention memory test, indicating that the neuronal network(s) underlying memory consolidation/retrieval in the Morris Water Maze task was altered by combining rhEpo therapy and environmental enrichment.

It has been demonstrated that enriched housing made it possible to recover from memory deficits in mice lacking NMDA receptor 1 subunit, likely by increasing synaptic density in area CA1 of the hippocampus (Rampon C, 2000b). It is then possible that protective effects of enrichment on learning and memory in rats subjected to Li-Pilo SE at weaning and then housed in enriched housing (without rhEpo administration) involved similar mechanisms. Since enriched housing after Li-Pilo SE in young rats has been shown to increase neurogenesis in the dentate gyrus, and has been proposed to mediate the positive effects of enriched housing on cognition in these rats (Faverjon S, 2002), it may be possible that "epileptic" rats with improved cognitive performances after housing in the Marlau™ cages also had increased hippocampal neurogenesis. However, the puzzling reason that may explain why rhEpo, which has positive effects on learning and memory (Adamcio B, 2008) and neurogenesis (Chen ZY, 2007; Shingo T, 2001; Tsai PT, 2006) interfered with environmental enrichment remains to be determined. It is noteworthy that 50% of the "epileptic" rats subjected to rhEpo treatment and housed in Marlau™ cages exhibited an abnormal increase in neocortical and hippocampal

vascularity. This result cannot explain however the cognitive decline observed in all rats within this experimental group. While using a cage that greatly standardized enrichment procedures, we show that tissue abnormalities are not reproducible among animals. This might be explained by the fact that Sprague-Dawley rats are not genetically homogenous (outbred strain) compared with inbred rats such as Fisher 344 or Brown Norway. Future studies will be performed in different inbred strains of rats to test whether variability in tissue abnormalities is genetically determined.

Part IV. Seizure development

Although we did not implant electrodes to perform EEG recordings and to assess the number of rats that exhibited an impaired EEG pattern, we report that: 1) either rhEpo administered in rats reared in standard cages or housing in Marlau™ cages decreased the number of rats that developed BSRS; 2) the combination of rhEpo administration and enriched housing highly increased the number of rats that developed BSRS. It has been reported that seizures induced changes in "place cell" physiology by rendering them less precise and less stable (Liu X, 2003). We can presume that altered performance in place navigation in the Morris Water Maze task when rats were injected with rhEpo and raised in Marlau™ cages might be caused by an increased seizure frequency. To support this hypothesis, 1) it has been shown recently that high vessel density in patients with temporal lobe epilepsy positively correlates with seizure frequency (Rigau V, 2007), and 2) we report that rhEpo increased angiogenesis in the neocortex and the hippocampus of rats reared in Marlau™ cages. However, while increased seizure frequency can be plausible to explain the altered performance in the retention test, this argument alone is not enough since only 50% of these rats exhibited an increased angiogenesis. In some patients with epilepsy, cognitive impairment was found not to be systematically related to the number of seizures; it may sometimes be a part or the result of epileptogenesis (Taylor J 2009).

Part V. Ending suggestions

One in one thousand screened drug compounds makes it through the pre-clinical phase to the clinical phase (Relman AS, 2002). Some reasons that may explain the existence of such a failure have recently been discussed. Numerous methodological problems were raised concerning: 1) the clinical relevance of animal models, such as the choice of the species, the strain and the gender; 2) the inadequacy of the pharmacological evaluation of drug effects, such as lack of complete dose response and/or failure to examine therapeutic windows (Faden AI, 2007). Based on our results, showing that the neuroprotective effects of rhEpo are dependent on the housing conditions of the rats, we propose that additional epigenetic factors may explain to some extent why drugs showing

desired pharmacological effects in pre-clinical studies have failed to meet expectations in clinical trials. Indeed, to date, all pre-clinical studies testing the efficacy of potential neuroprotective molecules have been conducted on animals housed in standard cages only. This choice was justified because enriched housing program was lacking standardized procedures in laboratories (Nithianantharajah J, 2006). Now, by launching Marlau™ cages as an easy way to provide standardized and validated enriched housing program for rodents, we strongly suggest, before moving to clinical trials, the followings:

1. All drugs/molecules should be tested in animals housed in standard and enriched environments.
2. If beneficial effect of a candidate molecule is observed in both the standard and enriched conditions, then the possibility to go through a clinical phase may be feasible, independently of the psycho-social context in which the patient lives.
3. If beneficial effect of a candidate molecule is observed in only one housing condition, then the possibility to go through a clinical phase may be evaluated considering the psycho-social context in which the patient lives.
4. Acquired pathological condition may be accompanied by a change (positive or negative) in the psycho-social environment of the patient. In this case, it may also be valuable to test the efficacy of candidate therapeutics in pre-clinical investigations when animals switch from one environment (standard or enriched) to the other once the disease is contracted or brain insults has occurred. Furthermore, it may happen that the life condition of a patients turns towards social isolation. This is also another psycho-social change to take into consideration in pre-clinical investigations.
5. All drugs/molecules that have shown beneficial effects in enriched housing conditions should be tested on the long term since: 1) enriched housing increase brain robustness and may delay the appearance of brain alterations, and 2) we have shown that negative effects of rhEpo treatment appeared late in Marlau™ cages.
6. If a molecule has shown promising effects in standard cages but failed in the enriched environment, we propose before excluding it to test different doses, since brain expression of many receptors in rats reared in the enriched environment may differ from that of rats reared in standard cages.
7. If a molecule failed to show beneficial effects in standard housing, we should test it in the enriched housing condition before excluding it since we have shown that the expression of the targeted receptor/molecule completely changes the efficacy of the tested neuroprotective compound. Modulation of such receptors may be achieved using physiological conditions such as enriched environment, or any condition that disrupts boredom in living conditions.

Part VI. Suggested perspectives in the short term

1. The neuroprotective effects of rhEpo require high doses to be administered, that may have unwanted side effects (increased hematocrit for instance). It has been shown that intranasal administration of rhEpo needed lower doses than intravenous or intraperitoneal administration to produce equivalent neuroprotective effects. In addition, intranasal co-administration of rhEpo and IGF-1 demonstrated highly beneficial effects on neurological functions in a model of stroke in mice, still observable at a long term (3 months) after the administration (Fletcher L, 2009). We will therefore test whether the intranasal infusion of rhEpo in combination or not with IGF-1 produces significant improvements on the neurological function in rats subjected to Li-Pilo SE at weaning.
2. We would like to test to which extent physiological hippocampal stimulation in Marlau™ cage is able to protect the functional outcome of rats following hippocampal lesion. For this, the model of non-convulsivant SE induced by perforant path stimulation developed by Robert S. Sloviter will be optimal since, at the difference with chemically-induced SE as used in adults rats, neuronal loss is restricted to the hippocampus and does not affect extra-hippocampal regions, such as the amygdala, the piriform cortex, the dorsal part of the thalamus and the insular agranular cortex.
3. We are going to test whether delaying gradually the time at which rats are housed in enriched conditions (in Marlau™ cages) after the initial insult will result or not in maintained rehabilitation of cognitive function. This study will help us to determine whether beneficial effects of positive changes in the psycho-social environment can be expected, even if they are initiated at advanced stages of the disease.

Reference List (n=310)

A

Acharya, M.M., Hattiangady, B., Shetty, A.K., 2008. Progress in neuroprotective strategies for preventing epilepsy. Prog Neurobiol 84, 363-404.

Abramov, U., Raud, S., Innos, J., Lasner, H., Kurrikoff, K., Turna, T., Puussaar, T., Okva, K., Matsui, T., Vasar, E., 2008. Different housing conditions alter the behavioural phenotype of CCK(2) receptor-deficient mice. Behav Brain Res 193, 108-116.

Adamcio, B., Sargin, D., Stradomska, A., Medrihan, L., Gertler, C., Theis, F., Zhang, M., Muller, M., Hassouna, I., Hannke, K., Sperling, S., Radyushkin, K., El-Kordi, A., Schulze, L., Ronnenberg, A., Wolf, F., Brose, N., Rhee, J.S., Zhang, W., Ehrenreich, H., 2008. Erythropoietin enhances hippocampal long-term potentiation and memory. BMC Biology 6, 37.

Adriani, W., Giannakopoulou, D., Bokulic, Z., Jernej, B., Alleva, E., Laviola, G., 2006. Response to novelty, social and self-control behaviors, in rats exposed to neonatal anoxia: modulatory effects of an enriched environment. Psychopharmacology (Berl) 184, 155-165.

Alafaci, C., Salpietro, F., Grasso, G., Sfacteria, A., Passalacqua, M., Morabito, A., Tripodo, E., Calapai, G., Buemi, M., Tomasello, F., 2000. Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage. Eur J Pharmacol 406, 219-225.

Andin, J., Hallbeck, M., Mohammed, A.H., Marcusson, J., 2007. Influence of environmental enrichment on steady-state mRNA levels for EAAC1, AMPA1 and NMDA2A receptor subunits in rat hippocampus. Brain Res 1174, 18-27.

Artola, A., von Frijtag, J.C., Fermont, P.C.J., Gispen, W.H., Schrama, L.H., Kamal, A., Spruijt, B.M., 2006. Long-lasting modulation of the induction of LTD and LTP in rat hippocampal CA1 by behavioural stress and environmental enrichment. Eur J Neurosci 23, 261-272.

Assaraf, M.I., Diaz, Z., Liberman, A., Miller, W.H., Jr., Arvanitakis, Z., Li, Y., Bennett, D.A., Schipper, H.M., 2007. Brain erythropoietin receptor expression in Alzheimer disease and mild cognitive impairment. J Neuropathol Exp Neurol 66, 389-398.

Auvergne, R., Lere, C., El Bahh, B., Arthaud, S., Lespinet, V., Rougier, A., Le Gal La Salle, G., 2002. Delayed kindling epileptogenesis and increased neurogenesis in adult rats housed in an enriched environment. Brain Res 954, 277-285.

B

Barlow, D.H., 2000. Unraveling the mysteries of anxiety and its disorders from the perspective of emotion theory. Am Psychol 55, 1247-1263.

Belayev, L., Khoutorova, L., Zhao, W., Vigdorchik, A., Belayev, A., Busto, R., Magal, E., Ginsberg, M.D., 2005. Neuroprotective effect of darbepoetin alfa, a novel recombinant erythropoietic protein, in focal cerebral ischemia in rats. Stroke 36, 1071-1076.

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., Yaniv, I., Pick, C.G., 2004. Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. Eur J Neurosci 20, 1341-1347.

Bennett, J.C., McRae, P.A., Levy, L.J., Frick, K.M., 2006. Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. Neurobiol Learn Mem 85, 139-152.

Berardi, N., Braschi, C., Capsoni, S., Cattaneo, A., Maffei, L., 2007. Environmental enrichment delays the onset of memory deficits and reduces neuropathological hallmarks in a mouse model of Alzheimer-like neurodegeneration. J Alzheimers Dis 11, 359-370.

Bernaudin, M., Marti, H.H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E.T., Petit, E., 1999. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 19, 643-651.

Bezard, E., Dovero, S., Belin, D., Duconger, S., Jackson-Lewis, V., Przedborski, S., Piazza, P.V., Gross, C.E., Jaber, M., 2003. Enriched environment confers resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and cocaine: involvement of dopamine transporter and trophic factors. J Neurosci 23, 10999-11007.

Bindu, B., Alladi, P.A., Mansooralikhan, B.M., Srikumar, B.N., Raju, T.R., Kutty, B.M., 2007. Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions. Neuroscience 144, 412-423.

Boehm, G.W., Sherman, G.F., Hoplight, B.J., 2nd, Hyde, L.A., Waters, N.S., Bradway, D.M., Galaburda, A.M., Denenberg, V.H., 1996. Learning and memory in the autoimmune BXSB mouse: effects of neocortical ectopias and environmental enrichment. Brain Res 726, 11-22.

Bouilleret, V., Ridoux, V., Depaulis, A., Marescaux, C., Nehlig, A., Le Gal La Salle, G., 1999. Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience 89, 717-729.

Bowling, S.L., Rowlett, J.K., Bardo, M.T., 1993. The effect of environmental enrichment on amphetamine-stimulated locomotor activity, dopamine synthesis and dopamine release. Neuropharmacology 32, 885-893.

Boyke, J., Driemeyer, J., Gaser, C., Buechel, C., May, A., 2008. Training-induced brain structure changes in the elderly. J Neurosci 28, 7031-7035.

Bredy, T.W., Humpartzoomian, R.A., Cain, D.P., Meaney, M.J., 2003. Partial reversal of the effect of maternal care on cognitive function through environmental enrichment. Neuroscience 118, 571-576.

Bredy, T.W., Zhang, T.Y., Grant, R.J., Diorio, J., Meaney, M.J., 2004. Peripubertal environmental enrichment reverses the effects of maternal care on hippocampal development and glutamate receptor subunit expression. Eur J Neurosci 20, 1355-1362.

Briellmann, R.S., Berkovic, S.F., Syngeniotis, A., King, M.A., Jackson, G.D., 2002. Seizure-associated hippocampal volume loss: a longitudinal magnetic resonance study of temporal lobe epilepsy. Ann Neurol 51, 641-644.

Brines, M., Cerami, A., 2005. Emerging biological roles for erythropoietin in the nervous system. Nature Reviews Neuroscience 6, 484-494.

Brines, M., Grasso, G., Fiordaliso, F., Sfacteria, A., Ghezzi, P., Fratelli, M., Latini, R., Xie, Q.W., Smart, J., Su-Rick, C.J., Pobre, E., Diaz, D., Gomez, D., Hand, C., Coleman, T.,

Cerami, A., 2004. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci U S A 101, 14907-14912.

Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de-Lanerolle, N.C., Cerami, C., Itri, L.M., Cerami, A., 2000. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 97, 10526-10531.

Briones, T.L., Suh, E., Jozsa, L., Woods, J., 2006. Behaviorally induced synaptogenesis and dendritic growth in the hippocampal region following transient global cerebral ischemia are accompanied by improvement in spatial learning. Exp Neurol 198, 530-538.

Briones, T.L., Woods, J., Wadowska, M., Rogozinska, M., 2006b. Amelioration of cognitive impairment and changes in microtubule-associated protein 2 after transient global cerebral ischemia are influenced by complex environment experience. Behav Brain Res 168, 261-271.

Brodie, M.J., Schachter, S.C. 2001. Epilepsy 2nd ed. Health Press, Oxford.

Bruel-Jungerman, E., Laroche, S., Rampon, C., 2005. New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. Eur J Neurosci 21, 513-521.

Brun, V.H., Otnass, M.K., Molden, S., Steffenach, H.A., Witter, M.P., Moser, M.B., Moser, E.I., 2002. Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. Science 296, 2243-2246.

C

Calapai, G., Marciano, M.C., Corica, F., Allegra, A., Parisi, A., Frisina, N., Caputi, A.P., Buemi, M., 2000. Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. Eur J Pharmacol 401, 349-356.

Cao, L., Jiao, X., Zuzga, D.S., Liu, Y., Fong, D.M., Young, D., During, M.J., 2004. VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet 36, 827-835.

Cao, X., Huang, S., Ruan, D., 2008. Enriched environment restores impaired hippocampal long-term potentiation and water maze performance induced by developmental lead exposure in rats. Dev Psychobiol 50, 307-313.

Carro, E., Trejo, J.L., Busiguina, S., Torres-Aleman, I., 2001. Circulating insulin-like growth factor I mediates the protective effects of physical exercise against brain insults of different etiology and anatomy. J Neurosci 21, 5678-5684.

Caston, J., Devulder, B., Jouen, F., Lalonde, R., Delhaye-Bouchaud, N., Mariani, J., 1999. Role of an enriched environment on the restoration of behavioral deficits in Lurcher mutant mice. Dev Psychobiol 35, 291-303.

Catania, M.A., Marciano, M.C., Parisi, A., Sturiale, A., Buemi, M., Grasso, G., Squadrito, F., Caputi, A.P., Calapai, G., 2002. Erythropoietin prevents cognition impairment induced by transient brain ischemia in gerbils. Eur J Pharmacol 437, 147-150.

Cavazos, J.E., Das, I., Sutula, T.P., 1994. Neuronal loss induced in limbic pathways by kindling: evidence for induction of hippocampal sclerosis by repeated brief seizures. J Neurosci 14, 3106-3121.

Celik, M., Gokmen, N., Erbayraktar, S., Akhisaroglu, M., Konakc, S., Ulukus, C., Genc, S., Genc, K., Sagiroglu, E., Cerami, A., Brines, M., 2002. Erythropoietin prevents motor

neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci U S A 99, 2258-2263.
Chamove, A.S., 1989. Environmental enrichment: a review. Anim Technol 40, 155–178.

Chapillon, P., Manneche, C., Belzung, C., Caston, J., 1999. Rearing environmental enrichment in two inbred strains of mice: 1. Effects on emotional reactivity. Behav Genet 29, 41-46.

Chavez, J.C., LaManna, J.C., 2002. Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: potential role of insulin-like growth factor-1. J Neurosci 22, 8922-8931.

Chen, X., Li, Y., Kline, A.E., Dixon, C.E., Zafonte, R.D., Wagner, A.K., 2005. Gender and environmental effects on regional brain-derived neurotrophic factor expression after experimental traumatic brain injury. Neuroscience 135, 11-17.

Chen, Z.Y., Asavaritikrai, P., Prchal, J.T., Noguchi, C.T., 2007. Endogenous erythropoietin signaling is required for normal neural progenitor cell proliferation. J Biol Chem 282, 25875-25883.

Chen, Z.Y., Warin, R., Noguchi, C.T., 2006. Erythropoietin and normal brain development: receptor expression determines multi-tissue response. Neurodegener Dis 3, 68-75.

Chin, K., Yu, X., Beleslin-Cokic, B., Liu, C., Shen, K., Mohrenweiser, H.W., Noguchi, C.T., 2000. Production and processing of erythropoietin receptor transcripts in brain. Molecular Brain Research 81, 29-42.

Choi, I.Y., Allan, A.M., Cunningham, L.A., 2005. Moderate fetal alcohol exposure impairs the neurogenic response to an enriched environment in adult mice. Alcoholism-Clinical and Experimental Research 29, 2053-2062.

Chu, K., Jung, K.H., Lee, S.T., Kim, J.H., Kang, K.M., Kim, H.K., Lim, J.S., Park, H.K., Kim, M., Lee, S.K., Roh, J.K., 2008. Erythropoietin reduces epileptogenic processes following status epilepticus. Epilepsia 49, 1723-1732.

Ciucci, F., Putignano, E., Baroncelli, L., Landi, S., Berardi, N., Maffei, L., 2007. Insulin-like growth factor 1 (IGF-1) mediates the effects of enriched environment (EE) on visual cortical development. PLoS ONE 2, e475.

Comeau, W., Gibb, R., Hastings, E., Cioe, J., Kolb, B., 2008. Therapeutic effects of complex rearing or bFGF after perinatal frontal lesions. Dev Psychobiol 50, 134-146.

Connor, J.R., Diamond, M.C., 1982. A comparison of dendritic spine number and type on pyramidal neurons of the visual cortex of old adult rats from social or isolated environments. J Comp Neurol 210, 99-106.

Cotman, C.W., Berchtold, N.C., 2007. Physical activity and the maintenance of cognition: Learning from animal models. Alzheimers & Dementia 3, S30-S37.

Crabbe, J.C., Wahlsten, D., Dudek, B.C., 1999. Genetics of mouse behavior: interactions with laboratory environment. Science 284, 1670-1672.

Cui, M., Yang, Y., Yang, J., Zhang, J., Han, H., Ma, W., Li, H., Mao, R., Xu, L., Hao, W., Cao, J., 2006. Enriched environment experience overcomes the memory deficits and depressive-like behavior induced by early life stress. Neurosci Lett 404, 208-212.

Cui, M.H., Yang, Y., Yang, J.L., Zhang, J.C., Han, H.L., Ma, W.P., Li, H.B., Mao, R.R., Xu, L., Hao, W., Cao, J., 2006. Enriched environment experience overcomes the memory deficits and depressive-like behavior induced by early life stress. Neurosci Lett 404, 208-212.

D

de Boer, H.M., Mula, M., Sander, J.W., 2008. The global burden and stigma of epilepsy. Epilepsy Behav 12, 540-546.

Del Arco, A., Segovia, G., Canales, J.J., Garrido, P., de Blas, M., Garcia-Verdugo, J.M., Mora, F., 2007b. Environmental enrichment reduces the function of D1 dopamine receptors in the prefrontal cortex of the rat. J Neural Transm 114, 43-48.

Del Arco, A., Segovia, G., Garrido, P., de Blas, M., Mora, F., 2007. Stress, prefrontal cortex and environmental enrichment: Studies on dopamine and acetylcholine release and working memory performance in rats. Behav Brain Res 176, 267-273.

Demers, E.J., McPherson, R.J., Juul, S.E., 2005. Erythropoietin protects dopaminergic neurons and improves neurobehavioral outcomes in juvenile rats after neonatal hypoxia-ischemia. Pediatr Res 58, 297-301.

Dhanushkodi, A., Bindu, B., Raju, T.R., Kutty, B.M., 2007. Exposure to enriched environment improves spatial learning performances and enhances cell density but not choline acetyltransferase activity in the hippocampus of ventral subicular-lesioned rats. Behav Neurosci 121, 491-500.

Diamond, M.C., Ingham, C.A., Johnson, R.E., Bennett, E.L., Rosenzweig, M.R., 1976. Effects of environment on morphology of rat cerebral cortex and hippocampus. J Neurobiol 7, 75-85.

Diamond, M.C., Krech, D., Rosenzweig, M.R., 1964. The Effects of an Enriched Environment on the Histology of the Rat Cerebral Cortex. J Comp Neurol 123, 111-120.

Digicaylioglu, M., Bichet, S., Marti, H.H., Wenger, R.H., Rivas, L.A., Bauer, C., Gassmann, M., 1995. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 92, 3717-3720.

Digicaylioglu, M., Garden, G., Timberlake, S., Fletcher, L., Lipton, S.A., 2004. Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. Proc Natl Acad Sci U S A 101, 9855-9860.

Ding, Q., Vaynman, S., Akhavan, M., Ying, Z., Gomez-Pinilla, F., 2006. Insulin-like growth factor I interfaces with brain-derived neurotrophic factor-mediated synaptic plasticity to modulate aspects of exercise-induced cognitive function. Neuroscience 140, 823-833.

Dobrossy, M.D., Dunnett, S.B., 2006. Morphological and cellular changes within embryonic striatal grafts associated with enriched environment and involuntary exercise. Eur J Neurosci 24, 3223-3233.

Duffy, S.N., Craddock, K.J., Abel, T., Nguyen, P.V., 2001. Environmental enrichment modifies the PKA-dependence of hippocampal LTP and improves hippocampus-dependent memory. Learn Mem 8, 26-34.

E

Ehrenreich, H., Fischer, B., Norra, C., Schellenberger, F., Stender, N., Stiefel, M., Siren, A.L., Paulus, W., Nave, K.A., Gold, R., Bartels, C., 2007. Exploring recombinant human erythropoietin in chronic progressive multiple sclerosis. Brain 130, 2577-2588.

Ehrenreich, H., Hasselblatt, M., Dembowski, C., Cepek, L., Lewczuk, P., Stiefel, M., Rustenbeck, H.H., Breiter, N., Jacob, S., Knerlich, F., Bohn, M., Poser, W., Ruther, E., Kochen, M., Gefeller, O., Gleiter, C., Wessel, T.C., De Ryck, M., Itri, L., Prange, H., Cerami, A., Brines, M., Siren, A.L., 2002. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 8, 495-505.

Ehrenreich, H., Hasselblatt, M., Knerlich, F., von Ahsen, N., Jacob, S., Sperling, S., Woldt, H., Vehmeyer, K., Nave, K.A., Siren, A.L., 2005. A hematopoietic growth factor, thrombopoietin, has a proapoptotic role in the brain. Proc Natl Acad Sci U S A 102, 862-867.

Ehrenreich, H., Hinze-Selch, D., Stawicki, S., Aust, C., Knolle-Veentjer, S., Wilms, S., Heinz, G., Erdag, S., Jahn, H., Degner, D., Ritzen, M., Mohr, A., Wagner, M., Schneider, U., Bohn, M., Huber, M., Czernik, A., Pollmacher, T., Maier, W., Siren, A.L., Klosterkotter, J., Falkai, P., Ruther, E., Aldenhoff, J.B., Krampe, H., 2007b. Improvement of cognitive functions in chronic schizophrenic patients by recombinant human erythropoietin. Mol Psychiatry 12, 206-220.

Eid, T., Brines, M.L., Cerami, A., Spencer, D.D., Kim, J.H., Schweitzer, J.S., Ottersen, O.P., de Lanerolle, N.C., 2004. Increased expression of erythropoietin receptor on blood vessels in the human epileptogenic hippocampus with sclerosis. J Neuropathol Exp Neurol 63, 73-83.

Ekstrand, J., Hellsten, J., Tingstrom, A., 2008. Environmental enrichment, exercise and corticosterone affect endothelial cell proliferation in adult rat hippocampus and prefrontal cortex. Neurosci Lett 442, 203-207.

Ekstrom, A.D., Kahana, M.J., Caplan, J.B., Fields, T.A., Isham, E.A., Newman, E.L., Fried, I., 2003. Cellular networks underlying human spatial navigation. Nature 425, 184-188.

El Rawas, R., Thiriet, N., Lardeux, V., Jaber, M., Solinas, M., 2009. Environmental enrichment decreases the rewarding but not the activating effects of heroin. Psychopharmacology (Berl) 203, 561-570.

F

Faden, A.I., Stoica, B., 2007. Neuroprotection: challenges and opportunities. Arch Neurol 64, 794-800.

Faherty, C.J., Shepherd, K.R., Herasimtschuk, A., Smeyne, R.J., 2005. Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. Molecular Brain Research 134, 170-179.

Falkenberg, T., Mohammed, A.K., Henriksson, B., Persson, H., Winblad, B., Lindefors, N., 1992. Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. Neurosci Lett 138, 153-156.

Fan, Y., Liu, Z., Weinstein, P.R., Fike, J.R., Liu, J., 2007. Environmental enrichment enhances neurogenesis and improves functional outcome after cranial irradiation. Eur J Neurosci 25, 38-46.

Faverjon, S., Silveira, D.C., Fu, D.D., Cha, B.H., Akman, C., Hu, Y., Holmes, G.L., 2002. Beneficial effects of enriched environment following status epilepticus in immature rats. Neurology 59, 1356-1364.

FELASA 2006, Working Group Standardization of Enrichment, Working Group Report.

Feng, R., Rampon, C., Tang, Y.P., Shrom, D., Jin, J., Kyin, M., Sopher, B., Miller, M.W., Ware, C.B., Martin, G.M., Kim, S.H., Langdon, R.B., Sisodia, S.S., Tsien, J.Z., 2001. Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 32, 911-926.

Ferrari, M., Matthews, W.S., Barabas, G., 1983. The family and the child with epilepsy. Fam Process 22, 53-59.

Ferriero, D.M., 2005. Protecting neurons. Epilepsia 46 Suppl 7, 45-51.

Fisher, R.S., van Emde Boas, W., Blume, W., Elger, C., Genton, P., Lee, P., Engel, J., Jr., 2005. Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46, 470-472.

Fletcher, L., Kohli, S., Sprague, S.M., Scranton, R.A., Lipton, S.A., Parra, A., Jimenez, D.F., Digicaylioglu, M., 2009. Intranasal delivery of erythropoietin plus insulin-like growth factor-I for acute neuroprotection in stroke. Laboratory investigation. J Neurosurg 111, 164-170.

Foley, A.G., Murphy, K.J., Regan, C.M., 2005. Complex-environment rearing prevents prenatal hypoxia-induced deficits in hippocampal cellular mechanisms necessary for memory consolidation in the adult Wistar rat. J Neurosci Res 82, 245-254.

Foster, T.C., Fugger, H.N., Cunningham, S.G., 2000. Receptor blockade reveals a correspondence between hippocampal-dependent behavior and experience-dependent synaptic enhancement. Brain Res 871, 39-43.

Foster, T.C., Gagne, J., Massicotte, G., 1996. Mechanism of altered synaptic strength due to experience: relation to long-term potentiation. Brain Res 736, 243-250.

Frank, L.M., Stanley, G.B., Brown, E.N., 2004. Hippocampal plasticity across multiple days of exposure to novel environments. J Neurosci 24, 7681-7689.

Franklin, T.B., Murphy, J.A., Myers, T.L., Clarke, D.B., Currie, R.W., 2006. Enriched environment during adolescence changes brain-derived neurotrophic factor and TrkB levels in the rat visual system but does not offer neuroprotection to retinal ganglion cells following axotomy. Brain Res 1095, 1-11.

Frick, K.M., Fernandez, S.M., 2003. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. Neurobiol Aging 24, 615-626.

Frick, K.M., Stearns, N.A., Pan, J.Y., Berger-Sweeney, J., 2003. Effects of environmental enrichment on spatial memory and neurochemistry in middle-aged mice. Learn Mem 10, 187-198.

Friske, J.E., Gammie, S.C., 2005. Environmental enrichment alters plus maze, but not maternal defense performance in mice. Physiol Behav 85, 187-194.

G

Gagne, J., Gelinas, S., Martinoli, M.G., Foster, T.C., Ohayon, M., Thompson, R.F., Baudry, M., Massicotte, G., 1998. AMPA receptor properties in adult rat hippocampus following environmental enrichment. Brain Res 799, 16-25.

Galani, R., Berthel, M.C., Lazarus, C., Majchrzak, M., Barbelivien, A., Kelche, C., Cassel, J.C., 2007. The behavioral effects of enriched housing are not altered by serotonin depletion but enrichment alters hippocampal neurochemistry. Neurobiol Learn Mem 88, 1-10.

Galani, R., Jarrard, L.E., Will, B.E., Kelche, C., 1997. Effects of postoperative housing conditions on functional recovery in rats with lesions of the hippocampus, subiculum, or entorhinal cortex. Neurobiol Learn Mem 67, 43-56.

Gaulke, L.J., Horner, P.J., Fink, A.J., McNamara, C.L., Hicks, R.R., 2005. Environmental enrichment increases progenitor cell survival in the dentate gyrus following lateral fluid percussion injury. Molecular Brain Research 141, 138-150.

Ghezzi, P., Brines, M., 2004. Erythropoietin as an antiapoptotic, tissue-protective cytokine. Cell Death Differ 11 Suppl 1, S37-44.

Giza, C.C., Griesbach, G.S., Hovda, D.A., 2005. Experience-dependent behavioral plasticity is disturbed following traumatic injury to the immature brain. Behav Brain Res 157, 11-22.

Glass, M., van Dellen, A., Blakemore, C., Hannan, A.J., Faull, R.L., 2004. Delayed onset of Huntington's disease in mice in an enriched environment correlates with delayed loss of cannabinoid CB1 receptors. Neuroscience 123, 207-212.

Gobbo, O.L., O'Mara, S.M., 2004. Impact of enriched-environment housing on brain-derived neurotrophic factor and on cognitive performance after a transient global ischemia. Behav Brain Res 152, 231-241.

Gobbo, O.L., O'Mara, S.M., 2005. Exercise, but not environmental enrichment, improves learning after kainic acid-induced hippocampal neurodegeneration in association with an increase in brain-derived neurotrophic factor. Behav Brain Res 159, 21-26.

Gorio, A., Gokmen, N., Erbayraktar, S., Yilmaz, O., Madaschi, L., Cichetti, C., Di Giulio, A.M., Vardar, E., Cerami, A., Brines, M., 2002. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci U S A 99, 9450-9455.

Gortz, N., Lewejohann, L., Tomm, M., Ambree, O., Keyvani, K., Paulus, W., Sachser, N., 2008. Effects of environmental enrichment on exploration, anxiety, and memory in female TgCRND8 Alzheimer mice. Behav Brain Res 191, 43-48.

Grasso, G., Buemi, M., Alafaci, C., Sfacteria, A., Passalacqua, M., Sturiale, A., Calapai, G., De Vico, G., Piedimonte, G., Salpietro, F.M., Tomasello, F., 2002. Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. Proc Natl Acad Sci U S A 99, 5627-5631.

Grasso, G., Sfacteria, A., Passalacqua, M., Morabito, A., Buemi, M., Macri, B., Brines, M.L., Tomasello, F., 2005. Erythropoietin and erythropoietin receptor expression after experimental spinal cord injury encourages therapy by exogenous erythropoietin. Neurosurgery 56, 821-827; discussion 821-827.

Greenough, W.T., Volkmar, F.R., Juraska, J.M., 1973. Effects of rearing complexity on dendritic branching in frontolateral and temporal cortex of the rat. Exp Neurol 41, 371-378.

Guilarte, T.R., Toscano, C.D., McGlothan, J.L., Weaver, S.A., 2003. Environmental enrichment reverses cognitive and molecular deficits induced by developmental lead exposure. Ann Neurol 53, 50-56.

H

Hasselblatt, M., Ehrenreich, H., Siren, A.L., 2006. The brain erythropoietin system and its potential for therapeutic exploitation in brain disease. J Neurosurg Anesthesiol 18, 132-138.

Hattiangady, B., Shetty, A.K., 2008. Implications of decreased hippocampal neurogenesis in chronic temporal lobe epilepsy. Epilepsia 49, 26-41.

Hattori, S., Hashimoto, R., Miyakawa, T., Yamanaka, H., Maeno, H., Wada, K., Kunugi, H., 2007. Enriched environments influence depression-related behavior in adult mice and the survival of newborn cells in their hippocampi. Behav Brain Res 180, 69-76.

Hellemans, K.G., Benge, L.C., Olmstead, M.C., 2004. Adolescent enrichment partially reverses the social isolation syndrome. Brain Res Dev Brain Res 150, 103-115.

Hengemihle, J.M., Abugo, O., Rifkind, J., Spangler, E., Danon, D., Ingram, D.K., 1996. Chronic treatment with human recombinant erythropoietin increases hematocrit and improves water maze performance in mice. Physiol Behav 59, 153-156.
Henshall, D.C., Simon, R.P., 2005. Epilepsy and apoptosis pathways. J Cereb Blood Flow Metab 25, 1557-1572.

Hermann, B., Jones, J., Sheth, R., Dow, C., Koehn, M., Seidenberg, M., 2006. Children with new-onset epilepsy: neuropsychological status and brain structure. Brain 129, 2609-2619.

Hermann, B., Seidenberg, M., 2002. Neuropsychology and temporal lobe epilepsy. CNS Spectr 7, 343-348.

Hesdorffer, D.C., Hauser, W.A., Annegers, J.F., Cascino, G., 2000. Major depression is a risk factor for seizures in older adults. Ann Neurol 47, 246-249.

Hesdorffer, D.C., Hauser, W.A., Olafsson, E., Ludvigsson, P., Kjartansson, O., 2006. Depression and suicide attempt as risk factors for incident unprovoked seizures. Ann Neurol 59, 35-41.

Hicks, R.R., Danielson, L., Deluao, V., Haberpointer, M., 2004. Environmental enrichment increases neural progenitors in the hilus following lateral fluid percussion brain injury. In: 22nd Annual National-Neurotrauma-Society Symposium, Mary Ann Liebert Inc, San Diego, CA, p. P33

Hicks, R.R., Zhang, L., Atkinson, A., Stevenon, M., Veneracion, M., Seroogy, K.B., 2002. Environmental enrichment attenuates cognitive deficits, but does not alter neurotrophin gene expression in the hippocampus following lateral fluid percussion brain injury. Neuroscience 112, 631-637.

Holguin, S., Huang, Y., Liu, J., Wurtman, R., 2008. Chronic administration of DHA and UMP improves the impaired memory of environmentally impoverished rats. Behav Brain Res 191, 11-16.

Holloway, R.L., Jr., 1966. Dendritic branching: some preliminary results of training and complexity in rat visual cortex. Brain Res 2, 393-396.

Hori, E., Tabuchi, E., Matsumura, N., Tamura, R., Eifuku, S., Endo, S., Nishijo, H., Ono, T., 2003. Representation of place by monkey hippocampal neurons in real and virtual translocation. Hippocampus 13, 190-196.

Huang, F.L., Huang, K.P., Wu, J., Boucheron, C., 2006. Environmental enrichment enhances neurogranin expression and hippocampal learning and memory but fails to rescue the impairments of neurogranin null mutant mice. J Neurosci 26, 6230-6237.

I

Ickes, B., Pham, T., Sanders, L., Albeck, D., Mohammed, A., Granholm, A., 2000. Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. Exp Neurol 164, 45-52.

Imanaka, A., Morinobu, S., Toki, S., Yamamoto, S., Matsuki, A., Kozuru, T., Yamawaki, S., 2008. Neonatal tactile stimulation reverses the effect of neonatal isolation on open-field and anxiety-like behavior, and pain sensitivity in male and female adult Sprague-Dawley rats. Behav Brain Res 186, 91-97.

Imanaka, A., Morinobu, S., Toki, S., Yamawaki, S., 2006. Importance of early environment in the development of post-traumatic stress disorder-like behaviors. Behav Brain Res 173, 129-137.

Irvine, G.I., Logan, B., Eckert, M., Abraham, W.C., 2006. Enriched environment exposure regulates excitability, synaptic transmission, and UP in the dentate gyrus of freely moving rats. Hippocampus 16, 149-160.

Iwata, E., Kikusui, T., Takeuchi, Y., Mori, Y., 2007. Fostering and environmental enrichment ameliorate anxious behavior induced by early weaning in Balb/c mice. Physiol Behav 91, 318-324.

J

Jaber, M., Robinson, S.W., Missale, C., Caron, M.G., 1996. Dopamine receptors and brain function. Neuropharmacology 35, 1503-1519.

Jacobs, M.P., Leblanc, G.G., Brooks-Kayal, A., Jensen, F.E., Lowenstein, D.H., Noebels, J.L., Spencer, D.D., Swann, J.W., 2009. Curing epilepsy: progress and future directions. Epilepsy Behav 14, 438-445.

Jankowsky, J.L., Melnikova, T., Fadale, D.J., Xu, G.M., Slunt, H.H., Gonzales, V., Younkin, L.H., Younkin, S.G., Borchelt, D.R., Savonenko, A.V., 2005. Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. J Neurosci 25, 5217-5224.

Jelkmann, W., 1992. Erythropoietin: structure, control of production, and function. Physiol Rev 72, 449-489.

Jones, J.E., Watson, R., Sheth, R., Caplan, R., Koehn, M., Seidenberg, M., Hermann, B., 2007. Psychiatric comorbidity in children with new onset epilepsy. Dev Med Child Neurol 49, 493-497.

Juul, S.E., Yachnis, A.T., Rojiani, A.M., Christensen, R.D., 1999. Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. Pediatr Dev Pathol 2, 148-158.

K

Kanaan, N.M., Collier, T.J., Marchionini, D.M., McGuire, S.O., Fleming, M.F., Sortwell, C.E., 2006. Exogenous erythropoietin provides neuroprotection of grafted dopamine neurons in a rodent model of Parkinson's disease. Brain Res 1068, 221-229.

Kawakami, M., Sekiguchi, M., Sato, K., Kozaki, S., Takahashi, M., 2001. Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. J Biol Chem 276, 39469-39475.

Kempermann, G., Brandon, E.P., Gage, F.H., 1998. Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. Curr Biol 8, 939-942.

Kempermann, G., Gast, D., Gage, F.H., 2002. Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. Ann Neurol 52, 135-143.

Kempermann, G., Jessberger, S., Steiner, B., Kronenberg, G., 2004. Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27, 447-452.

Kempermann, G., Kuhn, H.G., Gage, F.H., 1997. More hippocampal neurons in adult mice living in an enriched environment. Nature 386, 493-495.

Kempermann, G., Kuhn, H.G., Gage, F.H., 1998b. Experience-induced neurogenesis in the senescent dentate gyrus. J Neurosci 18, 3206-3212.

Kent, G.P., Schefft, B.K., Howe, S.R., Szaflarski, J.P., Yeh, H.S., Privitera, M.D., 2006. The effects of duration of intractable epilepsy on memory function. Epilepsy Behav 9, 469-477.

Keyvani, K., Sachser, N., Witte, O.W., Paulus, W., 2004. Gene expression profiling in the intact and injured brain following environmental enrichment. J Neuropathol Exp Neurol 63, 598-609.

Kobayashi, K., Ikeda, Y., Suzuki, H., 2006. Locomotor activity correlates with modifications of hippocampal mossy fibre synaptic transmission. Eur J Neurosci 24, 1867-1873.

Koh, S., Chung, H., Xia, H., Mahadevia, A., Song, Y., 2005. Environmental enrichment reverses the impaired exploratory behavior and altered gene expression induced by early-life seizures. J Child Neurol 20, 796-802.

Kolb, B., Gibb, R., 1991. Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. Cereb Cortex 1, 189-198.

Kondo, M., Gray, L.J., Pelka, G.J., Christodoulou, J., Tam, P.P., Hannan, A.J., 2008. Environmental enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome--Mecp2 gene dosage effects and BDNF expression. Eur J Neurosci 27, 3342-3350.

Koopmans, G.C., Brans, M., Gomez-Pinilla, F., Duis, S., Gispen, W.H., Torres-Aleman, I., Joosten, E.A., Hamers, F.P., 2006. Circulating insulin-like growth factor I and functional recovery from spinal cord injury under enriched housing conditions. Eur J Neurosci 23, 1035-1046.

Kronenberg, G., Bick-Sander, A., Bunk, E., Wolf, C., Ehninger, D., Kempermann, G., 2006. Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus. Neurobiol Aging 27, 1505-1513.

Kumral, A., Uysal, N., Tugyan, K., Sonmez, A., Yilmaz, O., Gokmen, N., Kiray, M., Genc, S., Duman, N., Koroglu, T.F., Ozkan, H., Genc, K., 2004. Erythropoietin improves long-term spatial memory deficits and brain injury following neonatal hypoxia-ischemia in rats. Behav Brain Res 153, 77-86.

L

Landi, S., Sale, A., Berardi, N., Viegi, A., Maffei, L., Cenni, M.C., 2007. Retinal functional development is sensitive to environmental enrichment: a role for BDNF. FASEB J 21, 130-139.

Laviola, G., Hannan, A.J., Macri, S., Solinas, M., Jaber, M., 2008. Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. Neurobiol Dis 31, 159-168.

Leal-Galicia, P., Saldivar-Gonzalez, A., Morimoto, S., Arias, C., 2007. Exposure to environmental enrichment elicits differential hippocampal cell proliferation: role of individual responsiveness to anxiety. Dev Neurobiol 67, 395-405.

Lee, S.T., Chu, K., Sinn, D.I., Jung, K.H., Kim, E.H., Kim, S.J., Kim, J.M., Ko, S.Y., Kim, M., Roh, J.K., 2006. Erythropoietin reduces perihematomal inflammation and cell death with eNOS and STAT3 activations in experimental intracerebral hemorrhage. J Neurochem 96, 1728-1739.

Leggio, M.G., Mandolesi, L., Federico, F., Spirito, F., Ricci, B., Gelfo, F., Petrosini, L., 2005. Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. Behav Brain Res 163, 78-90.

Lehmann, K., Butz, M., Teuchert-Noodt, G., 2005. Offer and demand: proliferation and survival of neurons in the dentate gyrus. Eur J Neurosci 21, 3205-3216.

Leist, M., Ghezzi, P., Grasso, G., Bianchi, R., Villa, P., Fratelli, M., Savino, C., Bianchi, M., Nielsen, J., Gerwien, J., Kallunki, P., Larsen, A.K., Helboe, L., Christensen, S., Pedersen, L.O., Nielsen, M., Torup, L., Sager, T., Sfacteria, A., Erbayraktar, S., Erbayraktar, Z., Gokmen, N., Yilmaz, O., Cerami-Hand, C., Xie, Q.W., Coleman, T., Cerami, A., Brines, M., 2004. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 305, 239-242.

Leonardi, M., Ustun, T.B., 2002. The global burden of epilepsy. Epilepsia 43 Suppl 6, 21-25.

Lewczuk, P., Hasselblatt, M., Kamrowski Kruck, H., Heyer, A., Unzicker, C., Siren, A.L., Ehrenreich, H., 2000. Survival of hippocampal neurons in culture upon hypoxia: effect of erythropoietin. Neuroreport 11, 3485-3488.

Li, S., Tian, X., Hartley, D.M., Feig, L.A., 2006. The environment versus genetics in controlling the contribution of MAP kinases to synaptic plasticity. Curr Biol 16, 2303-2313.

Liu, R., Suzuki, A., Guo, Z., Mizuno, Y., Urabe, T., 2006. Intrinsic and extrinsic erythropoietin enhances neuroprotection against ischemia and reperfusion injury in vitro. J Neurochem 96, 1101-1110.

Liu, X., Muller, R.U., Huang, L.T., Kubie, J.L., Rotenberg, A., Rivard, B., Cilio, M.R., Holmes, G.L., 2003. Seizure-induced changes in place cell physiology: relationship to spatial memory. J Neurosci 23, 11505-11515.

Lu, D., Mahmood, A., Qu, C., Goussev, A., Schallert, T., Chopp, M., 2005. Erythropoietin enhances neurogenesis and restores spatial memory in rats after traumatic brain injury. J Neurotrauma 22, 1011-1017.

Lupien, S.J., Evans, A., Lord, C., Miles, J., Pruessner, M., Pike, B., Pruessner, J.C., 2007. Hippocampal volume is as variable in young as in older adults: Implications for the notion of hippocampal atrophy in humans. Neuroimage 34, 479-485.

M

Maiese, K., Chong, Z.Z., Li, F., Shang, Y.C., 2008. Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies. Prog Neurobiol 85, 194-213.

Mala, H., Alsina, C.G., Madsen, K.S., Sibbesen, E.C., Stick, H., Mogensen, J., 2005. Erythropoietin improves place learning in an 8-arm radial maze in fimbria-fornix transected rats. Neural Plast 12, 329-340.

Mandolesi, L., De Bartolo, P., Foti, F., Gelfo, F., Federico, F., Leggio, M.G., Petrosini, L., 2008. Environmental enrichment provides a cognitive reserve to be spent in the case of brain lesion. J Alzheimers Dis 15, 11-28.

Margerison, J.H., Corsellis, J.A., 1966. Epilepsy and the temporal lobes. A clinical, electroencephalographic and neuropathological study of the brain in epilepsy, with particular reference to the temporal lobes. Brain 89, 499-530.

Marti, H.H., Wenger, R.H., Rivas, L.A., Straumann, U., Digicaylioglu, M., Henn, V., Yonekawa, Y., Bauer, C., Gassmann, M., 1996. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 8, 666-676.

Martinez-Cue, C., Rueda, N., Garcia, E., Davisson, M.T., Schmidt, C., Florez, J., 2005. Behavioral, cognitive and biochemical responses to different environmental conditions in male Ts65Dn mice, a model of Down syndrome. Behav Brain Res 163, 174-185.

McCagh, J., Fisk, J.E., Baker, G.A., 2009. Epilepsy, psychosocial and cognitive functioning. Epilepsy Res 86, 1-14.

McNair, K., Broad, J., Riedel, G., Davies, C.H., Cobb, S.R., 2007. Global changes in the hippocampal proteome following exposure to an enriched environment. Neuroscience 145, 413-422.

Meehan C.L., Mench J.A., 2003. The challenge of challenge: Can problem solving opportunities enhance animal welfare? Appl Anim Behav Sci 102, 246-261.

Melendez, R.I., Gregory, M.L., Bardo, M.T., Kalivas, P.W., 2004. Impoverished rearing environment alters metabotrophic glutamate receptor expression and function in the prefrontal cortex. Neuropsychopharmacology 29, 1980-1987.

Meshi, D., Drew, M.R., Saxe, M., Ansorge, M.S., David, D., Santarelli, L., Malapani, C., Moore, H., Hen, R., 2006. Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9, 729-731.

Mikati, M.A., El Hokayem, J.A., El Sabban, M.E., 2007. Effects of a single dose of erythropoietin on subsequent seizure susceptibility in rats exposed to acute hypoxia at P10. Epilepsia 48, 175-181.

Miu, A.C., Heilman, R.M., Pasca, S.P., Stefan, C.A., Spanu, F., Vasiu, R., Olteanu, A.I., Miclea, M., 2006. Behavioral effects of corpus callosum transection and environmental enrichment in adult rats. Behav Brain Res 172, 135-144.

Miu, A.C., Olteanu, A.I., Chis, I., Heilman, R.M., 2004. Have no fear, erythropoietin is here: erythropoietin protects fear conditioning performances after functional inactivation of the amygdala. Behav Brain Res 155, 223-229.

Mogensen, J., Miskowiak, K., Sorensen, T.A., Lind, C.T., Olsen, N.V., Springborg, J.B., Mala, H., 2004. Erythropoietin improves place learning in fimbria-fornix-transected rats and modifies the search pattern of normal rats. Pharmacol Biochem Behav 77, 381-390.

Morimoto, K., Fahnestock, M., Racine, R.J., 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73, 1-60.

Morishita, E., Masuda, S., Nagao, M., Yasuda, Y., Sasaki, R., 1997. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 76, 105-116.

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11, 47-60.

N

Nadam, J., Navarro, F., Sanchez, P., Moulin, C., Georges, B., Laglaine, A., Pequignot, J.M., Morales, A., Ryvlin, P., Bezin, L., 2007. Neuroprotective effects of erythropoietin in the rat hippocampus after pilocarpine-induced status epilepticus. Neurobiol Dis 25, 412-426.

Naka, F., Narita, N., Okado, N., Narita, M., 2005. Modification of AMPA receptor properties following environmental enrichment. Brain Dev 27, 275-278.

Naka, F., Shiga, T., Yaguchi, M., Okado, N., 2002. An enriched environment increases noradrenaline concentration in the mouse brain. Brain Res 924, 124-126.

Navarro, F.P., Fares, R.P., Sanchez, P.E., Nadam, J., Georges, B., Moulin, C., Morales, A., Pequignot, J.M., Bezin, L., 2008. Brain heparanase expression is up-regulated during postnatal development and hypoxia-induced neovascularization in adult rats. J Neurochem 105, 34-45.

Neugebauer, N.M., Cunningham, S.T., Zhu, J., Bryant, R.I., Middleton, L.S., Dwoskin, L.P., 2004. Effects of environmental enrichment on behavior and dopamine transporter function in medial prefrontal cortex in adult rats prenatally treated with cocaine. Brain Res Dev Brain Res 153, 213-223.

Newberry, R.C. 1995. Environmental enrichment: increasing the biological relevance of captive environments. Appl Anim Behav Sci 44, 229-244.

Nilsson, M., Perfilieva, E., Johansson, U., Orwar, O., Eriksson, P.S., 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J Neurobiol 39, 569-578.

Nithianantharajah, J., Barkus, C., Murphy, M., Hannan, A.J., 2008. Gene-environment interactions modulating cognitive function and molecular correlates of synaptic plasticity in Huntington's disease transgenic mice. Neurobiol Dis 29, 490-504.

Nithianantharajah, J., Hannan, A.J., 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7, 697-709.

Noguchi, C.T., Asavaritikrai, P., Teng, R., Jia, Y., 2007. Role of erythropoietin in the brain. Crit Rev Oncol Hematol 64, 159-171.

Nygren, J., Wieloch, T., Pesic, J., Brundin, P., Deierborg, T., 2006. Enriched environment attenuates cell genesis in subventricular zone after focal ischemia in mice and decreases migration of newborn cells to the striatum. Stroke 37, 2824-2829.

O

Oostrom, K.J., Schouten, A., Kruitwagen, C.L., Peters, A.C., Jennekens-Schinkel, A., 2003. Behavioral problems in children with newly diagnosed idiopathic or cryptogenic epilepsy attending normal schools are in majority not persistent. Epilepsia 44, 97-106.

Olsson, T., Mohammed, A.H., Donaldson, L.F., Henriksson, B.G., Seckl, J.R., 1994. Glucocorticoid receptor and NGFI-A gene expression are induced in the hippocampus after environmental enrichment in adult rats. Molecular Brain Research 23, 349-353.

P

Parent, J.M., 2007. Adult neurogenesis in the intact and epileptic dentate gyrus. In: Dentate Gyrus: a Comphrehensive Guide to Structure, Function, and Clinical Implications, vol. 163. Elsevier Science Bv, Amsterdam, pp. 529-540

Passineau, M.J., Green, E.J., Dietrich, W.D., 2001. Therapeutic effects of environmental enrichment on cognitive function and tissue integrity following severe traumatic brain injury in rats. Exp Neurol 168, 373-384.

Paylor, R., Morrison, S.K., Rudy, J.W., Waltrip, L.T., Wehner, J.M., 1992. Brief exposure to an enriched environment improves performance on the Morris water task and increases hippocampal cytosolic protein kinase C activity in young rats. Behav Brain Res 52, 49-59.

Pena, Y., Prunell, M., Dimitsantos, V., Nadal, R., Escorihuela, R.M., 2006. Environmental enrichment effects in social investigation in rats are gender dependent. Behav Brain Res 174, 181-187.

Pereira, L.O., Strapasson, A.C., Nabinger, P.M., Achaval, M., Netto, C.A., 2008. Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. Brain Res 1218, 257-266.

Pham, T.M., Ickes, B., Albeck, D., Soderstrom, S., Granholm, A.C., Mohammed, A.H., 1999b. Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. Neuroscience 94, 279-286.

Pham, T.M., Soderstrom, S., Winblad, B., Mohammed, A.H., 1999. Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. Behav Brain Res 103, 63-70.

Pinaud, R., Penner, M.R., Robertson, H.A., Currie, R.W., 2001. Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. Brain Res Mol Brain Res 91, 50-56.

Porter, B.E., Cui, X.N., Brooks-Kayal, A.R., 2006. Status epilepticus differentially alters AMPA and kainate receptor subunit expression in mature and immature dentate granule neurons. Eur J Neurosci 23, 2857-2863.

Prickaerts, J., Koopmans, G., Blokland, A., Scheepens, A., 2004. Learning and adult neurogenesis: Survival with or without proliferation? Neurobiol Learn Mem 81, 1-11.

Prusky, G.T., Reidel, C., Douglas, R.M., 2000. Environmental enrichment from birth enhances visual acuity but not place learning in mice. Behav Brain Res 114, 11-15.

Puurunen, K., Sirvio, J., Koistinaho, J., Miettinen, R., Haapalinna, A., Riekkinen, P., Sr., Sivenius, J., 1997. Studies on the influence of enriched-environment housing combined with systemic administration of an alpha2-adrenergic antagonist on spatial learning and hyperactivity after global ischemia in rats. Stroke 28, 623-631.

Q

Quiske, A., Helmstaedter, C., Lux, S., Elger, C.E., 2000. Depression in patients with temporal lobe epilepsy is related to mesial temporal sclerosis. Epilepsy Res 39, 121-125.

R

Rahman, S., Bardo, M.T., 2008. Environmental enrichment increases amphetamine-induced glutamate neurotransmission in the nucleus accumbens: A neurochemical study. Brain Res 1197, 40-46.

Rampon, C., Jiang, C.H., Dong, H., Tang, Y.P., Lockhart, D.J., Schultz, P.G., Tsien, J.Z., Hu, Y., 2000. Effects of environmental enrichment on gene expression in the brain. Proc Natl Acad Sci U S A 97, 12880-12884.

Rampon, C., Tang, Y.P., Goodhouse, J., Shimizu, E., Kyin, M., Tsien, J.Z., 2000b. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. Nat Neurosci 3, 238-244.

Rampon, C., Tsien, J.Z., 2000c. Genetic analysis of learning behavior-induced structural plasticity. Hippocampus 10, 605-609.

Ransome, M.I., Turnley, A.M., 2007. Systemically delivered Erythropoietin transiently enhances adult hippocampal neurogenesis. J Neurochem 102, 1953-1965.

Rasmuson, S., Olsson, T., Henriksson, B.G., Kelly, P.A., Holmes, M.C., Seckl, J.R., Mohammed, A.H., 1998. Environmental enrichment selectively increases 5-HT1A receptor mRNA expression and binding in the rat hippocampus. Brain Res Mol Brain Res 53, 285-290.

Relman, A.S., Angell, M., 2002. America's other drug problem: how the drug industry distorts medicine and politics. New Repub 227, 27-41.

Restivo, L., Ferrari, F., Passino, E., Sgobio, C., Bock, J., Oostra, B.A., Bagni, C., Ammassari-Teule, M., 2005. Enriched environment promotes behavioral and morphological recovery in a mouse model for the fragile X syndrome. Proc Natl Acad Sci U S A 102, 11557-11562.

Rigau, V., Morin, M., Rousset, M.C., de Bock, F., Lebrun, A., Coubes, P., Picot, M.C., Baldy-Moulinier, M., Bockaert, J., Crespel, A., Lerner-Natoli, M., 2007. Angiogenesis is associated with blood-brain barrier permeability in temporal lobe epilepsy. Brain 130, 1942-1956.

Rolls, E.T., 1999. Spatial view cells and the representation of place in the primate hippocampus. Hippocampus 9, 467-480.

Ronnback, A., Dahlqvist, P., Svensson, P.A., Jernas, M., Carlsson, B., Carlsson, L.M.S., Olsson, T., 2005. Gene expression profiling of the rat hippocampus one month after focal cerebral ischemia followed by enriched environment. Neurosci Lett 385, 173-178.

Rosenzweig, M.R., Bennett, E.L., Hebert, M., Morimoto, H., 1978. Social grouping cannot account for cerebral effects of enriched environments. Brain Res 153, 563-576.

Rossi, C., Angelucci, A., Costantin, L., Braschi, C., Mazzantini, M., Babbini, F., Fabbri, M.E., Tessarollo, L., Maffei, L., Berardi, N., Caleo, M., 2006. Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. Eur J Neurosci 24, 1850-1856.

Rutten, A., van Albada, M., Silveira, D.C., Cha, B.H., Liu, X., Hu, Y.N., Cilio, M.R., Holmes, G.L., 2002. Memory impairment following status epilepticus in immature rats: time-course and environmental effects. Eur J Neurosci 16, 501-513.

S

Sadamoto, Y., Igase, K., Sakanaka, M., Sato, K., Otsuka, H., Sakaki, S., Masuda, S., Sasaki, R., 1998. Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. Biochem Biophys Res Commun 253, 26-32.

Sakanaka, M., Wen, T.C., Matsuda, S., Masuda, S., Morishita, E., Nagao, M., Sasaki, R., 1998. In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A 95, 4635-4640.

Sale, A., Putignano, E., Cancedda, L., Landi, S., Cirulli, F., Berardi, N., Maffei, L., 2004. Enriched environment and acceleration of visual system development. Neuropharmacology 47, 649-660.

Salmenpera, T., Kononen, M., Roberts, N., Vanninen, R., Pitkanen, A., Kalviainen, R., 2005. Hippocampal damage in newly diagnosed focal epilepsy: a prospective MRI study. Neurology 64, 62-68.

Sambrook TD, and Buchanan-Smith HM, 1997. Control and complexity in novel object enrichment. Anim Welf 6, 207-216.

Sanchez, P.E., Fares, R.P., Risso, J.J., Bonnet, C., Bouvard, S., Le-Cavorsin, M., Georges, B., Moulin, C., Belmeguenai, A., Bodennec, J., Morales, A., Pequignot, J.M., Baulieu, E.E., Levine, R.A., Bezin, L., 2009. Optimal neuroprotection by erythropoietin requires elevated expression of its receptor in neurons. Proc Natl Acad Sci U S A 106, 9848-9853.

Sanchez, P.E., Navarro, F.P., Fares, R.P., Nadam, J., Georges, B., Moulin, C., Le Cavorsin, M., Bonnet, C., Ryvlin, P., Belmeguenai, A., Bodennec, J., Morales, A., Bezin, L., 2009b. Erythropoietin receptor expression is concordant with erythropoietin but not with common beta chain expression in the rat brain throughout the life span. J Comp Neurol 514, 403-414.

Sanchez, P.E., Thesis 2008. Optimization des effets neuroprotecteurs de l'erythropoiétine par l'augmentation de l'expression neuronale de son récepteur cible : Application à un modèle de Status Epilpeticus chez le rat.
http://physiologie.univ-lyon1.fr/recherche/theses/SanchezP/SanchezP.pdf

Schneider, J.S., Lee, M.H., Anderson, D.W., Zuck, L., Lidsky, T.I., 2001. Enriched environment during development is protective against lead-induced neurotoxicity. Brain Res 896, 48-55.

Schneider, T., Turczak, J., Przewlocki, R., 2006. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. Neuropsychopharmacology 31, 36-46.

Schrijver, N.C., Bahr, N.I., Weiss, I.C., Wurbel, H., 2002. Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. Pharmacol Biochem Behav 73, 209-224.

Schrijver, N.C., Pallier, P.N., Brown, V.J., Wurbel, H., 2004. Double dissociation of social and environmental stimulation on spatial learning and reversal learning in rats. Behav Brain Res 152, 307-314.

Segovia, G., Del Arco, A., de Blas, M., Garrido, P., Mora, F., 2008. Effects of an enriched environment on the release of dopamine in the prefrontal cortex produced by stress and on working memory during aging in the awake rat. Behav Brain Res 187, 304-311.

Segovia, G., Yague, A.G., Garcia-Verdugo, J.M., Mora, F., 2006. Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats. Brain Res Bull 70, 8-14.

Shimizu, E., Tang, Y.P., Rampon, C., Tsien, J.Z., 2000. NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. Science 290, 1170-1174.

Shingo, T., Sorokan, S.T., Shimazaki, T., Weiss, S., 2001. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci 21, 9733-9743.

Shorvon Simon: epilepsy, ONL 2009 (book)

Shum, F.W., Wu, L.J., Zhao, M.G., Toyoda, H., Xu, H., Ren, M., Pinaud, R., Ko, S.W., Lee, Y.S., Kaang, B.K., Zhuo, M., 2007. Alteration of cingulate long-term plasticity and behavioral sensitization to inflammation by environmental enrichment. Learn Mem 14, 304-312.

Siren, A.L., Knerlich, F., Poser, W., Gleiter, C.H., Bruck, W., Ehrenreich, H., 2001. Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. Acta Neuropathol (Berl) 101, 271-276.

Sloviter, R.S., Zappone, C.A., Bumanglag, A.V., Norwood, B.A., Kudrimoti, H., 2007. On the relevance of prolonged convulsive status epilepticus in animals to the etiology and neurobiology of human temporal lobe epilepsy. Epilepsia 48 Suppl 8, 6-10.

Sloviter, R.S., 2008. Hippocampal epileptogenesis in animal models of mesial temporal lobe epilepsy with hippocampal sclerosis: the importance of the "latent period" and other concepts. Epilepsia 49 Suppl 9, 85-92.

Sola, A., Rogido, M., Lee, B.H., Genetta, T., Wen, T.C., 2005. Erythropoietin after focal cerebral ischemia activates the Janus kinase-signal transducer and activator of transcription signaling pathway and improves brain injury in postnatal day 7 rats. Pediatr Res 57, 481-487.

Solinas, M., Chauvet, C., Thiriet, N., El Rawas, R., Jaber, M., 2008. Reversal of cocaine addiction by environmental enrichment. Proc Natl Acad Sci U S A 105, 17145-17150.

Solinas, M., Thiriet, N., El Rawas, R., Lardeux, V., Jaber, M., 2009. Environmental enrichment during early stages of life reduces the behavioral, neurochemical, and molecular effects of cocaine. Neuropsychopharmacology 34, 1102-1111.

Soliz, J., Gassmann, M., Joseph, V., 2007. Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. J Physiol (Lond) 583, 329-336.

Spandou, E., Papadopoulou, Z., Soubasi, V., Karkavelas, G., Simeonidou, C., Pazaiti, A., Guiba-Tziampiri, O., 2005. Erythropoietin prevents long-term sensorimotor deficits and brain injury following neonatal hypoxia-ischemia in rats. Brain Res 1045, 22-30.

Spires, T.L., Grote, H.E., Varshney, N.K., Cordery, P.M., van Dellen, A., Blakemore, C., Hannan, A.J., 2004. Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. J Neurosci 24, 2270-2276.

Spires, T.L., Hannan, A.J., 2005. Nature, nurture and neurology: gene-environment interactions in neurodegenerative disease. FEBS Anniversary Prize Lecture delivered on 27 June 2004 at the 29th FEBS Congress in Warsaw. Febs J 272, 2347-2361.

Susser, E.R., Wallace, R.B., 1982. The effects of environmental complexity on the hippocampal formation of the adult rat. Acta Neurobiol Exp (Wars) 42, 203-207.

T

Tang, Y.P., Wang, H., Feng, R., Kyin, M., Tsien, J.Z., 2001. Differential effects of enrichment on learning and memory function in NR2B transgenic mice. Neuropharmacology 41, 779-790.

Tashiro, A., Makino, H., Gage, F.H., 2007. Experience-specific functional modification of the dentate gyrus through adult neurogenesis: A critical period during an immature stage. J Neurosci 27, 3252-3259.

Taylor, J., Kolamunnage-Dona, R., Marson, A.G., Smith, P.E., Aldenkamp, A.P., Baker, G.A., 2009. Patients with epilepsy: Cognitively compromised before the start of antiepileptic drug treatment? Epilepsia.

Thiriet, N., Amar, L., Toussay, X., Lardeux, V., Ladenheim, B., Becker, K.G., Cadet, J.L., Solinas, M., Jaber, M., 2008. Environmental enrichment during adolescence regulates gene expression in the striatum of mice. Brain Res 1222, 31-41.

Thom, M., 2009. Hippocampal sclerosis: progress since Sommer. Brain Pathol 19, 565-572.

Thompson, P.J., 2000. Psychological aspects of epilepsy In: Duncan, J.S., Sisodiya, S.J.J., Smalls, J.E. (Eds.), Epilepsy 2000 from Science to Patient. I.L.A.E..

Toates, F. 2004. Cognition, emotion and action: a dynamic and vulnerable interdependence. Appl Anim Behav Sci 86, 173-204.

Toda, H., Hamani, C., Fawcett, A.P., Hutchison, W.D., Lozano, A.M., 2008. The regulation of adult rodent hippocampal neurogenesis by deep brain stimulation - Laboratory investigation. J Neurosurg 108, 132-138.

Torasdotter, M., Metsis, M., Henriksson, B.G., Winblad, B., Mohammed, A.H., 1996. Expression of neurotrophin-3 mRNA in the rat visual cortex and hippocampus is influenced by environmental conditions. Neurosci Lett 218, 107-110.

Torasdotter, M., Metsis, M., Henriksson, B.G., Winblad, B., Mohammed, A.H., 1998. Environmental enrichment results in higher levels of nerve growth factor mRNA in the rat visual cortex and hippocampus. Behav Brain Res 93, 83-90.

Toscano, C.D., McGlothan, J.L., Guilarte, T.R., 2006. Experience-dependent regulation of zif268 gene expression and spatial learning. Exp Neurol 200, 209-215.

Trejo, J.L., Carro, E., Torres-Aleman, I., 2001. Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. J Neurosci 21, 1628-1634.

Trouche, S., Bontempi, B., Roullet, P., Rampon, C., 2009. Recruitment of adult-generated neurons into functional hippocampal networks contributes to updating and strengthening of spatial memory. Proc Natl Acad Sci U S A 106, 5919-5924.

Tsai, P.T., Ohab, J.J., Kertesz, N., Groszer, M., Matter, C., Gao, J., Liu, X., Wu, H., Carmichael, S.T., 2006. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci 26, 1269-1274.

U

Ueda, S., Sakakibara, S., Yoshimoto, K., 2005. Effect of long-lasting serotonin depletion on environmental enrichment-induced neurogenesis in adult rat hippocampus and spatial learning. Neuroscience 135, 395-402.

Uzum, G., Sarper Diler, A., Bahcekapili, N., Ziya Ziylan, Y., 2006. Erythropoietin prevents the increase in blood-brain barrier permeability during pentylentetrazol induced seizures. Life Sci 78, 2571-2576.

V

Vaidya, V.A., Siuciak, J.A., Du, F., Duman, R.S., 1999. Hippocampal mossy fiber sprouting induced by chronic electroconvulsive seizures. Neuroscience 89, 157-166.

Van de Weerd, H.A., Aarsen, E.L., Mulder, A., Kruitwagen, C.L., Hendriksen, C.F., Baumans, V., 2002. Effects of environmental enrichment for mice: variation in experimental results. J Appl Anim Welf Sci 5, 87-109.

van Praag, H., Kempermann, G., Gage, F., 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2, 266-270.

van Rijzingen, I.M., Gispen, W.H., Spruijt, B.M., 1997. Postoperative environmental enrichment attenuates fimbria-fornix lesion-induced impairments in Morris maze performance. Neurobiol Learn Mem 67, 21-28.

van Vliet, E.A., da Costa Araujo, S., Redeker, S., van Schaik, R., Aronica, E., Gorter, J.A., 2007. Blood-brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain 130, 521-534.

Vazquez, B., Devinsky, O., 2003. Epilepsy and anxiety. Epilepsy Behav 4 Suppl 4, S20-25.

Verina, T., Rohde, C.A., Guilarte, T.R., 2007. Environmental lead exposure during early life alters granule cell neurogenesis and morphology in the hippocampus of young adult rats. Neuroscience 145, 1037-1047.

W

Wagner, A.K., Chen, X.B., Kline, A.E., Li, Y.M., Zafonte, R.D., Dixon, C.E., 2005. Gender and environmental enrichment impact dopamine transporter expression after experimental traumatic brain injury. Exp Neurol 195, 475-483.

Wainwright, P.E., Levesque, S., Krempulec, L., Bulman-Fleming, B., McCutcheon, D., 1993. Effects of environmental enrichment on cortical depth and Morris-maze performance in B6D2F2 mice exposed prenatally to ethanol. Neurotoxicol Teratol 15, 11-20.

Wallace, C.S., Withers, G.S., Weiler, I.J., George, J.M., Clayton, D.F., Greenough, W.T., 1995. Correspondence between sites of NGFI-A induction and sites of morphological plasticity following exposure to environmental complexity. Brain Res Mol Brain Res 32, 211-220.

Walsh, R.N., Budtz-Olsen, O.E., Penny, J.E., Cummins, R.A., 1969. The effects of environmental complexity on the histology of the rat hippocampus. J Comp Neurol 137, 361-366.

Wang, C.A., Lai, M.C., Lui, C.C., Yang, S.N., Tiao, M.M., Hsieh, C.S., Lin, H.H., Huang, L.T., 2007. An enriched environment improves cognitive performance after early-life status epilepticus accompanied by an increase in phosphorylation of extracellular signal-regulated kinase 2. Epilepsy Behav 11, 303-309.

Wang, H., Hu, Y., Tsien, J.Z., 2006. Molecular and systems mechanisms of memory consolidation and storage. Prog Neurobiol 79, 123-135.

Wang, L., Zhang, Z., Wang, Y., Zhang, R., Chopp, M., 2004. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke 35, 1732-1737.

Wang, Y., Zhang, Z.G., Rhodes, K., Renzi, M., Zhang, R.L., Kapke, A., Lu, M., Pool, C., Heavner, G., Chopp, M., 2007. Post-ischemic treatment with erythropoietin or carbamylated erythropoietin reduces infarction and improves neurological outcome in a rat model of focal cerebral ischemia. Br J Pharmacol 151, 1377-1384.

Ward G.E, and DeMille D., 1991. Environmental enrichment for laboratory mice (Mus musculus). Anim Technol 42, 149–156.

Wen, X., Huang, Y., Wang, J., 2006. Erythropoietin preconditioning on hippocampus neuronal apoptosis following status epilepticus induced by Li-pilocarpine in rats through anti-caspase-3 expression. Neurol India 54, 58-63; discussion 63.

Wolf, S.A., Kronenberg, G., Lehmann, K., Blankenship, A., Overall, R., Staufenbiel, M., Kempermann, G., 2006. Cognitive and physical activity differently modulate disease progression in the amyloid precursor protein (APP)-23 model of Alzheimer's disease. Biol Psychiatry 60, 1314-1323.

Wolfer, D.P., Litvin, O., Morf, S., Nitsch, R.M., Lipp, H.P., Wurbel, H., 2004. Laboratory animal welfare: cage enrichment and mouse behaviour. Nature 432, 821-822.

Wolff, M., Loukavenko, E.A., Will, B.E., Dalrymple-Alford, J.C., 2008. The Extended Hippocampal-Diencephalic Memory System: Enriched Housing Promotes Recovery of the Flexible Use of Spatial Representations After Anterior Thalamic Lesions. Hippocampus 18, 996-1007.

Wood, D.A., Buse, J.E., Wellman, C.L., Rebec, G.V., 2005. Differential environmental exposure alters NMDA but not AMPA receptor subunit expression in nucleus accumbens core and shell. Brain Res 1042, 176-183.

Wurm, F., Keiner, S., Kunze, A., Witte, O.W., Redecker, C., 2007. Effects of skilled forelimb training on hippocampal neurogenesis and spatial learning after focal cortical infarcts in the adult rat brain. Stroke 38, 2833-2840.

X

Xue, Y.Q., Zhao, L.R., Guo, W.P., Duan, W.M., 2007. Intrastriatal administration of erythropoietin protects dopaminergic neurons and improves neurobehavioral outcome in a rat model of Parkinson's disease. Neuroscience 146, 1245-1258.

Y

Yang, J., Huang, Y., Yu, X., Sun, H., Li, Y., Deng, Y., 2007. Erythropoietin preconditioning suppresses neuronal death following status epilepticus in rats. Acta Neurobiol Exp (Wars) 67, 141-148.

Yang, J.L., Hou, C.L., Ma, N., Liu, J., Zhang, Y., Zhou, J.S., Xu, L., Li, L.J., 2007. Enriched environment treatment restores impaired hippocampal synaptic plasticity and cognitive deficits induced by prenatal chronic stress. Neurobiol Learn Mem 87, 257-263.

Yamaji, R., Okada, T., Moriya, M., Naito, M., Tsuruo, T., Miyatake, K., Nakano, Y., 1996. Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. Eur J Biochem 239, 494-500.

Yatsiv, I., Grigoriadis, N., Simeonidou, C., Stahel, P.F., Schmidt, O.I., Alexandrovitch, A.G., Tsenter, J., Shohami, E., 2005. Erythropoietin is neuroprotective, improves functional recovery, and reduces neuronal apoptosis and inflammation in a rodent model of experimental closed head injury. FASEB J 19, 1701-1703.

Young, D., Lawlor, P.A., Leone, P., Dragunow, M., During, M.J., 1999. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5, 448-453.

Young, N.A., Wintink, A.J., Kalynchuk, L.E., 2004. Environmental enrichment facilitates amygdala kindling but reduces kindling-induced fear in male rats. Behav Neurosci 118, 1128-1133.

Yu, X., Shacka, J.J., Eells, J.B., Suarez-Quian, C., Przygodzki, R.M., Beleslin-Cokic, B., Lin, C.S., Nikodem, V.M., Hempstead, B., Flanders, K.C., Costantini, F., Noguchi, C.T., 2002. Erythropoietin receptor signalling is required for normal brain development. Development 129, 505-516.

Yu, Y.P., Xu, Q.Q., Zhang, Q., Zhang, W.P., Zhang, L.H., Wei, E.Q., 2005. Intranasal recombinant human erythropoietin protects rats against focal cerebral ischemia. Neurosci Lett 387, 5-10.

Z

Zald, D.H., Hagen, M.C., Pardo, J.V., 2002. Neural correlates of tasting concentrated quinine and sugar solutions. J Neurophysiol 87, 1068-1075.

Zald, D.H., Pardo, J.V., 1997. Emotion, olfaction, and the human amygdala: amygdala activation during aversive olfactory stimulation. Proc Natl Acad Sci U S A 94, 4119-4124.

Zambrana, C., Marco, E.M., Arranz, L., de Castro, N.M., Viveros, M.P., de la Fuente, M., 2007. Influence of aging and enriched environment on motor activity and emotional responses in mice. Ann N Y Acad Sci 1100, 543-552.

Zanardi, A., Ferrari, R., Leo, G., Maskos, U., Changeux, J.P., Zoli, M., 2007. Loss of high-affinity nicotinic receptors increases the vulnerability to excitotoxic lesion and decreases the positive effects of an enriched environment. FASEB J 21, 4028-4037.

Zhang, F., Signore, A.P., Zhou, Z., Wang, S., Cao, G., Chen, J., 2006. Erythropoietin protects CA1 neurons against global cerebral ischemia in rat: potential signaling mechanisms. J Neurosci Res 83, 1241-1251.

Zhang, F., Wang, S., Cao, G., Gao, Y., Chen, J., 2007. Signal transducers and activators of transcription 5 contributes to erythropoietin-mediated neuroprotection against hippocampal neuronal death after transient global cerebral ischemia. Neurobiol Dis 25, 45-53.

Zhao, L., Mattsson, B., Johansson, B., 2000. Environmental influence on brain-derived neurotrophic factor messenger RNA expression after middle cerebral artery occlusion in spontaneously hypertensive rats. Neuroscience 97, 177-184.

Zhao, S., Chai, X., Frotscher, M., 2007. Balance between neurogenesis and gliogenesis in the adult hippocampus: Role for reelin. Dev Neurosci 29, 84-90.

Zhu, J., Apparsundaram, S., Bardo, M.T., Dwoskin, L.P., 2005. Environmental enrichment decreases cell surface expression of the dopamine transporter in rat medial prefrontal cortex. J Neurochem 93, 1434-1443.

Zhu, J., Green, T., Bardo, M.T., Dwoskin, L.P., 2004. Environmental enrichment enhances sensitization to GBR 12935-induced activity and decreases dopamine transporter function in the medial prefrontal cortex. Behav Brain Res 148, 107-117.

Zhu, S.W., Yee, B.K., Nyffeler, M., Winblad, B., Feldon, J., Mohammed, A.H., 2006. Influence of differential housing on emotional behaviour and neurotrophin levels in mice. Behav Brain Res 169, 10-20.

RESUME

Dans de nombreux cas, les patients avec des épilepsies présentent d'importants troubles cognitifs et psychoaffectifs. Ces épilepsies sont parfois accompagnées de phénomènes neurodégénératifs qui sont la cible de certains agents thérapeutiques. Aujourd'hui, l'érythropoïétine apparaît comme l'un des agents neuroprotecteurs les plus prometteurs. En outre, des études de plus en plus nombreuses montrent que l'enrichissement (ou la complexification) des conditions d'hébergement diminue la vulnérabilité cérébrale face à des agressions d'origine différente. Dans cette thèse, nous montrons : 1) dans un modèle d'épilepsie accompagné de vastes lésions neuronales, que l'érythropoïétine protège les populations neuronales les plus vulnérables aux atteintes excitotoxiques, à la condition d'optimiser l'expression neuronale de son récepteur, préalablement à l'agression cérébrale ayant initié l'épilepsie ; 2) dans un modèle d'épilepsie associé à des phénomènes neurodégénératifs très discrets, que : i) l'érythropoïétine bloque le développement de l'anxiété et des troubles de l'apprentissage et de la mémoire spatiale, ii) l'enrichissement du milieu d'hébergement en cages Marlau™ est plus efficace que l'érythropoïétine, et iii) le traitement par l'érythropoïétine annihile les effets bénéfiques de l'enrichissement du milieu d'hébergement. Les résultats de cette thèse, obtenus dans des modèles expérimentaux d'épilepsies associés à des troubles cognitifs, démontrent que les effets bénéfiques d'un potentiel agent thérapeutique peuvent dépendre de la qualité de vie.

Mots-clés : neuroprotection, érythropoïétine, hypoxie, status epilepticus, pilocarpine, enrichissement du milieu de vie, cognition, epilepsie, cage Marlau™.

ABSTRACT

Patients with epilepsy often suffer debilitating cognitive and psycho-affective disorders. In some cases, epilepsy is associated to neurodegenerative processes that are the target of certain therapeutical agents. Today, erythropoietin is considered as one of the most promising neuroprotective agents. In addition, an increased body of studies provides evidence that enrichment (or complexity) of housing decreases the cerebral vulnerability in the context of diverse brain insults. In this thesis, we demonstrate: 1) in a model of epilepsy with large neuronal lesions, that erythropoietin protects the most vulnerable neuronal populations to excitotoxic injury, at the only condition that neuronal expression of its receptor is optimized prior to the primary insult causing epilepsy; 2) in a model of epilepsy associated with faint neuronal lesions that: i) erythropoietin prevents anxiety and impaired spatial learning and memory, ii) enriched housing in Marlau™ cages is more efficient than erythropoietin, and iii) erythropoietin treatment abolishes beneficial effects of enriched housing. These results, obtained in animal models of epilepsies associated with cognitive disorders establish that beneficial effects of a potential therapeutic agent may rely on quality of life.

Keywords: neuroprotection, erythropoiein , hypoxia, status epilepticus, pilocarpine, enriched housing, environmental enrichment, cognition, epilepsy, Marlau™ cage.

Laboratoire de Physiologie Intégrative Cellulaire et Moléculaire
UMR5123 CNRS et Université de lyon1. Dir. : Pr. Bruno Allard
Campus de la Doua – 43 boulevard du 11 novembre 1918 – 69100 Villeurbanne

Ecole Doctorale Interdisciplinaire Sciences et Santé « EDISS ». Dir. : Pr. Didier REVEL