Université de Strasbourg

Faculté des sciences de la vie

THESE

Discipline : Sciences de vivant

Spécialité : Aspects moléculaires et cellulaires de la biologie

Présentée par

Muhammad RAFIQ

Doctorat en Neurosciences

Post-operative dysregulation of Brain-Derived Neurotrophic Factor (BDNF) in cortex and hippocampus of rats

Soutenance le 13 septembre 2013

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Résumé

Présenté par
Muhammad RAFIQ

Dérégulation de la proteine BDNF (Brain-Derived Neurotrophic Factor) dans le cortex et l'hippocame à la suite d'une anesthésie générale : approches precliniques

Etat de la question

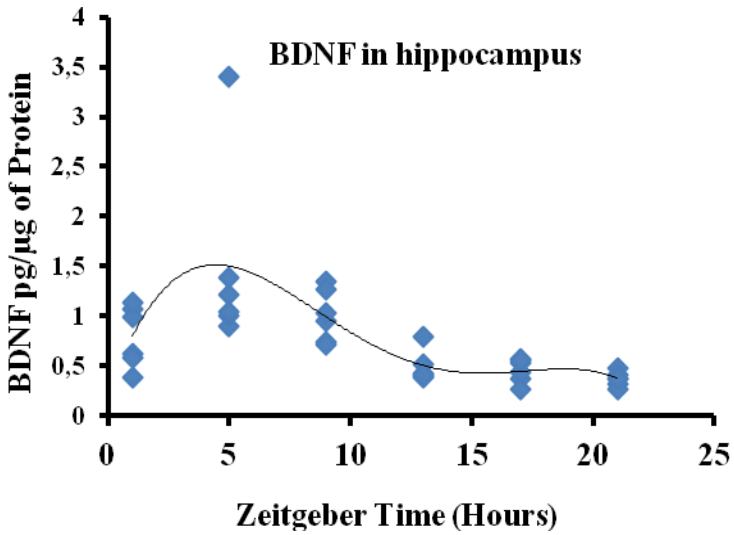
Les troubles attentionnels et mnésiques les jours suivants une anesthésie, et les troubles cognitifs plus prolongées (notamment chez les sujets âgés) surviennent même lorsque l'anesthésie est de courte durée, et sans chirurgie (et ses conséquences) associée. Les produits utilisés actuellement ont une demi-vie d'élimination très rapide, visant à une récupération plus rapide après anesthésie. Il n'était donc pas possible d'expliquer ces effets secondaires par un effet prolongé des drogues comme c'était le cas il y a une quinzaine d'années.

Nous avons alors émis l'hypothèse que les anesthésiques, de part leurs effets cérébraux, pouvaient de façon parallèle, déséquilibrer un certain nombre de systèmes de régulation cérébraux permettant la récupération ad integrum de l'organisme soumis à la séquence anesthésie et stress chirurgical. Aux niveaux cellulaire et sub-cellulaire, le BDNF (*brain-derived neurotrophic factor*) est une protéine qui assure une fonction cruciale dans les processus cognitifs et la plasticité cérébrale. Des données contradictoires issues de deux publications dans la littérature ont montré une augmentation ou une diminution du BDNF 24 heures après une intervention chirurgicale, sans que l'impact de l'anesthésie associée n'ait été etudié. Or, les anesthésiques généraux, quelque soit leur classe pharmacologique, ont un impact majeur sur les neurotransmissions gabaergique et cholinergique cérébrales, action pharmacologique qui pourraient retentir sur l'expression du BDNF. Notre travail de thèse a eu pour objectif de caractériser l'expression de la protéine BDNF dans deux structures cérébrales particulières, l(hippocampe et le cortex

préfrontal, à la suite d'une anesthésie générale, tout d'abord isolée puis associée à une chirurgie mineure ou une réaction inflammatoire majeure (LPS) chez le rat jeune.

Principaux résultats

Fluctuation nycthémérale des taux cérébraux de BDNF. Une première série d'expériences a été menée en vue de caractériser, chez le rat jeune adulte, le niveau basal de BDNF et son éventuelle fluctuation au cours du nycthémère. En effet, le BDNF intervenant dans la consolidation des souvenirs et cette consolidation étant, entre autres, dépendante du sommeil, l'hypothèse d'une fluctuation circadienne du taux de BDNF apparaissait pertinente. Le niveau d'expression de la protéine a été mesuré à l'aide d'une technique ELISA dans le surnageant de broyats d'hippocampes et de cortex préfrontal prélevés chez des rats euthanasiés à différents moments du cycle (ZT1, ZT5, ZT9, ZT13, ZT17 et ZT21 heures ; ZT, pour *Zeitgeber time*), sachant que la période d'activité correspondait à la fenêtre ZT12-ZT0. Le cycle comportait un éclairage de 12 h suivi d'une obscurité de 12 heures. Les résultats mettent en évidence une fluctuation circadienne du niveau d'expression du BDNF aussi bien dans l'hippocampe que dans le cortex (fig.1).



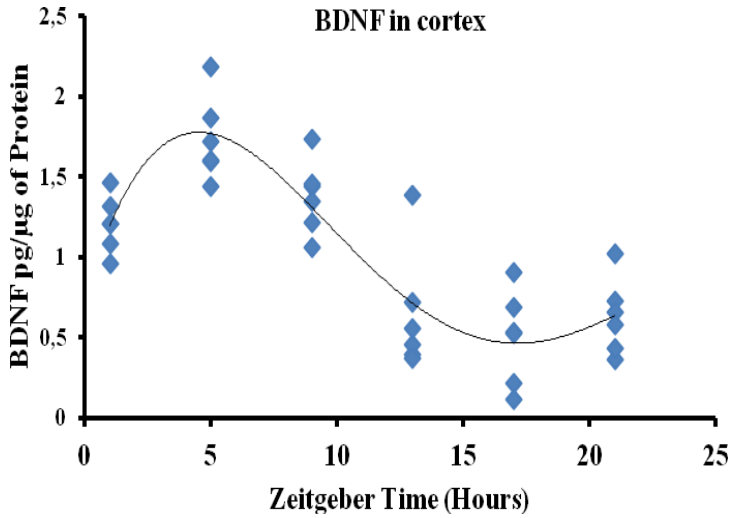


Fig.1. Protéine BDNF (pg / mg de protéine) dans le cortex préfrontal et l'hippocampe surnageant à différents moments de zeitgeber (ZT1, ZT5, ZT9, ZT13, ZT17, et ZT21) chez des rats Sprague Dawley. Cosinor analyse des données de la protéine BDNF pendant 24 h montre un rythme circadien de la protéine BDNF unimodal à la fois dans le cortex préfrontal et l'hippocampe.

Effet aigu d'une anesthésie au Propofol Des rats ont été anesthésiés au Propofol (120 mg/kg à ZT10 du nycthémère) et euthanasiés une heure plus tard. Le niveau d'expression de BDNF a été mesuré dans l'hippocampe et le cortex préfrontal. Comparativement à des rats témoins (ayant subi une injection de NaCl ou d'intralipide), on constate que, chez les rats anesthésiés, ce niveau a augmenté d'environ 20% dans le cortex et d'environ 55% dans l'hippocampe (fig.2).

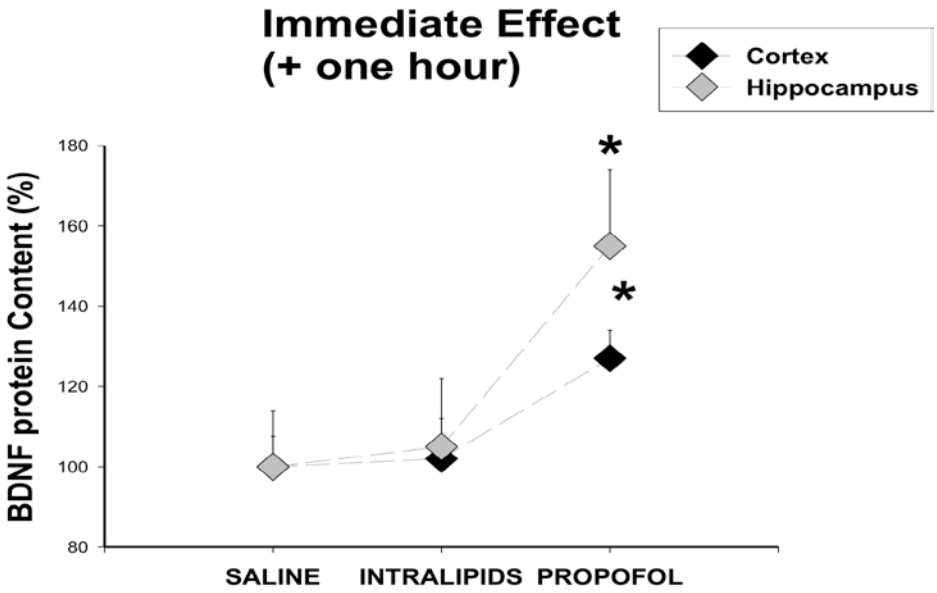


Fig.2. Contenu BDNF observée dans le cortex et l'hippocampe surnageant une heure après l'administration d'une dose anesthésique soit du propofol ou les lipidiques Intralipids la solution de contrôle. Les données sont présentées en tant que pourcentage de la valeur moyenne du groupe de contrôle (saline) obtenu pour chaque structure. Une importante augmentation de la protéine BDNF a été observée 1 heure après l'anesthésie dans le cortex préfrontal (+20%) et dans l'hippocampe (+ 56%).

Effet différé d'une anesthésie au Propofol Des rats ont été anesthésiés au Propofol (120 mg/kg à ZT10 du nycthémère) et euthanasiés à partir d'un délai post-anesthésique de 24 h, à raison d'un groupe toutes les 6 heures (ZT5, ZT11, ZT17, ZT23). Pour chaque délai, le niveau d'expression de BDNF a été mesuré dans l'hippocampe et le cortex préfrontal. Comparativement à des rats témoins (ayant subi une injection d'intralipide), on constate que, chez les rats anesthésiés, ce niveau ne présente plus la fluctuation circadienne dans le cortex et suit une fluctuation altérée dans l'hippocampe (Fig.3).

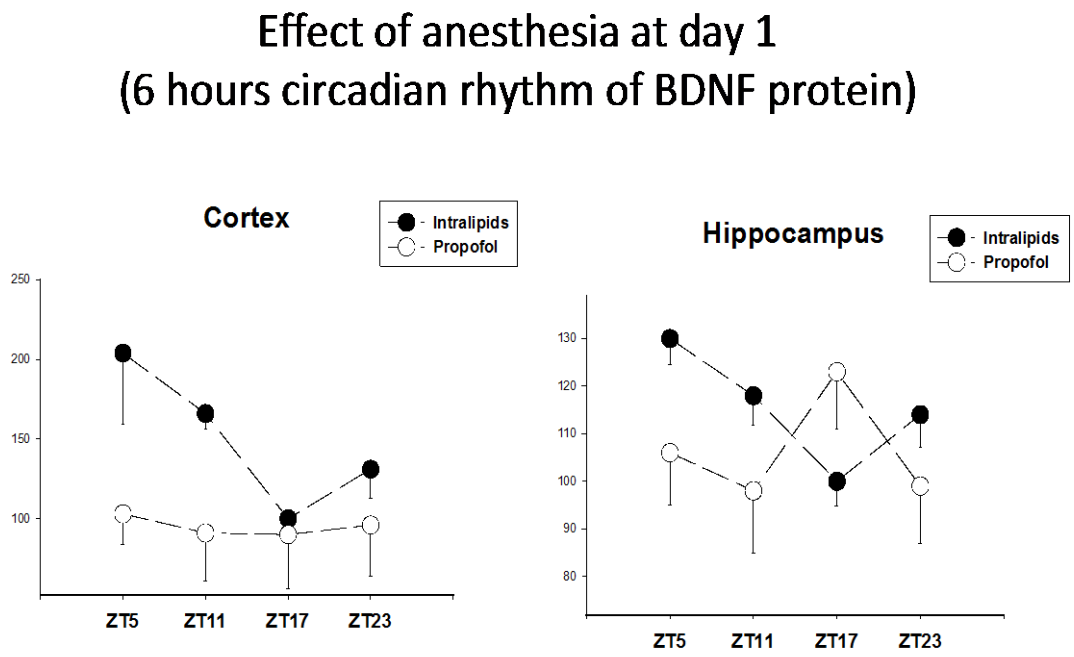


Fig.3. BDNF observée dans le cortex et l'hippocampe surnageant toutes les six heures pendant la journée suivant l'administration d'une dose anesthésique soit du propofol ou les lipidiques Intralipids la solution de contrôle. Les données sont présentées en pourcentage de la valeur moyenne du groupe témoin (Intralipids) observée à ZT17 pour chaque structure (cortex ou l'hippocampe). Une diminution significative de la protéine BDNF a été observé au lendemain de l'anesthésie dans le cortex préfrontal, qui est associée à la perte d'expression de la protéine rythmique chez les animaux recevant du propofol.

Effet prolongée d'une anesthésie au Propofol sur l'expression de BDNF et troubles de la mémoire. Afin d'étudier les effets mnésiques d'une anesthésie au Propofol, des rats ont été soumis à un protocole de conditionnement 1 jour, 3 jours ou 5 jours après l'anesthésie suivi d'une épreuve de rappel à un délai de 24 heures (test de mémoire récente) ou de dix jours (test de mémoire ancienne). Le test utilisé est le test d'évitement passif : les rats placés dans un compartiment éclairé subissent un choc électrique déplaisant administré au niveau des pattes lorsqu'ils pénètrent dans le compartiment sombre. Pour le test de rappel, ils sont replacés dans le compartiment éclairé et on mesure la latence d'entrée dans le compartiment sombre

au cours d'une unique séance de 900 secondes. Lorsque le conditionnement intervient 1, 3 ou 5 jours après l'anesthésie, la mémoire récente n'est pas altérée significativement. Par contre, pour un conditionnement intervenant 1 et 3 jours après l'anesthésie, mais plus lorsqu'il a lieu au délai de 5 jours, le souvenir a disparu au délai de 10 jours, suggérant que sa consolidation est compromise pendant les premiers jours qui suivent l'anesthésie (fig.4). Le niveau d'expression du BDNF a été mesuré aux délais de 1, 3 et 5 jours dans le cortex et l'hippocampe, toujours au même moment du nycthémère, à savoir ZT5. Au délai d'un jour, ce niveau est diminué. Au délai de 3 jours, il est augmenté. Au délai de 5 jours, il est redevenu quasi normal. Ces données laissent à penser que ce n'est pas la diminution ou l'augmentation du BDNF qui affecte la consolidation du souvenir, mais le dérèglement transitoire de la régulation du BDNF à l'issue de l'anesthésie (fig.5).

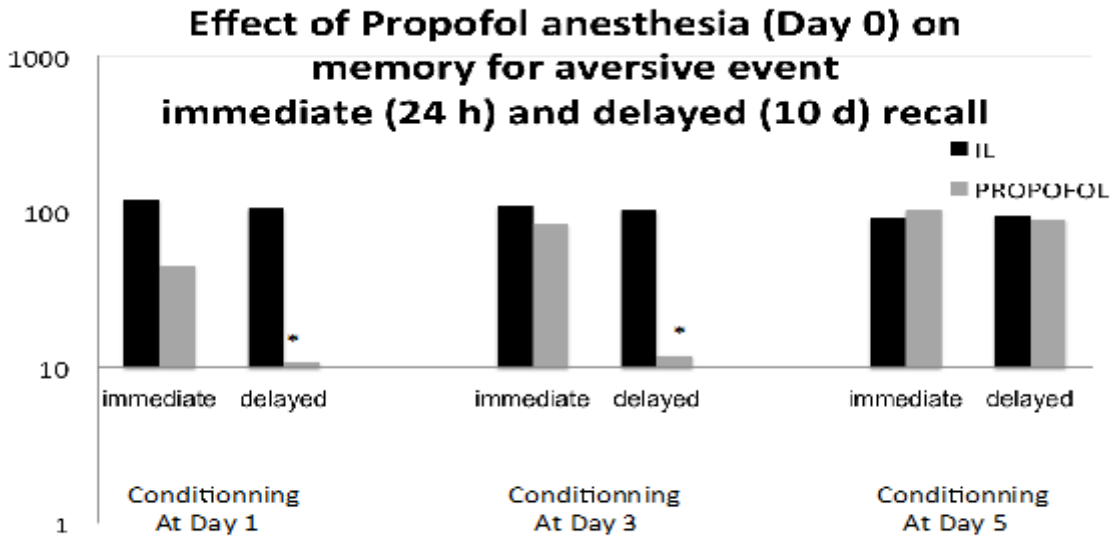


Fig.4. Effet de l'anesthésie au propofol effectuée au jour 0 de la mémoire de la peur (identifiez-vous latence en secondes pour entrer dans le compartiment sombre dans la peur conditionné paradigme). Conditionné a été effectuée soit au jour 1, jour 3 ou 5 jours. Mémoire immédiate a été évaluée en testant les animaux de peur 24 h après conditionné. Mémoire différée a été évaluée en testant les animaux de peur 10 jours après conditionné. Un effet significatif de l'anesthésie (effectué au jour 0) sur le rappel différé a été observée lors de

climatisation a été effectuée au jour 1 et 3. Aucun effet plus significative a été observée au jour 5.

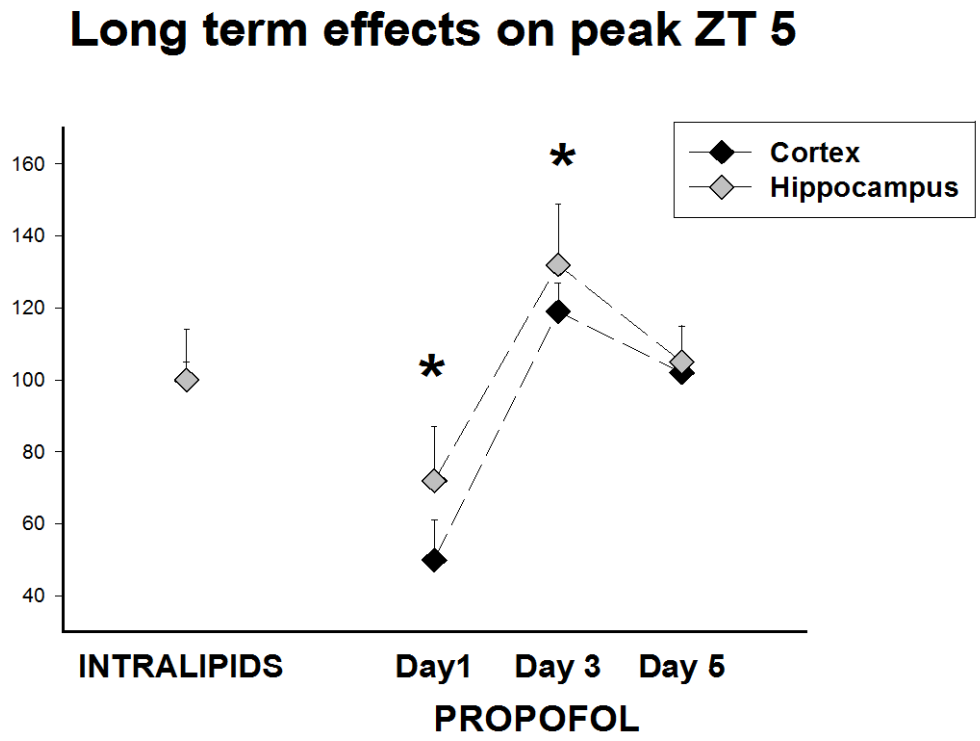
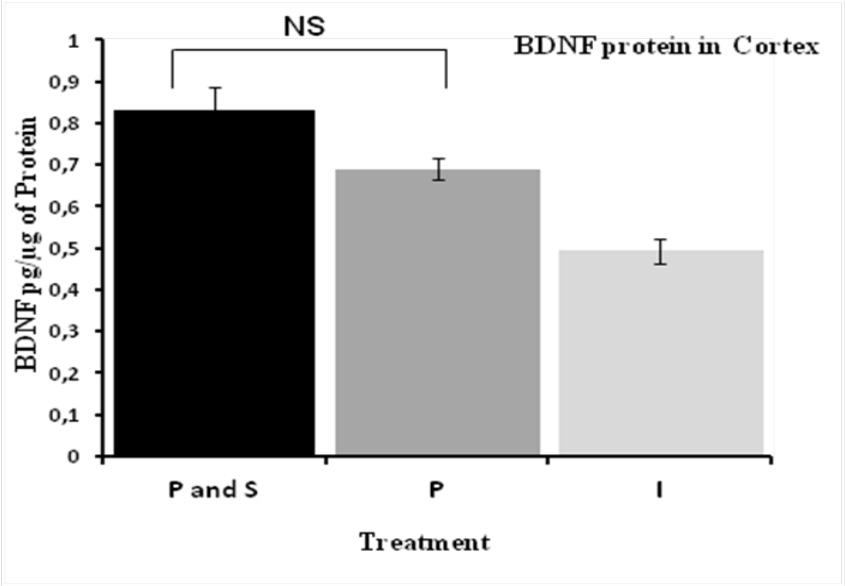


Fig.5. BDNF observée dans le cortex et l'hippocampe surnageant à ZT5 (pic de l'expression du BDNF) au Jour 1, Jour 3 et 5 jours. Au jour 0, les animaux ont reçu l'administration soit d'une dose anesthésique propofol ou de les lipidiques Intralipids la solution de contrôle. Les données sont présentées en pourcentage de la valeur moyenne du groupe témoin (Intralipids) obtenu pour chaque structure (voir le texte pour plus d'explications). Une diminution significative de la protéine BDNF a été observée au jour 1 alors qu'une augmentation significative de BDNF a été observée à jour 3.

Impact de l'anesthésie générale sur l'expression de BDNF induite par la chirurgie mineure ou l'administration de Lipopolysaccharide (LPS). Des rats ont été anesthésiés au Propofol (120 mg/kg à ZT10 du nycthémère) et euthanasiés à partir d'un délai post-anesthésique de 3 jours. L'association à une chirurgie mineure (coelioscopie avec anesthésie locale de complément) n'a eu aucun impact sur le niveau d'expression du BDNF induit par l'anesthésie seule dans le cortex et l'hippocampe surnageant (fig.6). Le déclenchement d'une réaction inflammatoire par

administration de Lipopolysaccharide a induit une augmentation de l'expression de BDNF, augmentation minorée lorsque l'injection de LPS était réalisée sous anesthésie générale dans le cortex et l'hippocampe surnageant (fig.7).



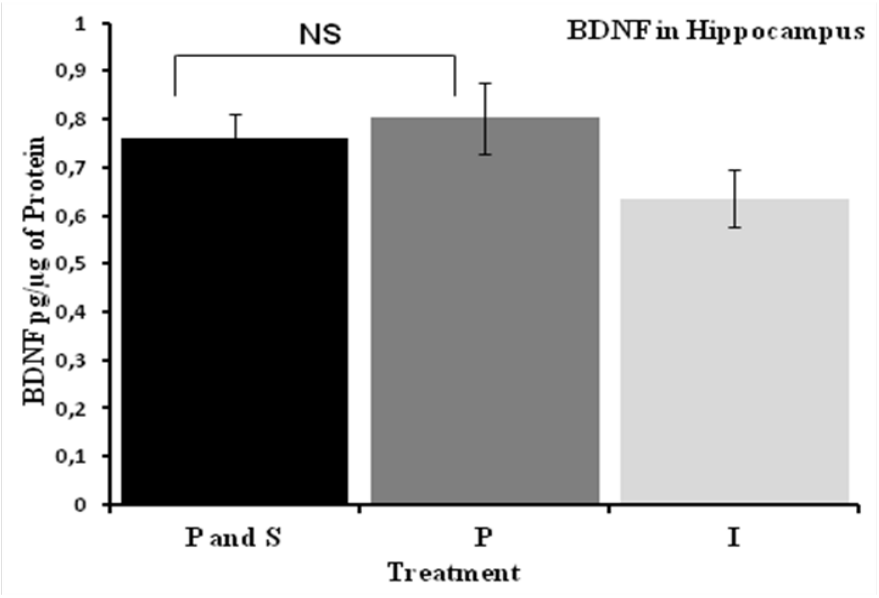
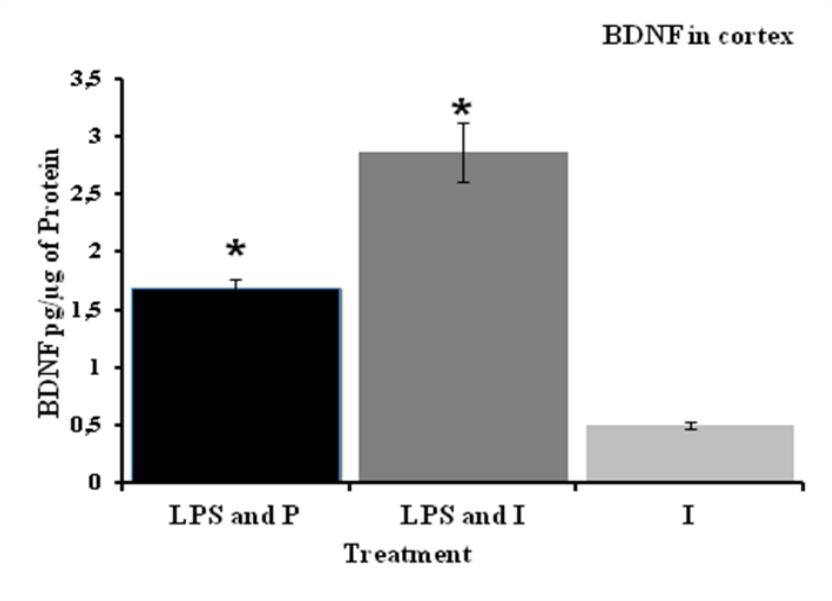


Fig.6. BDNF (pg / mg de protéine) observée dans le cortex et l'hippocampe surnageant traités par chirurgie mineure sous anesthésie propofol (P et S), propofol seul (P) ou avec intralipide que le contrôle (I).



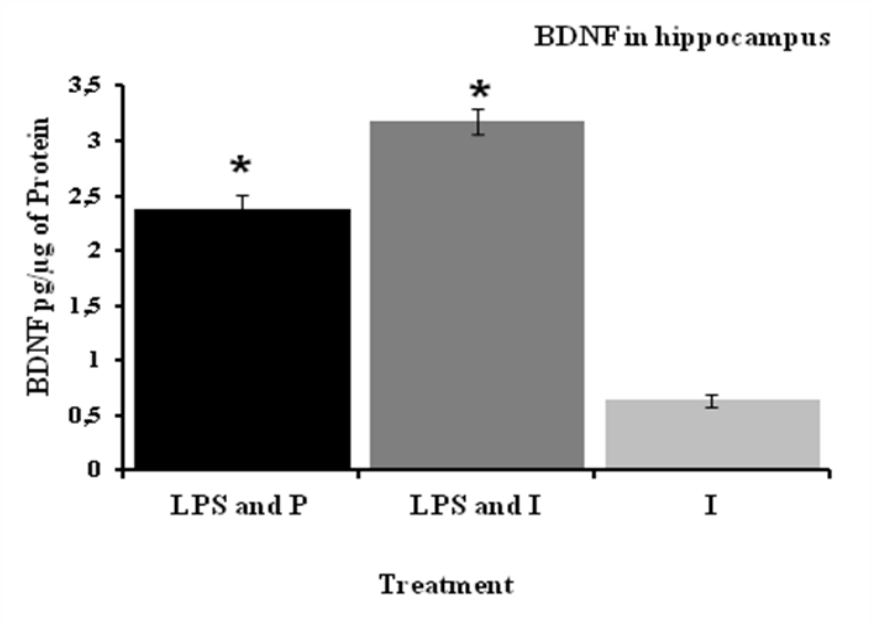


Fig.7. BDNF (pg / mg de protéine) observée dans le cortex et l'hippocampe surnageant traité avec LPS soit moins de propofol (LPS et P) ou avec Intralipids (LPS et I) seul ou avec intralipide que le contrôle (I).

Conclusions et perspectives

Nos travaux montrent pour la première fois à notre connaissance une dysrégulation prolongée (jusqu'à 3 jours) de l'expression de BDNF dans le cortex et l'hippocampe chez le rat jeune. Cette dysrégulation est observée en parallèle des troubles de la

consolidation de la trace mnésique les jours suivant une anesthésie générale mises en évidence chez le rat. Ces résultats ouvrent une nouvelle approche de recherches pour comprendre les mécanismes sous-tendant les troubles cognitifs observés après anesthésie générale. Des travaux récents suggérent les troubles cognitifs observés lors du vieillissement cérébral pathologique s'accompagneraient de modifications de la transcription exon-spécifique du BDNF. Une dysregulation plus prolongée et plus importante du BDNF induit par l'anesthésie chez le sujet âgé pourrait constituer une nouvelle piste de recherches pour comprendre les dysfonctions cognitives parfois majeures observées en post-opératoire.

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Dedication

I dedicate this thesis to my family, especially;

to my beloved parents for a lot that I can not mention;

to my brothers and sisters for their support and prayers at my each educational step;

to my wife and daughter who missed me a lot during my stay abroad

to all friends who supported me in any way!

Muhammad RAFIQ

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First of all, I am grateful to **The Almighty Allaha** for establishing me to complete this thesis. I am thankful to Higher Education Commission, Islamabad Pakistan on awarding me scholarship to make this thesis possible.

It is a pleasure for me to thank many people who made me finish this thesis;

First and foremost I offer my sincerest gratitude to my supervisor, **Dr Laure PAIN** who supported me throughout my thesis with her knowledge and experience. She also encouraged me to attend various trainings and workshops that boosted my knowledge and confidence. She supported me in all the ways and I can say like that simply one could not wish for a better and friendly supervisor. I say bundle of thanks to my co supervisor **Dr Yannick GOUMON** who made my experimental work possible and supported me in all the ways during my thesis.

I can not forget to mention Dominique CIOCCA, Alexis LAUX and Denise STUBER for their valuable support during experimental work.

It would be a long list to mention all the other friends I am indebted to. I gratefully thank all of them.

My parents and my siblings deserve a warm and special acknowledgement for their love and care.

Abbreviations

ACTH	Adrenocorticotropic hormone
AD	Alzheimer’s disease
ASPS	Advanced sleep-phase syndrome
BBB	Blood–brain barriers
BDNF	Brain-derived neurotrophic factor
BPD	Bipolar disorder
BSA	Bovine serum albumin
CD14	Cluster of Differentiation 14
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DSPS	Delayed sleep-phase syndrome
eIF4e	Eukaryotic translation initiation factor 4E
ELISA	Enzyme-Linked Immunosorbant Assay
ERK	Extracellular signal-regulated kinase
GABA	Gama aminobutyric acid
GAD	Glutamate decarboxylase
Grb2	Growth factor receptor-bound protein2
GTP	Guanosine-5'-triphosphate
HD	Huntington’s disease
HPA	Hypothalamo Pituitary Adrenal Axis
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
MD88	Myeloid differentiation 88

MDD	Major depressive disorder
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MSK	Mitogen and stress activated protein kinases
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
P75NTR	p75 neurotrophin receptor
PD	Parkinson’s disease
PERK1/2	Phosphorylated extracellular-signal-regulated kinase1/2
PI3K	Phosphatidylinositol 3-kinases
POCD	Postoperative cognitive dysfunction
PTSD	Posttraumatic stress disorder
RIP2	Receptor interacting protein-2
SCN	Supra chiasmatic nuclei
SOD	Superoxide dismutase
SOS	Son of sevenless
SP	Senile plaques
TLR4	Toll-like receptor 4
TNF	Tumour Necrosis Factor
TrkB	Tropomyosin-receptor kinase
VAChT	Vesicular acetylcholine transporter
ZT	Zeitgeber time

Contents

1. General Introduction

1.1: Brain Derived Neurotrophic Factor (BDNF)

1.1.1: Brain-derived neurotrophic factor:

Brain-derived neurotrophic factor (BDNF) protein is a member of the neurotrophin family that plays an important role in the development of the nervous system in human and other vertebrates (Bibel and Barde, 2000). BDNF is involved in the survival and differentiation of different neurons at embryonic levels and growing evidence indicates that BDNF also supports the neurons in adulthood for maintaining neuronal homeostasis and brain plasticity related processes such as memory and learning (Yamada et al., 2002). Dysregulation of BDNF expression in specific neuronal cells is responsible in various pathologies, including loss in cognitive abilities, dementia, depression, epilepsy as well as Alzheimer's, Huntington's, and Parkinson's diseases (Murer et al., 2001).

1.1.2: Brief History BDNF and other neurotrophins:

In 1982, BDNF, the second member of neurotrophic family of neurotrophic factors, was shown to promote survival of a sub-population of dorsal root ganglion neurons and was purified from pig brain (Barde et al., 1982). After the discovery of BDNF, other members of the neurotrophin family such as neurotrophin-3(NT-3), neurotrophin-4/5 (NT-4/5) have been described (Maisonpierre et al., 1990). Every neurotrophic factor has its own distinct effects on neurons in the central and peripheral nervous system.

1.1.3: Genetic Makeup of BDNF:

In human, the BDNF gene has been mapped to chromosome 11 that contains four 5′ exons (exons I-IV) and one 3′ exon (exon V) that encoding BDNF protein (Metsis et al., 1993). BDNF protein has near to 50% amino acid identity with NGF, NT-3 and NT-4/5, initially BDNF protein is produced as pro-neurotrophins having molecular weight ~30kDa. Pro-hormone convertases, such as furin, cleave the pro-BDNF to the mature neurotrophin having molecular weight ~14kDa (Chao and Bothwell, 2002).

Like human, rat has also the gene structure with four short 5′ non-coding exons (exons I–IV) containing separate promoters and one 3′ exon (exon V) encoding BDNF protein (Timmusk et al., 1993)(Fig.1.1). Each promoter and differential splicing generates four BDNF mRNAs with different 5′untranslated exons and the same coding exon.

In spite of this, each transcription unit uses two different polyadenylation signals at the 3′-end of exon V. Therefore, it generates eight different transcripts. It has also been

shown that the two polyadenylation signals are processed independently of splicing and initiation, since all four 5′ exon probes are hybridized to two BDNF mRN, as of 1.6 and 4.2 kb. These specific promoters seem to determine the tissue-specific expression of the rat BDNF gene (Timmusk et al., 1993). Hence, these transcripts are found to be differentially expressed throughout the whole brain areas. The schematic representation of BDNF gene is shown in the Fig.1.1 (adapted from (Givalois et al., 2001)).

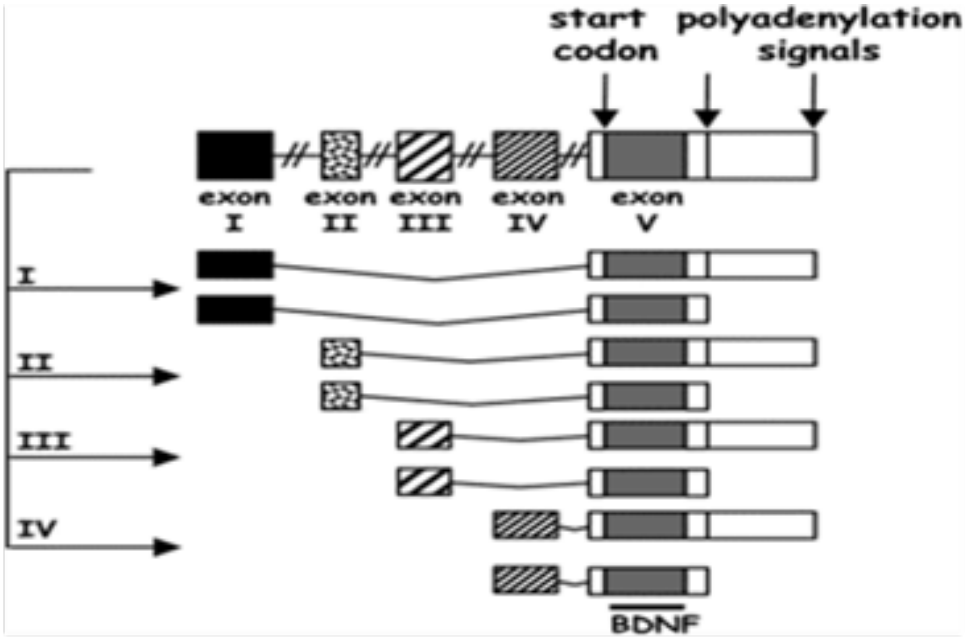


Fig.1.1: Schematic representation of the BDNF gene. Exons are represented as boxes and introns are lines. The eight possible transcripts from the gene are shown below the gene scheme, with lines indicating alternative splicing sites. The start codon and polyadenylation signals are indicated by arrows (adapted from (Givalois et al., 2012)).

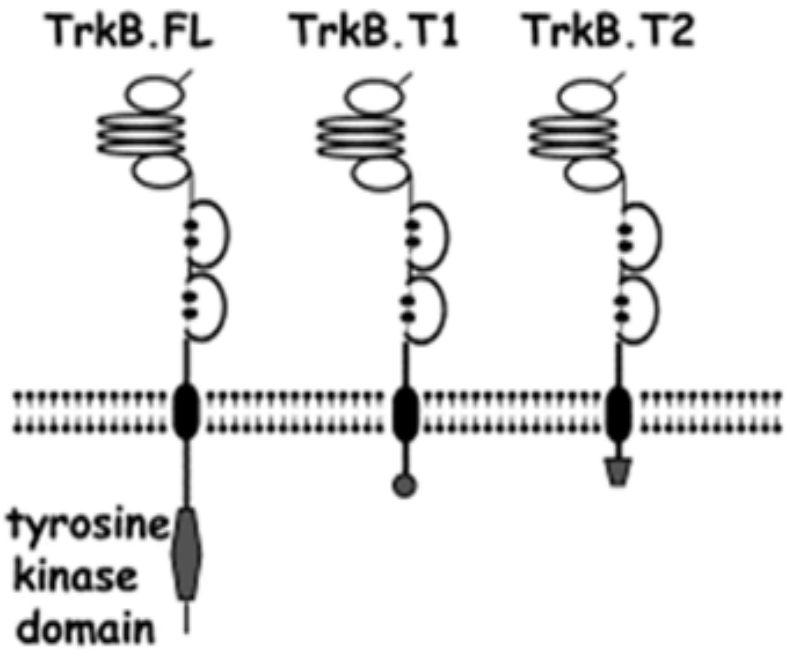


Fig.1.2: Schematic diagram of full-length (TrkB.FL) and truncated (TrkB.T1 and TrkB.T2) TrkB receptors in rodents (adapted from (Tapia-Arancibia et al., 2004).

1.1.4: BDNF receptor and its structure and physiology

BDNF mediate its effects through TrkB receptor and different TrkB isoform that are synthesised by alternative splicing of trkB mRNA. It generates at least three different TrkB receptors with different signalling capabilities in rodents, the full-length catalytic receptor (TrkB.FL) and two truncated forms that are TrkB.T1 and TrkB.T2 (Soppet et al., 1991). The isoform of TrkB are shown in the Fig.1.2.

The full length TrkB receptor contain tyrosine kinase domain but the truncated forms of TrkB receptor lack the intracellular tyrosine kinase domains and contain short specific cytoplasmatic tail (Middlemas et al., 1991). However, the truncated domains of the TrkB receptors are also biologically active since they trigger transduction signals and for this purpose, short isoform-specific intracellular sequences are required. There has been also reported that, during the signal transductions, the TrkB-T1 has a direct signalling role in mediating inositol-1,4,5-triphosphate-dependent calcium release in the glia cells (Rose et al., 2003). Besides, studies on the human TrkB gene have reported a new truncated isoform, TrkB-T-Shc, as it has a Shc-binding site in the juxta membrane domain that similar to the full length domain of the

Trk B receptor. However, TrkB-T-Shc lacks the kinase domain and has a unique truncated C terminus (Stoilov et al., 2002).

It has been shown that the full-length and truncated forms of TrkB have different functions. TrkB.T1 is abundantly expressed in non-neuronal cells lacking TrkB.FL in adult brain (Armanini et al., 1995). The physiological function of TrkB.T1 and TrkB.T2 isoforms is still unclear but it has been suggested that they may be involved as ligand trapping molecules in order to regulate the local bioavailability of neurotrophins (Biffo et al., 1995) or alternatively functioning as negative receptors of neurotrophin responsiveness by heterodimerization (Eide et al., 1996). Some studies have shown the role of truncated receptors in many cellular functions and processes like development, cognition, memory and injury paradigms (Saarelainen et al., 2000). Both truncated and full-length TrkB receptors isoform regulate distinct mode of dendritic growth in visual cortex (Yacoubian and Lo, 2000). It has also been shown that the transgenic mice over expressing truncated TrkB neurotrophin receptors in neurons have impaired long term spatial memory but normal hippocampal LTP (Saarelainen et al., 2000). In other hand, severe sensory neuron losses in knockout mice lacking the TrkB kinase domain have been observed, suggesting that truncated TrkB receptors negatively influence neuron survival by interfering with the function of catalytic TrkB receptors (Luikart et al., 2003). Studies have showed that TrkB receptors are highly sensitive to different experimental and physiological conditions including experimental brain injury, stress, depression, treatment with antidepressant drugs, cognitive performance. In this way, TrkB receptor is directly interfere with the action of BDNF leading in dysregulation of many biological processes that are dependent on BDNF. Interestingly, some studies indicate that BDNF expression vary as a function of development, age and cognitive performance (Croll et al., 1998). Finally, BDNF down regulates TrkB protein and or TrkB mRNA in neurons of different brain structures like hippocampus and cortex (Knusel et al., 1997).

1.1.5: Localization of BDNF in brain tissues:

Studies have shown a widespread distribution of BDNF mRNA and immunoreactivity in the central nervous system (CNS) (Conner et al., 1997). Immunoreactivity of BDNF protein expression has been shown to be high in the hippocampus (Conner et al., 1997). Along with the hippocampus, the hypothalamus is another structure of the brain

that displays highest BDNF protein and mRNA levels (Katoh-Semba et al., 1997). In hypothalamus, the paraventricular and supraoptic nucleus are found to be highly labelled with BDNF immunoreactivity while other hypothalamic nuclei are less labelled including arcuate, ventromedial, and dorsomedial nuclei (Kawamoto et al., 1996). In the hippocampus, the strongest staining has been detected in the pyramidal layers of CA regions, and in granular neuron cell bodies and dendrites of dentate gyrus (Yan et al., 1997) as well as among the Ca regions (CA2) (Kawamoto et al., 1996). Studies on adult rat brain show that BDNF mRNA is expressed in various brain regions like hippocampus, septum, hypothalamus and cortex (Katoh-Semba et al., 1997). Immunoreactivity of BDNF was found in various rat brain areas including cerebral cortex, hippocampus, basal forebrain, striatum, hypothalamus, brainstem, and cerebellum (Kawamoto et al., 2000). It appears that BDNF is predominately produced by neurons but is also synthesized and released by astrocytes under the control of neuronal activity (Juric et al., 2006). BDNF is not only restricted to brain and has been also found to be produced in the endothelial cells (Nakahashi et al., 2000), smooth muscle cells (Donovan et al., 1995) and eosinophils (Noga et al., 2003).

1.1.6: BDNF in blood:

BDNF protein has also been found in the blood (plasma and serum) and also been shown to pass the blood-brain barrier (Pan et al., 1998a). In blood, BDNF is stored in platelets (Yamamoto and Gurney, 1990) and released upon a platelet agonist stimulation by thrombin, Ca^{2+} ion, collagen (Fujimura et al., 2002). Blood BDNF is not only brain derived. Therefore, other peripheral sources of BDNF include the endothelial cells (Nakahashi et al. 2000), smooth muscle cells (Donovan et al. 1995) and eosinophils (Noga et al. 2003). Animal studies indicate a strong correlation between blood and brain BDNF during maturation and aging (Karege et al., 2002). Indeed, blood and brain BDNF levels are similarly altered by antidepressant treatment (Shimizu et al., 2003), in schizophrenia (Toyooka et al., 2002) and Alzheimer's disease (Laske et al., 2007).

1.1.7: Physiological Role of BDNF:

Shortly after the discovery of BDNF, researchers continued to know the physiological role of BDNF protein in different aspects and at different levels. Up till now, countless studies have evidenced the role of BDNF which are described below;

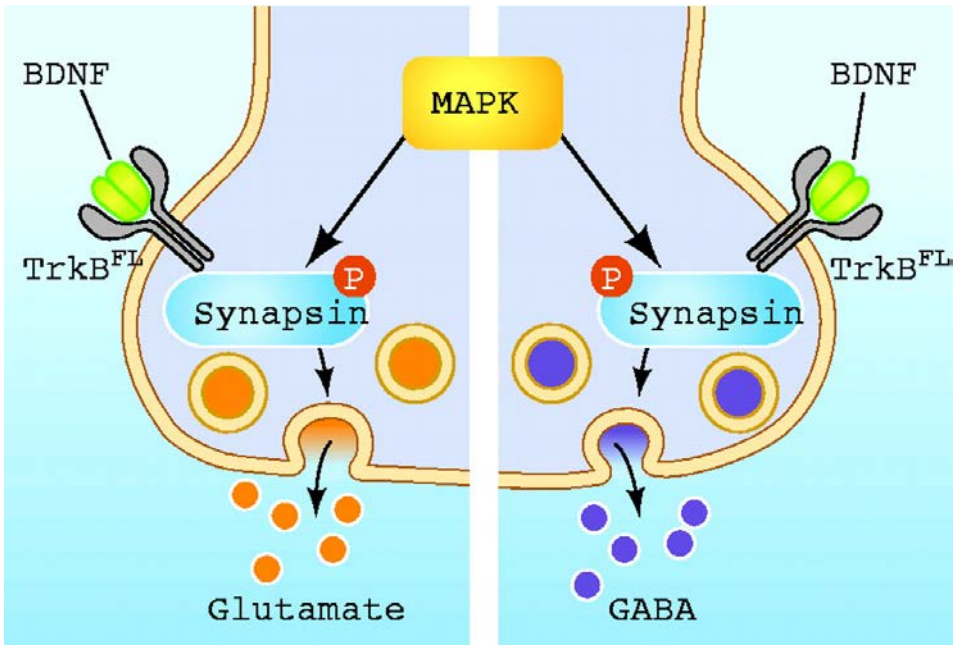


Fig. 1.3: Representation of the role of BDNF in regulating neurotransmitter (glutamate and GABA) release via TrkB/MAPK mediated phosphorylation of synapsin (Blum and Konnerth, 2005).

BDNF and neurotransmitter release:

The BDNF has shown to improve the synaptic transmission mediated by the TrkB receptors (Lohof et al., 1993). Neurotrophins have been shown to play an interesting role in the pre-synaptic release of neurotransmitters. Here a study on mammalian synaptosomes have evidenced that BDNF induces acetylcholine (Ach) release (Knipper et al., 1994). It is also reported that depolarization of synapses by the BDNF do not induce Ca^{2+} ions influx and it has been evidenced that pre-synaptic depolarization depends on BDNF intracellular signalling (Boulanger and Poo, 1999). The secretion of glutamate and gama aminobutyric acid (GABA) has been shown due to BDNF mediated mitogen activated protein kinase (MAPK) activity and phosphorylation of synapsin without any involvement of Ca^{2+} ions influx (Fig.1.3) (Blum and Konnerth, 2005).

BDNF and synaptic transmission:

BDNF and TrkB association plays important role for both excitatory and inhibitory synaptic transmission. During synaptic transmission, BDNF acts in two ways: acute, in which effects are developed in minutes and in chronic, effects are delayed up to days. BDNF is more associated in synaptic transmission in the hippocampal areas.

Therefore, studies have revealed that synaptic connectivity and pre-synaptic neurotransmitter release was increased in hippocampal cells (Takei and Nawa, 1998).

BDNF and cell survival and neuronal structure:

BDNF has been described to promote intracellular activities in the pyramidal neuronal cells of the hippocampus (Marsh and Palfrey, 1996) and high death rate has been observed in BDNF knockout mice hippocamal neurons as compared to wild type (Murphy et al., 1998). Neuronal survival through hippocampal as well as cortical neurons like dendritic density and axonal strength (Patel and McNamara, 1995).

BDNF is also shown to increase the amount of tyrosine hydroxylase (TH) and to protect the neurons from the neurotoxic agents. Studies on transgenic mice lacking of BDNF expression have revealed reduction of brain antitoxic enzyme tyrosine hydroxylase expressing cells which are calbindin neurons (Baquet et al., 2005).

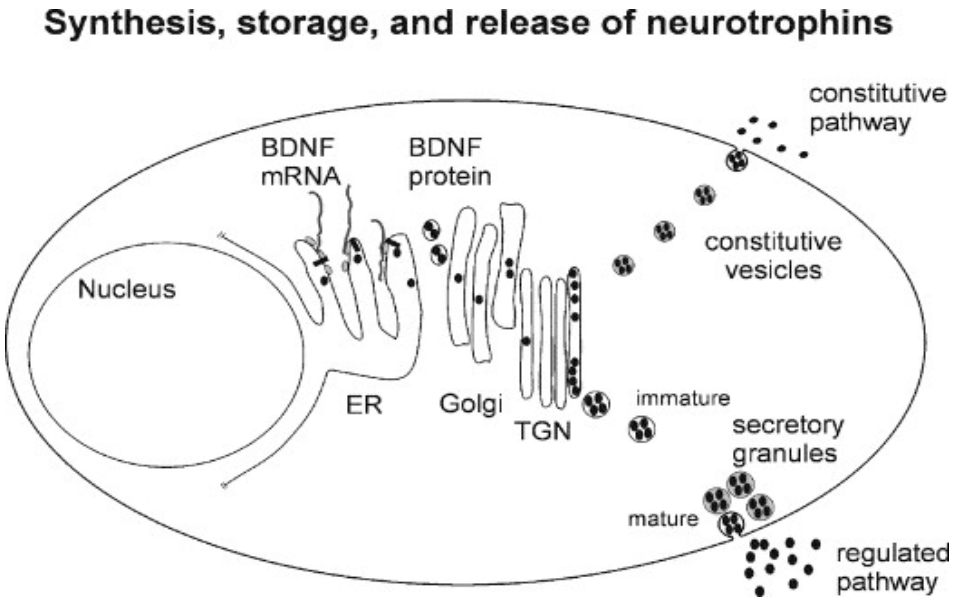


Fig.1.4: The schematic representation of BDNF synthesis and secretion (adapted from (Lessmann et al., 2003)).

1.1.8: BDNF protein synthesis and release:

Transcription for BDNF mRNA is done in the nucleus and translated into BDNF protein with the help of ribosomes of the rough endoplasmic reticulum (ER) and then the pre-pro-BDNF is targeted to the Golgi bodies in the form of vesicles for maturation. Protein convertases can cleave off the pro-sequence and then mature

BDNF is targeted to the membrane for its release upon triggering signals for regulated secretion (Fig. 1.4).

BDNF protein is synthesized as a precursor called pre-pro-BDNF protein that results after cleavage in a 32-kDa pro-BDNF protein via transcription of BDNF mRNA is cytoplasm. Pro-BDNF is cleaved intracellularly by enzymes like furin or pro-convertases and secreted as the 14 kDa mature BDNF or secreted as pro-BDNF. Pro-BDNF is then cleaved by extracellular proteases (metalloproteinases and plasmin) to mBDNF (Lessmann et al., 2003). Both pro-BDNF and mBDNF are sorted and packaged into vesicles of regulated secretory pathway. Localization of BDNF is predominantly somato dendritic but it is also present in the dendrites, where it is also synthesized from mRNA in close proximity to spines (Tongiorgi, 2008). BDNF is present in pre and post-synaptic compartments and it can follow the both retrograde and anterograde transport processes. Thus BDNF can act via autocrine and paracrine ways (Murer et al., 2001).

The release of BDNF follows three mechanisms dependent on the site of release: (i) Ca^{2+} ions influx-dependent release from postsynaptic sites, which is mediated by Ca^{2+} ions influx through ionotropic glutamate receptors and voltage gated Ca^{2+} ions channels (Hartmann et al., 2001), (ii) Ca^{2+} ions influx- dependent release from pre-synaptic sites (Balkowiec and Katz, 2002) and (iii) Ca^{2+} ions influx-independent release that relies on Ca^{2+} ions release from intracellular stores (Griesbeck et al., 1999). Neuron cell activity also shows to regulate the transport of BDNF mRNA and protein into dendrites (Tongiorgi, 2008).

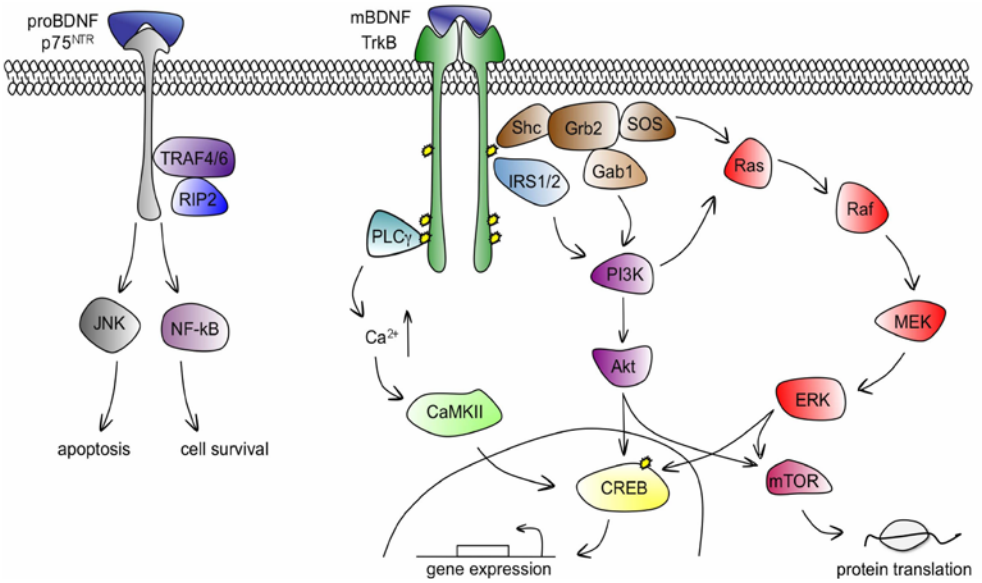


Fig. 1.5: Schematic representation of pro-BDNF-p75NTR and mBDNF-TrkB association is responsible for apoptosis and cell survival and gene expression and protein translation respectively. TrkB activation by BDNF is responsible for an intracellular three major cascades of signalling pathways: phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and a well-characterized cascade governed by extracellular signal-regulated kinases (ERK) that is an important member of the mitogen activated protein kinase (MAPK) family (Watson et al., 2001).

1.2: BDNF dependent signalling pathways:

BDNF action is mediated by two different transmembrane receptor proteins: the tropomyosin related kinase TrkB receptor with high affinity and the neurotrophin receptor p75NTR with low affinity (Fig.1. 5). Predominantly, all the synaptic effects of BDNF are attributed to TrkB activation and there is also evidence that pro-BDNF acts through by binding to p75NTR (Teng et al., 2005). BDNF–TrkB association is responsible for effects that are further responsible for synaptic plasticity and also for learning and memory. TrkB activation by BDNF is responsible for an intracellular three major cascades of signalling pathways: phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K) and a well-characterized cascade governed by extracellular signal-regulated kinase (ERK) that is an important member of the mitogen activated protein kinase (MAPK) family.

When BDNF activates TrkB receptor, the receptor dimerises and is autophosphorylated at specific tyrosine sites in the cytoplasm. Activation of this pathway results in the rise of intracellular Ca^{2+} ions via its release from intracellular stores i.e. endoplasmic reticulum and in the activation of the Ca-calmodulin dependent kinase, CaMKII.

The increase of Ca^{2+} ions in the cells is one of the most important outcomes of BDNF signalling in the postsynaptic cell and studies indicate that Ca^{2+} ions regulate mRNA translation that occurs locally at postsynaptic sites (Aakalu et al., 2001). This mechanism is responsible for the rapid and accurate expression of activity-induced gene products like BDNF at activated synapses.

Phosphorylation of TrkB Y490 upon BDNF binding permits the association of SH2 (src homology-type 2) linker proteins such as shc (src homology domain containing) and insulin receptor substrate 1 and 2 (IRS1, IRS2). This phosphorylation leads to the activation of the phosphatidylinositol 3-kinases (PI3K) and ERK signalling pathways. Src sequentially recruits an intermediary protein growth factor receptor-bound protein 2 (Grb2) and the guanine nucleotide exchange factor son of sevenless (SOS), initiating the Guanosine-5'-triphosphate (GTP) leading to the activation of ERK (extracellular signal regulated kinase) cascade. The phosphorylated ERK translocates to the nucleus to activate transcription factors such as CREB to regulate gene expression. Grb2 can also recruit another intermediary binding protein, Gab1 to activate PI3K and the downstream kinase Akt (also known as protein kinase B). BDNF can also activate the PI3K pathway via a direct interaction between IRS1/IRS2 and PI3K. The PI3K pathway was shown to be important that mediates the protective effects of BDNF in several neuronal cell types *in vitro,* including hippocampal neurons (Zheng and Quirion, 2004).

Besides, BDNF has been shown to be involved in the local translation of proteins in dendrites by the activation of mammalian target of rapamycin (mTOR) via PI3K (Schratt et al., 2004). Both mTOR and ERK regulate the assembly of the eukaryotic translation initiation factor 4E (eIF4e) complex and phosphorylation of S6K1, hence contributing to an enhanced mRNA translation initiation at active synapses (Bramham and Wells, 2007). The activation of p75NTR by pro-BDNF initiates the pro-survival of NF-kB and pro-apoptotic Jun kinase signalling cascades. The p75NTR mediated survival involves the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway via the association of p75NTR with tumour

necrosis factor receptor associated factor 4/6 (TRAF4/6) and receptor interacting protein-2 (RIP2). Through this signalling pathway, BDNF effects on synaptic transmission via acting at pre and post synaptic sites.

1.3 Circadian rhythms:

1.3.1: Circadian rhythms and their brief history:

Circadian (from the Latin word *circa*, meaning 'about', and *dies*, meaning 'day') rhythms are the rhythms occurring within a period of 24 hours and are endogenously driven in biochemical, physiological or behavioral processes. Circadian rhythms are entrained to the environment by the external cues called zeitgebers, the primary one of which is day light. Circadian rhythms have been widely observed in plants, animals, fungi and cyanobacteria. Different physiological activities of animals and plants vary during 24 hours. These different physiological activities may be at peak at one time, average at other time and may be down at other point of the 24 hours clock in a circadian fashion. Some of the physiological activities and their circadian fashion is shown in Fig.1.6. The formal study of biological rhythms such as daily, tidal, weekly, seasonal and annual rhythms is called chronobiology.

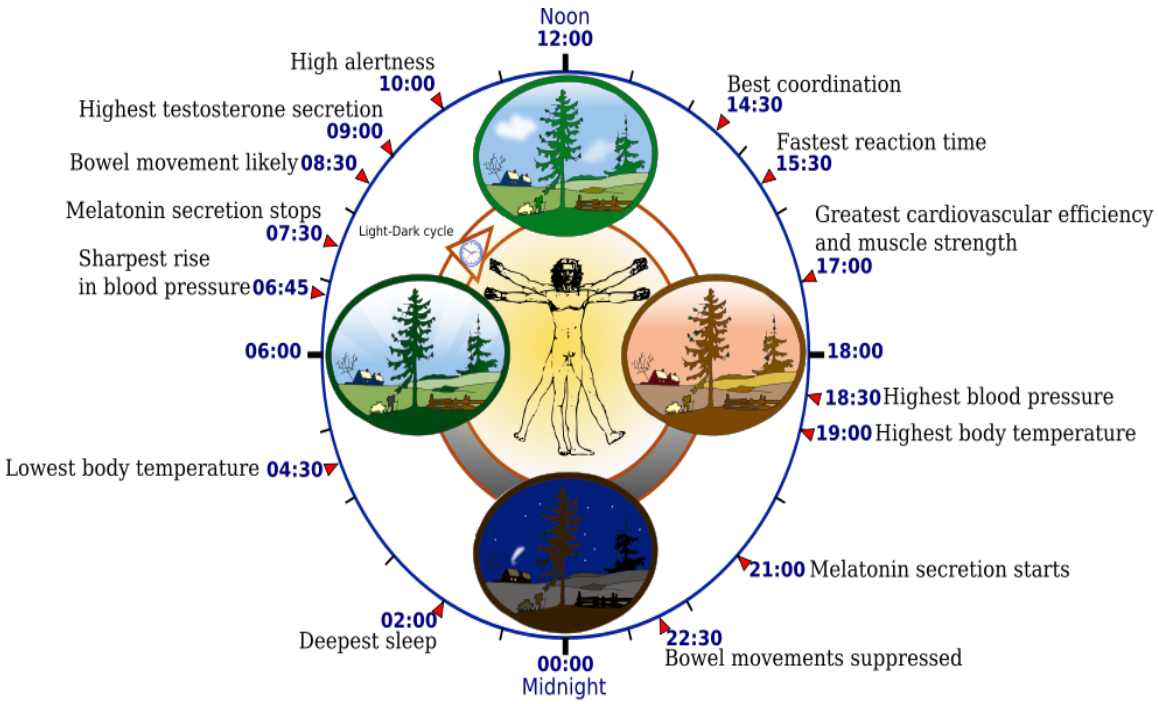


Fig 1.6: This diagram depicts the circadian patterns typical of someone who rises early in morning, eats lunch around noon and sleeps at night (10 p.m.). Although circadian rhythms tend to be synchronized with cycles of light and dark, other factors such as ambient temperature, meal times, stress and exercise can influence the timing as well (“The Body Clock Guide to Better Health" by Michael Smolensky and Lynne Lamberg; Henry Holt and Company, Publishers (2000)).

The first recorded observation of an endogenous circadian oscillation was done by the French scientist Jean-Jacques d'Ortous de Mairan in 1729. He noted that 24-hour patterns in the movement of the leaves of the plant *Mimosa pudica* continued even when the plants were kept in constant darkness, in the first experiment to attempt to distinguish an endogenous clock from responses to daily stimuli. In 1896, Patrick and Gilbert observed that during a prolonged period of sleep deprivation, sleepiness increases and decreases with a period of approximately 24 hours. In 1918, J.S. Szymanski showed that animals are capable of maintaining 24-hour activity patterns in the absence of external cues such as light and changes in temperature. Joseph Takahashi discovered the first mammalian 'clock gene' in 1994. The term "circadian" was proposed by Franz Halberg in the late 1950s.

1.3.2: Characteristics of Circadian rhythms:

A biological circadian rhythm must meet the following four general criteria:

1. The rhythms repeat once a day (they have a 24-hour period). In order to keep track of the time of day, a clock must be at the same point at the same time each day, i.e. repeat every 24 hours.

2. The rhythms persist in the absence of external cues (endogenous). The rhythm persists in constant conditions with a period of about 24 hours. The rationale for this criterion is to distinguish circadian rhythms from simple responses to daily external cues. A rhythm cannot be said to be endogenous unless it has been tested in conditions without external periodic input.

3. The rhythms can be adjusted to match the local time (entrainable). The rhythm can be reset by exposure to external stimuli (such as light and heat), by a process called entrainment. The rationale for this criterion is to distinguish circadian rhythms from other imaginable endogenous 24-hour rhythms that are immune to resetting by external cues and hence, do not serve the purpose of estimating the local time. Travel across time zones illustrates the ability of the human biological clock to adjust to the local time; a person will usually experience jet lag before entrainment of their circadian clock has brought it into sync with local time.

4. The rhythms maintain circadian periodicity over a range of physiological temperatures (exhibit temperature compensation). Some organisms live at a broad range of temperatures and the thermal energy will affect the kinetics of all molecular processes in their cells. In order to keep track of time, the organism's circadian clock must maintain a roughly 24-hour periodicity despite the changing kinetics, a property known as temperature compensation.

1.3.3: The circadian pacemaker resides in the brain

In 1948, Curt Richter discovered the approximate area where the principal circadian pacemaker is located in mammals. After a series of experiments in the rat brain, he was able to find only one area that had an effect on circadian rhythms. This region was the frontal part of the hypothalamus located at the base of the brain immediately above the optic chiasm (Fig.1.7). In 1972, two independent groups narrowed down this area

to the supra chiasmatic nuclei (SCN) (Fig. 1.8) which consist of two clusters (Moore and Eichler, 1972). They ablated the SCN bilaterally and observed a loss in circadian rhythmicity of corticosterone rhythm, drinking behaviour and locomotor activity. Later transplantation experiments demonstrated that circadian behaviour can be restored in SCN lesioned animals by SCN grafts (Sujino et al., 2003). Similar studies have been performed in a number of other mammalian species and they confirmed that the circadian pacemaker resides in the SCN in all mammals.

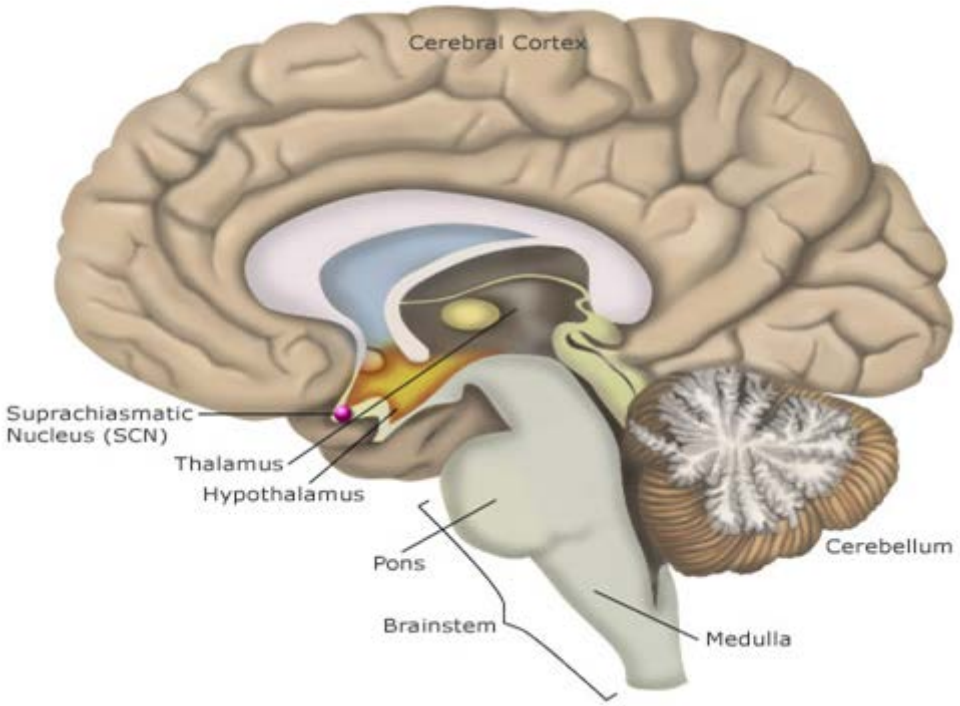


Fig.1.7: Brain section showing the position of Suprachiasmatic Nucleus (SCN) that is part of hypothalamus. Adapted from, ("The Body Clock Guide to Better Health" by Michael Smolensky and Lynne Lamberg; Henry Holt and Company, Publishers (2000)).

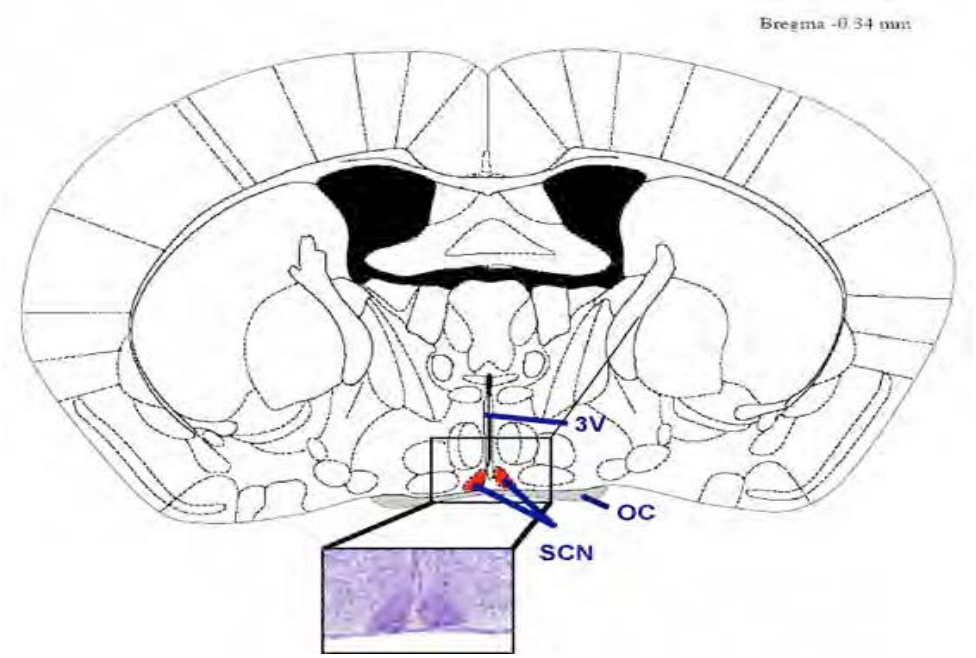


Fig.1.8: Localization of the suprachiasmatic nuclei (SCN) in the mouse brain. This coronal section shows the position of the SCN (red) on top of the optic chiasm (OC, gray) on both sides below the third ventricle (3V). The enlarged panel shows the SCN stained with cresyl violet (adapted from Paxinos and Franklin, 2001)).

1.3.4: Circadian clocks in mammals:

Circadian clocks are endogenous and self-sustained (meaning that rhythms can continue even in the absence of external cues) time-tracking systems that enable organisms to anticipate environmental changes, there by adapting their behaviour and physiology to the appropriate time of the day (Morse and Sassone-Corsi, 2002). The mammalian clock system is hierarchical, with a master clock located within the neurons of the SCN in the hypothalamus. The SCN receives signals from the environment and provides the principal timing cues for synchronizing the daily oscillations in peripheral tissues (Lowrey and Takahashi, 2000). The SCN pacemaker consists of multiple, autonomous single cell circadian oscillators, which are synchronized to generate a coordinated rhythmic output (Welsh et al., 1995).

1.3.5: Circadian rhythms and brain pathology:

The master circadian pacemaker located in the SCN controls many physiological and behavioural functions via the orchestrated function of clock-controlled genes that regulate the output rhythms throughout the central nervous system and periphery.

Disruptions in circadian clock genes that are considered the molecular basis of the circadian pacemaker may be implicated in mental disorders and increasing evidences suggest that alterations in circadian rhythms can have profound consequences on emotional behaviour and mental health. Abnormalities in different physiological rhythms have been implicated in depressive disorders, schizophrenia, bipolar disorder, anxiety disorders and a variety of other CNS disorders (Lamont et al., 2010). Latest data has shown that the disruption of one of the *clock* gene in the master circadian clock may be responsible for mania like behaviours (Roybal et al., 2007).

1.3.6: BDNF and circadian rhythms:

Literature suggests that BDNF central levels are influenced by light and dark cycle. Diurnal changes of BDNF mRNA contents have been demonstrated in the rat CNS. RT-PCR shows that in basal conditions, BDNF mRNA varies along with its TrkB receptor in both cortex and hippocampus at four different times of the day in adult rats during a 24-h cycle. The strongest variation was found in hippocampus (Bova et al., 1998). Plasma BDNF has been shown to vary at different times of the day in young male volunteers and shown to be significantly higher in the morning when compared with evening (Begliuomini et al., 2008). This study also shows a positive correlation between BDNF and cortisol variations during the day and strongly suggests presence of diurnal rhythm of BDNF in human. BDNF mRNA in hippocampal subfields (CA1, CA3, hilus and DG) also show variations observed at four different time points of the day in rats (Berchtold et al., 1999). *In situ* hybridization for BDNF mRNA expression significantly varies in different hippocampal subfields (DG, CA1 and CA3) at different time points during 12/12 h light/dark cycle (Schaaf et al., 2000).

1.3.7: Disturbances in Circadian Rhythm Pattern:

Disturbances in the circadian rhythm pattern can affect an organism. Circadian rhythm disturbances are categorized into transient disorders (e.g. jet lag; changed sleep schedule due to work, social responsibilities and illness) and chronic disorders like delayed sleep-phase syndrome (DSPS), advanced sleep-phase syndrome (ASPS) and irregular sleep-wake cycle.

Studies have highlighted the role of circadian rhythms in memory functions, as it is evidenced in behavioural studies in both rodents and man. Passive and active avoidance memory is impaired by the disruption of circadian rhythms of the adrenocorticotropic hormone in rats and mice (Tapp and Holloway, 1981). A variety of cognitive processes can also be affected by disruption of circadian rhythms. Therefore, the phase shifting that may change one of the external time cues in order to reset the SCN causes the normal animal to modify its behaviour and to adapt the novel schedule. During the water maze task, phase shifting has shown to interfere long term memory recall for the platform location two weeks later (Devan et al., 2001).

1.4: BDNF and learning/cognition

1.4.1: Synaptic Plasticity:

For the first time, Ramon y Cajal formulated that storage of informations in the brain is due to the modification of neuronal microscopic gaps called the synapses. So synaptic plasticity refers to any modification at the neuronal synapses leading to weakening or strengthening of synapses, in this way specific informations become available for specific physiological function. This process mostly occurs at the postsynaptic level (Sheng and Hoogenraad, 2007). On the basis of strengthening and weakening of the synapses, synaptic plasticity has been divided into two paradigms that are long term potentiation (LTP) and long term depression (LTD). It has been known that LTP generally exists into an early phase (E-LTP) and late phase (L-LTP) and it is generally accepted that E-LTP lasts for hours and it is dependent on the existing proteins (Malenka and Bear, 2004) whereas L-LTP last for days and this phase of LTP needs *de novo* protein synthesis (Kelleher et al., 2004).

1.4.2: BDNF and synaptic plasticity:

The mammalian brain adapts or modifies itself in response to experience and or environment depends on the plasticity of synaptic connections. Different evidences indicate that the number and strength of synapses is readily altered by neuronal activity. This process is known as synaptic plasticity that is important in several physiological properties that plays its role as a cellular correlate for multiple cognitive processes, including learning and memory. It is shown that BDNF is involved in many

of these physiologic processes including the increase of the excitatory postsynaptic currents (EPSCs) in *Xenopus* cultures (Lohof et al., 1993). Many studies have examined the actions of BDNF that appearing to strengthen excitatory (glutamatergic) synapses and weaken inhibitory (GABAergic) synapses. Rat hippocampal slices exposed to BDNF shown a long lasting potentiation of afferent input to hippocampal pyramidal cells (Kang and Schuman, 1995). Furthermore, many different studies have highlighted the role of BDNF in synaptic plasticity and LTP, like in case of hippocampal or cortical slices with TrkB inhibitors have evidenced the inhibition of LTP (Figurov et al., 1996) and in contrast to that hippocampal slices from BDNF knockout subjects have shown impaired LTP induction (Korte et al., 1995) which was restored by BDNF addition (Korte et al., 1996).

1.4.3: BDNF and LTP:

LTP is defined as activity dependent strengthening of the synapses and is the most known and discussed form of plasticity. Induction of LTP is responsible for the activation of various signaling cascades in the cell cytoplasm and nucleus like cascades by the BDNF. It has been studied that very low concentration of BDNF is responsible for action potential of hippocampal and cortical neurons in few milliseconds (Kafitz et al., 1999). Before this, only the classical neurotransmitters have such a rapid effect on the neuronal membrane potential and since then it has been evidenced the role of BDNF in LTP in hippocampus and dentate gyrus synapses (Messaoudi et al., 2002). Figurov et al. (1996) demonstrated that exogenous BDNF induce LTP in the young hippocamal slices. Alternately, LTP was restricted by the treatment of function blocking BDNF antibodies to the hippocamal slices (Figurov et al., 1996).

1.4.4: Synaptic plasticity and memory:

Modifications in synaptic strength are considered to be involved in the mechanisms underlying learning and memory. One of the main candidates responsible for modulating synaptic plasticity in learning and memory is BDNF (Yamada et al., 2002). It is evidenced that BDNF activating TrkB receptor by depolarizing neurons that enhances glutamatergic synaptic transmission and increases phosphorylation of the subunits of N-methyl-D-aspartate (NMDA) receptors in the hippocampus (Figurov et al., 1996). Some morphological studies have been shown that BDNF modulates the

dendritic growth and is involved in the complexity of the dendrites in the cerebral cortex (McAllister et al., 1995). Various evidences show the importance of BDNF in learning and memory. There is a good correlation between BDNF mRNA expression and behavioural performance in various learning and memory tests (Yamada et al., 2002). The hippocampal dependent learning in the Morris water maze, contextual fear, and passive avoidance tests is associated with a rapid and transient increase in BDNF mRNA expression in the hippocampus (Kafitz et al., 1999). Blocking the BDNF functions by anti-BDNF antibodies has shown to cause impairment of memory in the water maze (Mu et al., 1999).

1.4.5: BDNF and memory:

The synaptic plasticity correlates with learning and memory formation and it is well established that BDNF plays an important role in synaptic plasticity (Yamada and Nabeshima, 2003). The hippocampus responsible for learning and memory is the major site of BDNF action and it is also noted the expression of BDNF during learning process (Hall et al., 2000). In contrast to this, spatial learning was shown to be impaired when BDNF function was blocked through function inhibiting antibodies to BDNF (Mu et al., 1999). In primates, during tool use learning in monkeys, there has been shown an up regulation of BDNF in the parietal cortex (Ishibashi et al., 2002).

1.5: BDNF and diseases:

Neurotrophic factors especially the BDNF has been shown to be involved in various neuronal functions and regulate different processes during development as well as in adult life. In this way, neurotrophic factors are prominent in giving response to any kind of neuronal injury, so neurotrophic factors have been involved in the physiopathalogy of brain diseases.

1.5.1: BDNF and neurological disorders:

BDNF seems to be implicated in many neurological disorders. Many studies have evidenced less expression of BDNF than normal in degenerative disorders of the nervous system (Murer et al., 2001). Transcription for BDNF mRNA has been shown to decrease in hippocampus of the Alzheimer's disease (AD) patients (Phillips et al., 1991). AD is characterised by the loss of executive functions and memory. However the exact cause of this degenerative disease remains unknown but some studies on post mortem of brain have shown modifications of BDNF expression. It has been also studied that BDNF is expressed in two important parts of the brain, the entorhinal

cortex and hippocampus during adulthood which are structures of neuronal degeneration in AD. Many studies have noted the decrease of BDNF in brains of AD patients (Peng et al., 2005).

Parkinson's disease is characterized by the death of dopaminergic neurons in the substantia nigra (Howells et al., 2000). In Parkinson's disease, BDNF has been shown to be reduced in substantia nigra that plays an important role in movement.

Some studies have also been made on Huntington's disease (HD). In the HD disease, a protein called Huntingtin is mutated that upregulates BDNF transcription. Immunoreactivity has been shown to reduce in the brain areas like caudate neurons in HD specimens (Ferrer et al., 2000).

1.5.2: BDNF and psychiatric diseases:

BDNF may contribute in psychiatric diseases and, due to human BDNF polymorphism may increase the risk of mood disorders like bipolar disorder (BPD).

BPD also known as manic–depressive disorder is a category of mood disorders defined by the presence of one or more episodes of abnormally elevated energy levels and mood with or without one or more depressive episodes. The elevated moods are clinically referred to as mania or if milder, hypomania. It is reported that BDNF dysfunction and decrease levels of BDNF may be involved in the pathways which are important in the pathophysiology of BPD. Psychosocial factors like stress effect BPD and have been reported in the reduction of serum BDNF among the BPD patients (Kapczinski et al., 2008).

Dysregulation of synaptic plasticity and neurogenesis due to neurotrophic factors in hippocampus may result in malfunctioning of neural networks that may be involved in depressive disorders.

Major depressive disorder (MDD), a stress induced mental disorder characterized by loss of interest or pleasure in normally enjoyable activities is due to the failure of neurogenesis and dysregulated synaptic plasticity (Duman, 2002). Data on serum BDNF have suggested that MDD may be due to less expression of BDNF (Karege et al., 2002). Studies have reported that serum of drug free depressed patients contain less BDNF as compared to healthy individuals. A reciprocal relationship existed between severity of depression and serum BDNF levels. Antidepressant treatments are shown to recover the BDNF levels in depressed patients (Gonul et al., 2005). Studies

on brain post-mortem in suicide victims reported less BDNF levels compared to those victims who were treated with antidepressant drugs (Karege et al., 2005).

1.6: BDNF and inflammation:

1.6.1: Inflammation:

Inflammation:

Inflammation is process that protects the body from various dangerous stimuli like any injury and tissue ischaemia. It is also responsible for response to infections. Inflammation in the tissues is expressed by the five cardinal signs.

Cardinal signs:

The cardinal names come from Latin which are: Dolor (pain), Calor (heat), Rubor (redness), Tumor (swelling) and Functiolaesa (loss of function). Inflammation has also been defined in some other ways including invasion of lymphocytes and macrophages and expression of inflammatory stimulators (cytokines).

Inflammation and changes in microcirculation:

The microvasculature is between the venules and the terminal arterioles. There is capillary network between the respective venules and arterioles. In normal physiology, blood stream is fed through the capillary system when the pre-capillary sphincters are closed. There is also constant vasoconstriction and vasodilatation between these arterioles and venules. This process regulates blood flow and filtration pressure. During this process, many humoral and chemical substances are also important which are responsible for typical changes in the capillary form that occur in three phases:

First phase

During this phase, there is transient arteriolar vasoconstriction that last for seconds to few minutes and is not detectable in every inflammatory reaction. Scenario of this phase is that noxious agents enter the tissue and there is brief paling of the inflamed area. The faucet becomes off by the arteriolar vasoconstriction which is helpful in prevention of further noxious agents.

Second phase:

This phase starts a few minutes after the first phase which involves the inflammatory mediators which leads to vasodilatation of local vessels. This causes exudation of blood serum that leads to tissue swelling and stimulation of nociceptive nerves. In this phase noxious agent in the tissues that results in erythema, swelling and pain in the inflamed area.

Third phase:

This starts after some hours after the onset of inflammation and last for several hours. This phase involves vasodilatation of the capillaries and arterioles and then vasoconstriction of venules. Finally this process increases the permeability in the inflamed area. The noxious agent remains in the tissue and all faucets are turned and sealed off by vasoconstriction of the venules. The pathogenetic chain reaction to the slowed circulation in the microvasculature involves several events.

Sealing of vascular structures:

Group of erythrocytes change into homogeneous cylindrical vascular castings that appear red sludge under light microscope. This causes endothelial damage and also causes thrombocytes aggregation to form thrombosis that blocks the leaking vessels in the inflamed area.

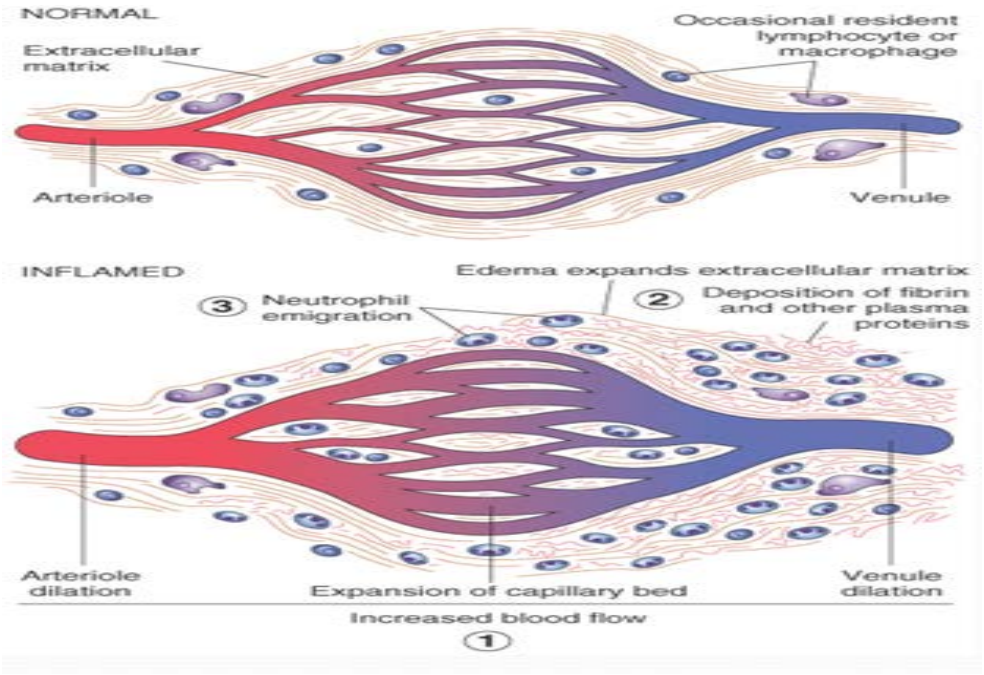


Fig.1.9: Diagramtic representation of a normal and inflamed arteriole, capillary and venule network.This shows increased blood flow (1), neutrophil emigration(2) and in extracellular matrix, deposition of fibrin and other plasma proteins (Riede / Werner, Color Atlas of Pathology © 2004 Thieme)

Leukocyte extravasation: Leukocytes start to migrate from the microvasculature and rush into inflamed area (Fig.1.9).

Causes of inflammation:

Inflammation may be caused by several agents and mechanisms. These include:

- Noxious physical agents
- Noxious chemical agents
- Micro-organisms
- Product of micro-organisms
- Accumulated product of metabolism
- Immunologic tissue injury
- Tissue injury from infection
- Tissue necrosis

Spread of Inflammation:

The inflammation that is limited to a circumscribed area of tissue near its part of entry is called the local inflammation. The spread of local inflammation is controlled by organ capsules and fascial septa. The following forms of dissemination are differentiated:

- Hematogenous dissemination occurs through blood vessels.
- Lymphatic dissemination occurs through lymph system.
- Neurogenic dissemination occurs via the flow of axoplasm in the nerves.
- Ductal dissemination occurs through ductal or canalicular systems in organs, leading to ascending inflammation.
- Direct contagion may also occur.

Metastatic inflammation:

When inflammation spreads from a local area to other organs and tissues, this kind of transmission is called metastasis. This ends in transmission of inflammatory pathogens to the tissue level where these pathogens can trigger a metastatic inflammation. Whereas in generalised kind of infection, the pathogen diffuses throughout the body.

1.6.2: Infection and inflammation:

Bacterial cells or their products entry is usually followed by an inflammatory response with increased secretion of a variety of mediators, such as cytokines, acute phase proteins and aggregation of leukocytes in affected tissue (Kilpatrick and Harris 1998).

Cytokines

Cytokines are polypeptides or glycoproteins with a molecular weight less than 30kD. They are secreted from monocytes and lymphocytes and also from non-immune cells like endothelial cells after activation by the micro-organisms. Cytokines bind to specific receptors at low concentration and are involved in signal transduction via intracellular messenger network and are responsible of the activation of various transcription factors. Cytokines have multiple effects, usually overlapping the effects of others. Cytokines mediate their action locally but also effect systemically. They play important roles in host defence. However, they may have detrimental effects if they are in high amounts (Waage et al., 1987).

Tumour Necrosis Factor (TNF):

TNF is a member of cytokine super family and is the main inflammatory mediator in autoimmune diseases and infection (Bazzoni and Beutler, 1996). Monocytes, tissue macrophages, play important role in producing TNF in response to various stimuli including infections, bacterial toxins, complement factor C5a, IL-1, IL-6 and TNF itself. TNF activates two receptors, p55 and p75, present on all cell membranes but may shed from cell membranes and become the part of soluble proteins in response to bacterial products like lipopolysaccharides (LPS) (Olsson et al., 1993).

Interleukin (IL):

IL-1 is synthesised by a variety of cells, including monocytes and macrophages in response to several stimuli including infections and bacterial toxins. IL-1β in combination with TNF plays a central role in inflammation and sepsis.

IL-6 is produced by B-lymphocytes and T-lymphocytes in different forms having different molecular weights. It is helpful in regulating immune system at the B and T cell levels and also responsible in stimulating the acute phase reaction (Heinrich et al., 1990).

IL-8 belongs to a large group of cytokines known as chemokines, so mainly involved in chemotactic actions. It exists in several forms and is produced by monocytes, macrophages and endothelial cells in response to endotoxins (LPS), TNF or IL-1.

Inflammatory response: **Local effects** of the inflammatory response are the accumulation and activation of leukocytes in the local affected area. Inflammatory response is initiated at the site of entry of any inflammatory agent. At the time of invasion, cytokines are produced and released by the macrophages. Pro-inflammatory cytokines IL-6 and chemokines like IL-8. IL-1 and IL-6 stimulate the process of blood formation and also involved in the release of neutrophils from the bone marrow. The aggregation of leukocytes in the local inflamed area is the result of a series of events:

(1) increased expression of adhesion molecules on endothelial cells; (2) establishment of leukocyte endothelial cell adhesion; (3) leukocytes migration through the endothelium and leukocyte migration through along a chemotactic gradient. Cytokines remain involved at each and every step of this process. As the inflammation progresses, TNF and IL-1 may also increase local production of vasodilators like nitric oxide and prostaglandins and vasoconstrictor like endothelins. **Systemic effects** are **s**everal physiologic, behavioural and biochemical changes involving organ system and these changes may involve inflammation (Gabay and Kushner, 1999). During this process of inflammation, pro-inflammatory cytokines may be responsible for fever and increased secretion of corticosteroids (Gabay and Kushner, 1999). The local and systemic inflammatory responses are maintained until regulatory mechanisms are activated and cytokine synthesis and biologic activities are attenuated. In most cases, the inflammatory response is successfully resolved. However, vigorous production of pro-inflammatory cytokines can lead to increasing systemic cytokine concentrations.

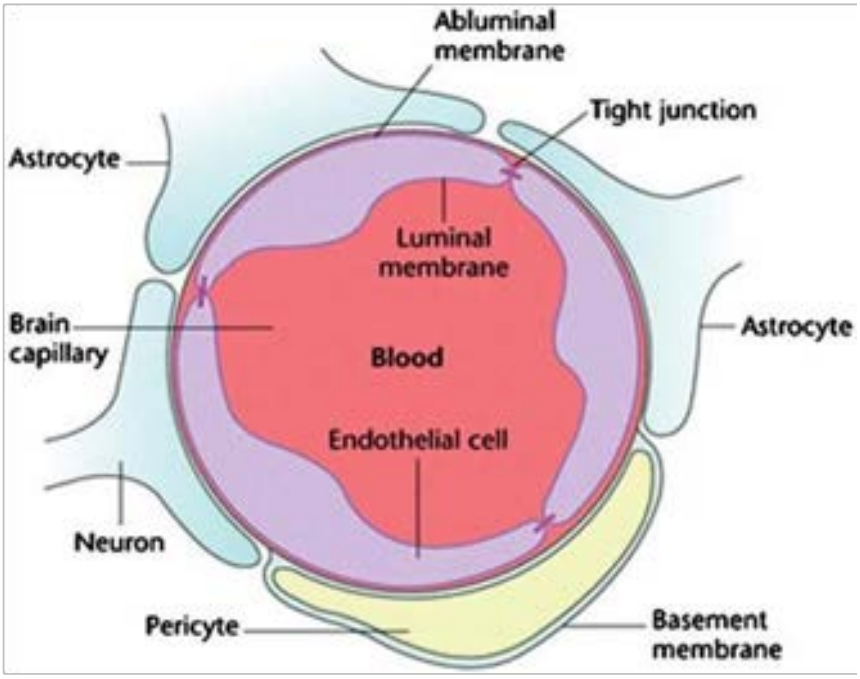


Fig.1.10: Schematic representation of Blood brain barrier composed of endothelial cells, basement membrane, astrocytes and pericytes (adapted from (Saunders et al., 2008)).

1.6.3: Inflammation and brain:

Blood brain barrier (BBB): In 1885, Ehrlich revealed the existence of the BBB. The BBB is an essential barrier for the normal functioning of the CNS. The BBB endothelial cells contain numerious tight junctions and spare pinocytic vesicular transport. Transport of hydrophilic molecules is limited via endothelial tight junctions, however small lipophillic substances such as O_2 and CO_2 cross passively the plasma membrane (Grieb et al., 1985). The BBB consist of a basement membrane, astrocytes and pericytes that are tightly packed in basement membrane (Saunders et al., 2008) (Fig. 1.10). All these components play their specific roles and are needed for the normal physiology and stability of the BBB.

Inflammation from peripheral to the CNS:

During the process of spreading inflammation from peripheral areas to the CNS, the inflammation follows two ways: either inflammation passes through or bypasses the BBB. Studies have suggested three processes for the spread of inflammation from peripheral areas to the CNS:

- Cytokine interaction that plays important role in the spread of inflammation (Banks et al., 1991).
- Cytokine interaction that is responsible for leukocyte migration (Pan and Kastin, 2002).
- Afferent neurons in the local inflamed area are stimulated that spread inflammation to CNS (Godbout et al., 2008).

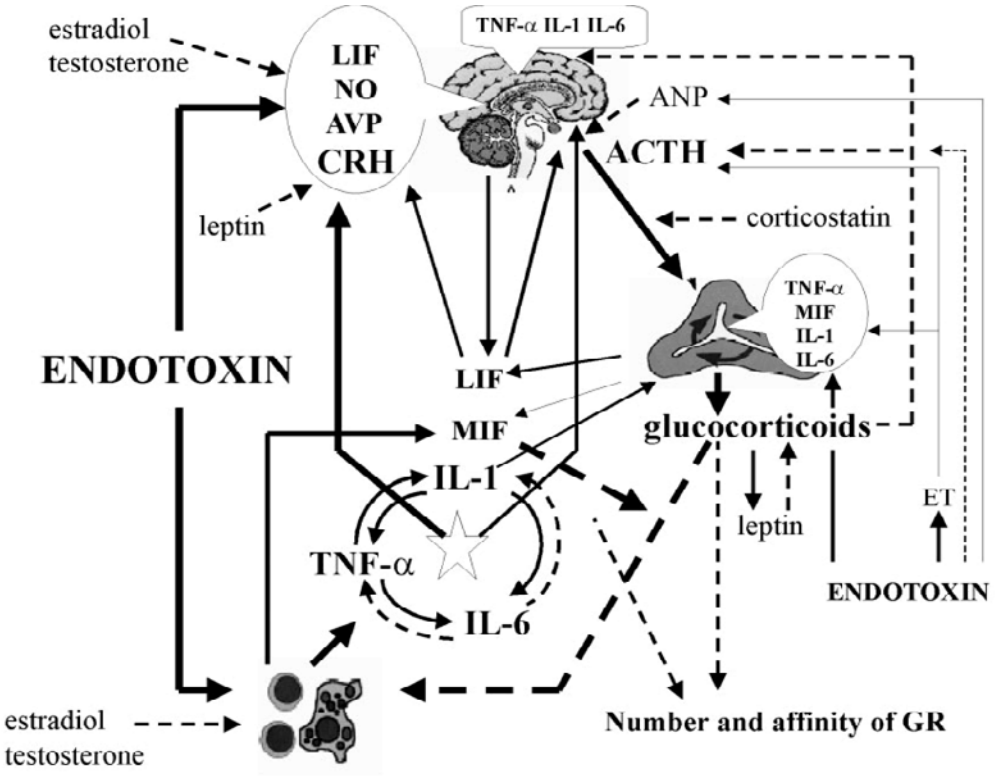


Fig. 1.11: Diagramatic representation of the effects of endotoxin on the different levels of the HPA axis, showing the interplay of different cytokines and other mediators (dottedlines, inhibitory effect continuous lines, stimulatory effect), (adapted from (Beishuizen and Thijs, 2003)).

1.6.4: Endotoxins and Hypothalamo Pituitary Adrenal Axis (HPA):

HPA Axis:

The HPA axis is a complex form of interactions between hypothalamus, the pituitary gland and the adrenal gland. The HPA axis plays a major role in the regulation of the immune system, the regulation of body temperature, the digestion, the mood and the sexuality. It is also considered important part of the system that play important role in giving response against dangerous stimuli and stress.

Mechanism of HPA Axis:

The main hypothalamic hormones corticotropin-releasing hormone (CRH) and vasopression are secreted from the external zone of median eminence (Munck et al., 1984) and they play role in the activation of release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Further ACTH is involved in the synthesis and secretion of cortisol from the adrenal cortex. So HPA axis controls the synthesis of glucocorticoids and through the negative feedback, the cortisol can inhibit the release

of CRH and ACTH (Fig.1.11). Studies have evidenced that cortisol acts on various tissues to control various physiologic functions including growth and various aspects of metabolism (Munck et al., 1984).

1.7: Lipopolysaccharide (LPS) and cognition:

1.7.1: Structure and composition of LPS:

Gram-negative bacteria contain very complex outer membrane and inner cytoplasmic membrane with a thin layer of peptidoglycan. The bilayered membrane contain proteins and phospholipids where as outer one contain a very important constituent called LPS or it is also known as endotoxin (Lugtenberg and Van Alphen, 1983).

LPS:

LPS, structurally and functionally plays very important role for gram-negative bacteria. LPS has also been evidenced as an important target of the immune system. Immune cells have the ability to recognise the LPS and there is release of pro-inflammatory mediators including TNF-α, IL-6 and IL-1. These pro-inflammatory mediators are helpful for the host by promoting inflammation and also important in activating the immune system that expels the infectious organisms. In severe cases, LPS comes in systems of the body through the blood stream, leading to systemic inflammation that may be responsible for organ failure, septic shock and finally death.

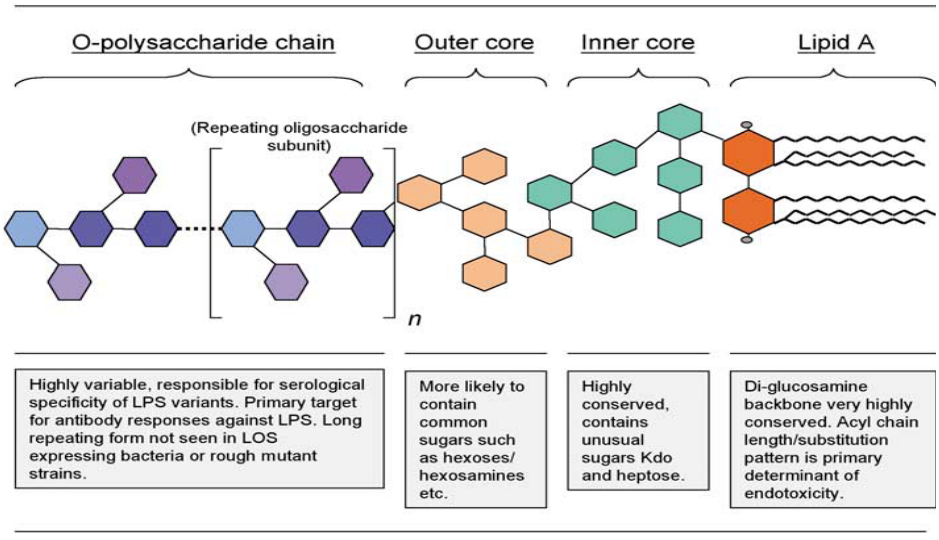


Fig. 1.12: Diagrammatic representation of general structure of Gram-negative LPS, adapted from (Erridge et al., 2002).

Structure of LPS:

LPS molecule can be divided into three separate regions (Fig. 1.12).

Lipid A is the highly hydrophobic and active part of the LPS. The endotoxicity of the LPS is due to its Lipid A region. Typically lipid A is composed of D-GlcN-(1-6)-α-D-GlcN disaccharide that contains two phosphoryl groups at position 1 and 4. Further four acyl chains are also attached to this structure by the ester or amide linkages. These chains are substituted by fatty acids to provide LPS molecule. (Fig. 1.13).

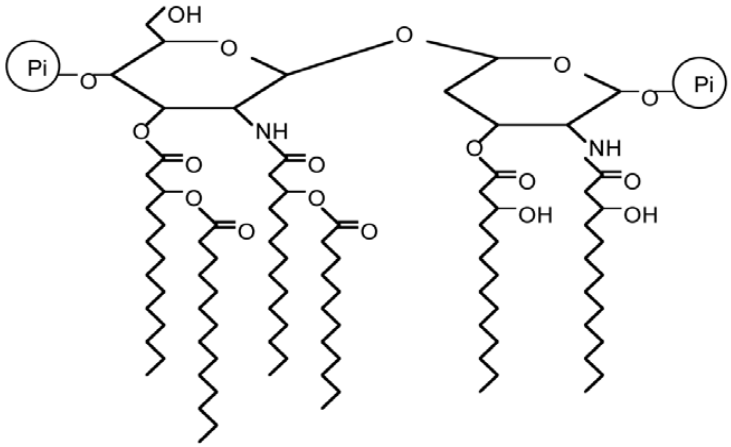


Fig. 1.13: Schematic diagram shows the chemical structure of *E. coli* lipid A (adapted from (Erridge et al., 2002)).

Lipid A is covalently bonded to the core region of the molecule which is further divided into inner and outer core. The inner core contains high proportion of sugars like L-glycero-D-mannoheptose and is situated proximal to the Lipid A. The outer core contains the more common sugars like hexoses and is projected further from the surface of bacteria.

***O*-polysaccharide** is also an important part of the LPS molecule that acts as antigen against which host immune system responses. *O*-chains are sometime called *O*-antigen because most of the host antibody responses are *O*-chain specific. The *O*-polysaccharide part of the LPS is also detected by the immune system that helps in playing an important role in the activation and inhibition of the complement activation (Joiner et al., 1986).

1.7.2: LPS signalling pathways:

LPS receptor:

LPS actions are mediated by binding to the Cluster of Differentiation 14 (CD14). CD14 may be present in two forms: embedded into the membrane by the glycosyl phosphatidylinositol (mCD14) or in soluble form (sCD14). CD14 receptors are

expressed on macrophages and neutrophil granulocytes. It is also expressed by dendritic cells. A soluble form sCD14 is secreted by the liver and monocytes and is sufficient in low concentrations to confer LPS-responsiveness to cells that otherwise do not express CD14. CD14 receptors are expressed on the micro glial cells and play important role in picking up the LPS through macropinocytosis (Vasselon et al., 1999).

LPS signalling pathways:

LPS follows two signalling pathways; CD14 dependent and CD14 independent. In CD14 dependent pathway, LPS binds to CD14 receptor in the presence of Toll-like receptor 4.

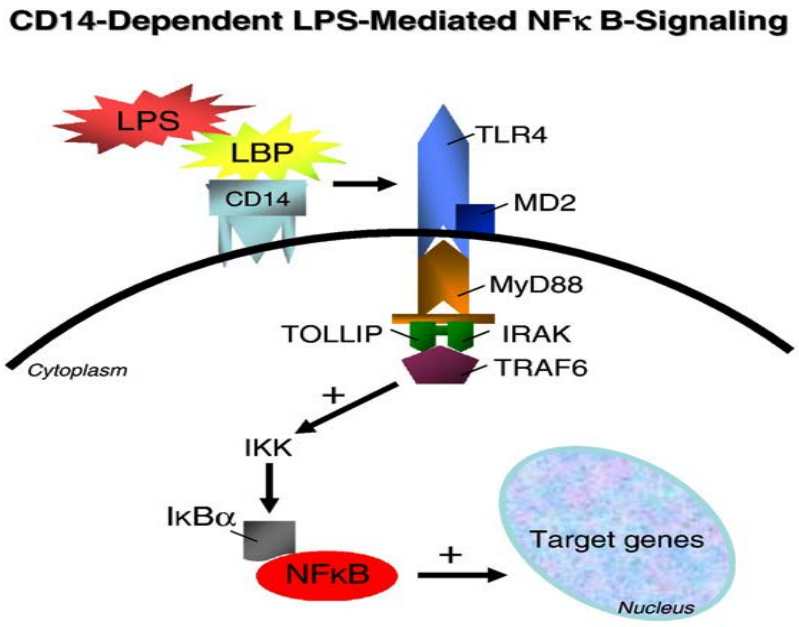


Fig.1.14 (A): Diagram illustrate the signalling interaction between CD14 and LPS (adapted from (Linde et al., 2007)).

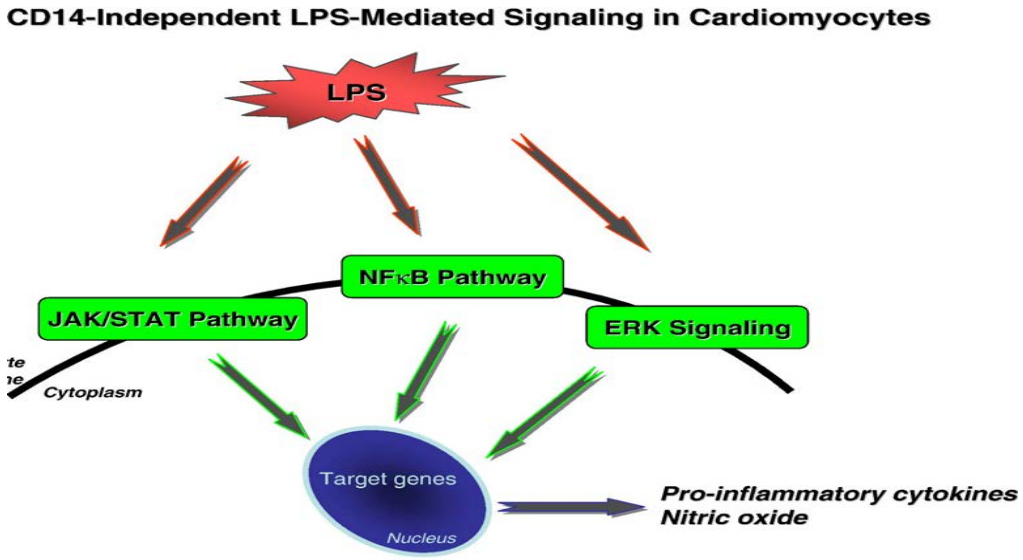


Fig.1.14 (B): Diagramtic illustration of CD14 independent LPS signalling (adapted from (Linde et al. 2007)).

(TLR4). CD14 receptor with TLR4 and MD2 for picking up the LPS and this happens always in the presence of lipopolysaccharide binding protein (LBP), (Kitchens, 2000). Then TLR4 uses the adaptor protein called the Myeloid differentiation 88 (MyD88) that activates the transcription factor called the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). NF-kB is the protein complex that controls the transcription of DNA. (Fig.1.14 A). NF-kB plays an important role in cellular responces to many stimuli including cytokines, ultraviolet irradiation and bacterial antigens (Gilmore, 2006). Dysregulation of NF-kB has been implicated in cancer, inflammatory diseases and improper immune development. It has been evidenced that NF-kB plays important role in synaptic plasticity and memory (Albensi and Mattson, 2000).

In the CD14 independent pathways, LPS has been shown to activate three pathways; the JAK/STAT pathway, NF-kB pathway and and ERK signalling pathway:

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway are usually involved in the transduction of development and homeostasis in almost all animals and it is also important mechanism for a variety of cytokines and growth factors. NF-kB pathway has been found in all cell types and is involved in

cellular responces to stimuli including cytokines, ultraviolet irradiation and bacterial antigens (Gilmore, 2006). Down regulation of ERK signalling to the nucleus, phosphorylates transcription factors and the mitogen and stress activated protein kinases (MSKs) (Deak et al., 1998). This down ERK signalling results in cellular growth and proliferation of proteins. This CD14 independent LPS signalling is demonstrated in Fig.1.14 (B).

1.8: Neuroinflammation and brain pathologies:

1.8.1: Cells involved in neuroinflammation:

Microglia is the type of the glial cells that are the macrophages of the CNS and represent approximately 20% of the glia population (Kreutzberg, 1995). These cells belong to the monocyte macrophage lineage and have mesodermal origin. Microglia has been shown to be predominated in the grey matter as well as the hippocampus, basal ganglia and substantia nigra (Block et al., 2007). The resting miroglia in normal circumstances, monitor and detect any changes in the surrounding area. Therefore, microglia are the first line of defence in the CNS (Gao and Hong, 2008). Infected or inflamed neurons cause resting microgila cells to change into activated form (Fig.1.15). The level of activation depends on the severity of neural injury like moderate kind of injuries cause hyper ramification of microglia (Wilson and Molliver, 1994). In case of neural death, microglial cells transform into brain macrophages, playing important role in removal of dead cells. It has been studied that microglial cells are involved in myelin repair, eliminate toxic proteins and help in neuroprotection (Gao and Hong, 2008).

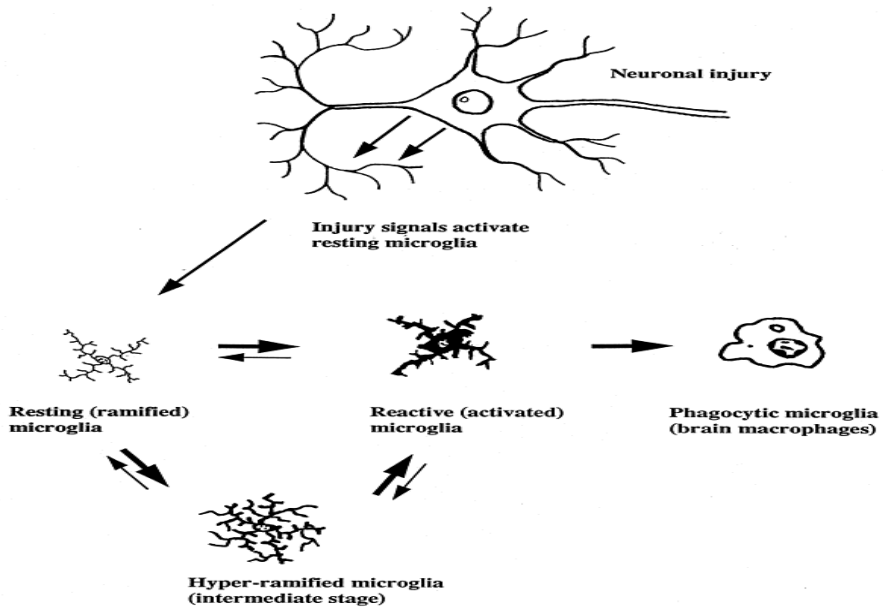


Fig.1.15: Functional plasticity of microglia. Injured or diseased neurons cause resting microglia to become activated (Wilson and Molliver, 1994).

Astrocytes are characterised by the star-like appearance and they make up about 50% of human brain volume. They are shown to involve in maintaining the extracellular environment and cell to cell communication in the CNS. These cells play significant role in metabolism in the brain and also involved in modulation of excitatory synaptic transmission (Maragakis and Rothstein, 2006). In the neuroinflammatory diseases like multiple sclerosis and AD, the activated astrocytes play significant role in leukocyte recruitment to the CNS through the increased production of leukocyte adhesion molecules and chemokine (Moynagh, 2005). Astrocytes have also been found in contribution of dysfunction of neurons in the diseased state (Maragakis and Rothstein, 2006).

1.8.2: CNS responses to infections and inflammation:

It is well known that the brain differs from other body tissues in the expression of response to peripheral insults. Inflammation or infection induce different response in the brain tissue, this has been shown in the leukocytes aggregation which is more fast in other tissues as compare to the brain where is delayed. In addition, brain responses include:

- Activation of glial cells.

- Formation of oedema.
- Expression of major histocompatibility complex (MHC).
- Expression of systemic acute phase response and synthesis of acute phase protein.
- Complement activation like membrane attack complex
- Synthesis of inflammatory mediators e.g. cytokines.
- Expression of adhesion molecules and invasion of immune cells.

The brain is able in sensing all kind of insults from the peripheral regions and can express responses in short period of time. These kind of afferent signals from peripheral inflamed or infected areas to the CNS have not yet been described but it has been shown the expression of inflammatory mediators like IL-6. Now, it has been accepted the importance of inflammation in the CNS, as it plays a significant role in the diseases including the acute brain injury, epilepsy, multiple sclerosis, motor disorders and Alzheimer's disease. In some recent studies, inflammation has shown to be involved in psychiatric disorders such as depression, anxiety and schizophrenia. The immune cells in the brain, the microglia actively participate in monitoring the environment and quickly respond to the external insults to induce cytotoxic factors like nitric oxide (NO) (Liu et al., 2002).

1.8.3: LPS and CNS:

LPS and Brain:

LPS is well known to cause the inflammation and the inflammatory mediators can cross the BBB, so LPS may be responsible for a number of effects on the CNS. Studies in rodents have shown that LPS activates the peripheral innate immune system and this plays important role in increasing inflammatory response by the brain of aged mice (Godbout et al., 2005). It is important to note that increased inflammatory response by the LPS in aged brain may be responsible for cognitive deficits that are commonly prevalent in elderly patients. It is reported that LPS bring changes through production of IL-1β and LPS injected centrally or peripherally, increases IL-1β concentrations in rat brain (Ilyin et al., 1998).

LPS and BDNF:

Studies have indicated that LPS may affect some specific brain areas by interfering with the neurotrophin expression and their functions. This is evidenced that intraperitoneal injections of LPS in rats are responsible in the reduction of BDNF

mRNA in the hippocampal formation (Lapchak et al., 1993). A recent study investigated a reduction in BDNF gene in mice synaptosomes within 1-6 days after LPS administration, with maximal reductions at day 3, this reduction in BDNF mRNA in the brain parallels the decrease in BDNF protein in the synaptosomes (Schnydrig et al., 2007). It has been studied that LPS not only decreases BDNF but also reduces NGF in different rat's brain structures like hippocampus , frontal cortex , parietal cortex, temporal cortex and occipital cortex at 7 h of injections of LPS (Guan and Fang, 2006).

1.9: LPS and neurodegenerative diseases:

1.9.1: Effect of inflammation on neurite outgrowth:

Activation of microglia by the high concentration of LPS has shown to induce cell death *in vitro* (Munch et al., 2003). However, the mechanism of cell death due to activated microglia is unknown. Münch et al. (2003) showed that LPS is involved in the reduction of neurite outgrowth via the activation of microglia cells. TNFα also plays role to reduce the neurite outgrowth and branching in the hippocampus via rho (a prokaryotic protein) dependent mechanism (Neumann et al., 2002). During the inflammation, the reduction in neural outgrowth may interfere with cytoskeleton reorganization. This change in reorganization can be responsible for learning and memory deficits (Gallagher et al., 1996).

1.9.2: Inflammation and neurogenesis:

Neurogenesis is the production of new neurons in the CNS. In some areas of the brain, this production of new neurons continues even throughout the whole life like the hippocampus. This production reflects its function especially learning and memory (Monje et al., 2003). Any damaging stimuli for the normal proliferating neural stem may be responsible for the disruption of neurogenesis that is further responsible for cognitive deficits. Monje et al. (2003) has demonstrated the effect of altered environment by injecting peripheral LPS into rodents and this has caused a 35% decrease in hippocampal neurogenesis. This disruption of neurogenesis by LPS was also shown to be responsible for spatial learning and memory deficits (Wu et al., 2007). The mechanisms responsible for the disruption of neurogenesis by the neuroinflammation is not fully understood. However, it is considered that cytokines like IL-6 inhibit cell proliferation and neurogenesis in the hippocampus by raising the glucocorticoids by the stimulation of HPA axis (Vallieres et al., 2002).

1.9.3: Effect of inflammation on LTP:

LTP is a type of synaptic plasticity that enhances neurotransmission in a continuous fashion following an adequate stimulus (Kerchner and Nicoll, 2008). Studies suggested that cytokines can abolish the action of LTP and peripheral LPS treatment can impair LTP in the hippocampus (Vereker et al., 2000). It has been also evidenced that LPS can impair LTP via IL-1β activation by increasing the stress activated kinases, the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), leading the impairment in neuronal function (O'Donnell et al., 2000).

Glutamate is considered an important neurotransmitter that plays a significant role in the propagation of LTP, so any disturbance in glutamate release and transport will lead to the impairment of LTP. It is evident that LPS disrupts glutamate release by activation of p38 and NF-kB (Kelly et al., 2003). Synaptosomes of rat dentate gyrus treated with IL-1β has shown a reduction in the amount of glutamate and its treatment with SB203580, a p38 inhibitor, has shown the reversal of this effect (Kelly et al., 2003). Studies on LPS show that LPS is sufficient to induce, not only neuroinflammation, but also impairment in LTP that is reflected in the cognitive deficit.

1.9.4: Neuroinflammation and cognition:

Hippocampus is important structure of the brain that plays a significant role in learning and memory and involved in cognitive disorders that are evident in patients with systemic infections. Studies on human have evidenced that acute cognitive impairment has been seen in elderly patients (Chiovenda et al., 2002). Activation of microglia cells stimulates the production of cytokines like IL-1 and TNF (Block et al., 1997). These cytokines are linked with cognitive deficits and in early AD, high levels of cytokines have been observed (Guerreiro et al., 2007, Magaki et al., 2007). Studies have reported that cytokines can be responsible in the dysregulation of synaptic plasticity in the hippocampal regions (Perry et al., 2007) and also involved in the disruption of hippocampal dependent learning and memory (Palin et al., 2004). Memory impairment has been reported in rats in adulthood when neonatal are exposed to *E.coli* (Bilbo et al., 2005). In addition, LPS in neonates, is reported to be associated in influencing reactivity to stress, immune regulation and susceptibility to diseases in adulthood (Shanks et al., 2000). Cognitive performance of AD patients has been shown to be decreased in the cases of chronic inflammation having high levels of TNFα (Holmes and Butchart, 2011). Elevated levels of IL-6 have been also shown in the nigrostriatal regions

(Holmes et al., 2009). Neuropathological changes have been observed in the transgenic animals having high levels of IL-6 (Akiyama et al., 2000). These studies may suggest a link between cognitive deficits and inflammation.

1.9.5: Contribution of inflammation in CNS diseases:

Neuroinflammation has been commonly seen in almost all the neurodegenerative diseases like Multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson disease (PD). High levels of inflammatory cytokines have been observed in AD and PD patients and these elevated levels of cytokines have played effects on cognition. It is also noted that over expressed cytokines like IL-6 have been observed to perform negatively in the cognitive tasks in the transgenic animals (Akiyama et al., 2000). Some of the neural conditions in which acute and chronic inflammation is involved are as follow;

Acute CNS injury:

Stroke and cerebral ischaemia is one of the leading causes of deaths in the Western countries and it is clear that inflammatory cytokines, especially the IL-1, are able to induce ischaemia. Experimentally, it is also shown that administration of anti IL-1 has inhibited the experimentally induced stroke damage in rats. Il-6 mRNA has also been shown to increase in the rodent brain in response to experimentally induced stroke injury. Experimental induced ischaemic injury has also been shown to increase by the administration of TNFa (Hallenbeck, 2002).

Traumatic brain injury (TBI) is a major cause of deaths and stimulates the CNS response by the production and release of pro-inflammatory cytokines. It is experimented that IL-1 injection at different time between 15min and 48h after experimental induced brain injury has reduced the CNS damage proving that IL-1 plays an important role in acute as well as delayed brain damage. Complement cascade may also play its role in the brain after TBI and also evidenced that experimentally induced damage is reduced in the mice which lack the complement 3 (C3) or complement 5 (C5). It is also studied that C5a receptor antagonists reduce the CNS damage (Sewell et al., 2004).

Epilepsy is much known and a common neurological disorder which affects around 50 million people in the world. It is characterised by seizures. The mechanism involved in these seizures is unknown. However, some evidences indicate that pro-inflammatory cytokines play important roles. These cytokines are significant in affecting the neuronal excitability directly or indirectly. Direct excitability is by action on the ionic

currents and indirect excitability is via gene transcription in glia and neurons. In rodents, seizures are induced by the injection of IL-1b and seizures are observed to be stopped or delayed with the treatment of IL-1ra (Interleukin-1 receptor antagonist) (Vezzani et al., 2002).

Chronic CNS diseases:

Chronic CNS diseases are more complex in case of aetiology than the acute brain and spinal cord injuries. The development of chronic CNS diseases is characterised as multifactorial interlinked with environment as well as genetic factors (Campbell, 2004). These factors may be responsible for inflammation of neurons, thus production of inflammatory mediators. Production of many of these inflammatory mediators is increased in chronic neurodegenerative diseases, activation of microglia and components of the complement system. Some of the CNS diseases due to chronic inflammation are discussed as follow;

***Multiple sclerosis** (MS)* is chronic disorder of the CNS in which inflammation plays a significant role. In MS, there is entry of T cells and macrophages in the CNS that induce damage to the myelin sheaths surrounding axons. This action of the T cells and macrophages leads to neuron's death. Clinical symptoms vary in different individuals because MS damages areas of the brain widely and may include fatigue and muscle weakness. Interestingly, the disease progresses in cycles of relapse and mostly linked with systemic infection and inflammation. It is evidenced that various inflammatory mediators become regulated in MS and become available to demyelinate the axons (Raivich and Banati, 2004).

Alzheimer's disease (AD) refers to untreatable and progressive disorder in which there is a loss of learning and memory due to the neuronal cell death in the hippocampus and frontal cortex. About four million people have been affected by this disease worldwide and the risk of AD increase with the age. AD is mainly characterised by major two hallmarks, the neurofibrillary tangles (NFT) and senile plaques (SP). NFT form of the AD consists of paired helical filaments containing Tau proteins, whereas in senile plaques, there is aggregation of amyloid peptides that being surrounded by the glia cells (Sheng et al., 1998). These cells then initiate a cyclic process and there is release of cytokines including IL-1, IL-6 and TNFa. These cytokines have been detected in the brain tissue as well as in cerebrospinal fluid (CSF) of AD patients and both TNFa and IL-1 have been reported to increase the expression of amyloid precursor protein. In addition, acute brain injury may be important in inducing

inflammation with an increase expression of MHC class II, IL-1 and TNFa. The most convincing role of inflammation in AD is the cause of association between the polymorphism in the genes of encoding the IL-1 family and AD (Rainero et al., 2004).

Parkinson's disease (PD) is the second most common neurodegenerative disorder and the most prevalent disorder of the aged people. PD is characterised by the tremors, rigidity and bradykinesia and the most important pathological finding is the loss of dopaminergic neurons in the substantia nigra. Various environmental agents like pesticides and infection may play significant role in the development of PD. It is also noted that activated microglia are expressed in close vicinity of the substantia nigral neurons of the PD patients and the role of inflammation in PD is also mentionable (Gao et al., 2003). CNS has been shown to express innate immune response in PD patients. There is shown an up regulation of pro-inflammatory cytokines in the CSF and substantia nigra of the PD patients (Nagatsu et al., 2000).

1.10: BDNF and GABAergic system:

1.10.1: GABAergic System:

The GABAergic system comprises the following parts;

- GABA
- GABA receptors
- Glutamic acid decarboxylase
- Vesicular GABA transporters

1.10.2: GABA as a neurotransmitter:

GABA has long been considered to be the important inhibitory neurotransmitter in the mammalian CNS. Its main role is the regulation of neuron's ability in two ways; either by hyperpolarizing the membrane or through shunting the excitatory inputs. In early studies, GABA has shown inhibitory effects when it was targeted to the nerve and muscle cells in both invertebrates and vertebrates (Krnjevic et al., 1966). However, a study suggests that GABA acts also as an excitatory neurotransmitter in the immature brain and is also shown to play an important role in regulating the rhythmic activities in neuronal networks (Ben-Ari and Holmes, 2005).

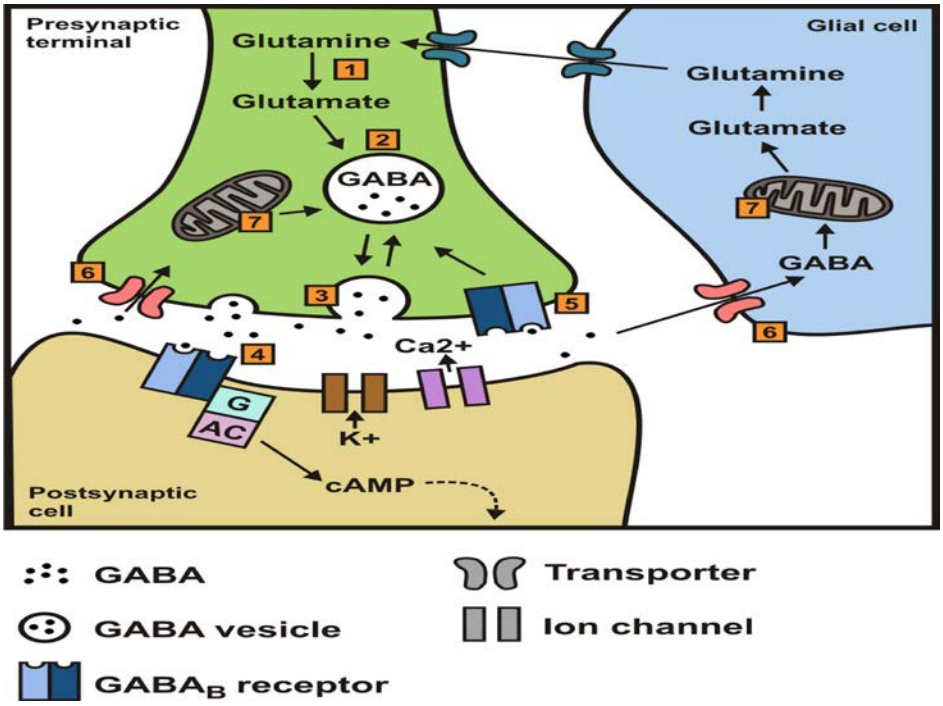


Fig.1.16: Schematic diagram showing GABA synthesis pathway. Synthesis of γ-aminobutyric acid (GABA) from glutamine/glutamate (catalyzed by l-glutamate decarboxylase (GAD); (2) transport and storage of GABA; (3) release of GABA by exocytosis; (4) binding to GABABreceptors and subsequent downstream effects mediated via a G protein and/or cAMP to K+ and Ca2+ channels; (5) binding to presynaptic receptors; (6) reuptake in presynaptic terminal and uptake by glia; (7) transamination of GABA to α-ketoglutarate (catalyzed by GABA transaminase, GABA-T), thereby regenerating glutamate and glutamine; glial glutamine then re-enters the neuron (adapted from (Hyland and Cryan, 2010)).

1.10.3: GABA synthesis:

Synthesis of GABA starts from glutamine/glutamate from the glia cells. The glutamate decarboxylase (GAD) catalyses the decarboxylation of glutamate to the GABA in the pre-synaptic neuron (Martin and Rimvall, 1993). After synthesis, GABA is stored into pre-synaptic terminal vesicales with the help of vesicular GABA transporters. GABA is released from the synaptic ends on stimulation through calcium-dependent exocytosis (Gaspary et al., 1998). In the synapse, GABA diffused freely and become available to interact with its receptor located on the postsynaptic membrane. There is also reuptake of GABA to the nerve terminals from the synaptic cleft that is achieved by GABA transporters (GATs) present in the membrane. By this process, GABA is returned to the GABAergic nerve terminals for next release (Conti et al., 2004).

Extracellular Na^+ and Cl^- play a significant role in the transport of GABA and it also noted that Na^+ and Cl^- are transported for each GABA molecule (Radian and Kanner, 1983). Conti et al. (2004) has also evidenced that GABA transporters have been found on nerve terminals and glia cell membranes of whole nervous system. Hence, there is also reuptake of GABA by the glia cells. The transamination of GABA to α-ketoglutarate that is catalyzed by GABA transaminase, (GABA-T); this regenerates glutamate and glutamine in the glia called the glial glutamine. Then this glutamate/glutamine becomes available for synthesis of GABA (Fig.1.16).

1.10.4: GABA receptors:

After synthesis and release, GABA acts as an inhibitory neurotransmitter through the GABA receptors present in both the pre-synaptic and post synaptic membranes of neurons and glia cells (Barakat and Bordey, 2002). The GABA receptors have been divided into three classes: $GABA_A$, $GABA_B$ and $GABA_C$ (Chebib and Johnston, 2000). $GABA_A$ receptor is localised in gray matter of the spinal cords as well as in glia cells. This receptor is an ionotropic ligand gated Cl^- channel. The Cl^- permeability increases upon the activation of $GABA_A$ receptor and post synaptic neurons are hyperpolarized, finally leading to increase the resting membrane conductance of the cell (Jensen et al., 2002). $GABA_B$ receptors are metabotrophic kind of receptors, localised superficially in the spinal cord dorsal horn in neurons as well as glia cells (Charles et al., 2003). It has been shown that $GABA_B$ receptor activation results in the inhibition of synaptic transmission in the spinal cord through decrease of calcium activity at the pre-synaptic end and hyperpolarization at the postsynaptic end via potassium ion conductance (Bowery et al., 1980). The GABAc receptor, actually is a subtype of the $GABA_A$ receptor and has been shown insensitive to benzodiazepine and it also seems to play a significant role in the cognition and neuronal processing as well (Johnston, 1996).

1.10.5: $GABA_A$ receptor characteristics:

The $GABA_A$ receptor belongs to ligand gated ion channels family (Stephenson, 1995). The $GABA_A$ receptor channels consist of five glycoprotein subunits to forming a functional Cl^- channel. $GABA_A$ receptor is available for regulation of opening and closing of channel pore on binding of GABA. $GABA_A$ receptor has also the property of containing agonist (GABA, barbiturates, propofol and anaesthetic steroids) as well as antagonist (bicuculline and flumazenil) recognition sites (Stephenson, 1995). It is shown that drugs have been involved to modify the transition rates between the closed,

open and desensitized states (Macdonald and Olsen, 1994). It is also shown that benzodiazepines, propofol and barbiturates have been shown to increase the affinity of $GABA_A$ receptor for GABA and thus rate of binding of GABA to $GABA_A$ receptors has been improved (Macdonald and Olsen, 1994). The $GABA_A$ receptor in the mammalian tissues has been divided into 20 subunits and grouped on the basis of their sequence. There are six α and four β, three γ, one δ, one ε, one π, one θ and three ρ subunits (Sieghart et al., 1999).

1.10.6: $GABA_A$ receptor and anaesthetics:

Aanaesthetic drugs play a significant role in influencing the $GABA_A$ receptor performance through various mechanisms. However, it is still not clear about the mechanism which is involved in the sedation, hypnosis and anaesthetic state process (Tanelian et al., 1993). It has been shown that barbiturates and propofol are able to activate the $GABA_A$ receptor. These drugs increase the GABA binding to the receptor that is further involved in desensitization (Orser et al., 1994).

1.10.7: Propofol's effects on $GABA_A$ receptor:

Propofol (2, 6-di-isopropylphenol) is a general anaesthetic for clinical use and its intravenous injection causes a reliable kind of consciousness without causing dependent tolerance, as it has been noted in other anaesthetics like barbiturates and benzodiazepines (Fassoulaki et al., 1994). Here use-dependent tolerance is the increasing dose requirement of the anaesthetics to regulate the same constant level of anaesthesia. Propofol is able to disturb the $GABA_A$ receptor performance indirectly by perturbing the plasma membrane as it is highly lipophilic (Tonner et al., 1992). It has been noted that propofol enhances the inhibitory synaptic transmission and potentiates the GABA induced depolarization in the rat olfactory cortex (Collins, 1988).

1.10.8: BDNF and GABA:

$GABA_A$ receptor's functions have been shown to be modulated by the BDNF. As an example, $GABA_A$ receptor responses are observed to be inhibited by the BDNF in the hippocampus of postnatal day 14 by increasing intracellular Ca^{2+} ions levels through the activation of TrkB receptors (Tanaka et al., 1997). However it has also been evidenced that in hippocampus; the postsynaptic $GABA_A$ receptor responses have been potentiated reversibly by the BDNF in the postnatal day 6 (Mizoguchi et al., 2003). Immunoreactivity for some subunits of $GABA_A$ receptor was shown to be

reduced with a BDNF treatment in the majority of neurons (Brunig et al., 2001). Early maturation of GABAergic inhibition in the visual cortex has been noted in the mice over expressing the BDNF (Huang et al., 1999).

1.11: Modulation of BDNF by perioperative conditions:

1.11.1: General anaesthetics and their mode of action:

Currently used anaesthetics have been divided into two groups on the basis of their mechanism of action: anaesthetics may act either by increasing the inhibition through the $GABA_A$ receptors like propofol, barbiturates, etomidate or act by decreasing the excitation via NMDA receptors like ketamine, nitrous oxide etc (Franks and Lieb, 1994, Jevtovic-Todorovic et al., 1998). Their suggested mechanism is to affect the synaptic transmission through the modification of GABA and NMDA receptors (Campagna et al., 2003). It has been shown that general anaesthetics function as allosteric positively or negatively modulators on ligand gated ion channels. It is also described that these ligand gated ions channels, especially the receptors for GABA and glutamate, are altered by the anaesthetics (Hemmings et al., 2005). Most of the inhaled anaesthetics are involved in increasing action of GABA receptor via channel opening and enhancing the inhibition at both pre-synaptic and postsynaptic receptors. In intravenous anaesthetics including propofol, an alteration of GABA receptor action occurs through the increasing gating of the receptors by the GABA.

1.11.2: General anaesthetics and BDNF:

General anaesthetics are known to induce neuronal depression and theoretically impact the synthesis of BDNF. It has been evident that BDNF is altered by the physical activity in rat brain areas (Neeper et al., 1996). In response to exercise in human, BDNF levels have been significantly elevated (Ferris et al., 2007). Only few data regarding anaesthetic effects of general anaesthetics on the modulation of BDNF in the brain or plasma are available. Some studies indicate that anaesthesia might modulate BDNF levels in the rodent brain and anaesthesia is involved in the modulation of BDNF activated apoptotic pathway in the two important brain regions, the cerebral cortex and thalamus (Lu et al., 2006). Another study has shown that anaesthesia causes a significant down regulation of BDNF protein in rat's thalamus 2 h compared to 0 h and 6 h of anaesthesia. But in cerebral cortex, anaesthesia exposure triggered significant up regulation of BDNF protein after 4 and 6 h compared to 0 h (Lu et al.,

2006). BDNF and TrkB cDNA has been shown to increase in response to ketamine in rat pups and this response may be due to ketamine induced injury (Ibla et al., 2009).

1.11.3: Surgery and BDNF:

In few studies, surgery has been shown to effect BDNF expression. Combined effect of propofol with minor surgery showed a decrease in plasma BDNF concentrations that were observed 15 minutes after propofol administration in patients undergoing minor surgery during the perioperative period (Vutskits et al., 2008). Another study also shows a decrease levels of BDNF in the hippocampus after orthopaedic surgery (Fidalgo et al., 2011).

1.13: Perioperative Pathologies:

Interestingly, elder people are at more risk for CNS dysfunction, especially cognitive dysfunction after anaesthesia and surgery.

1.13.1: Postoperative cognitive dysfunction (POCD):

POCD refers to a decline in cognitive performance and intellectual functioning including loss of memory and/or concentration (Moller et al., 1998). Cognitive dysfunctions are more prevalent over the age of 60 that is 15-25% and 10% have symptoms three months after operation (Olin et al., 2005). It is further studied that patients having post-operative cognitive disorders are more prevalent to lose further cognitive performance few years after operation (Lewis et al., 2007).

However, the mechanisms involved in POCD are not clear yet. Studies have suggested that over age, duration and type of anaesthesia and repetition of surgical procedures may be the risk factors of the POCD. However, there is clue about the individual contribution of anaesthetics and major surgery for POCD (Moller et al., 1998, Rohan et al., 2005). Some other factors like hypotension, hypoxia-ischemia do not show any evidence for their involvement in POCD that may further suggest that it is the surgical procedures themselves that are responsible for cognitive dysfunctions (Cook et al., 2007) and cognitive dysfunctions have been shown to be potentiated by surgical procedures rather than anesthesia (Cao et al., 2010).

1.13.2: Anaesthetics and memory impairment:

As it is known that anaesthetics cause sedation and further responsible for unconsciousness. This sedation may interfere with reception of information and this may be due to the reduction of perception (Smith et al., 1994). Studies have indicated that propofol is responsible for impairment of memory and it is suggested that

propofol induced sedation is responsible for this memory impairment (Veselis et al., 1992, Pang et al., 1993, Nordstrom and Sandin, 1996). However, some studies evidenced that propofol have amnesic effects, but these amnesic effects are independent of sedation (Veselis et al., 1997, Veselis et al., 2001). Propofol also impair the memory of aversive and non-aversive events with a low dose (non-sedative dose 9mg/kg) in rodents (Pain et al., 2002).

1.14: Postoperative behavioural changes:

1.14.1: Post traumatic stress disorder (PTSD):

PTSD refers to a group of symptoms that are expressed after an exposure to traumatic stressor and characterised by fear, helplessness or horror, persistent re-experiencing of the event and avoidance of the specific stimuli that is associated with the event and also unresponsive to the general stimuli. Minor surgeries or the conditions requiring surgery can be responsible in the induction of anxiety as well as PTSD. Sometimes, the preoperative anxiety in addition to PSTD, may persist during the postoperative period (Kain et al., 1999). As in tooth removal surgeries, 8% patients have been shown to develop the symptoms of the PTSD one month after the procedure (de Jongh et al., 2008).

1.14.2: Depression and post-operative fatigue:

Depression and fatigue are the post surgical behaviour that has been involved in the alterations of brain systems (Nickinson et al., 2009, Zargar-Shoshtari and Hill, 2009). Postoperative depression either develops immediately or some weeks after the surgery. About 50% of the orthopaedic patients have been found to be postoperatively depressed (Nickinson et al., 2009). Postoperative fatigue has been reported about four weeks after the surgery and it happens around 30% in surgical patients (Kehlet and Christensen, 1988). Certain surgeries like abdominal, gynaecological and cardiac are more literly to induce the postoperative fatigue syndrome (Rubin et al., 2004).

1.14.3: Chronic pain:

Due to unknown reasons, even minor surgeries may be responsible for the induction of chronic pain (Kehlet et al., 2006). Acute postoperative pain followed by persistent pain in 10-50% of the individuals after surgeries including inguinal hernia, mastectomy, thoracic surgery (Cunningham et al., 1996, Kehlet et al., 2006, Maguire et al., 2006). Severity of the chronic pain can be 2-10% among these patients, the main cause of the chronic pain has been reported to be iatrogenic nerve damages

(Kehlet et al., 2006). Development of persistent pain is linked with the severity of the acute postoperative pain (Kehlet et al., 2006). Some studies have highlighted the lack of knowledge about the endogenous pain modulation variation in different individuals that may become responsible for some patients to be at greater risk for the modulation of postoperative chronic pain (Yarnitsky et al., 2008).

1.14.4: Cognitive decline in the elderly:

naesthetics are responsible for damaging effects, not only on young brain but also on elderly brain. However anaesthetics effects on the aging brain are extremely studied currently under great investigation (Durieux, 2010). Anaesthetics roles have been implicated in the modulation of AD as well as POCD. Oligomers of the amyloid-β have been evidenced in the pathogenesis of neuronal damage in AD as well as in other neurodegenerative disorders. Inhaled anaesthetics have been described to interact with the amyloid-β and further become responsible for its oligomerization, suggesting pathogenic role of anaesthetics in AD (Tang et al., 2010). Usually, anaesthetics are given for a surgical operation. However, anaesthetics effects, along with surgery cause more severe POCD as compared to anaesthesia alone (Wan et al., 2007).

1.14.5: Stress during perioperative period:

During the perioperative period, acute stress has four main sources.

- Anxiety
- Pain
- The surgical stress response
- Neurotoxicity of anaesthetics

Anxiety is known to play an important role on brain functions, especially affecting the prefrontal cortex and amygdala. Studies in healthy volunteers evidenced that prefrontal cortex may be involved in the pathogenesis of the anxiety disorders (Spampinato et al., 2009). The hippocampus and anterior cingulate cortex have been affected in the clinical anxiety like PTSD (Ferrari et al., 2008). Changes in the size of amygdala and functional alteration in the amygdala and prefrontal cortex have been noted in the anxiety disorders (Etkin and Wager, 2007, Hayano et al., 2009).

Pain it has been noted that nociceptive inputs during the perioperative period may be responsible in altering the gene expression, leading to rapid neuronal sensitization that may play role in CNS damage (Costigan et al., 2009). Nociceptive inputs have also been shown to be associated with behavioural changes (Besson, 1999). Neuronal

insults like pain and neuronal injury in rat neonates have long lasting effects in their old age (Ruda et al., 2000).

Surgical stress response results of an afferent neuronal input from effected site to the hypothalamus that further results in the activation of endocrine and inflammatory responses, the strength and period of these responses is directly linked with the degree of the surgical injury. During the surgical stress response, cortisol may be excessively released under specific conditions. Hence, major surgery has been reported to induce cortisol release (Malatinsky et al., 1986). Similarly, cortisol secretion has also been reported in dental surgical treatment (Miller et al., 1995). The over secretion of cortisol, epinephrine, nor-epinephrine and growth hormone during preoperative cardiac surgery has been reported (Roth-Isigkeit et al., 1998).

Neurotoxicity induced by the anaesthetic agents in rats and other mammals including human has great impact on CNS. Recent investigations have reviewed the pathogenic changes induced by the anaesthetics (Stratmann et al., 2009, Creeley and Olney, 2010). Apoptotic neurodegeneration has been reported in the developing brain during synaptogenesis by the $GABA_A$ receptor agonists or NMDA receptor antagonists (Ikonomidou, 2009). A mixture of anaesthetics including midazolam, isoflurane and nitrous oxide has resulted in apoptotic neurodegeneration, hippocampal synaptic deficits and memory impairments in rodents (Jevtovic-Todorovic et al., 2003). Neurotoxicity by some other anaesthetics has also been reported including ketamine, diazepam and propofol (Patel and Sun, 2009).

2: Objectives

The main objective of the present study was to understand the post-operative effects on the modification of BNDF protein in the hippocampus and prefrontal cortex. For this purpose, the following specific objectives were pursued:

Is there any circadian rhythmicity of BDNF protein in the hippocampus and cortex in the nocturnal and diurnal rodents?

What is the effect of the short duration anaesthetics (propofol) on the circadian rhythmicity of BDNF protein in the hippocampus and cortex of rodents?

How BDNF protein is altered in rodent's brain when subjects are submitted to propofol anaesthesia and minor surgey under propofol anaesthesia.

Is there any effect of LPS on the BDNF protein expression in the hippocampus and cortex?

3. General Methodology

All the experiments were conducted in accordance with institutional guidelines complying with national (Council directive 87848, 19 October 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale) and international guidelines (NIH publication, no. 86-23, revised 1985).

3.1: Animals:

Young male Sprague Dawley rats obtained from Charles River were used in all experiments except *Arvicanthis ansorgei* which were used to study the circadian rhythmicity of BDNF protein as model for diurnal rodents. Data has showed that *A.ansorgei* is an interesting animal model for circadian studies in diurnal animals (Challet et al., 2002).

3.2: Circadian approach:

Animals were housed six rats per cage in a temperature (20±1C°) and humidity (40 ± 2%). Rats were kept in controlled environment for 15 days on a twelve-hour light/dark cycle (lights on 7am), with *ad libitum* access to food and water. To study the normal physiological circadian variation of the BDNF protein, subjects were sacrificed at different zeitgeber time (ZT). Zeitgeber comes from the German word Zeit means time and Geber means giver, so zeitgeber means time-giver. The term Zeitgeber was introduced into science about 1954 by Jurgen Aschoff and light is one of the zeitgebers. Zeitgeber time is a standard of time based on the period of a zeitgeber. Note: Under standard light-dark cycles, the time of lights on usually defines zeitgeber time zero (ZT 0) and the time of lights off defines zeitgeber time twelve (ZT 12). ZT0 and ZT12 are the time of onset of activity and off set of activity respectively in diurnal animals and vice versa in nocturnal rodents (Fig.3.0 and 3.1).

3.3: Short duration anaesthesia:

Animals were injected intraperitonaely either with propofol short duration (30min) anaesthesia (*Fresenius, France*) at the rate of 120mg/kg or with its intralipid control, Intralipid (*Fresenius, France*). Following injection of propofol, all animals lost their righting reflex in 5-8min, loss of righting reflex can be defined as the inability of the body to maintain its balance and the animal is unable to stand on its feet. Duration of loss of righting reflex has been shown to follow circadian rhythms during 24 hours of the day (Challet et al., 2007), Fig.3.2.

Recovery of loss of righting reflex was shown to be varied at different zeitgeber time ranging 35-40minutes. However, animals recovered not only the loss of righting reflex but they also recovered their spontaneous locomotion after one hour of propofol

anaesthesia. During anaesthesia state, animal's room temperature was increased up to 24C° to avoid hypothermia.

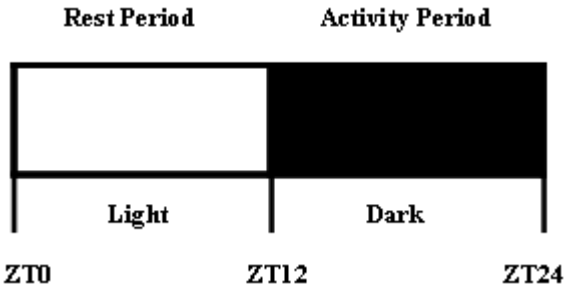


Fig.3.0: Schematic representation of rest and activity period in nocturnal rodents.

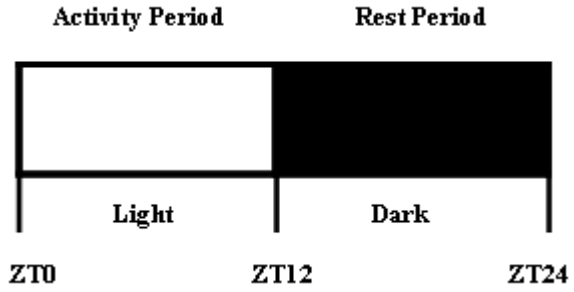


Fig.3.1: Schematic representation of rest and activity period in diurnal rodents.

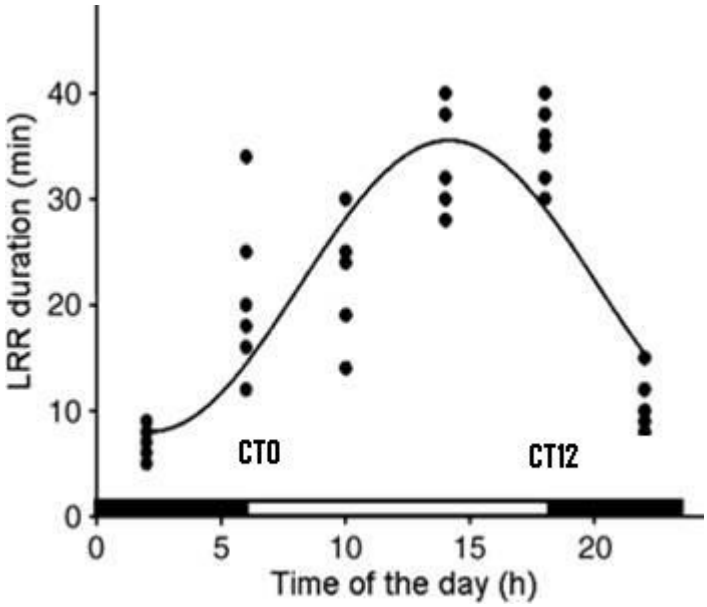


Fig.3.2: Represnts variation of loss of righting reflex following propofol injection in rats (adapted from (Challet et al., 2007)).

3.4: LPS treatment:

Male rats received an intra peritoneal injection of LPS from an *E.coli strain* (*E.coli* 0171: B_4, Sigma USA) at 1mg/kg with its intralipidic controls. Animals were injected at ZT10 and brains were removed after 3days post injections. To study the combined effect of LPS and anaesthesia, animals were injected with LPS plus propofol with their control intralipid.

3.5: Minor surgery:

For the purpose of minor surgery in rats, midline laparotomy was performed under short duration anaesthesia propofol at 120mg/kg intraperitoneally.

Protocol for laparotomy

Hair Removal

- Fur along the incision site was removed with the small clipper.
- Incision area was clipped as to ensure fur does not contaminate the wound and sufficient area that can be disinfected properly.
- It was also avoided to take off too much fur, because this may reduce the animal's ability to regulate its body temperature.

Antiseptic preparation of surgical site

- A solution consisting of three alternating scrubs of an iodophor or chlorhexidine and 70% alcohol was used as antiseptic.
- Cleaning with soaked cotton was done in circular motion.
- Cleaning was begun from the center of the shaved area to the periphery.

Laparotomy

- Animals under anaesthesia were placed on a small surgical table provided with proper light source.
- Hands were washed properly with antiseptic soap and sterile gloves were used.
- Local anaesthesia (Xylocaine^{R}10%) was sprayed at the incision site to reduce the pain and a midline incision of about 2cm was done with sharp surgical blade.
- Intestine was taken out from the incision site, was shaken well and was put back to its place.
- Small sterile drapes were used to clean the blood around the incision area.
- Then surgical incision was closed with surgical staples. 3-4 staples were used to close the incised area (comprehensive steps involved in laparotomy are elaborated in the Fig.3.3)

Perioperative care

- Normal body temperature was supported during the peri-operative period, as rats have high surface area and lose body heat by conduction process. A major cause of surgical mortality is not always the surgery or the anaesthesia but hypothermia. Body temperature drops precipitously under sedation or anaesthesia. Low body temperatures can cause irreversible shock and death.
- Animals were provided with a heat source during the pre, intra and post-operative periods. For this purpose, circulating hot water blankets were used.
- Animals were placed on insulating materials (folded drape) and heating lamps were placed at a distance to control the heating.

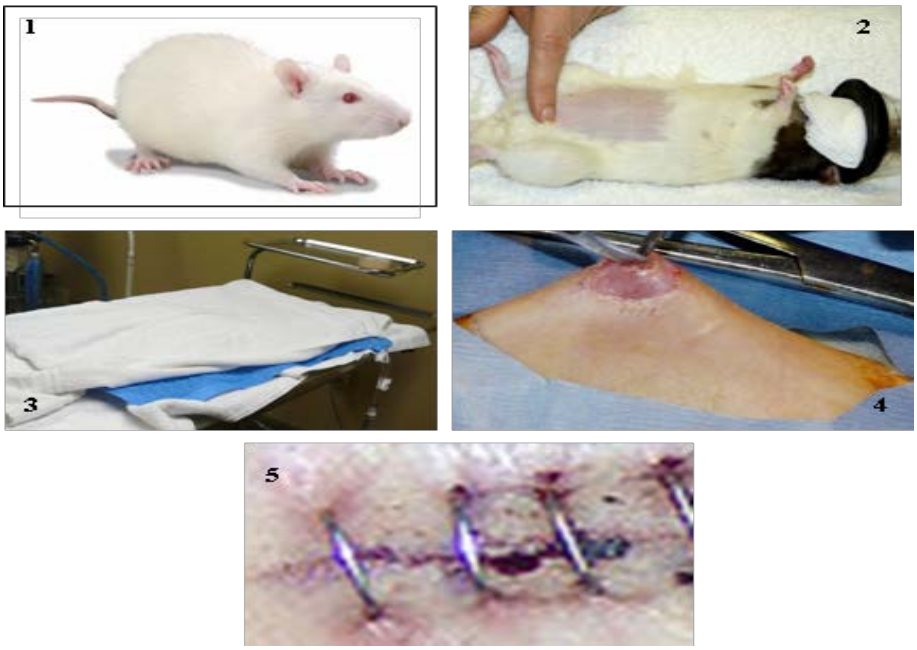


Fig. 3.3: Diagramatic steps of minor surgery (laparoscopy) in rat.

3.6: Preparation of animals:

After 15 days in a controlled environment, animals were physically examined. Healthy and normal subjects were considered for experiments. Rats fit for the experiments were sacrificed at six different zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17 and ZT21) to study the normal physiological circadian rhythms of BDNF. Effect of propofol on BDNF circadian profile was observed at four different points (ZT5, ZT11, ZT17 and ZT23). For short and long term effects of propofol anaesthesia, animals were sacrificed at ZT5 (peak of BDNF). Six to eight subjects were kept in each group in different experiments.

3.7: Preparation of samples:

Subjects of each group were sacrificed at their specific zeitgeber time after euthanasia under CO_2 at concentration of 0.5 bars for maximum one minute. After euthanasia by exposure of carbon dioxide; the brains were quickly removed on ice. Cortex and hippocampal structures for each subject were removed. Typical extraction buffer was used to preserve the desired tissues. Typical extraction buffer contains:

- TrisHCl 50mM
- $MgCl_2$ 5mM
- DDT 1mM

- PMSF 0.5mM
- EDTA 0.1mM
- EGTA 0.1mM and NaCl 0.9%

Cortex and hippocampal tissues were triturated with the buffer and centrifuged at the rate of 4000 rpm for 20 minutes. Supernatant of about 400µl for the cortex and hippocampus were collected and stored at -20 till quantification of BDNF protein using ELISA.

3.8: Enzyme-Linked Immunosorbant Assay (ELISA).

Briefly describing, prior to BDNF analysis, protein contents were determined using a protein assay kit (Bio-Rad CA, USA). BDNF proteins were quantified using a rat-BDNF ELISA kit (Merck-Millipore, Molsheim, France) according to manufacturer's recommendations. Briefly, 100µl of the samples diluted (1:2) with the sample diluent provided in the kit were loaded in the 96 wells ELISA plate and incubated for 12 h at 4C°. The plates were washed four times (300µl) with wash buffer provided in the kit. Then 100µl of anti-BDNF antibody (1:1000, v:v) were added in each well and let incubated for 3h at room temperature. Wells were washed four times with wash buffer and 100 µl of streptavidine HRP (1:1000, v: v) were added and let incubated for one hour at room temperature. Then, wells were washed four times with wash buffer and 100µl of TBM were added. Coloration was let developed during 15 min and blocked by adding 100µl of stop solution. ELISA plates were read at 450nm with a plate reader (Thermo Scientific Multi Scan FC, Courtaboeuf, France). BDNF standards were diluted in the solution of sample diluting buffer and extraction buffer (1:1, as for the sample). Standard and samples were loaded in duplicates. The CV values were between 0% to 7%. All samples with a higher CV value were retested in order to obtain a CV below or equal to 7%.

3.9: Memory performance test:

Animals were submitted to short duration propofol anaesthesia at 120 mg/kg intraperitoneally with its intralipid control. The anaesthesia was performed at ZT10 and conditioning was performed at ZT5, after day 1, day3 and day5. Animals were tested for memory either 24 hours after conditioning (immediate recall) or 10 days after conditioning (delayed recall) depending on the group allocated (n = 8 per group). For conditioning purpose, we used passive avoidance apparatus.

Passive avoidance apparatus consisted of two adjacent compartments, a bright large one and a small dark one, separated by a guillotine door (Fig.3.4). The bright compartment had white plexiglas walls (46 cm long, 42 cm large and 44 cm high) and was illuminated by a 25 W bulb fixed on the wall opposite to the entrance to the dark one. This latter compartment had black plexiglas walls and roof (30 cm long, 15 cm large and 15 cm high). The roof could be opened to place directly and observe the animal in this compartment. The floor of the two compartments was made of stainless steel bars (0.6 cm diameter) regularly spaced apart by 1.8 cm. Scrambled foot shocks could be delivered through the bars of the dark compartment. The scrambler was set to deliver two moderate foot shocks at t_1 = 2:00 and t_2 = 3:30 min during a 5-min session (effective intensity, 0.1 mA; 5 s: duration; 20 ms ON-140 ms OFF).

In this apparatus, naïve undrugged rats exhibit a spontaneous fear for the bright large compartment and escape quickly (within 10 s) from it to enter the adjacent dark small one. To assess the memory for an aversively loaded event, we used a "classic fear-conditioning preparation" for the acquisition phase of learning.

Classic fear conditioning preparation

- Each rat was placed (*via* opening the roof) and confined in the small dark compartment where it experienced two mild electrical foot shocks during a 5 minutes period.
- The animal was tested for memory of the aversive experience by assessing the passive avoidance of the small dark compartment. When initially shocked in the small dark compartment, the rat has to remember this event to refrain from its natural tendency to enter this compartment when subsequently exposed to the bright large compartment. The fact that an animal enters the small dark compartment while he has been previously drugged during the conditioning session indicates no memory of the initial training.
- The dependent variable was the latency to enter the small dark compartment (cutoff, 900 s). Statistical analysis was performed on the Log10 of this variable (Log latency) to better approximate the normal distribution necessary for the use of parametric statistics.

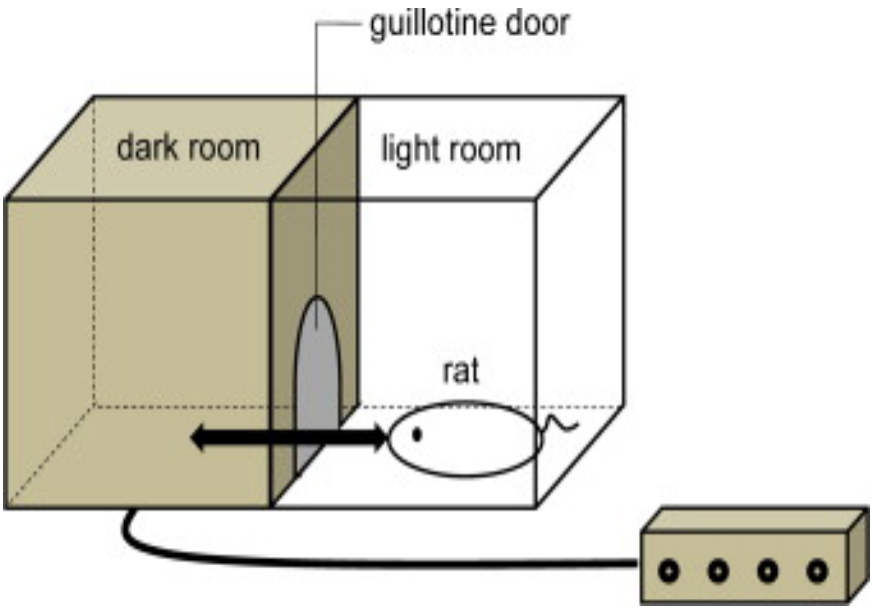


Fig.3.4: Passive avoidance apparatus

4. Experiments

4.1. Experiment 1

Time of the day expression of the BDNF protein in hippocampus and prefrontal cortex.

Pre-clinical and clinical studies have shown that BDNF vary according to the time of the day (Bova et al., 1998; Berchtold et al., 1999, Schaaf et al., 2000, Katoh-Simba et al. 2007, Begliuomini et al., 2008, Begliuomini et al., 2008) in young male volunteers. Pre-clinicalal data showed an increase of hippocampal BDNF mRNA (Bova et al., 1998; Berchtold et al., 1999) during the dark period as compared to the light period in rats. Conversely, Katoh-Simba et al. (2007) showed a clear increase of BDNF protein during the light period as compared to the dark period in both cortex and serum in rats. However, to what extent BDNF protein expression in hippocampus follows a circadian pattern remains undetermined. Moreover, the relationship between cortical and hippocampal BDNF has not been yet examined. From a physiological point of view, the expression of BDNF protein rather than mRNA is important to determine. So the present study was initiated to examine putative circadian rhythms of BDNF protein in prefrontal cortex and hippocampus in nocturnal (Sprague Dawley) and diurnal/crepuscular murid (*Arvicanthis ansorgei*) rodents. Indeed, *Arvicanthis ansorgei* appears a useful experimental model to understand the regulation of the circadian rhythms in day active species (Challet et al., 2002, Verhagen 2004).

Experiment 1A: Sprague Dawley rats

Specific Methodology

Forty-eight rats Sprague Dawley(Charles River) were divided into six groups on the basis of zeitgeber time (ZT) for six zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17 and ZT21). The BDNF protein expression was examined by ELISA in the supernatant of brain structures (hippocampus and prefrontal cortex) at every four hours during a 12/12 light-dark period of the day.

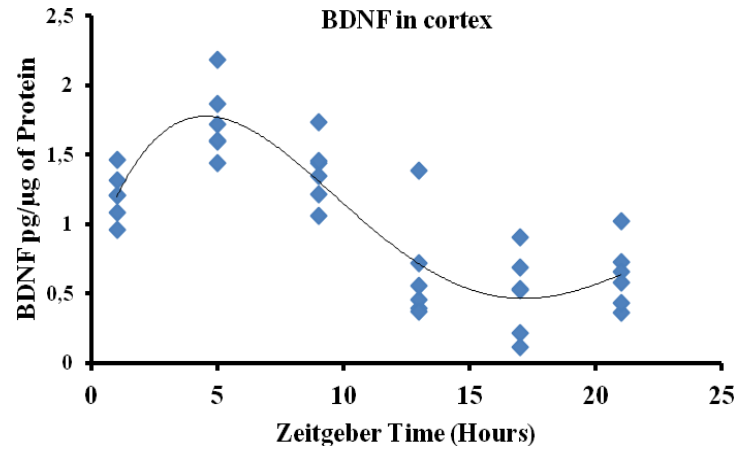
Results

Figure 1 shows value of BDNF protein in the cortex and hippocampus supernatant at different zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21). Two way analysis of variance on BDNF content (between factors structure and time evidenced that there are significant differences between structure (cortex or hippocampus; $F(1, 30) =$

8.862, P=0.006) and between time (ZT1, 5, 9, 13, 17 or 21; F (5, 30) = 18.72, $P<10^{-4}$), but no significant interaction between brain areas and time (F (5, 30) =0.44).

Cortex: One way analysis of variance detected a significant effect of time (F (5, 30) = 20.16; $P<10^{-4}$ on BDNF content in the cortex (Fig.1 A). Cosinar analysis showed circadian rhythm of BDNF protein with a peak cosinor parameter of 5 h 18 min. (F (2, 35) =48.81; $P<10^{-4.}$

Hippocampus: One way analysis of variance detected a significant effect of time (F (5, 30) = 5.971; $P<10^{-4}$ on BDNF protein content in the hippocampus (Fig.1A). Cosinar analysis showed circadian rhythm (F (2, 35) =12.39; $P<10^{-4)}$ of BDNF protein in hippocampus with a peak cosinor parameter of 5 h 42 min.



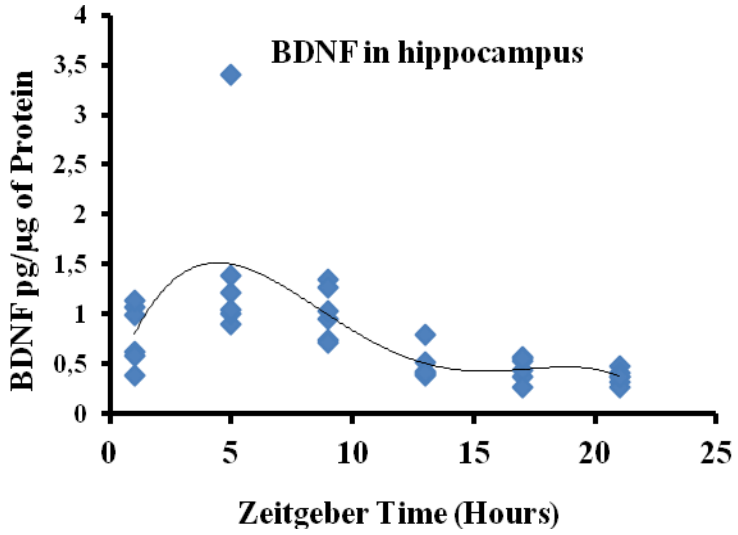


Figure1A. BDNF protein (pg/µg of protein) the prefrontal cortex and hippocampus supernatant at different zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21) in Sprague Dawley rats. Cosinor analysis of data of BDNF protein during 24 h shows a unimodal circadian rhythm of BDNF protein in both prefrontal cortex and hippocampus.

Experiment 1B: ***Arvicanthis ansorgei***

Specific methodology

Forty-eight animal were divided into six groups on the basis of Zeitgeber time (ZT) for six zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17 and ZT21).

The BDNF protein expression was examined by ELISA in the supernatant of brain structures (hippocampus and prefrontal cortex) at every four hours during a 12/12 light-dark period of the day.

Results

Two way analysis of variance on BDNF content (between factors structure and time evidenced that there are significant differences between structure (cortex or hippocampus; $F(1, 30) = 9.602$; $P<0.001$) and between time (ZT1, 5, 9, 13, 17 or 21; $F(5, 30) = 14.52$; $P<10^{-4}$), and a significant interaction between brain areas and time ($F(5, 30) =11,56$; $P< 0.001$). Fig 1B.

Cortex: One way analysis of variance detected a significant effect of time ($F(5, 29) = 2, 32$; $P<0.05$ on BDNF content in the cortex. Cosinor analysis showed circadian rhythm of BDNF protein with a peak cosinor parameter of 4 h 40 min. ($F(2, 34) = 4.75$; $P<0.01$.

Hippocampus: One way analysis of variance detected no significant effect of time ($F(5, 29) = 1.90$; $P=0.1768$ on BDNF protein content in the hippocampus. Cosinor analysis showed a statistical trend to fit a bimodal circadian rhythm ($P=0.09$) of BDNF protein in hippocampus.

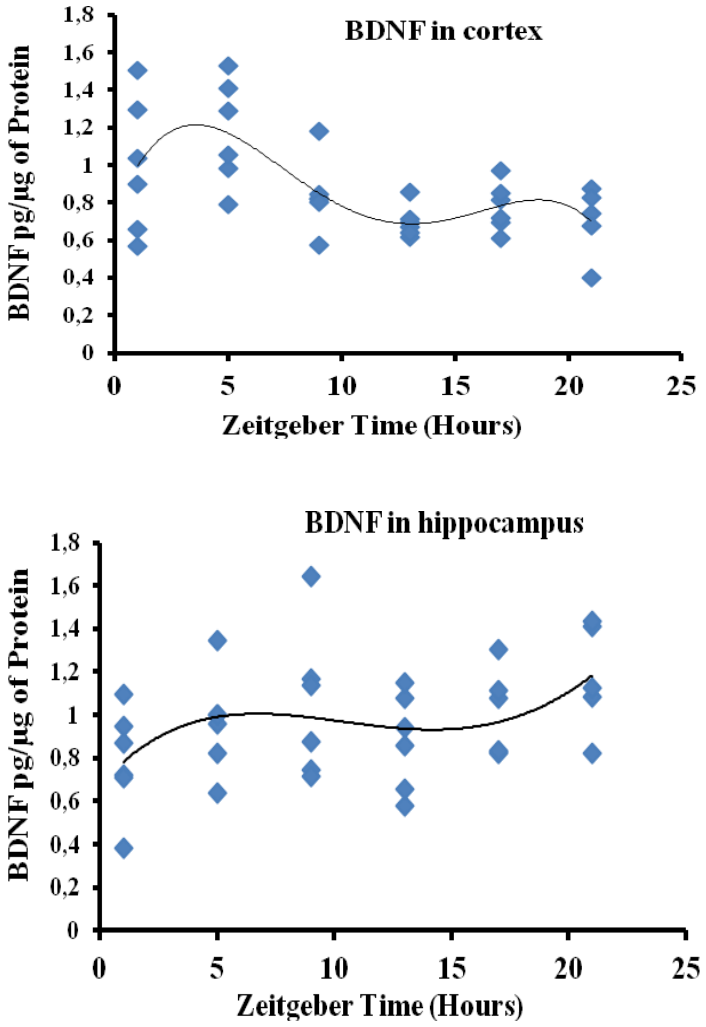


Figure1B. BDNF protein (pg/µg of protein) the prefrontal cortex and hippocampus supernatant at different Zeitgeber times (ZT1, ZT5, ZT9, ZT13, ZT17, and ZT21) in *Arvicanthis ansorgei*. Cosinor analysis of data of BDNF protein during 24 h shows an unimodal circadian rhythm of BDNF protein in the prefrontal cortex but not in the hippocampus.

Discussion of the data from experiment 1A and 1B

Whatever the temporal organisation of the rodent, nocturnal or diurnal, BDNF protein exhibits an unimodal circadian rhythm in prefrontal cortex. In *A.ansorgei*, we observed a circadian unimodal rhythm of BDNF protein in cortex, with a peak cosinor at 4 h 40 (after the light on) that mimicked the results obtained in SD nocturnal rats (5 h18 min.). Then BDNF is decreased gradually with a minimum at around ZT17. The similar results obtained on both nocturnal SD rat and *A.ansorgei* demonstrated that the expression of BDNF is increased according to the light –dark cycle and not according

to the activity-rest cycle. From our results, we may suggest that the expression of BDNF protein in cortical structures is under the influence of photic synchronisers via the SCN pathway.

Our data showed clearly that the temporal organization of the rodent, either nocturnal, or diurnal, is responsible for different circadian patterns of BDNF protein in hippocampus. We observed a bimodal rhythm of the hippocampal BDNF protein, with two peaks, ZT 5 and ZT 21, respectively, in *A.ansorgei*. Such results were globally in phase opposition to what we observed in SD rat in the same experimental conditions. Also in these species, the overall organization of the SCN was found to be fairly comparable to that of nocturnal species, although also some differences in circadian rhythms have been reported. In *A.ansorgei,* some circadian rhythms are bimodal and in phase opposition to nocturnal rats, such as locomotor activity, temperature and corticosterone (Verhagen et al 2004, Cuesta et al 2009). In comparison to the nocturnal behavioural activity in rats, general and wheel running activity occur essentially during day time in *A.ansorgei*, albeit with crepuscular bouts of activity (Challet et al, 2002).

4.2. Experiment 2

Effects of surgery and propofol anaesthesia on the expression of BDNF protein in hippocampus and prefrontal cortex.

Specific methodology

At ZT11, we performed a short duration (30min) anesthesia (propofol intraperitoneally and skin local anesthesia using lidocaine), a minor surgery procedure (mini laparoscopy) in adult rats, and we measured BDNF protein contents in tissue extracts removed at the third post-operative day. Because of the observed daily variations in brain and plasmatic BDNF, the examination of brain BDNF was made during the mid subjective day (peak level of BDNF), at the same circadian time (ZT5) for each animal to avoid any bias linked to circadian variability of BDNF. Rats were divided into three groups according to the treatment they received at day 0 at the specific zeitgeber time (ZT11), control (intralipids injection), propofol, and surgery (under propofol anesthesia). Eight animals were included in each group.

Results

We observed an increase of the BDNF content in the brain supernatants when animals were submitted to propofol anaesthesia alone or to surgery under propofol anaesthesia, in both cortex (Fig. 2A) and in hippocampus samples (2B) as compared to the lipidic controls. Two way analysis of variance (between factor: procedure; within factor: brain structures) showed a significant effect of the procedure (control, propofol anaesthesia, surgery under propofol anaesthesia; $F(2,21) = 9.86$; $P=0.001$). However, no significant effect of the brain structure (cortex versus hippocampus; $F(1,21)= 2.28$) and no significant interaction between procedure and structure ($F(2,21)=2.82$) on the BDNF content in supernatant was detected. Post-hoc analyses showed that animals submitted to propofol anesthesia or surgery under anesthesia differed significantly from the control group, in both cortical (all $P<0.01$) and hippocampal structures (all $P<0.05$). For the cortical structure, we observed a marginal difference ($P=0.0590$) between animals submitted to propofol anesthesia and to surgery under propofol anaesthesia.

The concentrations of BDNF in plasma were unchanged whatever the procedure performed, propofol anaesthesia or surgery under anaesthesia (Fig. 2C). One way analysis of variance revealed no significant effect of the procedure on plasmatic BDNF ($F (2, 21) =0.27$).

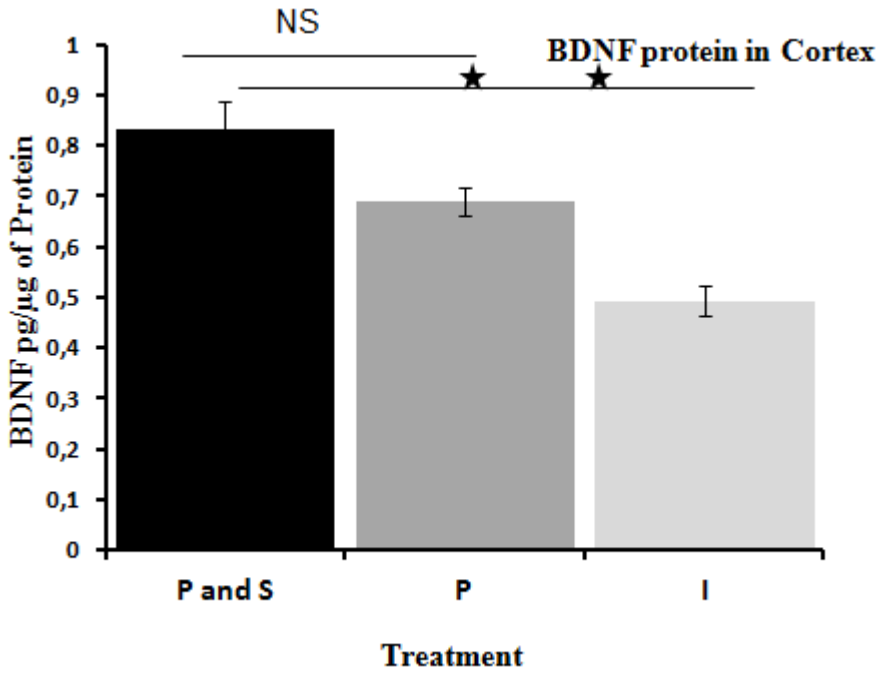


Figure 2A. BDNF (pg/µg of protein) observed in rat's cortex supernatent treated with minor surgery under propofol anaesthesia (P and S), propofol anaesthesia alone (P) or with intralipid as control (I).

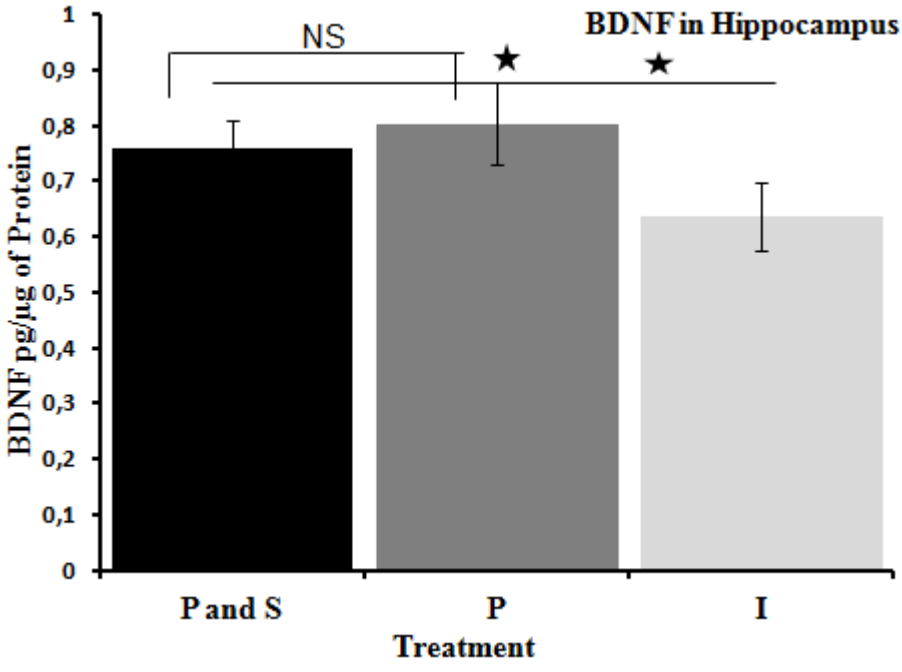


Figure 2B. BDNF (pg/µg of protein) observed in rat's hippocampus supernatent treated with minor surgery under propofol anaesthesia (P and S), propofol anaesthesia alone (P) or with intralipid as control (I).

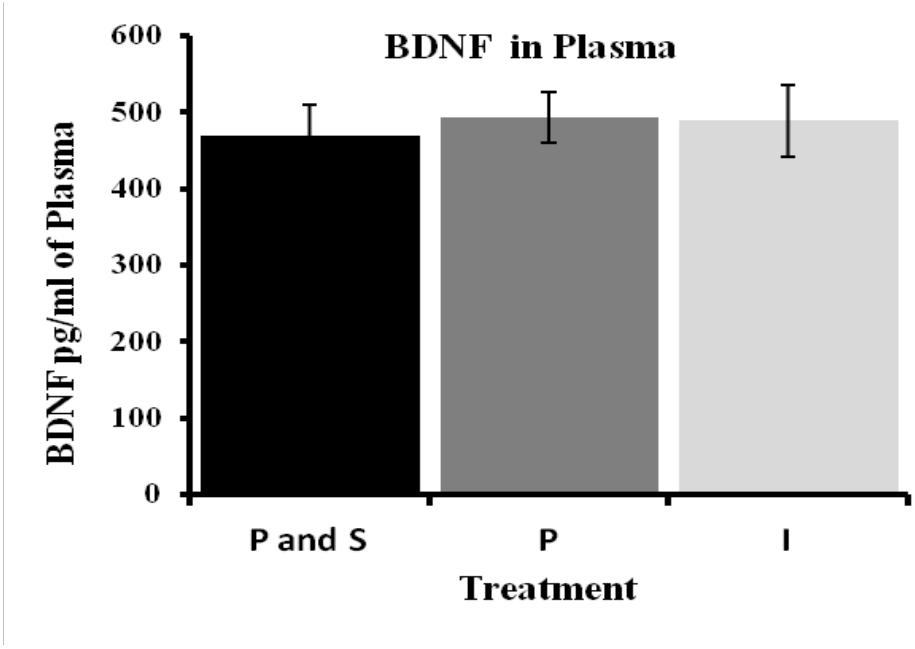


Figure 2C. BDNF (pg/ml) observed in rat's plasma treated with minor surgery under propofol anaesthesia (P and S), propofol anaesthesia alone (P) or with intralipids as control (I).

Discussion of the data

The data obtained here clearly demonstrate for the first time that the expression of the brain BDNF protein is increased at the third day following a short duration propofol anesthesia (30 min) in adult rats, thus excluding a unique role for surgery to impact brain BDNF during the post operative period.

Previous study investigating surgeries showed a post-operative decrease of BDNF proteins amounts. Indeed, tibia fracture under anesthesia has been associated with a decrease of BDNF protein in hippocampus at 24 h following surgery as compared to control naïve mice. Such a decrease was observed in parallel with learning deficit in animals submitted to surgery (Fidalgo et al., 2011). Here, we do not observe any main role for surgery as compared to the role of anesthesia. However, surgery performed in the present experiment was minor one, with no major inflammation reaction. Our data obtained here in rats following anesthesia, combined with previous ones on orthopaedic surgery and inflammation in mice, lead to suppose that the respective role

of both anesthesia and inflammation linked to surgery have to be taken into account when examining the changes of brain BDNF during the post operative period.

It must be noted that in this experiment, the increase in brain BDNF was observed with no change of the plasma BDNF concentrations sampled at the same time in animals. A previous study evidenced a decrease of plasma BDNF levels during the initial 24 h following surgery under anesthesia in patients. Based on a theoretical positive correlation between brain and blood levels of BDNF, plasma concentrations of BDNF have been then proposed as putatively reflecting the brain modifications of BDNF during the post operative period (Vutskits et al., 2008). From our results, BDNF plasma concentrations appear not reliable to inform from brain BDNF changes, at least in rodent's model.

4.3. Experiment 3

Effects of inflammation and propofol anesthesia on the expression of BDNF protein in the hippocampus and prefrontal cortex.

In experiment 2, we did not observe any main implication for surgery as compared to the role of anaesthesia. However, surgery performed in experiment 2 was minor one, with no major inflammation reaction. Both anaesthesia and inflammation linked to surgery may have an effect when examining the changes of brain BDNF during the post operative period.

To address this point, we examine here the effects of LPS (inflammatory process) and anaesthesia on BDNF protein levels in both prefrontal cortex and hippocampus.

Specific methodology

At ZT10, we performed an injection of LPS either under short duration (30 min) propofol anaesthesia or combined with the injection of the lipidic solvent of propofol in adult rats. A control group received intralipids alone. We measured BDNF protein levels in tissue extracts removed at the third post-operative day. Because of the observed daily variations in brain and plasmatic BDNF, the examination of brain BDNF was made during the mid subjective day (peak level of BDNF), at the same circadian time (ZT5) for each animal to avoid any bias linked to circadian variability of BDNF. Rats were divided into three groups according to the treatment they received at day 0 at the specific Zeitgeber time (ZT11): control (intralipids injection), propofol and LPS, intralipids and LPS. Eight animals were included in each group.

Results

We observed an increase of the BDNF content in the brain supernatants when animals were submitted to LPS injection in both propofol anaesthesia or intralipids administration conditions, in cortex (Fig. 3A) and in hippocampus samples (3B) as compared to the lipidic controls. Two way analysis of variance (between factor : procedure; within factor : brain structures) showed a significant effect of the procedure (control, LPS, LPS with propofol anaesthesia; $F(2,21) = 18.42$; $P<0.0001$) but no significant effect of the brain structure (cortex versus hippocampus; $F(1,21)= 1,88$) and no significant interaction between procedure and structure ($F(2,21)=2.32$) on the BDNF content in supernatant have been observed. Post-hoc analysis showed that animals submitted to LPS injection (propofol anesthesia or intralipids) differed significantly from the control group, in both cortical ($P<0.001$) and hippocampal structures ($P<0.001$). For both structures, we observed a significant decrease of the

BDNF expression (P<0.01) between animals submitted to LPS injection under propofol anesthesia and to LPS injection under intralipds.

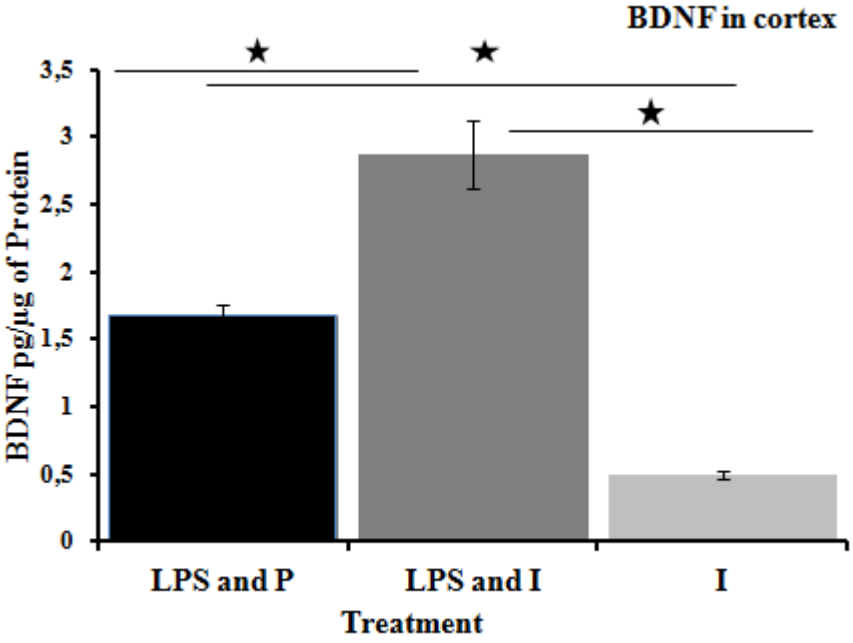


Figure3A. BDNF (pg/µg of protein) observed in rat's cortex supernatent treated with LPS either under propofol anesthesia (LPS + P) or with intralipids (LPS + I) with intralipid as control (I).

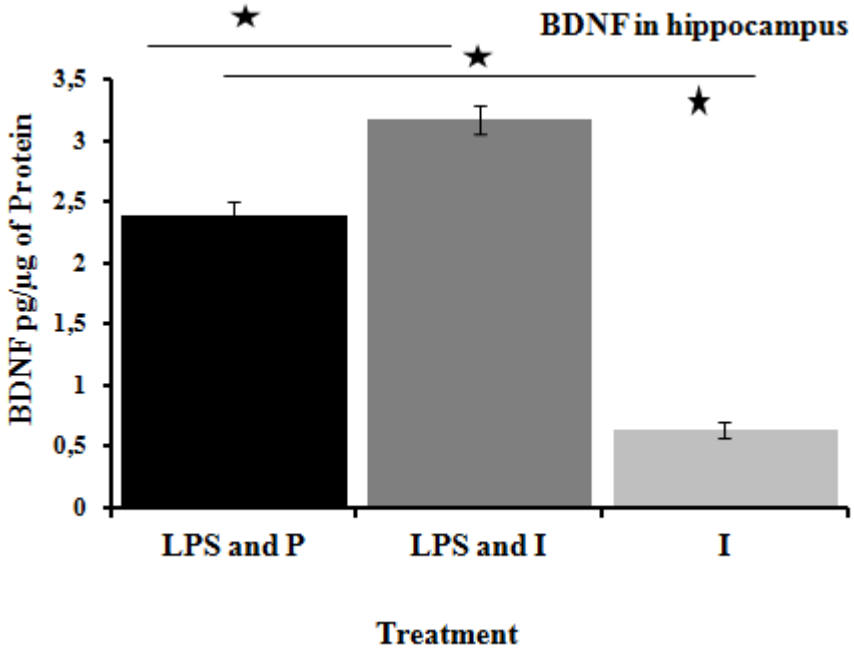


Figure 3B. BDNF (pg/µg of protein) observed in rat's hippocampus supernatent treated with LPS either under propofol anaesthesia (LPS and P) or with intralipids (LPS and I) alone and with intralipid as control (I).

Discussion of the data

The data obtained here clearly showed that the expression of the brain BDNF protein is largely increased at the third day following a single injection of LPS. The short duration propofol anesthesia (30 min) appeared to counteract slightly this increase. Data from experiment 2 and 3 suggest that both anaesthesia and inflammation have impact on brain BDNF during the post operative period.

Previous studies have revealed effects of LPS on neurotrophic factors as one study shows that LPS stimulates the synthesis of BDNF and causes an increased level of BDNF mRNA and BDNF immunoreactivity in the cultured microglia (Miwa et al., 1997). However, another study by Lapchak et al.(1993) shows decrease of BDNF mRNA just after 4 h of injection of either LPS at 500µg/kg or equal volume of saline as control. Intraperitoneal injections of LPS after 7 h also showed that reduced level of BDNF protein in the rat's hippocampus (Guan and Fang, 2006). This reduction in BDNF protein and mRNA few hours after LPS injection may be due to the immediate effect of LPS. Our present study differs in way that we observed effect of LPS and combined LPS with anaesthesia on BDNF protein after three days of treatments and at a specific zeitgeber time (ZT5) that has peak value of BDNF without any treatment. Furthermore anaesthesia combined with LPS decreases BDNF that may reflect the protective effect of propofol. However, the increased BDNF proteins levels may also be important, as some behavioural and chemical data shows the detrimental effects of over expression of BDNF in learning and memory (Cunha et al., 2009).

4.4. Experiment 4

Proper effects of propofol anaesthesia on the expression of BDNF protein in the hippocampus and prefrontal cortex.

The data obtained from experiment 2 evidenced a long-lasting effect (at day 3) of propofol anesthesia itself on the expression of BDNF protein in both pre-frontal cortex and hippocampus. Therefore, we examined more particularly the specific effect of propofol anesthesia alone in a series of experiments:

- The immediate effects of Propofol anesthesia on BDNF expression (experiment 4.a.).
- The effects on the circadian rhythmicity of the BDNF expression the day after propofol anesthesia (experiment 4.b.).
- The effects on the peak of BDNF expression (ZT5) during the days following anesthesia (experiment 4.c.).

Experiment 4.a. immediate effect of Propofol

Specific methodology

At ZT10, we performed a short duration (30 min) anesthesia (propofol intraperitoneally in adult rats, and we measured BDNF protein in tissue extracts removed one hour after the injection of propofol. Rats were divided into three groups according to the treatment they received: saline, intralipids or, propofol. Eight animals were included in each group. Statistical analysis was performed on values of BDNF content using two way ANOVA (independent factor: treatment, within factor: structure). For sake of clarity, the results are shown in figure as the percentage of the control situation (mean obtained in rats receiving the saline injection) for each structure (cortex or hippocampus).

Results

We observed an increase of the BDNF content in the brain supernatants when animals were submitted to propofol injection in both cortex and hippocampus samples (4A) as compared to the saline and lipidic controls. Two way analysis of variance (between factor : treatment; within factor : brain structures) showed a significant effect of the treatment (saline, intralipids, propofol) $F(2,21) = 16,84$; $P<0.0001$), a significant effect of the brain structure (cortex versus hippocampus; $F(1,21)= 3,66$) and a significant interaction between treatment and structure ($F(2,21)=4;95$) on the BDNF content in supernatant extracts. Post-hoc analysis showed that BDNF contents differed significantly ($P<10^{-4}$) between cortex and hippocampus for the propofol group.

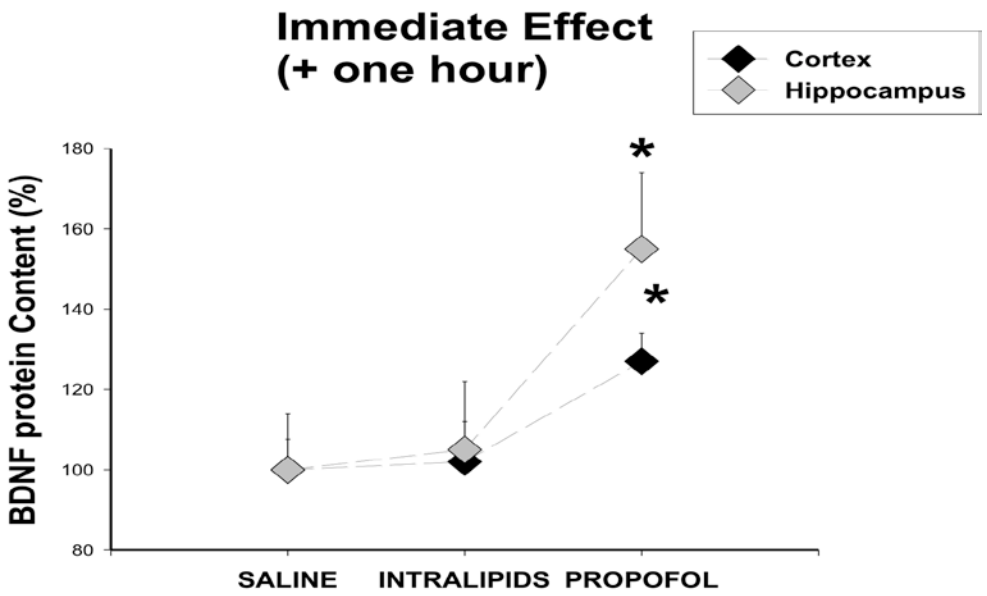


Figure 4A. BDNF contents observed in cortex and hippocampus supernatant one hour after the administration of either an anaesthetic dose of propofol or the control lipidic solution intralipids. Data are shown as percentage of the mean value of the control group (saline) obtained for each structure. A significant increased of BDNF protein was observed 1 hour after anesthesia in prefrontal cortex (+20 %) and in hippocampus (+ 56 %).

Experiment 4.b. Effect of propofol on BDNF circadian rhythmicity at Day 1.

Specific methodology

At ZT10, we performed a short duration (30 min) anesthesia (propofol intraperitoneally in adult rats at Day 0). We measured BDNF protein in tissue extracts removed every six hours (ZT5, ZT11, ZT17 and ZT23) at Day 1. Rats were divided into eight groups according to the treatment (intralipids or propofol) and the Time (ZT5, ZT11, ZT17 and ZT23). Six animals were included in each group. Statistical analysis was performed on values of BDNF content using two way ANOVA (between factors: time, treatment) each time for each structure (cortex or hippocampus). For sake of clarity, the results are shown in figures as the percentage of the mean level of BDNF at ZT17 obtained in rats receiving intralipids, for each structure (cortex or hippocampus).

Results

In prefrontal cortex, we observed a decrease of the BDNF content in the brain supernatants in animals submitted to propofol anesthesia. There is still a daily variation of the BDNF content, in animals reciving intralipids injection whereas there is no more daily variation in animals submitted to propofol anesthesia. Two way ANOVA showed a significant effect of time ($F(3, 44)=6,96$), a significant effect of treatment ($F(1,44)=4,56$) and a significant interaction between time and treatment. Post-hoc analysis showed that BDNF contents did not differ at ZT5, ZT11, and ZT23 as compared to ZT17 in animals receiving propofol injection (all $P>0.05$), whereas there is significant differences for ZT5 and ZT11 as compared to ZT17 in animals receiving intralipids (all $P<0.05$).

In hippocampus, we observed daily variations of the BDNF content that differed according to the treatment animals received. Two way ANOVA showed a significant effect of time ($F(3,44)=11.75$), a marginal effect of treatment ($F(1,44)=2.15$), but a significant interaction between time and treatment. Post-hoc analysis showed that BDNF contents at ZT17 in animals receiving propofol differed significantly from ZT5, ZT11 and ZT23 in propofol conditions but also from ZT17 in animal receiving intralipids.

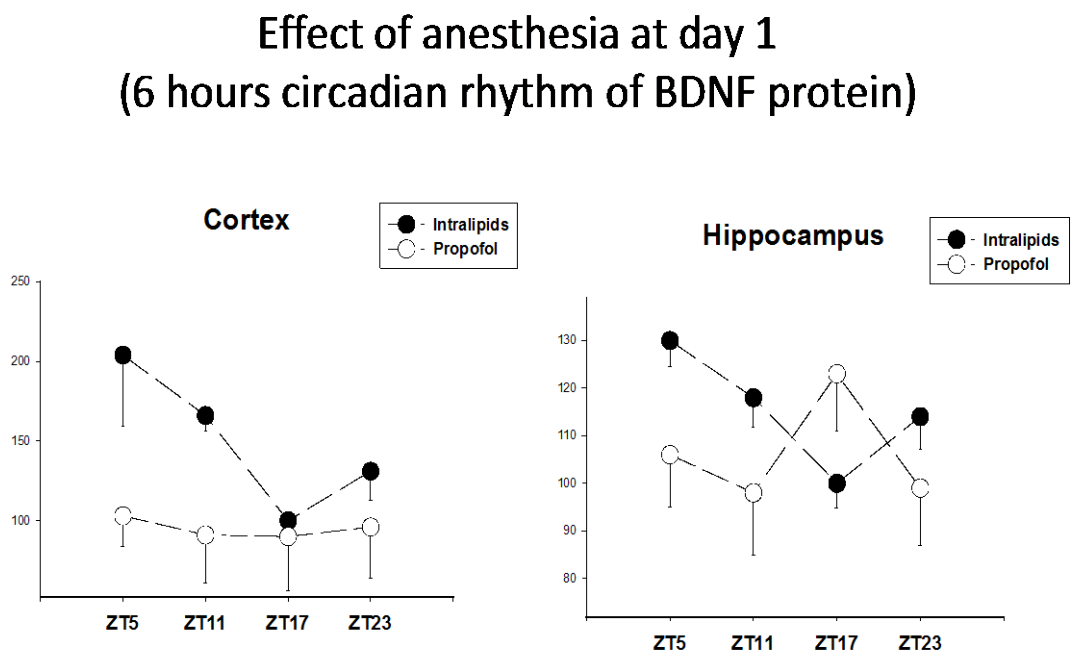


Figure 4B. BDNF observed in cortex and hippocampus supernatant every six hours during the day following the administration of either an anesthetic dose of propofol or the control lipidic solution intralipids. Data are shown as percentage of mean value of the control group (intralipids) observed at ZT17 for each structure (cortex or hippocampus). A significant decrease of BDNF protein was observed the day after anesthesia in prefrontal cortex, which is associated with the loss of rhythmic protein expression in animals receiving propofol.

Experiment 4.c. Effect on the peak of BDNF at day 1, day 3 and day 5.

Specific methodology

At ZT10, we performed a short duration (30 min) anesthesia (propofol intraperitoneally 120mg/kg) in adult rats at day 0. We measured BDNF protein contents in tissue extracts removed at ZT5 the days following propofol anesthesia (day 1, day 3, and day 5). Rats were divided into six groups according to the treatment (intralipids or propofol) and the time (day 1, day3 and day 5). Eight animals were included in each group. Statistical analysis of the absolute values of BDNF content in each groups was performed using three way ANOVA (between factors: time, treatment; within factor: structure). For sake of clarity, the results are shown in figures as the percentage of the mean values of BDNF obtained in rats receiving intralipids, for each structure (cortex or hippocampus). Because of no statistical difference (ANOVA) for BDNF content obtained at day 1, day 3 and day 5 in animals receiving intralipids, all data were used to set the mean value of intralipids control for each structure (cortex or hippocampus) to set the figure.

Results

We observed modifications (decrease at day 1 and increase at day3) of the BDNF content in the structure supernatants when animals were submitted to propofol injection as compared to their respective lipidic controls in both cortex and hippocampus samples (4C). Three way analysis of variance showed a significant effect of the treatment (intralipids, propofol) $F(1,44) = 8,86$), a significant effect of time ($F(1,44)=6,66$), no significant effect of the brain structure (cortex versus hippocampus), but a significant interaction between treatment and time ($F(2,44)=5;97$) on the BDNF content in supernatant. Post-hoc analysis showed that BDNF content differed significantly ($P<10-4$) between intralipids and propofol at ZT1 and ZT3 for both cortex and hippocampus. No statistical difference was observed at ZT5 between intralipids and propofol in cortex as well as in hippocampus.

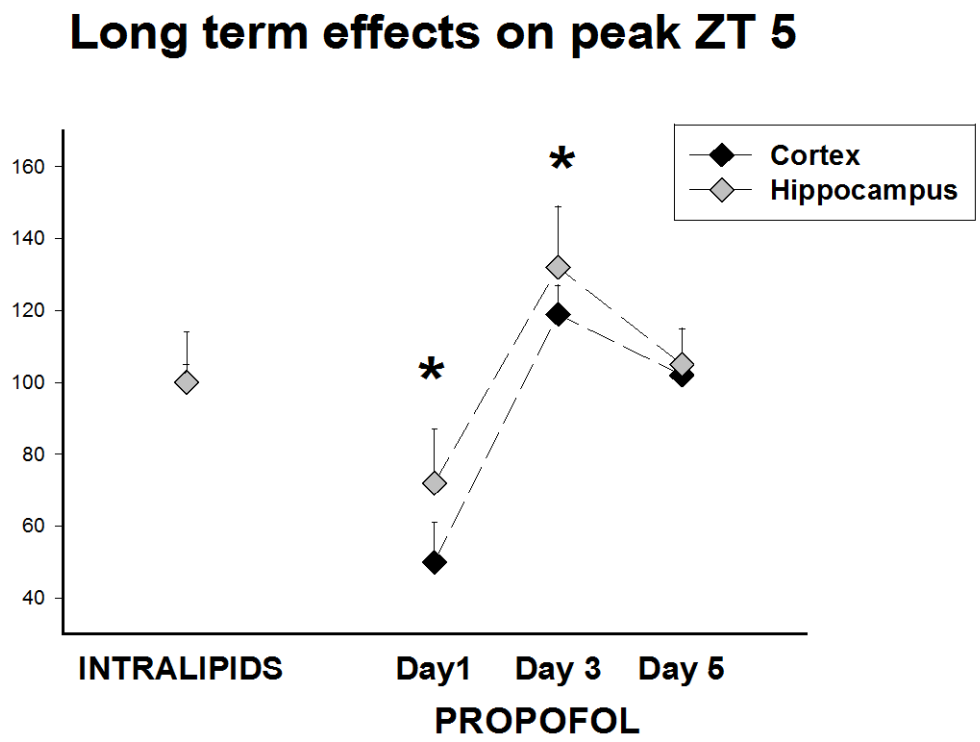


Figure 4C. BDNF observed in cortex and hippocampus supernatent at ZT5 (peak of the BDNF expression) at day 1, day 3 and day 5. At day 0, the animals have received the administration of either an anaesthetic dose of propofol or the control lipidic solution intralipids. Data are shown as percentage of the mean value of the control group (intralipids) obtained for each structure (see text for more explanation). A significant decrease of BDNF protein was observed at day 1 whereas a significant increase of BDNF was observed at day 3.

Discussion of the data

The data obtained in experiment 4 reproduce and extent some of the results we observed in experiment 2. Indeed, we reproduce here (experiment 4c) the increase of BDNF induced by propofol observed at day 3 (experiment 2). Conversely, a decrease of BDNF induced by propofol was observed at day 1 in both experiments 4b (ZT5 value) and in experiment 4c. From a methodological point of view, our series of experiment were performed exactly in the same conditions (propofol administration at ZT 10) and time of removal (ZT5). Indeed, we set our experimental design for examining the effects of propofol on the results obtained in experiment 1 as regards to the rhythmicity of the brain BDNF expression in our laboratory conditions.

All these results suggested that the modifications of BDNF induced by propofol anesthesia are varying during the post operative period. No more modification could be observed at day 5. Data obtained in experiment 4c did not evidence a main difference of the effect of propofol between the two brain structures we examined.

However, our data showed that not only propofol alter the expression of BDNF differently in a day-to-day basis, but also alter the daily rhythmicity of its expression (experiment 4b).

From our results of experiment 4, we may conclude that propofol anesthesia have rather disrupted the cyclic expression of BDNF protein, than decrease/increase the BDNF content.

4.5. Experiment 5:

Effects of Propofol anesthesia on fear memory.

Propofol has been shown to impair the memory of aversive and non-aversive event with a low dose (non-sedative dose 9mg/kg) in rodents (Pain et al., 2002). Memory impairment induced by propofol appeared to be of short duration (Veselis et al., 2001). No data are available on the type and duration of memory impairment following propofol anesthesia in rodents. To address this point, we examined the long term effects of propofol anaesthesia (same regimen as in previous experiments) on both the immediate recall and delayed recall following a classical fear learning performed at different periods (day 1, 3, and 5) during the post anaesthesia period.

Specific methodology

Short duration anesthesia propofol or intralipids (control) was performed at ZT10 (Day 0). Conditioning phase was performed at ZT5 in three different conditions:

- The day after anesthesia Day 1 (n= 32)
- Three days after anesthesia Day 3 (n= 32)
- Five days after anesthesia Day 5 (n=32)

Animals were tested for memory either 24 h after conditioning (immediate recall) or 10 days after conditioning (delayed recall) depending of the group allocated (n = 8 per group)

Statistical analysis was performed using a NEWMAN-KEUL Test on Log latency to enter the compartment (see general methodology above).

Results

We observed that the amount of conditionning (Log latency to enter the dark compartment) was significantly increased in groups receiving propofol anesthesia, as compared to intralipids control when conditionning was performed at day 1 and day 3 and when testing was performed in a delay manner (10 days). Satistical annalysis showed that only the groups conditionning day1/10 days recall and conditionning day 3/10 days recall differed significantly ($P<10\text{-}4$).

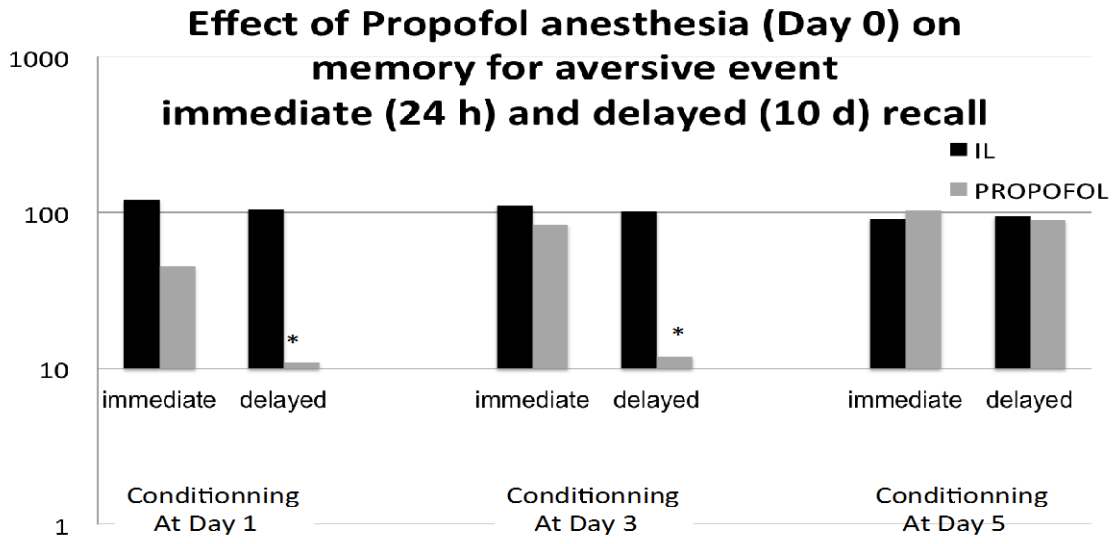


Figure 5. Effect of propofol anesthesia performed at day 0 on the fear memory (Log latency in seconds to enter the dark compartment in fear conditionning paradigm). Conditionning was performed either at day 1, day 3 or day 5. Immediate memory was assessed by testing the animals for fear 24 h after conditionning. Delayed memory was assessed by testing the animals for fear 10 days after conditionning. A significant effect of anesthesia (performed at day 0) on delayed recall was observed when conditioning was performed at day 1 and 3. No more significant effect was observed at day 5.

Discussion of the data.

As previously observed in the same laboratory (Pain et al., 1998), propofol anesthesia has no significant effect on the immediate memory when learning is performed above 24 h following anesthesia. However the data obtained here on the delayed memory showed clearly that propofol anesthesia has a deleterious effects on the delayed memory until at least, in our experimental conditions, three days after anesthesia (day 1 and day 3). Delayed memory is supposed to reflect the consolidation phase of memory and may involve the expression of BDNF.

In experiments 4 and 5, we observed a similar time course (day 1 and day 3, with no modification at day 5) of the alteration induced by propofol on both the BDNF expression and the delayed memory.

4.6. Supplemental Experiment

Effect of Propofol on ERK1/2 expression on the rat hippocampus

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Abstract

Considering that anaesthesia is responsible for many effects on the central nervous system, we hypothesized that it may also have different effects at different circadian times. Propofol (general anaesthesia) has showed circadian variation in duration of effects. To characterize the effects of propofol on ERK1/2 in hippocampus at two different times of the day. Male rats received either an intra peritoneal injection of 120 mg/kg of propofol to set short-duration anaesthesia state (20-30 minutes) or the equivalent amount of the lipidic solvent as controls. For both groups of animals, anaesthesia or control, the injections were performed either at ZT0 or at ZT12. One hour following the injection, the animals were euthanized; the brains were removed and immediately frozen. The amount of ERK1/2 was assessed by using immunohistochemistry on brain sections. The amount of ERK1/2 density was significantly decreased in CA1, CA2 and CA3 areas of the hippocampus, but only when anaesthesia was performed at ZT12. Our current results evidenced that the impact of propofol anaesthesia on hippocampus may vary depending on the zeitgeber time.

Introduction

Propofol (2, 6-diisopropylphenol) is a highly effective IV anaesthetic and is widely used for general anaesthesia and for sedation with local anaesthesia (Sebel and Lowdon, 1989). Propofol produces rapid sedation with quick onset and offset and relatively few serious side effects (bradycardia, hypotension and dose-dependent respiratory depression). This property has made propofol a drug of choice for many procedures where general anaesthesia is not required. Like other intravenous anaesthetics, propofol produces amnesia in addition to sedation, hypnosis and general anaesthesia. Propofol induced amnesia could be observed not only at low dose under its administration in both animal models and in human volunteers but also at recovery from anaesthesia (Veselis, 1999, Pain et al., 2002). However, the detailed mechanisms

underlying the amnesic effect of propofol are still under examination. The amnesic effect of propofol is considered to be related to an inhibition of long term potentiation (LTP) at Schaffer collateral-commissural pathway to CA1 (Cornu Ammonis area) pyramidal cell synapses in the hippocampus (Wei et al., 2002, Takamatsu et al., 2005, Fibuch and Wang, 2007). Psychologists and neuroscientists generally agree that the hippocampus has an important role in the formation of new memories about experienced events (episodic or autobiographical memory) and consolidation (Squire, 1992).

Several behavioral and cellular studies show that ERK1/2 has a very important role in controlling LTP and memory formation in the adult brain (Sweatt, 2004, Thomas and Huganir, 2004). ERK1/2 activation facilitates transcriptional events and as a result, it regulates distribution and functions of synaptic proteins to control many forms of synaptic plasticity, including LTP and memory in the hippocampus (Wei et al., 2002, Takamatsu et al., 2005). A concentration dependent increase was seen in both the membrane and cytosolic fractions of cortical cells in culture; these changes in ERK1/2 phosphorylation being induced by the binding of propofol to the $GABA_AR$ (Gama amino butyric acid type A receptor) complex (Oscarsson et al., 2007). Another study shows that propofol inhibited NMDAR dependent activation of MAPK/ERK cascade in hippocampal cell cultures (Kozinn et al., 2006). Propofol has showed circadian effect for its hypnotic properties in rats (Challet et al., 2007). Is there any effect of propofol on ERK1/2 at different zeitgeber times?

The goal of the study was to examine the effect of *in vivo* administration of propofol on ERK1/2 protein in the hippocampus at two different zeitgeber times; ZT0 (start of rest period) and ZT12 (start of activity period). For this, we used an *exvivo* technique; the immunostaining of ERK1/2 on brain sections collected one h after the injection of propofol.

Material and Methods

All the experiments were conducted in accordance with institutional guidelines complying with national (Council directive 87848, 19 October 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale;) and international guidelines (NIH publication no. 86-23, revised 1985). Male rats of 8 weeks (*Long Evans, Charles River*) were housed in 22-24C° and they were kept in12:12 h light–dark cycle with *ad libitum* for 15 days before the experiments.

Two treatments were submitted at two different zeitgeber times; ZT0 and ZT12 offset and onset periods of activity respectively in rodents. Propofol (Propofol Fresenius®1% manufactured by Fresenius *Kabi, France*) or the lipidic solvent Intralipid (Intralipid® manufactured by *Fresenius Kabi, France*) as a control were given intra peritoneally at the dose of 120mg/kg. Eight animals were used in each group and the duration of anaesthesia in adult rats was about 20-30 minutes.

Animals were euthanized by exposure of carbon dioxide; the brains were quickly removed on ice and frozen in isopentane at -40°C. Sequential 25 µm-thick brain coronal sections were cut at -18°C on a cryostat at -3.14 coordinate from bregma according to the atlas of Paxinos and Watson (1998).

Briefly, immunohistochemistry steps were carried out at room temperature (18-22 C°) unless otherwise specified. All washes were carried out under gentle agitation for ten minutes and repeated three times in buffer, 0.02M TBS (Tris Buffer Saline). Incubations were carried out at flat slides in humid chambers and 400-500µl of reagent was provided per each slide depending upon the surface area of the tissues. Tissues were fixed in 4% paraformaldehyde for seven minutes. After being fixed by the paraformaldehyde, slides were placed in 0.3% hydrogen peroxide diluted in TBS for 15 minutes to quench endogeneous peroxidases. Slides were incubated for 1 hour in 5% goat serum *(Vector Lab Birlingame CA USA)*, then incubated in rabbit ERK1/2 antibody (*Cell Signalling Technology California USA*), in the dilution ratio1:500 over night at 4C°. The secondary antibody was anti-rabbit made in goat (*Vector Lab Birlingame CA USA)*. Sections were incubated with Avidine –Biotin peroxides complex *(Vectastain Elite Kit, Vector Laboratories)*. Both Avidin-Biotins were used as 3 µl per ml of TBS 0.01%. Sections were stained with a chromagen DAB (3, 3-diaminobenzidine tetrahydrochloride by company *SIGMA, Missouri USA*), then sections were dehydrated and cover slipped and mark for the ERK1/2 were quantified

The variable examined was the cell count immunostaining per micrometer square. For each area, a global two way analysis of variance was performed, between factors and time: ZT0 versus ZT12 and treatment: Intralipids versus Propofol. If a significant effect was observed, post hoc analyses were performed using a t-test with Bonferonni correction for multiple comparisons (Systat 8.0 software).

Results

These rats were divided into four groups on the basis of treatment and time of administration (n=6 per group). Following the injection of propofol, all animals lost

their righting reflex in a 5-8 minutes delay. The mean time in minutes for ZT0 and ZT12 to recover the righting reflex following the injection of propofol was 28 and 35 respectively. One hour after the injection, all animals have recovered not only their righting reflex but also their spontaneous locomotion.

Analysis of variance in CA1 area of the hippocampus demonstrated that there are significant effects of treatment ($F(1, 12) = 25.88$, $P<10^{-4}$) and time ($F(1.12) = 7.48$, $P=0.017$) with a significant interaction between treatment and time on the ERK1/2 immunostaining in CA1 area of the hippocampus. Indeed post hoc analysis revealed that there are significant differences only at ZT12, intralipid versus propofol ($P=0.03$) and for propofol, ZT0 versus ZT12 ($P=0.05$). General analysis of variance in CA2 area demonstrated that there are a significant effect of treatment ($F(1, 12) = 17.84$, $P<10^{-4}$) and no effect of time ($F(1.13) = 2.13$, $P=0.167$) on the ERK1/2 immunostaining in CA2 area of the hippocampus. Indeed post hoc analysis revealed that there is a significant difference only at ZT12, intralipid versus propofol ($P=0.03$) and a marginal effect for ZT0 versus ZT12 in the propofol group ($P=0.057$). There is similar significant effects of treatment ($F(1. 12) = 9.61$, $P=0.003$) and no effect of time ($F(1.12) = 1.232$, $=0.287$) on the ERK1/2 immunostaining in CA3 area of the hippocampus. Indeed post hoc analysis revealed that there is a significant difference only at ZT12, intralipid versus propofol ($P=0.014$) and a marginal effect for ZT0 versus ZT12 in the propofol group ($P=0.107$). There is no significant effect of treatment ($F(1, 12) = 2.48$, $P=0.141$) but a significant effect of time ($F(1.12) = 7.139$, $P=0.020$) on the ERK1/2 immunostaining in DG area of the hippocampus (Fig.1 and 2).

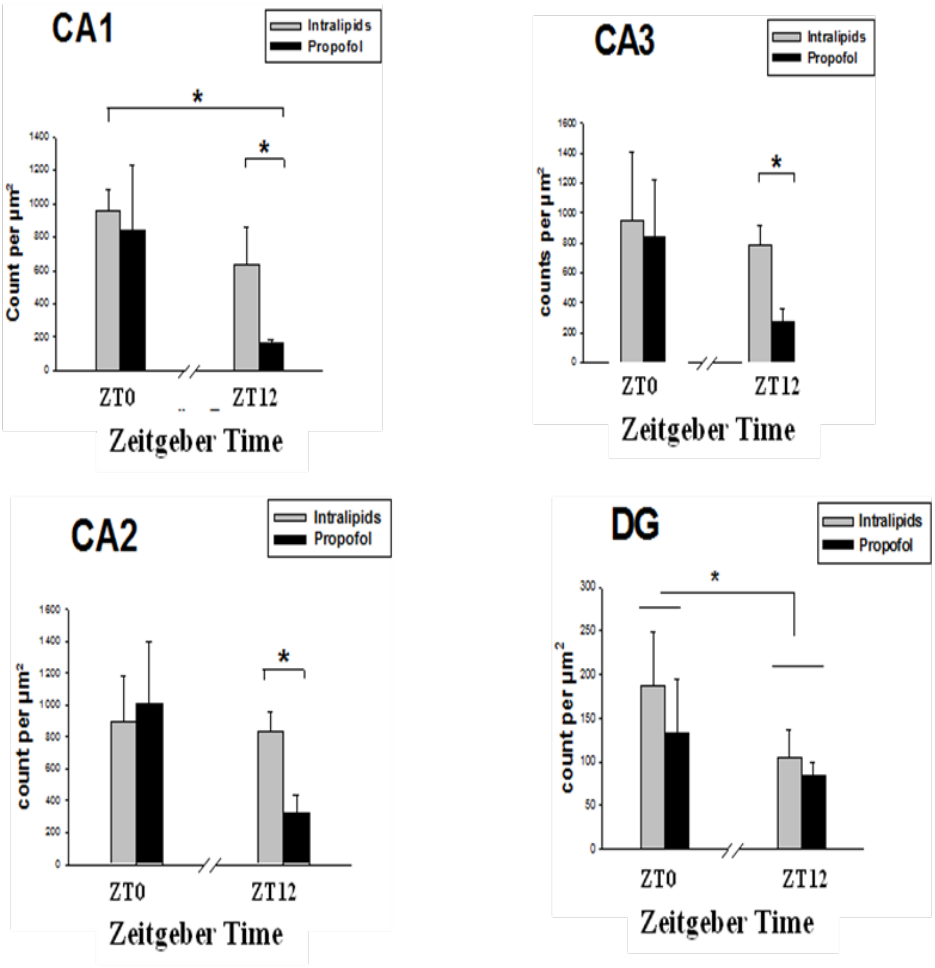

Fig.1: Effects of propofol and the lipidic solvent (Intralipids) on cell counts ERK1/2 immunostaining per micrometer square in CA1, CA2, CA3 and Dentate Gyrus (DG) areas of hippocampus at the two different circadian times of injection (ZT0, and ZT12, respectively offset and onset of activity period). * *means P<0.05*

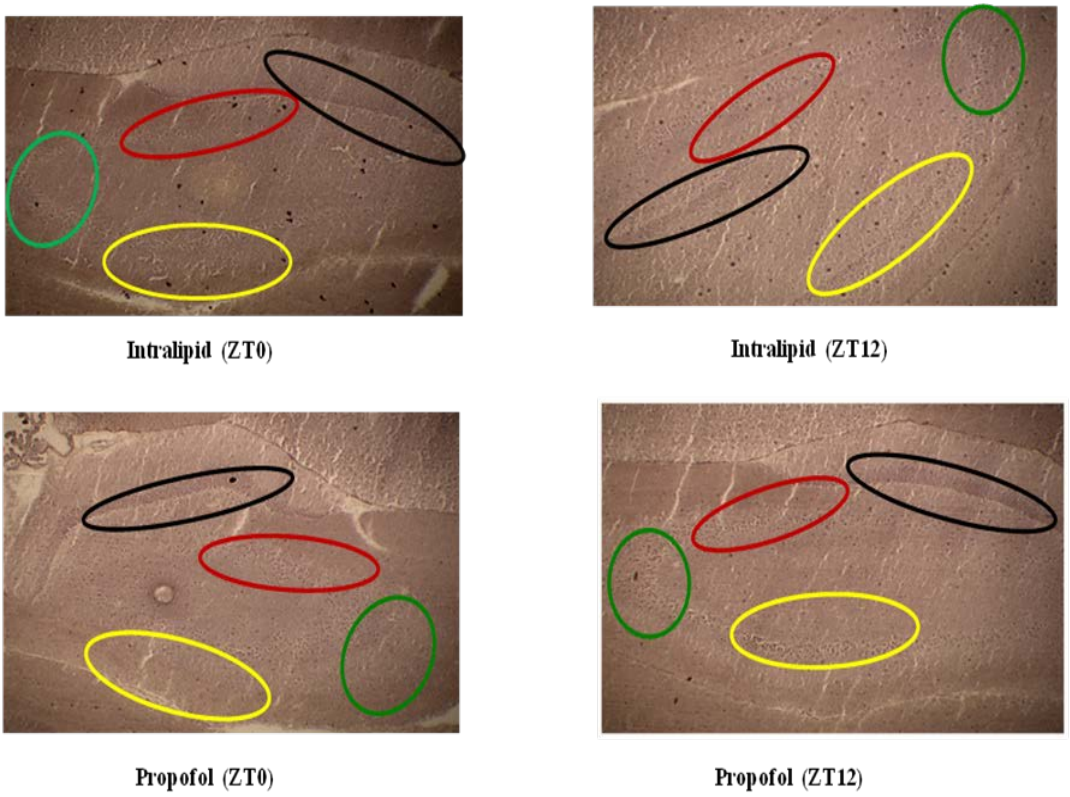

Fig.2: ERK1/2 immunostaining in different hippocampal areas with propofol and intralipid treatments here yellow, green,red and black circles represent CA1,CA2,CA3 and DG respectively.

Discussion

In the present study, we observed the effect of propofol on ERK1/2 protein at two different zeitgeber times (ZT0 and ZT12). We used male rats and the brains were removed after one hour injection of propofol (120mg/kg) at two different times (ZT0 and ZT12). Here, we used immunohistochemistry that permits to have good localization of the ERK1/2 protein. Our main results show remarkable decrease in the ERK1/2 protein at ZT12 in hippocampal areas (CA1, CA2 and CA3) without any change in the Dentate Gyrus induced by propofol.

Previous studies have shown that propofol may disturb the ERK1/2, in brain cell cultures, a treatment with propofol, induced a concentration-dependent increase of ERK-1/2 phosphorylation in both the membrane and cytosolic fractions of the cell; this increase being blocked by pre- incubation with bicuculline, a GABA antagonist, suggesting that the observed changes in ERK-1/2 phosphorylation were induced by the binding of propofol to the $GABA_AR$ complex (Oscarsson et al., 2007). Conversely, in

cultured rat hippocampal neurons, propofol reduced the NMDAR dependent activation of MAPK/ERK cascade (Kozinn et al., 2006). NMDA receptors are phosphorylated at Ser897 and Ser896 positions and these sites for phosphorylation are blocked by propofol (Kingston et al., 2006). Therefore, it has been suggested that propofol may affect NMDAR–dependent ERK phosphorylation by affecting NMDA phosphorylation (Fibuch and Wang, 2007). Our results obtained at recovery from anaesthesia are in agreement with those obtained in vivo by Kozinn et al. (2006) and evidence a long lasting inhibitory effect of propofol on ERK1/2 phosphorylation in hippocampus is in opposition with the results obtained by Oscarson et al. (2007) in brain cell culture. However, the methodology used in this last study differs largely from our study. Firstly, we examined the ERK1/2 contents at recovery from anaesthesia, one hour after propofol administration while Orscarson examined ERK1/2 phosphorylation soon after the administration of propofol at concentration clinically relevant for an anaesthesia state.

Decrease of hippocampal ERK1/2, appears to us important to understand the cellular mechanisms underlying the well described inhibitory effect of propofol on LTP and probably its amnesic effect. Indeed, a significant contribution of ERK1/2 phosphorylation to synaptic plasticity and memory formation in the adult brain has been established in behavioural as well as in cellular studies (Adams and Sweatt, 2002, Sweatt, 2004). ERK1/2 is required for the induction of stable LTP while LTP inducing high frequency stimulation of schaffer- collateral inputs to area CA1 lead to the activation of ERK1/2 in CA1 (English and Sweatt, 1996). Such a role of activation of ERK1/2 in memory has also been observed in vivo; the performance in a fear learning task in rodents being largely decreased when animals received MEK inhibitors while, fear learning was associated with a significant activation of ERK1/2 in hippocampus in trained animals (Atkins et al., 1998). As mentioned above, propofol has amnesic effect at low dose, high anaesthetic dose and at recovery from anaesthesia (Veselis, 1999, Pain et al., 2002). The amnesic effect of propofol is considered to be related to its known inhibition of LTP at Schaffer collateral-commissural pathway to CA1 pyramidal cell synapses in the hippocampus (Wei et al., 2002, Takamatsu et al., 2005, Fibuch and Wang, 2007). From our results, we can suggest that a specific inhibitory effect of propofol on ERK1/2 in hippocampus might be one of the transcriptional targets to explain the inhibitory effect of propofol on hippocampal LTP.

Our results focusing on hippocampus evidenced clearly and for the first time to our knowledge that propofol has an inhibitory effect on ERK1/2 and this inhibitory effect depends on the time of the day administration of propofol.

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5. Discussion

In our studies, the major findings: BDNF protein followed a circadian rhythmicity during the 24hour of day in the rat's hippocampus and cortex and the concentration of BDNF protein has been observed at peak around ZT5 whether the animals were nocturnal or diurnal. In addition, we found that BDNF protein content is altered in the hippocampus and cortex of rats when they were submitted to short duration propofol anaesthesia, LPS and minor surgery under propofol anaesthesia; however minor surgery alone has no effect on alteration of BDNF protein in the hippocampus and cortex as well. Finally, we were able to describe a similar time course of BDNF dysregulation and cognitive dysfunction (aversive delayed memory) in animals submitted to equivalent short duration propofol anesthesia in the same experimental conditions (performed at ZT5).

The BDNF protein levels in the cortex and hippocampal supernatants as well as in the blood were determined using an ELISA technique. Alternatively, memory was tested by fear conditioning using the classic fear conditioning preparation (using passive avoidance apparatus) for the acquisition phase learning. Classic fear conditioning preparation has been shown to test memory for aversive events specifically in rodents (Pain et al., 2002).

Intralipid is a lipidic emulsion that is used intravenously as a nutrient for patients in the intensive care units (Ulrich et al., 1996) and also been used as vehicle for lipid soluble compounds such as propofol. For this reason, we used lipidic emulsion as control to ensure that any alteration of BDNF protein in the brain after propofol treatment is due to the anaesthesia and not the vehicle. Furthermore, lipidic emulsion does not interact with $GABA_A$ receptors and therefore interact with anaesthetic effect (Orser et al., 1994).

5.1: Circadian rhythmicity of BDNF

BDNF is considered as major biomarker involved in the cognitive processes and various behavioural and cellular studies have evidenced that hippocampus and cortical structures have great importance in cognitive functions, learning and memory. Our aim was to examine the effect of short duration anaesthesia, LPS and minor surgery on the BDNF protein level alterations in the hippocampus and cortex. Before to achieve this goal, we have considered necessary to know the peak of BDNF during the day and for this we examined the daily variation of BDNF protein at different time points during 24 h without any treatment.

Only few data regarding the circadian variation of BDNF in the brain is available. *In situ* hybridization, BDNF mRNA expression significantly varies in dentate gyrus (DG) of the hippocampus at four different time points during 12/12 h light/dark cycle and the peak is observed between ZT1-ZT5 (Schaaf et al., 2000). It is also described that BDNF mRNA in hippocampal subfields (CA1, CA3, hilus and DG) show variations observed at four different time points of the day (noon, 6 pm, midnight and 6 am) in rats and basel level of BDNF was at peak at 6 am (Berchtold et al., 1999). Studies in rodents have been performed at maximum four time points showing variation of BDNF mRNA with a peak during the light period that is in agreement with our data showing peak of BDNF at ZT5 (five h after the light on). Older studies did not show a clear circadian rhythmicity as studies have been performed at four points. Our results are also in agreement with the study of Begliuomini et al.(2008) that showed that plasma BDNF varies at different times of the day in human males volunteers and plasma BDNF is noted higher in the morning (around 8 h, is activity period for human) and lowest concentration was observed at mid night (24 h).

We assumed that it is the light that maintains the circadian variations of the BDNF protein levels in the hippocampus and cortex. Light appears to regulates BDNF mRNA in the visual cortex. This was evidenced by keeping the animals in the dark that the BDNF mRNA and mRNA of its receptor trkB was decreased in the visual cortex and light exposure again restored BDNF mRNA to the normal level (Castren et al., 1992).

Different evidences that are in agreement with our results and support our data of BDNF protein circadian rhythmicity. Therefore, various extracellular and intracellular proteins that are involved directly and indirectly in the transcription of BDNF have showed circadian rhythmicity, like glutamate, the major excitatory neurotransmitter in the mammalian brain has been shown to increase the transcription and release of BDNF (Zafra et al., 1991). Furthermore, it has also been described that glutamate follows circadian rhythms in rodents with a peak of glutamate at transient onset of light period (Castaneda et al., 2004). These results are in favour of our data of BDNF protein circadian rhythmicity during 24 h of the day.

ERK1/2 belongs to a family of mitogen activated protein kinases (MAPKs) that refers to a large family of cytosolic and nuclear serine/ threonine kinases that are involved in a variety of cellular activities in mammalian cells (Peyssonnaux and Eychene, 2001). Several behavioural and cellular studies have shown that ERK1/2 has a very important

role in controlling LTP and memory formation in the adult brain (Sweatt, 2004, Thomas and Huganir, 2004). ERK1/2 activation facilitates transcriptional events, and as a result, regulates distribution and functions of synaptic proteins to control many forms of synaptic plasticity, including LTP and memory in the hippocampus (Wei et al., 2002, Takamatsu et al., 2005). Synaptic plasticity correlates with learning and memory formation and it is also well established that BDNF plays an important role in synaptic plasticity, especially in activity dependent one (Yamada and Nabeshima, 2003). This strongly suggests that BDNF might have a great role in learning and memory. Hippocampus responsible for learning and memory is the major site of BDNF action and it is also noted that the expression of BDNF during learning process (Hall et al., 2000). ERK1/2 has been shown to activate the BDNF during intracellular signalling (Ludwig et al., 2011) and it has evidenced that ERK1/2 protein show a circadian rhythm in the hippocampus (Eckel-Mahan et al., 2008). An ERK1/2 protein peak is observed at around ZT5 and training and testing in mice has resulted in better memory at ZT4 rather than other zeitgeber times. This circadian rhythmicity of ERK1/2 is parallel to the circadian memory performance (Roth and Sweatt, 2008), as the maximum memory performance is observed around ZT5. This study together with our results that show a circadian rhythmicity of BDNF protein and with a BDNF peak at ZT5.

However, some data in the literature show controversies with our results, as *in situ* hybridization BDNF mRNA expression significantly varies during 24 h of the day observed at four points in CA3 area of hippocampus and peak of BDNF is noted between ZT13-ZT17 (Schaaf et al., 2000). Daily changes of BDNF mRNA contents have been demonstrated in the rat prefrontal cortex and hippocampus. RT-PCR showed that BDNF mRNA vary during 24 h time period that was observed at four time points (Bova et al., 1998). In this study, mRNA is at peak at CT13 (one hour at start of activity period) and minimum at CT5 (Five hours at start of rest period). These two studies show peak of BDNF mRNA during the dark period that is different from our data which shows peak of BDNF during the light period. This may be due to the posttranscriptional regulation as well as differences in mRNA and protein turnover rates, as the central dogma of molecular biology states that DNA transcribes mRNA and mRNA is translated into proteins, suggesting that there is a direct relationship between mRNA and protein levels but it is also observed that biologically, variation between mRNA and protein expression level can be caused by posttranscriptional

regulation as well as differences in mRNA and protein turnover rates (Cox et al., 2005). It may be possible that variation between mRNA and protein level may be caused by circadian time.

We examined BDNF protein circadian rhythmicity in the cortex and the hippocampus and we observed a similar pattern of circadian rhythms of BDNF protein levels in both the structures. It is examined that dominant levels of BDNF have been noted in the two structures and immunoreactivity of BDNF protein expression has been shown high in the hippocampus (Conner et al., 1997), as well as in the cortex compared to other structures (Kawamoto et al., 2000). It is also suggested that the hippocampus and prefrontal cortex are involved in parallel for processing the short term spatial working memory and it is also suggested that inactivating one of these structures, have affected the short term working memory performance (Lee and Kesner, 2003). Bova et al. (1998) evidenced that both the cortex and hippocampus follow a similar pattern of BDNF mRNA variation during a 24 h of the day.

We also examined plasma of the rats in order to determine if a circadian variation of BDNF protein exists. We did not observe any circadian rhythmicity of BDNF protein in the plasma in the same rodents which showed circadian rhythmicity in the hippocampal and cortical structures of the brain. Actually, there is only few data regarding plasma and serum BDNF protein levels in both rodents and human. Study using three time points shows no diurnal variation of BDNF protein in plasma of women and men (Choi et al., 2011). Piccinni et al. (2008) has also evidenced that there is no diurnal variation of BDNF protein in plasma of women. Our results on the plasma BDNF are in disagreement with the study of Begliuomini et al. (2008) that shows a significant variation of plasma BDNF in men that is at peak at 08:00 hours and minimum at 22:00 hours (Piccinni et al., 2008).

Our data can not conclude about any circadian variation of precursor form of the BDNF that is called pro-BDNF. BDNF is initially synthesized as a precursor protein (pre pro-BDNF) in the endoplasmic reticulum. Following cleavage of the signal peptide, pro-BDNF is transported to the Golgi bodies where it is processed either constitutive or regulated secretory vesicles. Then Pro-BDNF may be converted into mature BDNF intracellularly in the Golgi bodies by members of the subtilisin–kexin family of endoproteases, such as furin, or in the immature secretory granules by pro-protein convertases (Lu et al., 2005). It is also necessary to examine the circadian profile of pro-BDNF because data emerging from recent works suggests that pro-BDNF

is not only a precursor of the mature and active form of BDNF but it also seems to elicit important biological functions. This hypothesis firstly derives from the evidence that pro-NGF, another neurotrophin precursor, is able to bind p75NTR with high affinity and to promote neuronal apoptosis (Lee et al., 2001). Recently, it has been demonstrated that pro-BDNF was responsible for the induction of apoptosis in cultured sympathetic neurons through the activation of receptor p75NTR. In addition, pro-BDNF was also linked to synaptic plasticity and in especially it was established that it is able to enhance LTD following binding to p75NTR (Woo et al., 2005). Taken together, these findings reveal that cell survival and synaptic plasticity seems to depend on the right balancing of these two forms. A study has also investigated the parallel role of both pro and mature BDNF in the cognitive performance and their levels were reduced in the pre-clinical stages of AD (Peng et al., 2005).

In order to characterise the profile of circadian rhythms of BDNF protein levels in the diurnal animals, for this purpose, we selected *Arvicanthis ansorgei* as diurnal rodents. *A. ansorgei* are considered best rodents as diurnal animals and excellent model for circadian studies (Challet et al., 2002). Samples were collected at the same six time points as we did for nocturnal rodents.

We observed a biphasic pattern of circadian variation of BDNF protein in hippocampus and cortical structures of brains of *A. ansorgei*. Two peaks of BDNF protein activation were observed during this biphasic circadian variation, one peak was noted in the light period and second peak was noted during the dark period during the 24 h of the day in both the hippocampus and cortex.

Literature on circadian studies in diurnal animals is very rare the diurnal variation of protein, especially the brain proteins in the diurnal rodents (Abe et al., 1995). One of the reasons may be the lack of suitable diurnal rodent models which can be used for research purposes. However, we used *A.ansorgei* for the first time to examine the circadian profile of BDNF protein in the brain, as Challet et al (2002) evidenced that *A. ansorgei* are suitable rodents for research studies as diurnal models. Some studies on circadian rhythms in *A. ansorgei* that are compatible with our results of biphasic circadian rhythms of BDNF protein in the cortex and hippocampus indirectly, like activity noted during the wheel running tasks in *A.ansorgei* has been varied during the 24 h and two peaks of activity have been observed at the transitions of light and dark periods (Slotten et al., 2005). It is also likely that activity or exercise is responsible for the increase of hippocampal BDNF that is further responsible for the formation of

spatial memory (Khabour et al., 2010). If there is biphasic activity during 24 h of the day, it may lead to biphasic expression of BDNF protein.

BDNF has been shown to induce the expression of various genes in hippocampal cells and one of the most prominently affected genes is the activity-regulated cytoskeletal associated protein, commonly called as Arc (Yin et al., 2002). Arc is the product of an immediate early gene involved in regulation of actin cytoskeletal dynamics underlying consolidation of LTP and contributes to activity dependent synaptic plasticity and adaptive behaviour such as memory storage. Biphasic expression of Arc protein has been observed in the hippocampal subfields CA1, CA3 and DG with the initial wave from 30min to 2h and second wave of Arc protein expression is from 8 to 24h (Ramirez-Amaya et al., 2005). Arc protein traffics towards dendrites and accumulates at the site of synaptic activity (Steward et al., 1998). Alternatively, it is also suggested that Arc protein is locally synthesised in the dendrites (Moga et al., 2004). These characteristic features of Arc lead to the coupling synaptic activity to synaptic plasticity and it is also evidenced that Arc synthesis plays an important role in maintaining the activity of key translation factors during LTP consolidation, this consolidation is activated by the secretory neurotrophin BDNF (Bramham et al., 2008).

Furthermore, corticosterone levels have also show a biphasic peaks during the 24 h of the day in *A.ansorgei* and these two peaks are observed around the transitory light and dark periods (Verhagen et al., 2004). This pattern of biphasic variation of corticosterone is compatible with the biphasic pattern of activity studied by Slotten et al. (2005). In nocturnal rodents, Schaaf et al (2000) has also evidenced that BDNF mRNA and corticosterone levels also show daily variation. So, these studies may be compatible with our results showing for the first time biphasic circadian variation of BDNF protein in the cortex and hippocampus of diurnal rodent *A.ansorgei*.

5.2: Anaesthesia and BDNF

In order to study the effect of anaesthesia on BDNF protein expression in brain, we used short duration propofol anaesthesia. Propofol (2, 6-di-isopropylphenol) is being used for the induction of anaesthesia as well as maintenance of anaesthesia because of its rapid onset short acting anaesthetic properties and lesser side effects. The proposed mechanism of action of propofol is the inhibition of neural activity and is based on the activation of $GABA_A$ receptor. However the molecular mechanisms of the effects of propofol on neural activity remain elusive (Krasowski and Harrison, 1999). It has been

evidenced that GABA mediated neuronal activity is important for the development of brain and it has been proposed that exposure to general anaesthetics interfere with normal maturation and continual behavioural deficits of the brain (Varju et al., 2001).

We observed for the first time a dysregulated pattern in circadian rhythmicity of BDNF protein in both the cortical and hippocampal structures of the brain when animals were submitted to short duration anaesthesia with propofol. There is a lack of data on the effects of anaesthetics on the circadian variations of neurotrophic factors, especially circadian rhythms of BDNF protein either in rodents or human. However few data in the literature evidenced that anaesthetics affect circadian rhythmicity of physiological activities. It has been suggested that the normal melatonin circadian rhythms are disturbed by the general anaesthesia with conjunction of surgery (Karkela et al., 2002). Melatonin is secreted by the pineal gland and is commonly used as a circadian phase marker and exogenous melatonin has been shown to affect the circadian clock (Pevet, 2003). It may suggest that any alteration in the circulating melatonin lead to alter the functioning of the supra chiasmatic clock.

One of the prominent changes observed in animals after general anaesthesia is the transient hypothermia (Kurz, 1997). Changes in temperature can be responsible for phase shift circadian neuronal rhythms of supra chiasmatic nuclei (Herzog and Huckfeldt, 2003) and transient hypothermia has been shown to set back the biological clock of thermoregulation in rats (Hopfer and Sunderman, 1988). The *period 2* gene play key roles in photo entrainment of the circadian clock to light pulses (Akiyama et al., 1999) and the *Period2 (Per2)* gene circadian rhythmicity is examined altered in lizards when they were exposed to low temperature and when lizards were exposed to high temperature, *Per2* gene rhythmicity was again reestablished after 2 days of adaptation (Magnone et al., 2005).

Propofol is described to inhibit the phosphorylation of ERK1/2, the main intracellular kinase (Nagata et al., 2001) and phosphorylation of ERK1/2 is involved in synaptic plasticity and memory performance. Besides, it has been studied that PERK1/2 protein show a circadian rhythm as well (Eckel-Mahan et al., 2008). PERK1/2 protein peak is observed at around ZT5 (parallel to peak of BDNF protein at ZT5). This circadian rhythmicity of PERK1/2 is parallel to the circadian memory performance (Roth and Sweatt, 2008), as the maximum memory performance is observed at around ZT5. In addition to this, ERK1/2 is shown to regulate the BDNF in glial cells and astrocytes and any imbalance of the ERK1/2 contributes in the pathogenesis of neurocognitive

disorders (Su et al., 2011). So, it can be hypothesised that propofol can dysregulate the BDNF protein in the brain.

As we were able to observe peak of BDNF protein level around ZT5 (five hours after the onset of light), we have take this time point and we examined the short and long term effect of propofol anaesthesia. Only few data regarding the effect of propofol on the expression of BDNF are available. However some studies are in agreement with our data and have also discrepancies as well. Most of the studies do not indicate the time of sample collection, as BDNF protein varies significantly during the 24 h of the day, so it is important to mention the time of experiment.

We evidenced an immediate effect of propofol anaesthesia on the alteration of BDNF protein in the rat's cortex and hippocampus structures when subjects were sacrificed an hour after the anesthesia. Interestingly, this initial increase was followed by a dysregulation long lasting. The study of Popic et al. (2012) shows that propofol has no effect on the BDNF protein in the cortex at first four hours after the anaesthesia, but the authors did not mention the time of experiment and they used propofol at 25mg/kg in developing brain. The main downstream effector kinase ERK1/2 that is involved in the neurotrophic factor signalling pathway remained unchanged during the first four hours of propofol anaesthesia (Popic et al., 2012). However, there has been noted a significant effect of propofol anaesthesia on BDNF protein in the cortex and thalamus of post natal day14 (PND14) brain of rats over 24 h (Popic et al., 2012). This study shows that BDNF protein is shown to be reduced from 8 to 24 h after the anaesthesia, accompanied with the reduction of TrkB receptors over 24 h after the anaesthesia. Another study in neonate mice shows a significant reduction of BDNF by the propofol anaesthesia in brain (Ponten et al., 2011). These studies are in agreement with our data, except we used the adult rat brains with propofol 120mg/kg rather than developing brain with lower propofol dose.

The main downstream effector phosphrylated ERK1/2 that is involved in the neurotrophic factors signalling pathway was reduced 4 h after the propofol anaesthesia (Popic et al., 2012). In addition to ERK1/2, protein kinase B (PKB), commonly known as Akt, is a serine/threonine specific protein kinase that plays a key role in multiple cellular processes such as apoptosis, cell proliferation, transcription and acts downstream from neurotrophin receptors (Dolcet et al., 1999). Akt is down regulated in the cortex during 24 h after propofol anaesthesia (Popic et al., 2012) and this is

roughly parallel with our data of down regulation of BDNF protein in cortex and hippocampus.

Different studies described an alteration of proteins and genes when animals have received anesthesia. However, few data in the literature focusing on developing brain demonstrated that anaesthesia alone may impact in a complex manner on the biosynthesis of BDNF. In addition, a general long duration anaesthetic triggers an alteration of BDNF protein in brain of rat pups (Lu et al., 2006). It is evidenced that different hippocampal genes are altered (some genes are up regulated and some are down regulated) in rats with long duration anesthesia using isoflurane or nitrous oxide when animals were sacrificed 48 h after the anesthesia (Culley et al., 2006). It has also been described that plasma BDNF concentrations were reduced 24 h after lumber or cervical discotomy under general anaesthesia with propofol and/or thiopental-isoflurane in male patients (Vutskits et al., 2008). Usually anaesthesia is associated with surgery and Fidalgo et al. (2011) also noted that plasma BDNF was decreased 15 min after the induction of anaesthesia with 2% isoflurane in the oxygen enriched air and buprenorphine (0.1 mg/kg, i.p).

In humans, the pharmacokinetics of propofol has been widely studied after either injections or tablet doses (Bryson et al., 1995) and the elimination kinetics is characterized by a rapid metabolic clearance and elimination half life varies between 1 to 3 h (Fulton and Sorkin, 1995). We hypothesise that is there any effect of propofol anaesthesia on BDNF protein even after its elimination. For this, we studied the long term effect of propofol on BDNF protein.

We observed for the first time that short-duration propofol has increased BDNF protein at day 3 after anaesthesia and BDNF protein was again lowered to normal level at day 5. We hypothesise that BDNF protein expression is up regulated at day 3 that may be due to a struggling activity of the animals to recover from the anaesthetic effects that may be responsible for a robust increase of BDNF protein, as a decrease level was observed at day 1. At day 5 after anaesthesia, the increased level of BDNF at day 3 was reduced to a baseline in parallel to the control group. This may be the result of complete elimination of anaesthetic effects from the brain.

No data were available on the long term effect of short duration anaesthesia on BDNF protein in the brain. However, few studies show a long term effect of long duration anaesthetics on brain protein, as changes have been reported in brain proteins 3 days after the submission of long duration anaesthesia and recovery of brain protein is also

observed after weeks (Kalenka et al., 2007). Volatile anaesthetic desflurane has induced changes in protein expression in the brain that persist up to 72 h after anaesthesia (Futterer et al., 2004). As it is well established that synaptic plasticity correlates with learning and memory formation and it is evidenced that BDNF plays an important role in synaptic plasticity (Yamada and Nabeshima, 2003), we may hypothesised that BDNF might have a great role in learning and memory.

5.3: Post-operative cognitive defecit and BDNF

Protein synthesis is required for the long term memory formation which is initiated at the time of training and ends for a few hours afterwards (Davis and Squire, 1984). It has been determined that after an initial phase, there comes a second wave of increased protein synthesis (Ramirez-Amaya et al., 2005). Signalling pathways proteins and mRNA have also shown biphasic activity in various brain regions including hippocampus and cortical regions (Igaz et al., 2002, Trifilieff et al., 2006). Hippocampal subfields immunohistochemistry experiments in mice after conditioning have confirmed the presence of the biphasic augmentation of the pERK in CA1 subfield with an early phase indentified at 1h post training and the second transient phase was observed at around 9h after conditioning (Trifilieff et al., 2007). As it was discussed earlier that pERK plays important role in the synaptic plasticity, it has been suggested that multiple waves of neural plasticity play important role in memory consolidation (Rose, 2000). Clinical studies on postoperative cognitive dysfunction have revealed that surgery under general anaesthesia is associated with cognitive deficits, these cognitive deficits persisting even for months (Moller et al., 1998). Various studies have demonstrated the role of post-operative condition on cognitive performance. As soon as cognition is examined in the post-operative period, two main components of the operative procedure have to be taken into account: anesthesia and surgery.

In this field, some articles are focused on the role of anesthesia. General anaesthetics are associated with the impairment of cognitive and psychomotor performance that is due to the incomplete drug clearance (Moller et al., 1993) and magnitude of this cognitive impairment depends on the type of anaesthetics and their duration. Some studies have evidenced that general anaesthetics are responsible for long lasting cognitive effects. Hence, halothane and nitrous oxide anaesthesia have shown learning deficits and delayed behavioural development during the perinatal period (Levin et al., 1991). The target receptor for nitrous oxide NMDA receptor blocked by dizocilpine

has resulted in long lasting memory deficits for weeks (Lukoyanov and Paula-Barbosa, 2000).

Behavioural studies have investigated that anaesthesia with isoflurane and nitrous oxide produced a long lasting effect on spatial memory and sustained memory impairment has been observed even after 8 weeks in aged rats (Pain et al., 2002, Culley et al., 2003). However, recent data showed no effect of isoflurane on spatial learning at day 1, day 3 and day 7 following anesthesia. In humans, cognitive deficits by the propofol have been observed 3 h after the anaesthesia and these cognitive deficits have been shown to be recovered 6 h after the end of anaesthesia (Sanou et al., 1996).

We examined the long term effect of propofol anaesthesia on memory impairment at 1^{st}, 3rd and 5^{th} day after submission of anaesthesia at 120mg/kg intraperitonealy. We evidenced that delayed memory impairment was observed at 1^{st} and 3^{rd} day after anaesthesia. Whereas no effect was noticed at day 5^{th}. A study on propofol has shown that spatial working memory is intact in aged rats two days after anaesthesia (Lee et al., 2004). Our data revealed that propofol anesthesia has similarly no effect on immediate memory for fear conditioning. This appears in agreement with the short duration amnestic effect of propofol previously described (Veselis, 1999, Pain et al., 2002). However, we highlighted a long lasting deleterious effect of propofol anesthesia on the delayed memory for fear conditioning. Indeed, there are discrepancies in the literature about the proper effects of propofol or other anesthetics on cognition the days following the anesthesia. The mechanisms and the need for reconsolidation of the learning differ from one task to another one, especially in spatial and fear learning (McGaugh, 2000). Recent work in this field suggests that de novo protein synthesis could play a key role for the reconsolidation of persistent memory. Reconsolidation is one of the mechanisms underlying delayed memory as compared to immediate memory. Spatial working memory and learning are linked with BDNF expression in primates. So during tool use learning, an up regulation of BDNF in the parietal cortex has been determined (Ishibashi et al., 2002) and it is evidenced that spatial learning was shown to be impaired when BDNF function was blocked through function inhibiting antibodies to BDNF (Mu et al., 1999). To date, BDNF is involved in consolidation of many types of memories and is critical for late LTP, persistence of LTM storage in the hippocampus. BDNF appears therefore ***"to be a major player in the mechanisms governing the dynamics of memory and may be relevant to***

counteracting a natural process of memory decay" (Bekinschtein et al., 2008). As suggested by our results, any dysregulation of BDNF protein synthesis may impact the memory consolidation and therefore performance of a delayed memory task.

As anaesthesia is associated with surgery, we also examined the effect of minor surgery under propofol anaesthesia, comparing with propofol alone with its intralipidic control on the expression of BDNF protein. After 3rd day of minor surgery under propofol anaesthesia, no alteration of BDNF protein in both the hippocampus and cortex. We observed no effect of minor surgery on BDNF protein expression because minor surgery is unable to produce sufficient inflammation to alter the protein expression. However, with propofol alone, we observed an increase of BDNF protein in the cortex and hippocampal supernatants.

There is few data regarding the effect of surgery on BDNF expression. However, Vutskits et al. (2008) showed that plasma BDNF concentrations were reduced 24 h after lumber or cervical discotomy under general anaesthesia in male patients. Fidalgo et al. (2011) also noted that plasma BDNF was decreased 15 min after the induction of anaesthesia and before the beginning of surgery procedure and surgery has decreased hippocampal BDNF 24 h after tibia fracture performed under general anaesthesia in mice. These two studies are not in agreement to our results. Such difference can be due to the degree of surgeries, as the above studies are made on major surgeries that could induce a sufficient inflammation to alter the protein expression in the brain. LPS that is known to induce inflammation can alter the synthesis of BDNF (Miwa et al., 1997). Inflammatory response induced by the peripheral orthopaedic surgery that increases the level of IL-1β which is shown to impair memory in mice (Cibelli et al., 2010).

5.4: Inflammation and BDNF

Inflammation is one of the main effects associated with post-operative conditions. To further investigate the effect of inflammation on the expression of BDNF protein in the hippocampus and cortical region of rat's brain, we used LPS, as LPS has been used as an inflammation inducer in the previous studies (Merrill et al., 2011, Moreno et al., 2011) and for this purpose, we used *E.coli* LPS to induce systemic inflammation. We observed an increase (about 400%) of BDNF protein in the hippocampal and cortical structures when subjects were submitted with i.p. injections of LPS, compared with intralipidic controls.

Previously, it has been reported that the release of BDNF from hippocampal slices was not changed by the i.p. injection of LPS in rats and it was not possible to find any differences in BDNF alterations after LPS treatment due to different reasons (Shaw et al., 2001). We measured the BDNF proteins in hippocampus and cortical samples homogenized with typical extraction buffer, where as Shaw et al. measured depolarization induced release of BDNF from hippocampal slices prepared from the animals that received i.p. injection of LPS. They incubated hippocampal slices in Krebs solution, applied KCl_2 to depolarize the slice. It is not clear to what extent the amounts of BDNF released into the incubation fluid reflected the actual intracellular BDNF levels in their study. In addition, Shaw et al. tested the effect of LPS with low dose (0.1 mg/kg). Authors suspected that this dose was too low to induce alterations in BDNF. This appeared to be the case since we observed that the effect of LPS with higher dose (1mg/kg). Besides, our data is partially consistent with the observation made by Miwa et al.(1997) who determined that LPS stimulates the synthesis of BDNF and causes an increase of BDNF mRNA and BDNF immunoreactivity (Miwa et al., 1997). However, authors were able to observe effect of LPS only in the cultured microglia. Lapchak et al. (1993) also evidenced that BDNF in the hippocampus was not significantly reduced by LPS at 0.1 and 0.3 mg/kg doses, but reduced by 1.0 mg/kg dose. Our results are not consistent with the observation that systemic LPS at a 1 mg/kg dose reduced BDNF mRNA in the hippocampus. This may be because Lapchak et al. studied the effect of LPS 4 h after the injection that may be responsible an immediate decrease of the BDNF mRNA. In addition, authors studied LPS's effect in the subfields of hippocampus and can not conclude LPS's effect on whole hippocampal and cortex homogenate. A similar study also shows a decrease of BDNF protein in hippocampus as well as in the cortical areas 7 h after injection of LPS (Guan and Fang, 2006). Alternatively, the effects of a single LPS injection on changes in pro-BDNF and mBDNF were studied and showed a transient decrease of BDNF from day 1^{st} - 3^{rd} day. In this case, BDNF was recovered at day 6^{th} after the injections of LPS (Schnydrig et al., 2007). This longer persistent effect at day 3^{rd} may be due to a higher dose of LPS (3mg/kg), compared to our 1mg/kg dose. In addition, these studies investigated LPS's effect on whole brain rather than a specific brain area.

5.5: Role of cytokines in post-operative period

It has been speculated that pro-inflammatory cytokines can play an important role in the development of cognitive deficits that may usually follow surgery and by

extension anaesthesia (Shenkin et al., 1989). Surgery procedure increased IL-6 levels within 2 h of surgery and was remained up to 3rd day (Cruickshank et al., 1990). The magnitude of cytokines elevation is dependent on the extent of surgery, as there is higher elevation of IL-6 after abdominal aortic and colorectal procedures than hip replacement (Baigrie et al., 1992). Peripheral cytokines are shown to exert effects within the CNS through the indirect and direct means. Thus it has been evidenced that in the periventicular regions, IL-1 and TNF-α can enter into the CNS throught the relatively permeable BBB (Gutierrez et al., 1993, Gaykema et al., 1998). Directly, IL-1 can interact with their receptors that are present on the endothelial cells within the brain microvasculature where they play role in expressing a central inflammatory response (Van Dam et al., 1996). Indirectly, changes within the CNS are induced by the cytokines via the vagal afferent nerves (Laye et al., 1995).

It is still not clear how the neurotrophic factors including BDNF mRNA or proteins in the blood or brain are modified by the peripheral LPS injections. However, pro-inflammatory cytokines may be responsible for changes in BDNF protein contents, as LPS is a potent stimulator of pro-inflammatory cytokines and i.p. injections of LPS have been shown to increases pro-inflammatory cytokines in the blood and brain (Hansen et al., 2000, Turrin et al., 2001). Lapchak et al. (1993) has characterised that various effects of LPS are mediated by IL-1, TNF, and IL-6. IP injection of IL-1β also reduces BDNF mRNA in the hippocampus. It is well established that neurotrophins can cross the BBB and peripheral neurotrophins can be transported to the brain (Pan et al., 1998b).

Vagal sensory nerves terminate in the dorsal vagal complex (DVC) of the brainstem, a region that functions to integrate visceral and vagal inputs, are known to mediate some of the effects of peripheral immune challenges including LPS on the brain (Marvel et al., 2004). However, the influences of vagal inputs on brain neurotrophins have not been examined yet. Peripherally derived neurotrophins may also cross the BBB, transported to the brain. In addition, LPS may increase brain neurotrophic factors levels by promoting monocytes and macrophage infiltration into the brain (Zito et al., 2001). It is likely that the brain neurotrophic factors can be increased through such mechanisms since LPS has been shown to increase the production of NGF by monocytes and macrophages (Caroleo et al., 2001).

At cellular and molecular level, Miwa et al. (1997) showed that LPS stimulates secretion of BDNF and NGF in microglia cultures, whereas IL-1 stimulates the

synthesis and release of NGF in microglia and astrocytes (Heese et al., 1998, Juric and Carman-Krzan, 2001). These studies suggest that LPS may be responsible in changing the BDNF protein levels in the brain indirectly in a possible way that the IL-1 may impair cholinergic activities of the neurons which further up regulate BDNF and NGF (Knipper et al., 1994). On other way, decrease of neurotrophic factors may result of the activation of HPA axis, as LPS is a potent stimulator of pro-inflammatory cytokines and injection of LPS increases pro-inflammatory cytokines in the blood and brain (Beishuizen and Thijs, 2003). Hence, TNF plays an important role in stimulating HPA axis after single dose of endotoxin administration (Hadid et al., 1996).

5.6: Role of glucocorticoids in post-operative period

During the postoperative period, inflammation is the most prevalent side effect and is a host defence process involving the recruitment and activation of cells of immune system in response to infection or injury (Chapman et al., 2009). Glucocorticoids not only act as potent anti-inflammatory but also act as immunomodulatory and depending on their concentration they can enhance or suppress the immune reactions (Staab and Maser, 2010). During the inflammatory process, HPA axis is activated by the circulating pro-inflammatory cytokines like IL-6, IL-1β which are further responsible for the increased production of cortisol (Staab and Maser, 2010).

Glucocorticoids have various effects on the brain, in particular the hippocampus that expresses high levels of mineralocorticoid receptor and glucocorticoid receptors. These receptors are the targets of the glucocorticoid actions, such actions are anti-inflammatory and immunomodulatory in nature (Staab and Maser, 2010). These basal and permissive effects include the inhibition of neural death in the hippocampal regions, stimulation of synaptic plasticity and improvement of hippocampal dependent cognitive performance. Glucocorticoids have shown neuroprotective effects during the innate immune response that was induced by the single dose of LPS. It induced a transient production of different cytokines including TNF-α and these events are down regulated by the glucocorticoids without such endogenous response by the glucocortiocids, microglial TNF-α may become highly neurotoxic that may responsible for cell death through the caspase and nitric oxide pathways (Nadeau and Rivest, 2003).

However, chronic glucocorticoid excess has shown deleterious effects on the hippocampus. In human, high levels of circulating cortisol levels are associated with poor memory, hippocampal damage and shrinkage and neuronal loss (Newcomer et

al., 1994, Lupien et al., 1998). High levels of glucocorticoids are also associated with various diseases including Cushing's syndrome (Sonino and Fava, 2001) and various neurodegenerative diseases like Alzheimer's disease and chronic actions of glucocorticoids have shown in promoting the aging of the brain, especially the hippocampal neurons (Porter and Landfield, 1998).

Data in the literature have indicated that glucocorticoids play important role in regulating neurotrophin mRNA expression both in the cortex and in the hippocampus and it is suggested that the effects of glucocorticoids on the brain for neuronal survival could be due to the alterations in the levels of neurotrophins in the brain (Barbany and Persson, 1992). Levels of BDNF mRNA are reduced up to -70% as compared to its control in both the cortex and hippocampus 3rd day after the injection of single dose of dexamethasone at 5 mg/kg (Barbany and Persson, 1992). Previously, it has also been evidenced that exogenous corticosterone decreased neurotrophins in astrocytes and mixed culture (Gubba et al., 2004) and subcutaneous injections of corticosterone are shown to suppress the expression of BDNF mRNA in the hippocampus (Hansson et al., 2003).

It has been well established from above studies that exogenous glucocorticoids play important role as anti-inflammatory and immunomodulatory agents. However, endogenous glucocorticoids have been described for their protective importance against TNF-α induced increase in anxiety like behaviour in mice when the subjects were infected with virus (Silverman et al., 2007).

5.7: A possible protective effect of propofol anaesthesia on inflammation ?

Previously, different studies have suggested protective effects of anesthetics on the brain. Propofol has decreased neuronal damage and improves neurologic outcome when compared to fentanyl/N_2O in incomplete cerebral ischemia (Kochs et al., 1992). Propofol also shows the scavenging of free radicals that resembles endogenous antioxidant alpha-tochopherol also called vitamin E (Murphy et al., 1992). Hence, in comparison with midazolam, propofol may be antioxidant (Tsuchiya et al., 2001). As propofol mediates its action via activation of $GABA_A$ receptor and it is indicated that propofol inhibit ischemia induced neural death (Ito et al., 1999). Propofol is also shown to reverse partially the neural death induced by the oxygen–glucose deprivation in primary mixed cultures of neurons and astrocytes in the rat's cerebral cortex (Velly et al., 2003).

Propofol has also been shown to exhibit immunomodulatory effects by decreasing the production of pro-inflammatory cytokines and altering the biosynthesis of nitric oxide (Marik, 2005, Takemoto, 2005). Early post treatment of propofol after endotoxin injection has shown to reduce the mortality rate of rats. In addition, propofol has shown to attenuate their cytokine response after endotoxin injections and attenuated the production of TNF- α (Taniguchi et al., 2002). Our data on early post treatment of propofol after the LPS has reduced the BDNF levels that were increased by the LPS alone after 3rd day of treatment. We hypothesised that it may reflect the protective effect of propofol.

6: Conclusion

The purpose of our studies was to investigate the post-operative cognitive dysfunction through the quantification of BDNF protein in two important structure of the brain (hippocampus and prefrontal cortex). The hippocampus that plays key role in learning and memory and the prefrontal cortex is implicated in major executive functions. Initially, we performed study to assess the peak level of BDNF protein during 24 h of the day and it was observed for the first time that hippocampal BDNF protein follows a circadian rhythm with a peak of BDNF expression at ZT5 (five hours after the light on period) either in nocturnal or diurnal rodents. These data urge us to perform experiments at specific time in order to standardise the experiments. Operative conditions were created mainly including inflammation, anaesthesia state and surgery itself. In order to induce anaesthesia state, inflammation and surgery, short duration propofol anaesthesia, bacterial LPS and minor abdominal surgery were performed.

Intraperitoneal injections of short duration propofol anaesthesia were able to alter the BDNF protein concentrations in both the hippocampus and cortex (until day 3). Immediate and long term effects of propofol anesthesia on the expression of BDNF protein were observed. After an initial increase, there were reduced levels of BDNF at day 1^{st} followed by an increased expression at day 3^{rd}. At day 5^{th} BDNF recovers the levels of controls. Our data clearly showed, for the first time, a dysregulation of the expression of BDNF following short duration anesthesia induced by propofol.

For normal functioning of the body, it is always needed a balanced quantity of specific proteins. These proteins become detrimental if they are reduced or increased in quantity. Study by Cunha et al., 2009 has shown that increased levels of BDNF in the CNS have shown detrimental effects on learning and memory. Behavioral studies also show that short duration propofol anaesthesia has long term effect on memory impairment. Inflammation is the major post-operative effect that is responsible for variety of detrimental effects in all the body systems including the nerous system. However, minor surgery alone was unable to alter the BDNF protein levels in both the cortex and hippocampus, as minor surgery did not induce sufficient inflammation.

Intraperitoneal administration of LPS was shown to increse BDNF in both the cortex and hippocampus at day 3 after the injections. In response to LPS, there is induction of an inflammatory response in the CNS and elevated TNFα and MHCII levels are

observed. These pro-inflammatory mediators produced in response to LPS are hypothesised to affect cognition. Cytokines such as TNFα have been shown to affect learning and memory processes such as LTP by affecting glutamatergic transmission in various ways like affecting the number of receptors, transporters and glutamate release (Vereker et al., 2000, Zou and Crews, 2005). Furthermore, excessive glutamate present in the synaptic cleft due to the alteration in transmission is able to induce neural toxicity (Hardingham et al., 2002). It is not limiting to glutamatergic transmission, LPS treatment has also been shown to affect cholinergic innervations. Long term cognitive impairment and reduced expression of vesicular acetylcholine transporter (VAChT) in the cortex suggested a loss of cholinergic innervations (Semmler et al., 2007). VAChT is correlated as well as affected by BDNF (Takei et al., 1997).

In addition, it was also evidenced the protective effect of short duration propofol anaesthesia that show a reduction of BDNF protein level that was over expressed by LPS in both the hippocampus and cortical homogenates. This suggests that anaesthetic dose of propofol is less damaging for the neuronal tissue and has less effects on learning and memory as compared to other anaesthetics. Our data on behavioural studies have also highlighted that propofol impairs memory at day 1^{st} and 3^{rd} but no at day 5^{th}. Previous studies have also been shown neuroprotective effects of propofol due to its antioxidant action (Bao et al., 1998) and has anti-inflammatory properties as well as it might protect brain from ischemic injury (Kotani et al., 2008). As detrimental effects are concerned, few studies describe the neurodegenerative effects only in developing brains. Commonly used general anaesthetics may be responsible in the induction of neurodegeneration. Apoptotic action of propofol has been studied in 7 day old brain of rats that has shown down regulation of NGF mRNA and protein expression in the thalamus and cortex during 24 h after the propofol treatment. This anaesthetic dose (25mg/kg) of propofol was responsible in inducing complex changes that were accompanied by the cell death in both the structures cortex and thalamus of the developing brain (Pesic et al., 2009). So our results might suggest a possible protective effect of the short duration propofol anaesthesia against neuroinflammation induced by LPS.

7: Future Perspectives

A study suggesting the role of pro-BDNF in cellular dysfunction and synaptic loss underlying cognitive impairment in AD (Peng et al., 2005). Pro-BDNF levels in the parietal cortex are decreased 40% in end-stage AD compared with age matched control brain (Michalski and Fahnestock, 2003). It should be important to know whether pro-BDNF follow circadian rhythms like mBDNF or not. Is pro-BDNF also altered post-operatively?

Our data can not conclude the postoperative effects on different hippocampal subfields, as we determined BDNF protein in the hippocampal homogenate. Hippocampal subfields may vary in protein concentrations. The strongest staining has been detected in the pyramidal layers of CA regions, and in granular neuron cell bodies and dendrites of dentate gyrus (Yan et al., 1997). Therefore, it could be possible to determin the BDNF protein in individual subfields of the hippocampus using immunohistochemistry on the brain slices. In addition, we studied the effect of minor surgery and we can not conclude the effect of major surgery on the expression of BDNF protein, as magnitude of the inflammation is dependent on the extent of surgery.

Alteration and dysregulation of BDNF has been reported in various pathologies, including loss in cognitive abilities, dementia, depression, epilepsy, and Alzheimer's, Huntington's, and Parkinson's diseases (Murer et al., 2001). Our main results on the effect of short duration anaesthesia evidenced that propofol has dysregulated the circadian profile of BDNF protein (observed at six different time points). As BDNF has been evidenced important for learning and memory performance and this memory performance has been shown to follow a circadian profile (Roth and Sweatt, 2008) in parallel with the circadian rhythmicity of PERK1/2 (Eckel-Mahan et al., 2008). To determine if any dysregulation of ERK1/2 and memory performance rhythmicity post-operatively can be our future perspective.

Our data did not demonstrate postoperative effects on the aged brains, as our studies are focused on young brains. It is investigated that age of the brain is considered important in regard of after effects of anaesthetics, as it is evidenced that general

anaesthetics are responsible for cognitive deficits in aged rodents while young rodents have no memory impairment when general anaesthetics are used (Culley et al., 2003). There may be different consequences of short duration anaesthetics on BDNF alteration in aged brains, as Culley et al. (2003) evidenced that general anaesthetics are responsible for cognitive impairment in aged rats while no effect on young rats. It has also been shown that BDNF alteration is effected by the aging (Shi et al., 2010). Conner et al. (1997) has shown wide spread distribution of BDNF in CNS and high levels of BDNF protein are observed in the hippocampus. It has also been described that higher loss of hippocampal neurons is related with aged rats as compared to young ones, that may be responsible for increased cognitive deficits (Hattiangady et al., 2011). The possible post-operative effects on alteration of BDNF protein expression in the aged brains can be investigated using ELISA.

Data in the literature have highlighted the importance of cortisol along with BDNF and it has been evidenced that BDNF and cortisol play important role for neurogenesis in mammals (Jacobs et al., 2000). It has also been noted that cortisol levels follow a circadian profile observed in healthy males at different points during the day (Begliuomini et al., 2008). It could be interesting to investigate anesthetic effects on cortisol rhythmicity and post-operative effects on alteration of cortisol expression if any using ELISA.

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Résumé : Le Brain-derived Neurotrophic Factor (BDNF) est impliqué dans les processus cognitifs impliquant l'hippocampe et les structures corticales. Cette étude avait pour but d'analyser les effets postopératoires sur l'expression du BDNF dans ces structures cérébrales. Ainsi, les effets d'une anesthésie de courte durée au propofol, d'une chirurgie mineure et du lipopolysaccharide (LPS) sur l'altération de l'expression du BDNF ont été analysée dans l'hippocampe et le cortex de deux modèles de rongeurs nocturnes et diurnes, correspondant respectivement des jeunes rats Sprague Dawley mâles et des *Arvicanthis ansorgei.*

Dans un premier temps, la rythmicité nycthémérale de l'expression du BDNF a été analysée. Les quantités de BDNF présentes dans le cortex et l'hippocampe ont été déterminées par une technique ELISA. Il s'est avéré que, dans l'hippocampe et le cortex de rat et d'*A. ansorgei*, le BDNF suit une rythmicité sur 24 heures. La quantité de BDNF atteint un maximum à ZT5 (*i.e.*, 5 heures après le début de l'exposition à la lumière).

Dans un deuxième temps, les effets sur l'expression du BDNF ont été analysés après administration de propofol et/ou LPS, ainsi que lors d'une chirurgie légère. Parallèlement, l'impact de ces traitements sur la mémoire a été testé à l'aide d'un test d'évitement passif.

Nos résultats indiquent que la quantité de BDNF est régulée positivement dans l'hippocampe et le cortex de rats lorsque les animaux ont subit une anesthésie de courte durée au propofol en présence ou absence de LPS. A l'opposé, une chirurgie mineure (sous anesthésie propofol) n'a aucun effet sur les quantités de BDNF.

En conclusion, ces études mettent en évidence des effets majeurs d'une anesthésie sur l'expression du BDNF, ainsi que les effets protecteurs du propofol sur la neuroinflammation induite par le LPS.

Abstract: Brain-derived neurotrophic factor (BDNF) is involved in cognition and hippocampus and cortical structures are important in cognition. The present study was designed to analyse the post-operative effects on BDNF. For this purpose, we examined the effects of short duration propofol anaesthesia, LPS and minor surgery on the BDNF protein alteration in the hippocampus and cortex.

Young male Sprague Dawley rats were used in all experiments except *Arvicanthis ansorgei* which were used to study the circadian rhythmicity of BDNF protein as model for diurnal rodents. The quantity of BDNF protein present in the cortex and hippocampal supernatants was determined with an ELISA technique. Memory was tested by fear conditioning using the classic fear conditioning preparation (passive avoidance apparatus).

The major finding on BDNF protein in control conditions is that the BDNF protein followed a circadian rhythmicity during the 24 hours of day in the rat's hippocampus and cortex. The concentration of BDNF protein has been observed to reach a maximum at ZT5 (5 hours after the light on period) whether the animals were nocturnal or diurnal. In addition, we found that BDNF protein amount is upregulated in the hippocampus and cortex of rats when they were submitted to short duration propofol anaesthesia, as well as LPS and no effect when minor surgery under propofol anaesthesia was performed.

In conclusion, these studies illustrate the dramatic effects of post-operative conditions and neuroinflammation induced by LPS on cognition and the potential mechanism involved. This study also suggests the protective effects of the short duration propofol anaesthesia against neuroinflammation induced by LPS.