THE ROLE OF *DREAM* IN THE REGULATION OF FORMALIN INDUCED PAIN IN RAPID EYE MOVEMENT (REM) SLEEP DEPRIVED RATS

by

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This thesis is a special dedication to my beloved brother,
Allahyarham Sorhaizi Bin Siran
(1973 – 1992)

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LIST OF ABBREVIATIONS

5-HT	: serotonin
A_{260}	: absorbance at 260 nm
A_{280}	: absorbance at 280 nm
ABC	: avidin-biotin complex
AC	: adenylyl cyclase
Ach	: acetylcholine
ACTH	: adrenocorticotropic hormone
ANOVA	: analysis of variance
APS	: ammonium persulphate
ARAS	: ascending reticular activating system
ATP	: adenosine-5'-triphosphate
BSA	: bovine serum albumin
BWg	: body weight gain
Ca^{2+}	: calcium
$CaCl_2$	: calcium chloride
cAMP	: cyclic adenosine monophosphate
cDNA	: complementary DNA
CER I	: cytoplasmic Extraction Reagent I
CER II	: cytoplasmic Extraction Reagent II
CMN	: cellular-molecular-network
CSF	: cerebrospinal fluid
C_T	: threshold cycle
DAB	: diaminobenzidine
DDR	: DNA damage response
DEPC	: diethyl pyrocarbonate
dH_2O	: deionized water
DNA	: deoxyribonucleic acid
DOW	: disk over water
DPN	: DREAM positive neuronal cells
dR	: fluorescence value
DRE	: downstream response element

DREAM : down regulatory antagonist modulator
EDTA : ethylenediaminetetraacetic acid
EEG : electroencephalogram
EF1 : first EF hand
EF2 : second EF hand
EF3 : third EF hand
EF4 : fourth EF hand
EMG : electromyogram
EOG : electrooculogram
ER : endoplasmic reticulum
Fc : food consumption
FMC : free moving control rats
FMCf : free moving control with formalin test rats
GABA : γ-aminobutyric acid
$GABA_A$: γ-aminobutyric acid-A
$GABA_B$: γ-aminobutyric acid-B
GAPDH : glyceraldehyde 3-phosphate dehydrogenase
GFAP : glial fibrillary acidic protein
H_2O_2 : hydrogen peroxide
HCl : hydrochloric acid
HRP : horseradish peroxidise
IASP : international association for the study of pain
IDV : integrated density values
IL-1β : interleukin-1 beta
InsP3R : inositol-1,4,5-triphosphate receptor
IP_3 : inositol trisphosphate
IPSP : inhibitory postsynaptic potential
kb : kilobase
KchIPs : Kv channel-interacting proteins
KCl : potassium chloride
kDa : kilodalton
Kv : voltage-gated potassium
LARUSM : laboratory animal research unit, Universiti Sains Malaysia

LC	: locus coeruleus
LDT	: lateral dorsal tegmentum
Mg^{2+}	; magnesium
$MgCl_2$	: magnesium chloride
mGluRs	: metabolic glutamate receptors
MORs	: mu-opioid receptors
mPRF	: medial pontine reticular formation
mRNA	: messenger RNA
NA	: noradrenaline
Na_2HPO_4	: disodium hydrogen phosphate
$Na_2HPO_4.7H_2O$	: sodium phosphate heptahydrate
NaCl	: sodium chloride
$NaH_2PO_4.H_2O$	: sodium dihydrogen phosphate dehydrate
$NaHCO_3$	: sodium bicarbonate
NaOH	: sodium hydroxide
NER	: nuclear extraction reagent
NGS	: normal goat serum
N-NOC	: non nociceptive
NPY	: neuropeptide Y
NREM	: non rapid eye movement
NREs	: negative regulatory elements
NSAIDs	: non steroidal anti-inflammatory drugs
PACAP	: pituitary adenylate cyclase-activating polypeptide
PAGE	: polyacrylamide gel electrophoresis
PB	: phosphate buffer
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PFA	: paraformaldehyde
PGD2	: prostaglandin D2
PGO	: ponto-geniculo-occipital
PI	: protease inhibitors
PKA	: protein kinase A
PLC	: phospholipase C

POMC	: pro-opiomelanocortin
PPT	: pedunculopontine tegmentum
PS-2	: presenilin-2
PVDF	: polyvinylidene fluoride
qRT-PCR	: real time quantitative polymerase chain reaction
REM	: rapid eye movement
REMs	: rapid eye movements
REMsd	: REM sleep deprivation rats
REMsdf	: REM sleep deprivation with formalin test
RG	: recovery rats
RGf	: recovery with formalin test rats
RN	: raphe nuclei
RNA	: ribonucleic acid
Rnase	: ribonuclease
rpm	: revolutions per minute
rRNA	: ribosomal RNA
RT-PCR	: real time PCR
S.E.M	: standard error mean
SDS	: sodium dodecyl sulphate
SDS-PAGE	: SDS-Polyacrylamide gel electrophoresis
SI	: primary somatosensory
SII	: secondary somatosensory
SN	: specific nociceptive
SNc	: substantia nigra compacta
SPSS	: statistical package of social sciences software
SWS	: slow wave sleep
TBE	: tris-borate-EDTA
TBS	: tris-buffered saline
TBST	: tris-buffered saline Tween-20
TC	: tank control rats
TCf	: tank control with formalin test rats
TEMED	: N'-N' Tetramethylenediamine
TNF-α	: tumour necrosis factor alpha

Tris-HCl	: tris-hydrochloric acid
tRNA	: transfer RNA
TUNEL	: terminal deoxynucleotidyl transferase dUTP nick end labelling
UCP1	: uncoupling protein 1
VB	: ventrobasal thalamic complex
VPI	: ventroposterioinferior
VPL	: ventroposterolateral
VPM	: ventroposteromedial
VTA	: ventral tegmental area
WDR	: wide dynamic range
xg	: times gravity
αCREM	: alpha cAMP response element modulator

PERANAN PROTEIN '*DREAM*' DALAM PENGAWALATURAN KESAKITAN DI BAWAH ARUHAN FORMALIN PADA TIKUS YANG KEKURANGAN TIDUR REM

ABSTRAK

Kekurangan tidur REM telah terbukti mengurangkan paras kesakitan selepas beberapa stimulasi kesakitan. Protein 'Downstream Regulatory Element Antagonist Modulator' (DREAM) merupakan penindas transkripsi bagi gen *prodynorphin*. Kajian ini dijalankan untuk mengkaji perubahan DREAM selepas kekurangan tidur, kesakitan dibawah aruhan formalin atau gabungan keduanya, dan perkaitannya dengan kelakuan tindak balas kesakitan di bawah aruhan formalin. Tikus Spraque Dawley jantan (250-300 g) dibahagikan kepada empat rawatan utama iaitu bebas bergerak tanpa apa-apa rawatan (n=36), kekurangan tidur REM (n=36), kawalan tangki (n=36) dan pemulihan tidur (n=36). Kekurangan tidur dirangsangkan selama 72 jam menggunakan teknik pasu terlangkup. Setiap rawatan utama seterusnya dibahagikan kepada dua kumpulan yang tidak atau disuntik dengan formalin 2.5% secara subkutan. Kelakuan tindak balas kesakitan dibawah aruhan formalin direkodkan selama 1 jam untuk kumpulan yang disuntik formalin. Bahagian kompleks ventrobasal talamik, (VB) dikeluarkan dari setiap kumpulan untuk analisis immunohistokimia (n=6), Western blot (n=6) dan real-time PCR (n=6) secara berasingan. Teknik pasu terlangkup telah disah mengaruhkan kekurangan tidur REM dengan memaparkan corak klasik hiperfagia dan kehilangan berat badan kepada kumpulan kekurangan tidur REM dan pemulihan tidur. Terdapat kekurangan kesakitan yang ketara pada fasa kedua kesakitan di bawah aruhan formalin pada tikus

yang kekurangan tidur REM. Kekurangan tidur REM mengaruh perubahan morfologi dan mengurangkan bilangan neuron yang positif kepada DREAM (DPN) pada kedua-dua sisi. Terdapat peningkatan kepada ekstrak nuklear DREAM. Selepas pemulihan tidur selama 72 jam, perubahan kepada DPN tidak kembali ke paras dasar serta terdapat pengurangan pada mRNA DREAM di kedua-dua sisi. Kesakitan di bawah aruhan formalin mengurangkan bilangan DPN di kedua-dua sisi dan peningkatan pada ekstrak DREAM nuklear di sisi kontralateral kepada kedudukan suntikan formalin. Kekurangan tidur REM dan ujian formalin meningkatkan DPN, ekstrak sitoplasma dan nuklear DREAM pada kedua-dua sisi, di mana peningkatan adalah lebih tinggi di sisi kontralateral kecuali pada ekstrak nuclear. Terdapat pengurangan pada mRNA DREAM pada sisi ipsilateral. Walaubagaimana pun, perubahan tersebut berpotensi untuk dipulihkan kerana tiada perubahan dapat dilihat pada DPN, ekstrak DREAM dan mRNA selepas pemulihan tidur. Sebagai kesimpulan, kekurangan tidur REM dan kesakitan di bawah aruhan formalin, secara berasingan menghasilkan pengaruh tersendiri ke atas DREAM. Walaupun demikian, kombinasi kedua-dua rawatan menghasilkan perubahan dinamik intraselular yang mencerminkan keupayaan kelangsungan hidup sel neuron, sekurang-kurangnya dengan melindungi fungsi asasnya. Memandangkan protein DREAM bertindak sebagai perencat transkripsi gen bagi prodynorphin, keupayaan kelangsungan hidup neuron ditunjukkan melalui kekurangan tindak balas kesakitan selepas kekurangan tidur REM dan kesakitan di bawah aruhan formalin.

THE ROLE OF *DREAM* IN THE REGULATION OF FORMALIN INDUCED PAIN IN RAPID EYE MOVEMENT (REM) SLEEP DEPRIVED RATS

ABSTRACT

Rapid eye movement (REM) sleep deprivation has been shown to decrease pain threshold after various pain stimuli. Down regulatory antagonist modulator (DREAM) is a transcriptional repressor of *prodynorphin* gene. This study evaluates the effect on DREAM in relation to REM sleep deprivation, formalin induced pain or combination of both; and its relationship to the formalin induced pain behavioural responses. Male Sprague Dawley rats (250-300 g) were divided into four major treatments; free moving control (n=36), REM sleep deprivation (n=36), tank control (n=36) and sleep recovery (n=36). REM sleep deprivation was elicited for 72 hours using the inverted flower pot technique. Each group was further divided into two groups which consisted of rats that were either not or subjected to 2.5% formalin subcutaneous injection. Food consumption and body weight gain were measured before and after the treatments. The formalin induced pain behavioural responses were recorded for one hour for rats that subjected to formalin injection. The ventrobasal thalamic complex of brain (VB) were removed from each group for immunohistochemistry (n=6), Western blot (n=6) and real-time PCR analysis (n=6) separately. The 'inverted flower' pot technique was confirmed to induce REM sleep deprivation in the REM sleep deprived and sleep recovered rats by the classic pattern of hyperphagia with converse loss of body weight. There is a marked hypoalgesia demonstrated in the second phase of formalin induced pain in the REM sleep deprived rats. REM sleep deprivation per se did induce morphological change and reduced the number of DREAM positive neurons (DPN) bilaterally. There was an

increase in nuclear DREAM extraction bilaterally. After 72 hours sleep recovery, the morphological changes still persisted with reduction of the DREAM mRNA bilaterally. Formalin induced pain reduced the number of DPN bilaterally and increased the nuclear DREAM extraction contralateral to formalin injected site. Interestingly, REM sleep deprivation with formalin test increased the number of DPN, cytoplasmic and nuclear DREAM extraction bilaterally which was more on the contralateral side except for nuclear extraction. There was a significant decrease of DREAM mRNA ipsilaterally. However the changes seem to be reversible as no change is seen in DPN, DREAM extractions and mRNA in sleep recovery group with formalin induced pain. . In conclusion, REM sleep deprivation and formalin induced pain per se generated their own distinct effects on DREAM. Nevertheless, the combination of both treatments resulted in dynamic intracellular changes which reflected the survival ability of neuronal cells, at least by preserving its basic functions. As DREAM is a transcriptional regulator of prodynorphin, the functional survivability of the neuronal cells was reflected behaviourally by the significant hypoalgesia after both REM sleep deprivation and formalin induced pain.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Sleep is an active process and consists of slow wave sleep (SWS) and rapid eye movement (REM) sleep. The progression of sleep depends on the activities of the wake promoting systems as well as the relative changes in the neurotransmitters of the brain. REM sleep is a crucial component of sleep and extensive studies prove that lack of REM sleep resulted in morbidities behaviourally (Gulyani & Mallick, 1995, Rechtshaffen & Bergmann, 1995, Suchecki *et al*., 2003) as well as to induce intracellular changes such as alteration in intracellular calcium (Ca^{2+}), membrane bound ATPase activity, receptor sensitivities and gene expressions (Mallick & Gulyani, 1996, Mallick *et al*., 2000, Majumdar & Mallick, 2003, Terao *et al*., 2003, Koban & Swinson, 2005, Koban *et al*., 2006, Das & Mallick, 2008). The REM sleep induced morbidities reflected how important REM sleep to human and animal is. However, the morbidities and intracellular changes are valuable when one is conducting a research in relation to REM sleep functions.

Currently, it is understood that while pain disrupts sleep, nevertheless, more significantly, sleep deprivation affects pain perception. There have been some studies which addressed this issue by specifically studying pain perception changes in relation to sleep disturbance in humans (Lentz *et al*., 1999, Onen *et al*., 2001). REM sleep deprivation induces significant increases in the animal pain behavioural responses in certain pain stimuli (Onen *et al*., 2000, Onen *et al*., 2001, Hakki Onen *et*

al., 2001, May *et al*., 2005). However, no molecular study has been conducted to clarify these observations which were related to REM sleep and pain. No doubt that pain is one of the major public health concerns and understanding pain is important in order to improve our decision making skills in relation to pain treatment. Sleep deprivation and pain are co-existed in patients who suffered in some major illnesses (Okifuji & Hare, 2011). The co-existence of both symptoms results in difficulties in term of diagnosing and managing not only the pain but also the illness. Hence, it is crucial to understand pain especially in relation to REM sleep by studying the molecular mechanisms mediating the interactions between the two factors. Formalin test is one of the best animal pain models that can be used to mimic clinical pain. Thus, by manipulating formalin induced pain on REM sleep deprived rats, the relationship between pain and REM sleep can be clarified.

The recent discovery of down regulatory antagonist modulator (DREAM) (Carrión *et al*., 1999) as a protein that may be involved in pain modulation (Carrión *et al*., 1999, Cheng *et al*., 2002) has disclosed even more research opportunities related to the study of pain. Moreover, previous study has shown that DREAM is a transcriptional gene repressor which binds directly to Ca^{2+} in order to exert its intracellular functions (Carrión *et al*., 1999). As mentioned earlier, REM sleep deprivation induces changes in intracellular Ca^{2+} concentrations (Mallick & Gulyani, 1996). Due to the facts that Ca^{2+} is bind directly to DREAM and REM sleep deprivation induces the intracellular Ca^{2+} changes; this study in conducted to primarily elucidate the role of DREAM in the regulation of formalin induced pain in REM sleep deprived rats. Consequently, the molecular mechanism between pain and sleep is delineated by the dynamic changes of intracellular DREAM and DREAM

mRNA. The molecular mechanism posed contributes to knowledge and assists any other preliminary efforts which are trying to define the cutting edge between sleep deprivation and pain. Subsequently, DREAM may be a potential protein marker which can be adopted later in clinical setting in order to demarcate causes of sleep deprivation, pain or combination of both in a patient. Furthermore, DREAM may represent as a potential alternative target for pain management strategies in patients with or without sleep deprivation.

1.2 Sleep

Sleep is one of the basic characteristics of all living organisms. It is perceived that sleep is a passive state by which living organisms "repress" their activities and become "reenergised" for future activities. Sleep is a unique phenomenon where a person who is asleep is in a passive state of unconsciousness and when awake, is unable to recall any mental activities that occurred while he was asleep. These observations lead scientists and philosophers to assume that sleep is a state of rest.

However, sleep is actually an active process. In mammals, in addition to conserving energy, sleep is also involved in other body functions such as a defence mechanism against oxidative stress (Everson *et al.*, 2005), facilitating neurogenesis (Guzman-Marin *et al.*, 2007, Guzman-Marin *et al.*, 2008), enhancing brain protein synthesis (Nakanishi *et al.*, 1997) as well as altering gene expression (O'Hara *et al.*, 1999, Cirelli *et al.*, 2004, Cirelli *et al.*, 2006). Sleep is characterised by a decrease of motor activity with a typical rest posture.

The eyes are usually closed and there is muscular hypotonia. At the early stage of sleep, mammals are alert to external stimuli but as the sleep deepens the responses become less obvious (Datta, 2010).

1.2.1 Studies of sleep patterns

The electroencephalogram (EEG) of both humans and animals while awake, asleep and dreaming, were first defined in the 1930's (Loomis *et al*., 1935a,b, Klaue, 1937, reviewed in Datta & Maclean, 2007). By studying the EEG patterns in the cat, Klaue found that there were two distinct EEG patterns : first was a period of light sleep in which there were slow cortical brain waves, followed by a period of deep sleep in which the waves sped up (Klaue, 1937, Datta & Maclean, 2007).

It was not until 1953, that the phenomenon of REM sleep was first observed in studies of sleep cycles in humans (Aserinsky & Kleitman, 1953) where the eye movements, as well as cortical EEG, pulse, respiratory rates and gross body movements were recorded in adult subjects (Aserinsky & Kleitman, 1953). It was in this study that Aserinsky and Kleitman first discovered REM sleep from observations of clusters of jerky, rapid eye movements (REMs), EEG patterns together with the increased respiratory and heart rate in sleeping humans (Aserinsky & Kleitman, 1953). These clusters of REMs appeared in cycles with variation in periods, lengthened as the sleep deepened (Aserinsky & Kleitman, 1953) and were related to dreams (Dement & Kleitman, 1957). The occurrences of REMs during this particular period of sleep resulted in this phase of sleep to be described as the 'REM sleep' phase.

REM sleep in the animal model was first established in sleeping cats (Dement, 1958). Two different patterns of cortical EEG activities were observed, one was a low voltage, fast EEG and the other consisted of slow waves and spindles as well as some muscle activities in the legs and eyeballs (Dement, 1958). The 'paradoxical' phenomena between EEG and electromyogram (EMG) activities were noticed in the same year by Jouvet and Michel as reviewed by Datta and Maclean in 2007. Subcortical electrodes were implanted and an EEG recording obtained from three main areas: the ventral hippocampus, midbrain reticular formation and pontine-reticularis caudalis nucleus (Datta & Maclean, 2007). Electrodes were also implanted in the neck muscles for EMG recording (Datta & Maclean, 2007). The disappearance of EMG activities during REM sleep coincided with the increment in awake-like EEG activities (Datta & Maclean, 2007). This paradoxical phenomenon was seen during REM sleep which was now alternatively known as the 'paradoxical' sleep phase (Jouvet, 1965).

1.2.2 Types of sleep

Sleep can be divided into two types: non rapid eye movement (NREM) and REM sleep. These sleep stages can be monitored and differentiated by polysomnography studies, which consist of electroencephalography, electromyography and electrooculography. During wakefulness or sleep, there are some discrepancies in the EEG, EMG and electrooculogram (EOG) patterns (Figure 1.1). In a waking state, there is low amplitude synchronization of the fast oscillation patterns in the EEG (Campbell, 2009) and presence of muscle tone detected by the EMG (Mileva-Seitz *et al.*, 2005). During NREM sleep (or slow wave sleep (SWS)), the patterns change slowly and the NREM sleep stages can be indentified.

In human, there are four stages of NREM sleep (Pizza *et al.*, 2011) (Figure 1.1). As the sleep deepens, there is high voltage but low frequency wave activity. Stage III and IV are the deepest NREM sleep stages and during these stages, only low-frequency wave activity is present. The waveforms in stage II are different as it is characterised by slow oscillation with distinctive sleep spindles and K-complex (a negative sharp wave followed immediately by slower positive component). However, in animals there are only two stages of NREM sleep which are stage I and II (Datta & Maclean, 2007) (Figure 1.1). During stage I, there is a presence of sleep spindles in the cortical EEG. Stage II or deep sleep is identified by the presence of high-amplitude, low-frequency waves in the cortical EEG.

There are 3 main characteristics that differentiate REM from NREM sleep (Hobson, 2005): 1) in the cortical EEG, there is low-amplitude synchronization of fast oscillations, theta waves; 2) in the EMG, there is very low muscle tone especially in antigravity muscles; and 3) in the EOG, there are singlets and clusters of REMs. There are also other associated signs such as myotonic twitches, flunctuations in cardio-respiratory rhythms and core temperature, penile erection and clitoral tumescence.

1.2.3 Wake-promoting systems of the brain

It is crucial to discuss the wake-promoting systems of the brain in order to understand the generation of sleep. The wake-promoting systems are responsible in maintaining the alert status of a person.

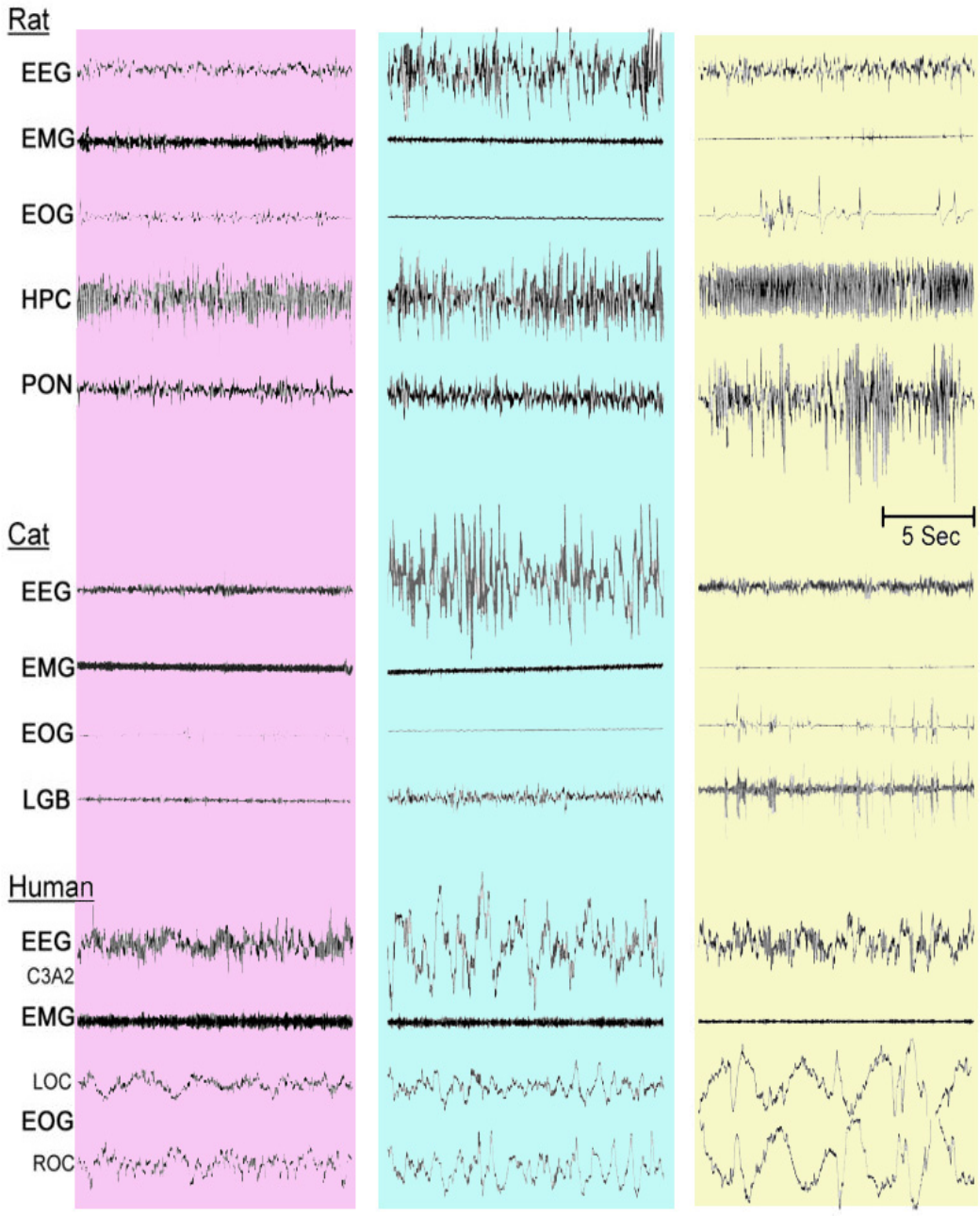

Figure 1.1: Typical polygraphic appearances showing similarities in behavioural state-specific physiological signs across the rat, cat and human.
Pink: wakefulness, blue: NREM sleep, and yellow: REM sleep (adapted from Datta & Maclean, 2007).

Most of these wake promoting neurons are located in the hypothalamus and the brainstem (Murillo-Rodríguez *et al.*, 2009), and produce their own type of neurotransmitters (Datta & Maclean, 2007, Murillo-Rodríguez *et al.*, 2009, Datta, 2010).

In brief, wake-promoting systems consist of some groups of cells that are located within the ascending reticular activating system (ARAS). The groups are: 1) noradrenergic neurons in locus coeruleus (LC), 2) serotoninergic neurons in raphe nucleus (RN), 3) cholinergic neurons in pedunculopontine tegmentum (PPT), 4) glutamatergic neurons in midbrain, and 5) dopaminergic neurons in substantia nigra compacta (SNc) and ventral tegmental area (VTA) (Sakai & Crochet, 2003, Datta, 2010). The levels of activity of these groups determine the level of thalamo-cortical relay cells that are in 'ready' state to transfer the sensory information to the cortex (Sakai & Crochet, 2003, Datta, 2010).

1.2.4 Initiation of sleep

There are many theories related to initiation of sleep. One of the most accepted theories is the reticular deactivation theory. In the reticular deactivation theory, it was assumed that the waking state requires a certain level of brain activity, and this activity is maintained by a steady flow of ascending impulses arising in the brainstem reticular formation (Moruzzi, 1972, Datta & Maclean, 2007, Datta, 2010). The reduction of these impulses results in sleep.

Recently, Datta and his colleague proposed that a passive process is involved in the initiation of sleep. This metabolic homeostasis theory consists of a passive process that depends on the homeostatic regulation of the levels of activity-dependent metabolites (Datta & Maclean, 2007) (Figure 1.2). During wakefulness, there is an increase in neuronal activity in the brain which results in an increase in endogenous metabolites. The accumulation of metabolites reaches a certain threshold and this leads to withdrawal of wakefulness and initiation of sleep (Figure 1.2). The withdrawal of wakefulness reduces the rate of metabolite production and the accumulated metabolites are cleared from the body, returning it to basal level (Figure 1.2).

Hence, the oscillation between accumulated and basal metabolites level determines the generation between wakefulness and sleep. The metabolites that have been recognised to be involved in the generation of sleep are adenosine, γ-aminobutyric acid (GABA), glycine, prostaglandin D2 (PGD2) and cytokines such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) (Datta, 2010).

1.2.5 Neural mechanisms of NREM sleep (or SWS)

NREM sleep occurs soon after the initiation of sleep. The initiation of NREM sleep depends on the activity of wake promoting systems that depends on the neuronal activity in thalamo-cortical networks and the level of metabolites in the brain. The wake promoting systems together with the level of metabolites are responsible in controlling the incoming sensory signals from the thalamus to cerebral cortex (Datta, 2010).

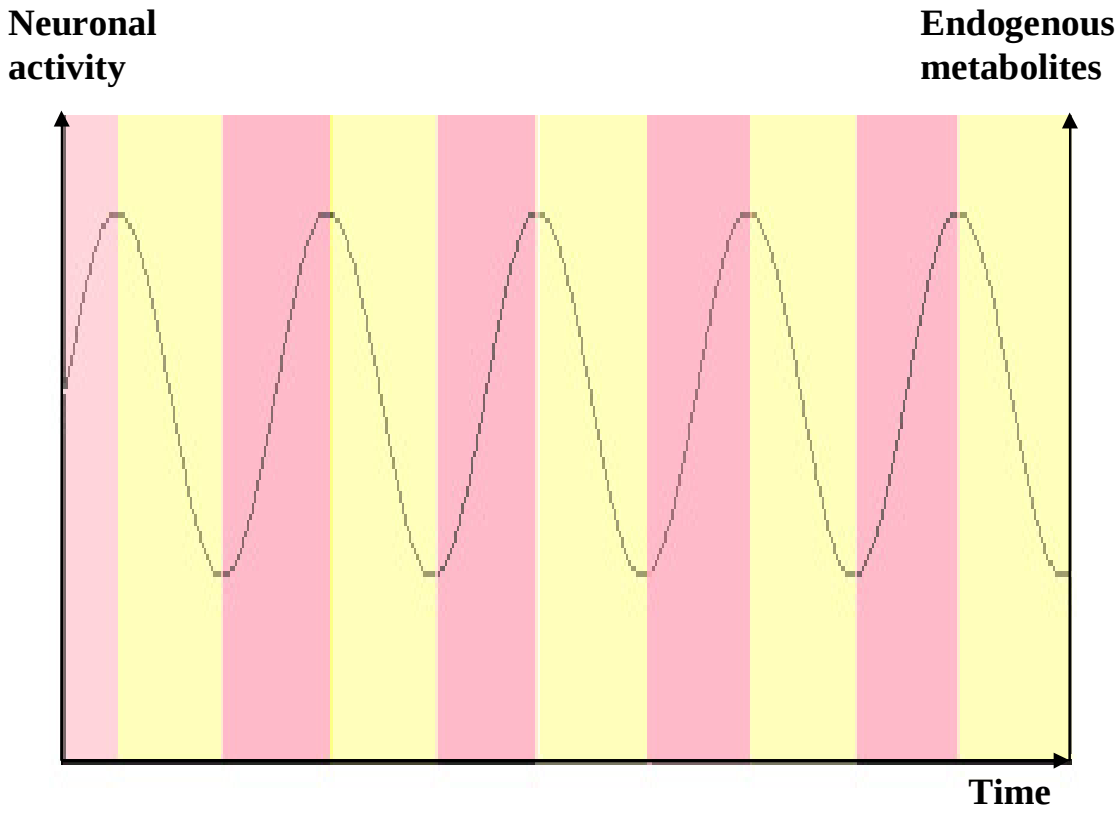

Figure 1.2: Metabolite homeostatic model of physiologic mechanisms for the initiation of sleep.
The oscillations of accumulated and basal metabolites level determine the generation between wakefulness and sleep. Red: wakefulness, yellow: sleep (adapted from Datta & Maclean, 2007).

The thalamus consists of two types of neurons which differ in function to generate NREM sleep: thalamo-cortical relay neurons which relay sensory information to the cortex, and thalamic reticular neurons which, when activated, prevent thalamo-cortical relay neurons from transferring sensory information to the cortex (Steriade *et al.*, 1993, Steriade & Timofeev, 2003, Datta, 2010). During NREM sleep generation, the activity of the wake promoting system is reduced and there is an increment of metabolites leading to the excitation of thalamic reticular cells and γ-aminobutyric acid-B ($GABA_{B)}$ inhibition of thalamo-cortical relay cells (Datta & Maclean, 2007, Datta, 2010). The thalamo-cortical relay cells are hyperpolarised by the activity of GABAergic neurons, thus blocking the transmission of sensory impulses to the cortex (Datta, 2010).

1.2.6 Neural mechanisms of REM sleep

The generation of REM sleep is a more complex process compared to NREM sleep. In general, the occurrence and intensity of REM sleep depend on the activity of REM promoting cell groups. As the REM-promoting neuronal activity reaches a certain threshold, the full set of REM signs occurs. The underlying neuronal activity is a continuous function. The cholinergic neurotransmitter acetylcholine (ACh) is thought to be important in REM sleep production, acting by exciting the populations of brainstem reticular formation neurons to produce the set of REM signs. The aminergic neurotransmitter serotonin (5-HT) and noradrenaline (NA) are released by another neuronal population called REM off neurons (McCarley, 2007) (Figure 1.3).

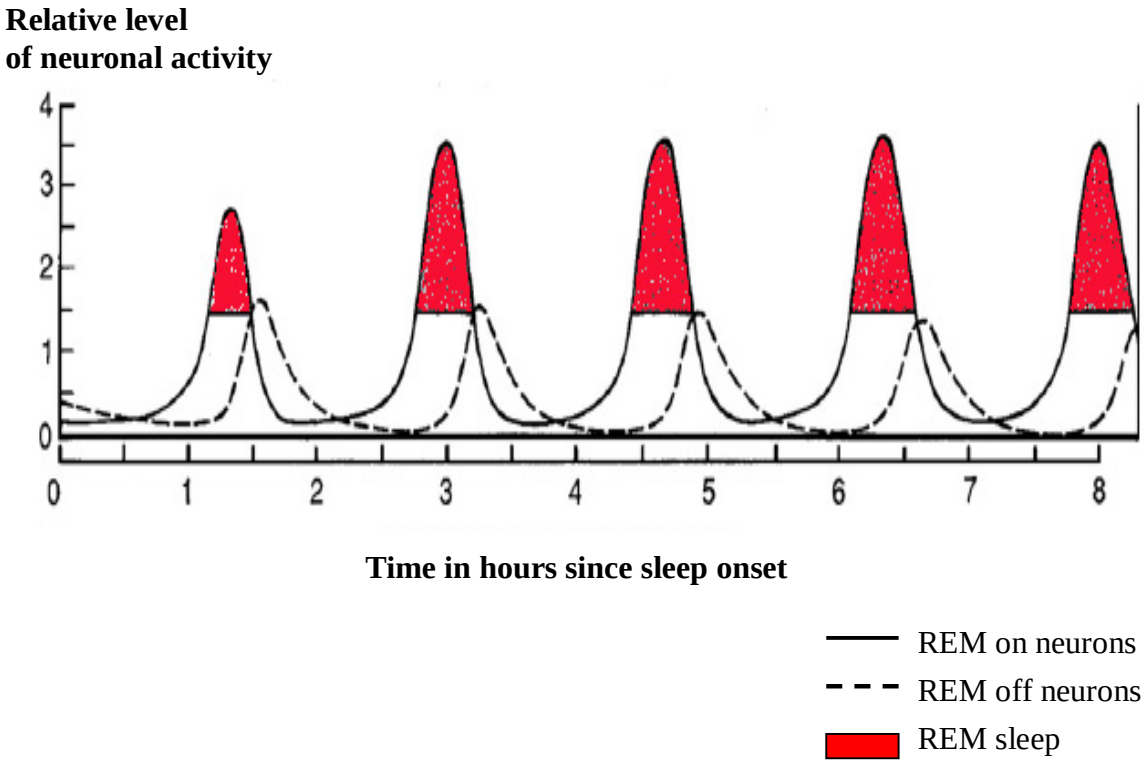

Figure 1.3: The underlying neuronal activity of REM sleep generation is a continuous function and involves REM-on and REM-off neurons (reprinted from Sleep Medicine, 8(4):302-30, R.W. McCarley, Neurobiology of REM and NREM sleep, (page 303), © (2007), with permission from Elsevier).

Recent knowledge on the mechanism of REM sleep generation is explained in detail by the cellular-molecular-network (CMN) model of REM sleep regulation (Datta & Maclean, 2007, Datta, 2010). As mentioned earlier, REM sleep is characterized by a sequence of events, generated by their own distinct group of cells located in the pons and midbrain (Table 1.1) (Datta & Maclean, 2007, Datta, 2010). These groups of cells that are responsible for generation of REM sleep signs are affected by the oscillating changes between cholinergic and aminergic neurotransmitters (Datta & Maclean, 2007, Datta, 2010). In order to generate REM sleep signs, the groups are excited by the increased level of cholinergic neurotransmitter released from the cholinergic neurons in the PPT and lateral dorsal tegmentum (LDT) (Datta & Maclean, 2007, Datta, 2010). Consequently, the release of aminergic neurotransmitter from the noradrenergic neurons in the LC and serotonergic neurons in the RN are reduced or nearly absent (Datta & Maclean, 2007, Datta, 2010).

1.2.6.1 The initiation of REM sleep

During initiation of REM sleep, the increased level of glutamate activates the kainate receptors in the cholinergic neurons in PPT and LDT (Datta & Maclean, 2007). This event leads to increased release of post-synaptic ACh. The ACh activates each of the groups that generate REM sleep signs and REM sleep inducing site in the medial pontine reticular formation (mPRF). While PPT and LDT cholinergic cells are activated, the noradrenergic neurons in the LC and serotonergic neurons in the RN are inhibited by GABAergic cells located in those areas.

Table 1.1: Cell groups in the brainstem which generate various components of REM sleep signs (adapted from Datta & Maclean, 2007).

Cells group in brainstem	REM sleep sign
• Mesencephalic reticular formation • Medullary magnocellular nucleus	• Cortical EEG activation
• Locus coerulus alpha	• Muscle atonia of postural muscles
• Periabducens reticular formation	• REMs
• Caudal-lateral peribrachial area (predator mammals) • Nucleus subcoeruleus (prey mammals)	• Ponto-geniculo-occipital (PGO) spikes in occipital cortex • Field potentials in the pons (P-wave)
• Pontis oralis	• Hippocampal theta rhythm
• Parabrachial nucleus	• Increase in brain temperature and cardio-respiratory fluctuations

This GABA-mediated inhibition of aminergic cells reduces or stops the release of aminergic neurotransmitter to the REM sleep sign-induced cells (Datta & Maclean, 2007, Datta, 2010).

1.2.6.2 The maintenance of REM sleep

The maintenance and episodes of REM sleep depend on the ratio of cholinergic and aminergic neurotransmitters within the cell groups (Datta & Siwek, 2002, Datta & Maclean, 2007). In wakefulness or NREM sleep, the ratio of aminergic and cholinergic neurotransmitter is 1:1 (Datta, 2010). The activity of aminergic and cholinergic neurons is approximately the same during wakefulness, but during NREM sleep the activities are equally reduced (Datta, 2010).

During REM sleep, the activity of aminergic neurons is reduced and the activity of cholinergic neurons is comparatively high where the ratio is 0:0.65 (Hobson, 1999, Datta & Siwek, 2002, Datta, 2010). The activity of cholinergic cells in PPT and LDT is maintained by the continuous activation from glutamate that is released from the ACh induced mPRF activity (Datta, 2010). The glutamate also activates the aminergic and GABAergic cells in the LC and RN; however the inhibition of LC and RN precedes the activation of aminergic cells due to the local release of GABA in the LC and RN (Datta & Maclean, 2007, Datta, 2010).

1.3 REM sleep deprivation

Sleep is critical for the maintenance of health and support of life. Considerable research interests have focused on the selective effects of REM sleep loss and the REM sleep deprivation animal model is accepted as a potential useful strategy for studying the regulation and functions of REM sleep.

1.3.1 REM sleep deprivation paradigm

The REM sleep deprivation paradigm can be elicited in animals using a classic platform method, which is either a single platform or multiplatform technique. The single inverted flower pot technique or platform was first used by Cohen and Dement in 1965 on sleep deprived rats (Cohen & Dement, 1965). In this paradigm, the rat is placed on a single platform; usually 6.5 cm in size surrounded by a confined tank filled with water. The surrounding water is at a specified level such that the rat will come into contact with the water when it undergoes REM sleep induced muscle atonia. The contact with water awakens the animal, thus preventing the occurrence of REM sleep (Tufik *et al*., 2009). This method has resulted in some morbidity related to REM sleep (Tufik, 1981, Seabra & Tufik, 1993, Hoshino, 1996) and it has been shown that during REM sleep deprivation, the NREM sleep loss is minimum (Machado *et al.*, 2005).

In a multiplatform paradigm, a rat is placed inside a large water tank that contains several platforms (Van Hulzen & Coenen, 1981). The presence of more than one platform reduces the movement restriction and allows the rat to ambulate (Van Hulzen & Coenen, 1981).

Even though the multiplatform paradigm does exclude immobilization stress, it does not however, exclude stress due to social isolation. Hence, a modified multiplatform technique is carried out in order to minimise the social isolation induced stress.

The modified multiplatform technique involves many disposable platforms and suppresses sleep simultaneously in a group of rats (Suchecki *et al*., 2000, Suchecki & Tufik, 2000, Machado *et al*., 2004, Machado *et al*., 2005). Interestingly, this technique results in more stress than the single platform method. At the same time, the occurrences of sleep episodes in the modified multiplatform technique were lower than in the single platform method (Medeiros *et al*., 1998). Nevertheless, socially induced awakenings and the social interactions induced between the rats in the tank caused a significant increase in the awakening time (Medeiros *et al*., 1998).

In order to study chronic sleep deprivation, a method called disk-over-water (DOW) method was designed (Rechtschaffen & Bergmann, 1995). An experimental rat was placed on one side of a divided horizontal disk that was suspended over a shallow tray of 2 to 3 cm deep water (Rechtschaffen & Bergmann, 1995). EEG, EMG and theta activity were continuously monitored to detect sleep states (Rechtschaffen & Bergmann, 1995). When the rat started to sleep, the disk was automatically rotated at low speed, awakening the rat and forcing it to walk in an opposite direction to the disk rotation in order to avoid falling into the water (Rechtschaffen & Bergmann, 1995). This method has been shown to selectively deprive REM sleep by 99 % (Kushida *et al*., 1989, Rechtschaffen & Bergmann, 1995) and 86 % (Landis *et al*., 1992, Rechtschaffen & Bergmann, 1995).

Nevertheless, the main issue here is how to exclude the stress that may be generated during these deprivation methods, which can be the causative factor of the REM sleep deprivation induced co-morbidities. Stress induced by social isolation in animals exposed to the single platform technique increases the plasma adrenocorticotropic hormone (ACTH) (Suchecki *et al*., 1998), plasma corticosterone (Suchecki *et al*., 1998) as well as aggressiveness (Gulyani & Mallick, 1995). It has been shown that immobilisation induced sleep changes which could interrupt not only REM, but also NREM sleep (Pawlyk *et al*., 2008).

In a multiplatform paradigm, it has been shown that there was an increase in the weight of the adrenal gland and a reduction in the thymus weight due to stress (Coenen & van Luijtelaar, 1985). The multiplatform technique reduces the social isolation and locomotor restrictions but the forced awakening secondary to social interactions results in stress with an associated increase of plasma corticosterone and ACTH levels (Suchecki *et al*., 1998, Suchecki *et al*., 2000). Interestingly, in the large multiplatform technique the ACTH levels of the rats were elevated even though it was not as high as in rats exposed to the small multiplatforms (Suchecki *et al*., 1998). Hence, the social interactions per se in the water tank can generate some form of stress.

1.3.2 Morbidities related to REM sleep deprivation paradigm

1.3.2.1 Morbidities related to animal behaviours

Significant morbidities developed when animals were exposed to REM sleep deprivation. These symptoms: debilitated appearance (Rechtshaffen & Bergmann,

1995), aggressive behaviour (Gulyani & Mallick, 1995), hyperphagia (Rechtshaffen & Bergmann, 1995, Suchecki *et al*., 2003, Hipólide *et al*., 2006, Koban *et al*., 2008), weight loss (Rechtshaffen & Bergmann, 1995, Suchecki *et al*., 2003, Hipólide *et al*., 2006, Koban *et al*., 2008), elevated metabolic rate (Koban & Swinson, 2005), elevated energy expenditure, increased plasma catecholamines, hypothyroidism, reduced core temperature (Rechtshaffen & Bergmann, 1995) and reduced levels of anabolic hormones (Everson & Crowley, 2004) are consistent and some are reproducible. These morbidities are harmful but at the same time useful as an alternative to assess if the inverted flower pot technique was able to produce rats that were selectively REM sleep deprived.

One of the most important characteristics of REM sleep deprivation is that REM sleep deprived rats developed a degree of hyperphagia that was conversely associated with a loss of body weight (Rechtshaffen & Bergmann, 1995, Suchecki *et al*., 2003, Koban & Swinson, 2005, Hipólide *et al*., 2006, Koban & Stewart, 2006, Koban *et al*., 2008, Galvão *et al*., 2009). Most studies have demonstrated changes in neuropeptide Y (NPY), pro-opiomelanocortin (POMC) and leptin (Koban & Swinson, 2005, Koban *et al*., 2006), in accordance with hyperphagia after REM sleep deprivation.

It has been known that hypothalamic NPY, POMC and leptin are the components of food intake regulation (Koban & Swinson 2005, Koban *et al*., 2006). The NPY gene expression was markedly increased within the hypothalamus (Koban *et al*., 2006) with a pronounced decrease in POMC gene expression (Koban *et al*., 2006), the counterpart to NPY.

In a study, serum leptin decreased more than 50 percent in rats which demonstrated hyperphagia and lost of body weight after 5 days of REM sleep deprivation (Koban & Swinson, 2005).

It has been shown that the body weight loss despite the hyperphagia is a result of an increase in resting metabolic rate in REM sleep deprived rats (Koban & Swinson, 2005). This hypermetabolic state is said to be mediated via Uncoupling Protein 1 (UCP1) in brown adipose tissue (Koban & Swinson, 2005). UCP1 is a 32 kDa inner mitochondrial membrane protein that plays a major role in cellular respiration by allowing proton leakage. The movement of protons resulted in thermodynamic energy which dissipated as heat rather than adenosine-5'-triphosphate (ATP) formation. This resulted in a hypermetabolic state with an associate weight loss despite the hyperphagia. REM sleep deprivation also leads to increased energy expenditure.

REM sleep deprived rats showed increased use of fats and proteins but with a normal glucose uptake (Rechtschaffen & Bergmann, 1995). Other studies have also confirmed that it was the accelerated use of fat that resulted in the loss of body weight (Hipólide *et al*., 2006). Studies have also shown that the increased energy loss was not due to increased waste products or weight loss from dehydration (Bergmann *et al*., 1989).

1.3.2.2 Morbidities related to morphological neuronal changes

The morphology of neuronal induced REM sleep deprivation changes has been specifically studied since more than a century ago. As quoted by Majumdar and

Mallick 2005, back in 1912, Pieron described the neuronal morphological changes due to REM sleep deprivation as 'become shrunken, the nucleus moving to periphery, joining the cell membrane. The nucleolus is also generally ectopic. There is vacuolization of the protoplasm, the cell seeming to proceed to autophagy'. The author also mentioned fragmentation and the disappearance of Nissl neurofibrillae (Pieron, 1912, Majumdar & Mallick, 2005).

Recent studies served to further confirm that neuronal morphology and structure were both affected by REM sleep deprivation (Majumdar & Mallick, 2005). It was observed that after REM sleep deprivation the size of the neurons in rat brain cells were reduced and the changes were dependent on the physiological function and neurotransmitter content of the neuronal cells (Majumdar & Mallick, 2005). Further studies confirmed that neuronal apoptosis occurred within the brain after 6 days of REM sleep deprivation (Biswas *et al.*, 2006).

1.3.3 REM sleep deprivation and pain

Many studies have been conducted to observe the effects of REM sleep deprivation on various pain modalities (Hakki Onen *et al.*, 2001, May *et al.*, 2005). In animals, REM sleep deprivation has been shown to induce a significant increase in the pain behaviour responses to mechanical (Hakki Onen *et al.*, 2001), thermal (Hakki Onen *et al.*, 2001, May *et al.*, 2005) and electrical noxious stimuli in rats (Hakki Onen *et al.*, 2001) but no significant differences have been found in the reaction to formalin noxious stimuli (Hakki Onen *et al.*, 2001).

1.4 Pain

The International Association for the Study of Pain (IASP) has defined pain as a sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries (Merskey & Bogduk, 1994). Pain is an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Loeser & Melzack, 1999).

1.4.1 Mechanisms of pain

In general, pain information is transmitted through peripheral nerve endings to the spinal cord, than carried by ascending tracts to the thalamus and primary somatosensory (SI) cortex.

1.4.1.1 Nociceptors

Nociceptor is a word from Latin *nocere*, meaning 'to hurt' (Bear *et al*., 2007). Nociceptors are peripheral receptors widely found in peripheral organs such as skin. Structurally, nociceptors are free nerve endings and represent the more distal part of a first-order peripheral afferent fibre (Almeida *et al*., 2004). In general, there are two general nociceptors: mechanical or thermal nociceptors and polymodal nociceptors (Nadeu *et al*., 2004, Bear *et al*., 2007).

The mechanical or thermal nociceptors transmit localised pain sensations to the central nervous system via the peripheral afferent fibres, Aδ fibres. The polymodal nociceptors respond to exogenous chemical stimuli (e.g. formalin, carrageenan), endogenous chemical stimuli (e.g. bradykinin, histamine) and thermal

stimuli and transmit to the central nervous system via another type of peripheral afferent fibre called C fibre (Nadeu *et al*., 2004). There is another fibre, Aβ fibre which is not responsive to noxious stimulus in normal conditions (Almeida *et al*., 2004).

1.4.1.2 First order afferent fibres

First order afferent fibres are classified in accordance to their structure, diameter, and conduction velocity. Aδ fibres which are myelinated, have a diameter from 2.0 to 6.0 μm and conduction velocity of 12-30 m/s. C fibres are unmyelinated, ranging in diameter from 0.4 to 1.2 μm and having a velocity of 0.5 – 2.0 m/s. There are also Aβ fibres which are myelinated, with a diameter of more than 10 μm and a velocity of 30-100 m/s and do not propagate noxious potentials in normal situations (Millan, 1999, Almeida *et al*., 2004, Schaible, 2007). Hence, the C fibres are the smallest and with the slowest conduction velocity and the Aβ fibres are the largest and fastest fibres, with the Aδ fibres being the intermediate between the two.

First order afferent fibres respond to the specific stimuli that activate the receptive field of the respective receptors. The Aδ fibres are classified into two groups: type I and II. The type I of Aδ fibres has mechanoreceptors that respond to thermal or chemical stimuli. Type II are fibres with mechanothermal receptors that respond to high temperatures (45-53 ºC) and some receptors for intense cold (-15 ºC) and later sensitized to vigorous mechanical stimuli at nonnoxious threshold (Millan, 1999, Almeida *et al*., 2004, Schaible, 2007).

There are three types of C fibres. These subtypes are different in terms of their responses to certain stimuli via their related nociceptors. One type of the C fibres is activated by tissue damage via its polymodal nociceptors. Another type of the C fibres is involved in detection of the burning sensation via its mechanosensitive nociceptors. The third type is the C fibres which respond to the presence of inflammation, detected by silent receptors, a group of receptors which does not respond to noxious stimuli in general (Millan, 1999, Almeida *et al*., 2004, Schaible, 2007).

1.4.1.3 The spinal cord organization

The primary afferent fibres reach the spinal cord via the dorsal root ganglion, organize themselves in the ventrolateral bundle of roots, become part of Lissauer Tract and synapse with the second order neurons. The second order neurons are distributed along the dorsal horn of the spinal cord and are organized according to the Rexed laminae (Almeida *et al*., 2004, Bear *et al*., 2007). The dorsal horn of the spinal cord consists of neurons that become the second order afferent fibres and ascending pathways originating from the spinal cord; as well as the intrinsic neurons which interact with the afferent and efferent nociceptive stimuli (Almeida *et al*., 2004).

There are three distinct groups of neurons that become the ascending pathways: the specific nociceptive (SN), wide dynamic range (WDR) and non nociceptive (N-NOC) neurons. The SN neurons respond exclusively to noxious stimuli from Aδ and C fibres. They are in laminae I (somatotropic organization), II (external), V and VI (Almeida *et al*., 2004). The WDR neurons respond to

mechanical, thermal and chemical stimuli coming from Aδ, C and Aβ fibres and found in lamina I, II (external), IV, V, VI, X and in the anterior horn. The N-NOC neurons respond to innocuous stimuli (Millan, 1999, Almeida *et al*., 2004).

1.4.1.4 Afferent nociceptive pathways of the spinal cord

After the interactions, the axons of the second order afferent fibres become part of the anterolateral fascicle or posterior fascicle forming afferent bundles that transmit the nociceptive impulse to the brain including among others, the thalamus, periaqueductal substance, parabrachial region and reticular formation of the medulla (Almeida *et al*., 2004).

The afferent bundles that transmit the sensory impulse to the thalamus are called the spinothalamic tract. There are three forms of spinothalamic tract. The first is the ventral spinothalamic tract which directly projects to the nuclei of the lateral complex of the thalamus involved in the sensory-discriminative component of pain. The dorsal spinothalamic tract projects to the nuclei of the posterior medial and intralaminar complex of the thalamus and is involved in the motivational-affective aspects of pain. Finally, another spinothalamic pathway projects directly to the medial central nucleus of the thalamus and is involved in the affective component of the painful experience (Almedia *et al*., 2004). Most of the cells project to the contralateral thalamus although a small fraction projects ipsilaterally (Willis & Westlund, 1997).