BENEFICIAL EFFECTS OF MELATONIN ON POST TRAUMATIC STRESS DISORDER AFTER SPINAL CORD INJURY STUDY IN EXPERIMENTAL ANIMAL MODELS

PAWARISA SAPPRASERT

MASTER OF SCIENCE
IN
ANTI-AGING AND REGENERATIVE SCIENCE

SCHOOL OF ANTI-AGING AND REGENERATIVE MEDICINE
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Pawarisa Sapprasert

Independent Study Title	Beneficial Effects of Melatonin on Post Traumatic Stress Disorder after Spinal Cord Injury Study in Experimental Animal Models
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ABSTRACT

Spinal cord injury (SCI) produces an inflammatory response in the CNS, leading to prolong stress involved HPA axis, stress-related psychopathologics such as post traumatic stress disorder (PTSD). It might play a key role in the down regulation of neurogenesis in adult brain. Whereas anti-inflammatory treatments could prevent this effect, Melatonin is a powerful antioxidant that can cross cell membrane and blood brain barrier (Reiter, Manchester & Tan, 2010). Antioxidants are reported to play an essential role in the survival of neuronal cells and it could also influence the production of new cells (Ott, Gogvadze, Orrenius & Zhivotovsky, 2007).

This research study concentrated in the therapeutic effects of melatonin against post traumatic stress disorder (PTSD) after spinal cord injury that induced by crush SCI in animals model. Female mice (3 months old) are randomly assignment to three different groups of animals; control group, SCI, and SCI+Melatonin. To study of post traumatic stress disorder (PTSD), induction of SCI will perform following the standardized severe crush method briefly, mice will be anesthetized and a laminectomy will perform at the T12. Using Dumont forceps to compress the cord laterally from the sides for 5 sec made crush injury model as previously described by Krityakiarana et al. (2010). For melatonin

treated mice, melatonin (10 mg/kg b.w./day) will deliver by intra-peritoneal injection for 14 days after SCI induction. After 14 days mice were sacrificed and brain tissue was removed for the histochemical procedures study for numbers of newly generated cells, GFAP and Ki-67 use for labeling the newly glial cells. Appropriated statistical analysis use to compare the effects of melatonin among groups.

The fact that reduction in inflammation were achieved via an anti-inflammatory effect of melatonin induction as seen in our study, the number of GFAP-positive cell was decreased in SCI-M group (107 $\pm$ 67.8) comparing with SCI group (175.8 $\pm$ 71.5) without reaching significance ($p = 0.747$) even though, it is not possible to claim that melatonin was responsible for the lack of effect so, dose dependent manner of melatonin and more sample size is necessary for future studies to evaluate the anti-inflammatory effects of melatonin on SCI model.

In our study, significantly difference of slightly gliogenesis, represent in Ki-67 labeling, was seen in hippocampus of SCI-M group (16.25 $\pm$ 12.2) comparing with SCI group (49.8 $\pm$ 26.4) ($p < 0.001$). These results can be confirm the neuroregenerative effect of melatonin do not seem to express in the hippocampus of SCI animal. Moreover, neuroprotective effect may exert around the traumatic lesion of spinal cord as derive from the study of melatonin effect on the functional recovery from SCI animal (Schiaveto-de-Souza, da-Silva, Defino & Del Bel, 2013). Finally, the dose response relationship studies for melatonin and large sample size could promise better results.

Keywords: Gliogenesis/Spinal Cord Injury/PTSD/melatonin

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ABBREVIATION AND SYMBOLS

SCI	Spinal cord injury
PTSD	Post traumatic stress disorder
MDD	major depressive disorder
HPA	hypothalamic-pituitary-adrenal axis
DG	dentate gyrus
CNS	central nervous system
PNS	peripheral nervous system
IL	interleukin
TNF	tumor necroting factor
EC	entorhinal cortex
PFC	prefrontal cortex
CA	cornu ammonis
SVZ	subventricular zone
BDNF	brain-derived neurotropic factor
GDNF	glial cell line derived neurotropic factor
PBS	Phosphate Buffer Saline
BPD	bipolar disease
GFAP	glial fibrillation acidic protein
DCX	double cortin
SD	Standard deviation
***	$p < 0.001$

CHAPTER 1

INTRODUCTION

1.1 Background

"Stress defined as a condition that seriously perturbs the physiological and psychological balance of an individual. Stress-related psychopathologies such as major depressive disorder (MDD), anxiety, conduct disorders, and posttraumatic stress disorder (PTSD) perturb behavioral, cognitive, and social domains and exacerbate one's reactivity to stressful events. Traumatic stress, however, does not affect everyone similarly. While susceptible individuals poorly adapt to stressors and express inappropriate responses that can become persistent states of stress" (Del Giudice, Ellis, & Shirtcliff, 2011). "Stress affects the hypothalamic-pituitaryadrenal (HPA) axis, with a severity that depends on the gestational stage of stress exposure, and the sex of the animal. The HPA axis is a highly adaptive neuroendocrine system strongly implicated in stress resilience and vulnerability" (Franklin, Saab & Mansuy, 2012)

"The hippocampus is one of the major brain areas that exert strong regulatory control over the HPA axis. It is also itself modulated by stress hormones. In rats and humans, hippocampus stimulation decreases glucocorticoid secretion while hippocampal lesion elevates basal glucocorticoid level, especially during the stress recovery phase, which is the most reliant on negative feedback" (Jankord & Herman, 2008). "Facilitated glutamatergic plasticity in the dentate gyrus (DG) enhances exploratory activity in mice" (Sab et al., 2009). "The hippocampus plays an important role in learning ability and memory capability" (Milner, Lee, Aicher & Rosin, 1998). "The neurons in the hippocampus are especially vulnerable to the PTSD" (Hendriksen, Prins, Olivier & Oosting, 2010). In humans, dysfunctions of glutamatergic neurotransmission, maladaptive structural and functional changes in hippocampal circuitry, and decreased hippocampal volume have been associated with stress-related conditions such as PTSD.

“Post-traumatic stress disorder (PTSD) is a condition which occurs after a person has experienced unusual stress. People with PTSD become very anxious whenever reminded of the incident which has caused the initial distress. Frequently they have nightmares or become fearful, depressed and irritable and function less well at work or in social situations” (Halligan, Michael, Wilhelm, Clark & Ehlers, 2006). “Symptoms related to traumatic cues are expressed as conditioned or sensitized fear responses” (Siegmund & Wotjak, 2007). “The animal models of PTSD resemble the animal models of neurodegenerative disease” (Hendriksen et al., 2010; Tamaki, Kamakura, Nakamichi, Taniura & Yoneda, 2008) and severe traumatic injury in the central nervous system especially spinal cord injury (SCI) (Krause, Saunders & Newman, 2010) . SCI is one of the major problems in neurological deficit and causes permanent paralysis in patients. . Recently, there is the evidence reported that PTSD is the result from the occurrence cascade of SCI (Han, Yan & Shi, 2013).Moreover, Spinal cord injury also leading to gliosis and limited cellular regeneration (Horner et al., 2000).

“Traumatic spinal cord injury (SCI) causes neuronal and glial cell damage and tissue disruption, leading to neurological dysfunction. Two major pathological stages occur in SCI: The primary injury involves free radical production (Rolls, Shechter & Schwartz, 2009), inflammation, mechanical force-mediated cell necrosis and tissue damage, and the secondary injury results in a cascade of biochemical events that produce progressive destruction on the spinal cord tissues” (Blight, 1992; Blight, 1988; Juurlink & Paterson, 1998; Anderson & Hall, 1993). Together, “the death of neurons, astroglia, and oligodendroglia in and around the lesion site disrupts neural circuitry and leads to neurological dysfunction” (Beattie & Farooqui, 2000). Although the biochemical events leading to prolong stress involved HPA axis encouraged low regeneration rate of the central nervous system (CNS).

“Neurogenesis encompasses cell proliferation, survival, migration, and neuronal differentiation. Newborn neurons in the hippocampus is associated with the learning ability and memory function, and neurogenesis in the hippocampal dentate gyrus is known to be enhanced by many factors, such as enriched environment, neurotrophic factors, and exercise” (Zheng et al., 2012; Ozdemir et al., 2005).

One of the neurotrophic factors in our body is melatonin. “Melatonin secretion into the blood from the pineal gland varies in a daily cycle, thereby allowing the regulation of sleep and circadian rhythm” (Rivera-Bermúdez, Gerdin, Earnest & Dubocovich, 2003). “Adult hippocampal neurogenesis is affected by circadian rhythms and sleep deprivation” (Holmes, Galea, Mistlberger & Kempermann, 2004; Guzman-Marin et al., 2005), thus implying a significant role for melatonin. “Melatonin’s effect on neuronal cell survival were recapitulated in vivo by application of exogenous melatonin (8 mg/kg) and demonstrated antidepressant behavior in mice” (Ramírez-Rodríguez, Klempin, Babu, Benítez-King & Kempermann, 2009). “Melatonin also increases neuritogenesis and dendritogenesis with agonist treatment, leading to greater complexity of the dendritic tree” (Ramirez-Rodriguez, Ortíz-López, Domínguez-Alonso, Benítez-King & Kempermann, 2011). “Melatonin has also been shown to improve the irradiation induced decline in adult hippocampal neurogenesis, suggested to occur through its ability to scavenge free radicals” (Manda, Ueno & Anzai, 2009).

“Several studies have implicated the abnormal accumulation of free radicals in neurodegenerative disorders. Free radical scavengers have been shown to protect against cell death” (Reiter, Guerrero, Garcia & Acuña-Castroviejo, 1998; Miller et al., 1996). “Melatonin is a highly potent free radical scavenger” (Tan et al, 1993; Matuszak, Reszka & Chignell, 1997).

The hypothesis of this study is if melatonin elicited therapeutic effects against post traumatic stress disorder (PTSD) after spinal cord injury that induced by crush SCI in animals model, then it could exert protective actions on glial cells (gliogenesis) in the hippocampus under immunohistochemical study. Furthermore, the present study can support for the emerging view that glial cells are equally active participants as neurons in the maintenance of a functional central nervous system.

1.2 Objectives

Study the effects of melatonin treatment on gliogenesis in the hippocampus after post traumatic stress disorder (PTSD) caused by SCI in animal model.

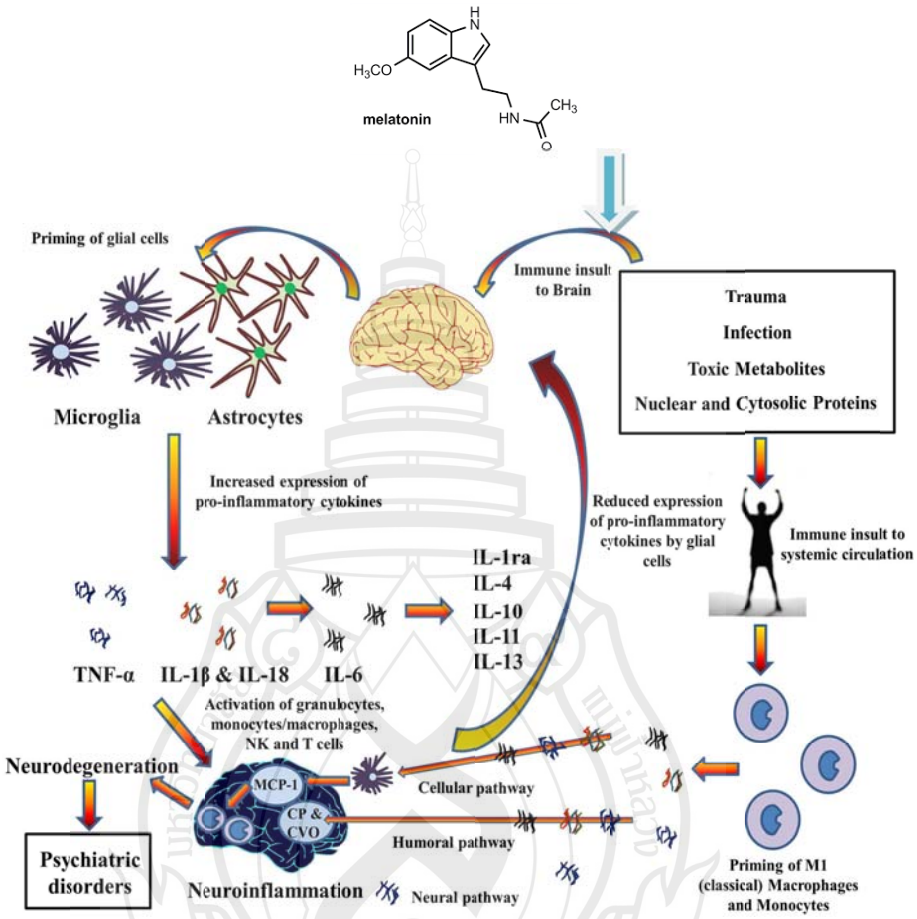
1.3 Hypothesis

Melatonin can improve gliogenesis in post traumatic stress disorder (PTSD) after SCI study in animal model.

1.4 The Scope of the Research

This research study concentrated in the therapeutic effects of melatonin against post traumatic stress disorder (PTSD) after spinal cord injury that induced by crush SCI in animals model. Female mice (3 months old) are randomly assignment to four different groups of animals; control group (n=1, 4 slides sample), SCI (n=1, 4 slides sample), SCI+Melatonin (n=1, 4 slides sample) and sham (n=1, 4 slides sample). Each SCI, sham and SCI+Melatonin mouse is kept individual cage. To study of post traumatic stress disorder (PTSD), induction of SCI will performe following the standardized severe crush method Briefly, mice will be anesthetized and a laminectomy will perform at the T12. Using Dumont forceps to compress the cord laterally from the sides for 5 sec made crush injury model as previously described by Krityakiarana et al. (2010). For melatonin treated mice, melatonin will deliver by intra-peritoneal injection after SCI induction for 14 days. Mice are maintained in a temperature-controlled environment during recovery. Manual bladder expression will perform 3 times a day. Antibiotics and pain-killer are administered after operation for 7 days and 3 days, respectively. After 14 days mice were sacrificed and brain tissue was removed for the histochemical procedures study for numbers of newly generated cells, glia cell. Appropriated statistical analysis use to compare the effects of melatonin among groups.

1.5 Conceptual Framework



Source Gaurav Singhal (2014)

Figure 1.1 Schematic Representation of Neuroinflammation: Implications in Comorbidity of Systemic Illnesses with Psychiatric Disorders

1.6 Operational Definition

Histological analysis: After dissected brain tissue from mice, tissue is then frozen in freezing media and brains were cut coronally to contain the entire hippocampus from the rostral to the ventral pole using a cryostat (20 μm). Ki67 and GFAP will be used as specific marker for newly generated glial cell. The number of colocalization of Ki67-positive cells and GFAP will be determined in the representative hippocampal sections. To present the number of immunopositive cells as cells/mm^2 in the hippocampus. Serial images stained sections are examined and photographed using fluorescence miscroscope.

CHAPTER 2

REVIEW LITERATURE

2.1 Spinal Cord Injury (SCI)

"Spinal cord injury (SCI) is characteristically accompanied by a period of secondary cellular degeneration that occurs in injured tissue over a course of hours and days after the initial insult, and that affects both glia and neurons" (Rudge & Silver, 1990; Springer, Azbill, Knapp, 1999). "Some neurons cannot be recovered or regenerated they continued to die for hours after traumatic SCI" (Kaptanoglu et al., 2000). "Intervention to reduce inflammation or to block specific membrane receptors during the first hours after SCI can partially protect cells and preserve function in both human patients and experimental animals" (Agrawal & Fehlings, 1997). "Although certain potential triggers of secondary degeneration have been identified, the cell biology of the response to SCI is not well understood, and the roles played by different cell types at different times in the progression of secondary degeneration are not well defined. Reactive astrocytes are a prominent feature of the cellular response to SCI. (Dergham et al., 2002). A role for reactive astrocytes has long been postulated in CNS wound healing" (Faulkner et al., 2004). "Astrocytes clear glutamate and potassium ions from the extracellular space, are a potential energy source, and produce numerous growth factors and cytokines" (Kettenmann & Ransom, 1995). "Astrocytes exert essential protective activities for neurons, oligodendrocytes, and myelin after SCI" (Faulkner et al., 2004). "Numbers of glial call were found in CNS remarkable from ratio between astrocytes and glial cell were 1: 10 in human brain" (Kandel, 2000), the former was interestingly to study of their role after SCI the later, glial cell was disregard from researcher. Nowadays, there has no report about relationship or role of gliogenesis after SCI.

2.2 Spinal Cord Injury Model

Spinal cord injury (SCI) models have proved indispensible not only for investigating the efficacy of therapeutic interventions but also for better understanding the molecular pathways involved. These models result in distinct neural deficits than thoracic injuries and should be considered in terms of therapeutic approaches. Based on the mechanism of injury, SCI models can be classified as contusion, compression, distraction, dislocation, transection or chemical. Compression models are characterized by compression of the spinal cord over an extended period of time (Dunham, Siriphorn, Chompoopong & Floyd, 2010).

The majority of behavioral outcome studies in mice with SCI used only a single strain and focused on locomotor recovery using a 5- or 6-point rating scale. The features of locomotor evaluation include weight supported plantar stepping, coordination of the forelimbs (FL) and hindlimbs, consistent position of the paw during stepping, adequate toe clearance, and maintenance of a stable trunk (Basso et al., 2006).

Locomotion in normal mice, regardless of strain, is typified by consistent weight supported stepping, a steady trunk and hindlimbs (HL) movement. Normal mice typically display a reproducible stepping pattern in which a step of the FL is coincident with a step of the contralateral HL and tail, and an easily recognizable pattern of FL (Basso et al., 2006)

The three phases of locomotor recovery depending on the severity of the injury. The first phase of locomotion was characterized by a period of either paralysis characterized by no hindlimb (HL) movement or paresis wherein only isolated joint movements below the level of the injury occurred. In the second phase, plantar placing of the paw and weight support in stance was followed by the onset of stepping and increasing frequency of stepping. In the third phase, improvements in the fine details of locomotion such as coordination and paw position were evident. Based on frequency analysis of the number of mice exhibiting these type of behaviors collapsed across the 42-day recovery period (Basso et al., 2006)

2.3 Post-Traumatic Stress Disorder (PTSD)

When in danger, it's natural to feel afraid. This fear triggers many split-second changes in the body to prepare to defend against the danger or to avoid it. This, fight-or-flight response is a healthy reaction meant to protect a person from harm. But in post-traumatic stress disorder (PTSD), this reaction is changed or damaged. People who have PTSD may feel stressed or frightened even when they're no longer in danger. PTSD was first brought to public attention in relation to war veterans, but it can result from a variety of traumatic incidents, such as mugging, rape, torture, being kidnapped or held captive, child abuse, car accidents, train wrecks, plane crashes, bombings, or natural disasters such as floods or earthquakes.

Currently, many scientists are focusing on genes that play a role in creating fear memories. Understanding how fear memories are created may help to refine or find new interventions for reducing the symptoms of PTSD. For example, PTSD researchers have pinpointed genes that make: Stathmin, a protein needed to form fear memories. In one study, mice that did not make stathmin were less likely than normal mice to freeze, a natural, protective response to danger, after being exposed to a fearful experience. They also showed less innate fear by exploring open spaces more willingly than normal mice.

GRP (gastrin-releasing peptide), a signaling chemical in the brain released during emotional events. In mice, GRP seems to help control the fear response, and lack of GRP may lead to the creation of greater and more lasting memories of fear. Traumatic stress can lead to chronic symptoms of posttraumatic stress disorder (PTSD), which are mediated by changes in brain structure and function. Brain areas involved in memory, including the hippocampus, prefrontal cortex, and amygdala, mediate symptoms of PTSD and associated memory dysfunction.

One such brain structure is the amygdala, known for its role in emotion, learning, and memory. The amygdala appears to be active in fear acquisition, or learning to fear an event (such as touching a hot stove), as well as in the early stages of fear extinction, or learning not to fear. Storing extinction memories and dampening the original fear response appears to involve the prefrontal cortex (PFC) area of the brain,

involved in tasks such as decision-making, problem-solving, and judgment. Certain areas of the PFC play slightly different roles. For example, when it deems a source of stress controllable, the medial PFC suppresses the amygdala an alarm center deep in the brainstem and controls the stress response. The ventromedial PFC helps sustain long-term extinction of fearful memories, and the size of this brain area may affect its ability to do so.

"One of the key players in the pathophysiology of posttraumatic stress disorder (PTSD) is the hippocampus" (Jatzko et al., 2006). "Animal research indicates that the hippocampus may be damaged by stress. Uno et al in 1989 performed postmortem examinations on monkeys who died spontaneously after a period of sustained social stress. All showed evidence of marked and preferential hippocampal degeneration" (Uno, Tarara, Else, Suleman & Sapolsky, 1989). Watanabe, Gould, Cameron, Daniels and McEwen (1992) reported that "repeated daily restraint stress in rodents caused atrophy of the apical dendrites of CA3 hippocampal pyramidal neurons" (Watanabe et al., 1992). Mizoguchi, Kunishita, Chui and Tabira (1992) found significant loss of hippocampal CA3 and CA4 neurons in castrated rats stressed by restraint and water immersion (Jatzko et al., 2006).

"The birth of new neurons within the hippocampal region of the central nervous system continues throughout life, and the amount of neurogenesis correlates closely with the hippocampal functions of learning and memory" (Shors, Wood & Bevlin, 2001; Feng et al., 2001). "The generation of new neurons within the hippocampus is mediated by proliferating neural stem or progenitor cells (NPC)" (Cameron & McKay, 1998; Cameron, Tanapat & Gould, 1998; Gage, Kempermann, Palmer, Peterson & Ray, 1998; Palmer, Takahashi & Gage, 1997). "that are widespread within the adult brain but instructed by local signaling to produce neurons only in discrete areas" (Suhonen, Peterson, Ray & Gage, 1996; Zigova, Pencea, Wiegand & Luskin, 1998) "Alterations in the microenvironment of the stem cell may allow ectopic neurogenesis to occur" (Nakatomi et al., 2002; Magavi, Leavitt & Macklis, 2000) "or even block essential neurogenesis, leading to deficits in learning and memory" (Cameron et al., 1998; Madsen, Kristjansen, Bolwig & Wörtwein, 2003; Monje, Mizumatsu, Fike & Palmer, 2002). "such as that observed in patients who receive therapeutic cranial radiation therapy" (Monje & Palmer, 2003) "In animal models, cranial irradiation ablates

hippocampal neurogenesis, in part by damaging the neurogenic microenvironment, leading to a blockade of endogenous neurogenesis" (Monje et al., 2002; Monje & Palmer, 2003) "Injury induces pro-inflammatory cytokine expression both peripherally and within the central nervous system and induces stress hormones,such as glucocorticoids, that inhibit hippocampal neurogenesis" (Cameron et al., 1998). "The extensive microglial inflammation and release of proinflammatory cytokines that accompanies this irradiation-induced failure suggests that inflammatory processes may influence neural progenitor cell activity" (Monje et al., 2002; Picard-Riera et al., 2002)

Individual differences in these genes or brain areas may only set the stage for PTSD without actually causing symptoms. Environmental factors, such as childhood trauma, head injury, or a history of mental illness, may further increase a person's risk by affecting the early growth of the brain. More research may show what combinations of these or perhaps other factors could be used someday to predict who will develop PTSD following a traumatic event (Golier & Yehuda, 2002).

2.4 Hippocampus

"The hippocampus is located in the medial temporal lobe of the brain. In this lateral view of the human brain, the frontal lobe is at left, the occipital lobe at right, and the temporal and parietal lobes have largely been removed to reveal the hippocampus underneath. The hippocampus is a major component of the brains of humans and other mammals. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation. Like the cerebral cortex, with which it is closely associated, it is a paired structure, with mirror-image halves in the left and right sides of the brain. In humans and other primates, the hippocampus is located inside the medial temporal lobe, beneath the cortical surface" (Amaral & Lavenex, 2006)

"The entorhinal cortex (EC), the greatest source of hippocampal input and target of hippocampal output, is strongly and reciprocally connected with many other parts of the cerebral cortex, and thereby serves as the main, interface between the hippocampus and other parts of the brain. The superficial layers of the EC provide the most prominent input to the hippocampus and the deep layers of the EC receive the most

prominent output. Within the hippocampus, the flow of information is largely unidirectional, with signals propagating through a series of tightly packed cell layers, first to the dentate gyrus, then to the CA3 layer, then to the CA1 layer, then to the subiculum, then out of the hippocampus to the EC. Each of these layers also contains complex intrinsic circuitry and extensive longitudinal connections" (Amaral & Lavenex, 2006). "Several other less salient connections play important roles in hippocampal function" (Joëls, 2008). "Beyond the output to the EC, additional output pathways go to other cortical areas including the prefrontal cortex, and another large output goes to the lateral septal area. The hippocampus receives modulatory input from the serotonin, norepinephrine, and dopamine systems, and from nucleus reuniens of the thalamus (Larner, Johnson & Keynes, 1995). A very important projection comes from the medial septal area, which sends cholinergic and GABAergic fibers to all parts of the hippocampus. The inputs from the septal area play a key role in controlling the physiological state of the hippocampus: destruction of the septal area abolishes the hippocampal theta rhythm, and severely impairs certain types of memory" (Winson, 1978).

2.5 Stress

"Stress defined as a condition that seriously perturbs the physiological and psychological balance of an individual. Stress-related psychopathologies such as major depressive disorder (MDD), anxiety, conduct disorders, and posttraumatic stress disorder (PTSD) perturb behavioral, cognitive, and social domains and exacerbate one's reactivity to stressful events. Traumatic stress, however, does not affect everyone similarly. While susceptible individuals poorly adapt to stressors and express inappropriate responses that can become persistent states of stress" (Del Giudice et al., 2011). "Stress affects the hypothalamic-pituitaryadrenal (HPA) axis, with a severity that depends on the gestational stage of stress exposure, and the sex of the animal. The HPA axis is a highly adaptive neuroendocrine system strongly implicated in stress resilience and vulnerability" (Franklin et al., 2012)

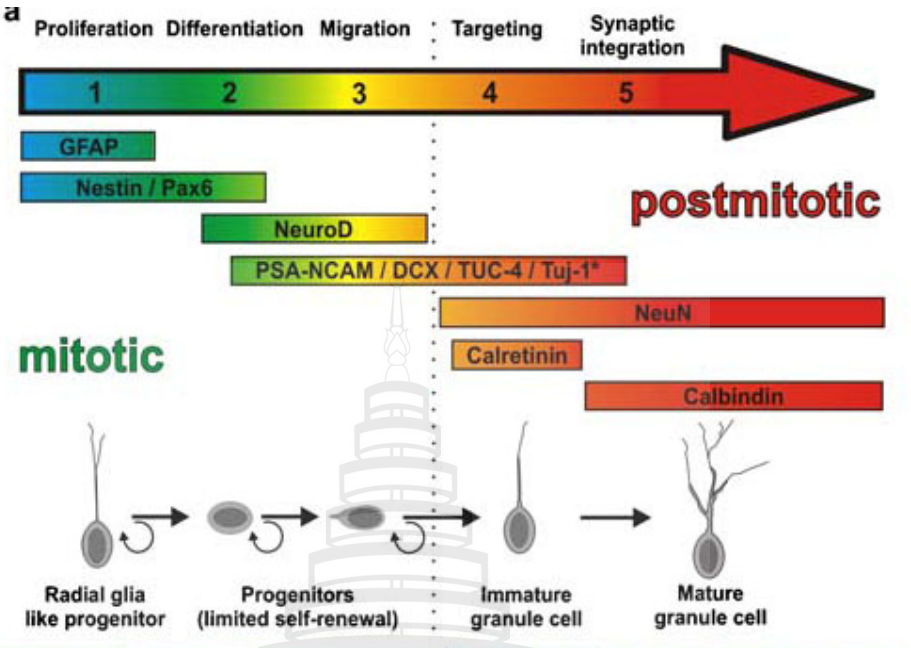
"The hippocampus is one of the major brain areas that exert strong regulatory control over the HPA axis. It is also itself modulated by stress hormones. The hippocampus plays an important role in learning ability and memory capability" (Milner, Lee, Aicher & Rosin, 1998). "The hippocampus contains high levels of glucocorticoid receptors, which make it more vulnerable to long-term stress than most other brain areas" (Joels, 2008). "Stress-related steroids affect the hippocampus in at least three ways: first, by reducing the excitability of some hippocampal neurons; second, by inhibiting the genesis of new neurons in the dentate gyrus; third, by causing atrophy of dendrites in pyramidal cells of the CA3 region. (Mizoguchi et al., 1992). There is evidence that humans who have experienced severe, long-lasting traumatic stress (for example, Holocaust survivors) show atrophy of the hippocampus, more than of other parts of the brain. These effects show up in post-traumatic stress disorder, and they may contribute to the hippocampal atrophy reported in schizophrenia and severe depression. The neurons in the hippocampus are especially vulnerable to the PTSD" (Hendriksen et al., 2010). "In humans, dysfunctions of glutamatergic neurotransmission, maladaptive structural and functional changes in hippocampal circuitry, and decreased hippocampal volume have been associated with stress-related conditions such as PTSD. A recent study has also revealed atrophy as a result of depression, but this can be stopped with anti-depressants, even if they are not effective in relieving other symptoms" (Campbell & MacQueen, 2004).

2.6 Immunohistological Markers

Biologists long believed that, once development is completed, no new neurons are produced in the forebrain. However, as is now firmly established, new neurons can be produced at least in two specific forebrain areas: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampal formation. Neurogenesis within the adult DG occurs constitutively throughout postnatal life, and the rate of neurogenesis within the DG can be altered under various physiological and pathophysiological conditions. The process of adult neurogenesis within the DG is a multi-step process (proliferation, differentiation, migration, targeting, and synaptic integration) that ends with the

formation of a post-mitotic functionally integrated new neuron. Various markers are expressed during specific stages of adult neurogenesis. The availability of such markers allows the time-course and fate of newly born cells to be followed within the DG in a detailed and precise fashion. Several of the available markers (e.g., PCNA, Ki-67, PH3, MCM2) are markers for proliferative events, whereas others are more specific for early phases of neurogenesis and gliogenesis within the adult DG (e.g., nestin, GFAP, Sox2, Pax6) (Eng & Ghirnikar, 1994). In addition, markers are available allowing events to be distinguished that are related to later steps of gliogenesis (e.g., vimentin, BLBP, S100 beta) or neurogenesis (e.g., NeuroD, PSA-NCAM, DCX) (von Bohlen Und Halbach O, 2007)

The process of neurogenesis occurs in several different stages, beginning with proliferation of early progenitor cells, and ending with synaptic integration, where the newly generated cell becomes a functionally integrated neuron. At different time-frames, various markers are expressed that correspond with the separate steps of the process. While different markers label for different things, certain markers tend to overlap one-another, so one must use precaution when drawing any definitive conclusions. In order to avoid some of these confounds, a double labeling technique is used so as not to confuse specific cell morphologies. For example, the majority of research designs regarding neurogensis use the double labeling technique with BrdU and NeuN. BrdU labels for newly generated cells, and NeuN is a specific label for adult neurons. If a cell expresses both of these labels, the results are more definitive and the use of specific markers allows for the advancement of scientific research to investigate the time course and fate of neurons during adult neurogenesis (von Bohlen Und Halbach O, 2007).



Source von Bohlen Und Halbach O (2007)

Figure 2.2 Immunohistological Markers for Staging Neurogenesis in Adult Hippocampus

2.7 Gliogenesis

"Glial cells were first identified as non-neuronal elements in the nineteenth century by the anatomist R. Virchow" (Kettenmann & Ransom, 2005). "In the past two decades, research has changed this perception and provided evidence for glia being important dynamic partners of neuronal cells actively participating in brain metabolism, synaptic neurotransmission and communication between neurons" (Volterra & Meldolesi, 2005). "The discovery of new glial functions coincides with growing evidence for the involvement of glia in the neuropathology of neurological" (Seifert, Schilling & Steinhäuser, 2006). "Glia are the most numerous cells in the human brain, outnumbering neurons by a ratio of ten to one" (Kandel, 2000). "This ratio drops to one-to-one in rodents" (Kandel, 2000) "implying that increases in glia over neurons are

associated with the progressive development of higher brain functions" (Nedergaard , 1994)

"Glial cells provide multiple functions to both the central nervous system (CNS) and the peripheral nervous system (PNS). Subsequent differentiation of glial cell populations results in function-specialized glial lineages. Glial cell-derived astrocytes are specialized lineages responsible for modulating the chemical environment by altering ion gradients and neurotransmitter transduction. (Butt, Hamilton, Hubbard, Pugh & Ibrahim, 2005). Similarly derived, oligodendrocytes secrete myelin for axon insulation and electric signal transduction. Finally, microglial cells are derived from glial precursors and carry-out macrophage like properties to remove cellular and foreign debris within the central nervous system" (Baumann & Hauw, 1979).

"During development, the CNS originates from the neuroepithelium, pseudostratified epithelial cells that maintain contact with both the ventricular and pial surfaces. As brain thickness increases, neuroepithelial cells transforminto radial glia" (Ihrie & Álvarez-Buylla, 2011; Haubensak, Attardo, Denk & Huttner, 2004). "Recent studies have shown that radial glia cells behave as stem cells, leading to the genesis of astrocytes, neurons" (Malatesta, Hartfuss & Götz, 2000; Noctor, Flint, Weissman, Dammerman & Kriegstein, 2001) "and to a lesser extent, oligodendrocytes" (Merkle, Tramontin, García-Verdugo, Alvarez-Buylla, 2004). "Thus radial glia cells not only serve as progenitors for many neurons and glial cells soon after birth, but also give rise to adult SVZ stem cells that continue to produce neurons throughout life" (Seri, García-Verdugo, Collado-Morente, McEwen & Alvarez-Buylla, 2004; Merkle et al., 2004). "A connection to radial glia cells has been suggested even in the hippocampal SGZ" (Eckenhoff & Rakic, 1984) "On the other hand, gliogenesis persists throughout the CNS in the form of parenchymal cell genesis capable of creating new oligodendrocytes and, to a lesser extent, astrocytes, throughout life" (Horner et al., 2000). "Most of this gliogenic activity is attributed to synantocytes / polydendrocytes (Ng2+ cells) which are widespread in the CNS.In the past, neurogenesis and gliogenesis had always been kept separate, the latter being considered less important than the former. In recent years, adult gliogenesis has been reevaluated as many populations of progenitor cells with glial-like features and proliferative capacity have been shown to exist in the mature mammalian CNS" (Reh & Levine, 1998; Dawson, Polito, Levine &

Reynolds, 2003; Nishiyama, Komitova, Suzuki & Zhu, 2009). “As a matter of fact, parenchymal cell genesis in the so-called nonneurogenic regions is mainly gliogenic. In most regions of the CNS, parenchymal progenitors assure a slow process of constitutive gliogenesis leading to renewal of oligodendrocytes and, to a lesser extent, astrocytes” (Horner et al., 2000; Nishiyama et al., 2009). “In rodents, the major population of cycling progenitors located outside the germinal niches are Ng2+ cells morphologically, antigenically, functionally distinct from mature astrocytes, oligodendrocytes, and microglia” (Horner et al., 2000; Nishiyama et al., 2009). Nevertheless, “many polydendrocytes remain as a resident cell population of Ng2-expressing cells in the mature white and grey matter after oligodendrocytes are generated. Thus it is widely accepted they represent the fourth CNS major glial population” (Nishiyama et al., 2009), “representing 2–9% of total cells during the last two decades, sometimes leading to excessive emphasis about theoretical correlations between neuro-glio-genic processes and brain repair. Focusing on the real neurogenic/gliogenic potential of the mammalian CNS should avoid to turn an exciting biological discovery into a therapeutic illusion” (Bonfanti, 2013)

2.8 Melatonin

“Melatonin secretion into the blood from the pineal gland located in the center of the brain varies in a daily cycle, thereby allowing the regulation of sleep and circadian rhythm” (Rivera-Bermúdez, Gerdin, Earnest & Dubocovich, 2003). “Adult hippocampal neurogenesis is affected by circadian rhythms and sleep deprivation” (Holmes et al., 2004; Guzman-Marin et al., 2005), thus “implying a significant role for melatonin. Besides its function as synchronizer of the biological clock, melatonin is a powerful free-radical scavenger and wide-spectrum antioxidant as discovered in 1993” (Tan et al., 1993). “In many less complex life forms, this is its only known function” (Tan, Manchester, Terron, Flores & Reiter, 2007). “Melatonin is an antioxidant that can easily cross cell membranes and the blood–brain barrier” (Reiter, Manchester & Tan, 2010). “This antioxidant is a direct scavenger of radical oxygen and nitrogen species including OH, O2−, and NO” (Tan et al., 1993). “Melatonin works with other

antioxidants to improve the overall effectiveness of each antioxidant. Melatonin has been proven to be twice as active as vitamin E, believed to be the most effective lipophilic antioxidant. Different from other classic antioxidants such as vitamin C and vitamin E, melatonin has amphiphilic properties. When compared to synthetic, mitochodrial-targeted antioxidants (MitoQ and MitoE), melatonin proved to be a better protector against mitochondrial oxidative stress. Melatonin's effect on neuronal cell survival were recapitulated in vivo by application of exogenous melatonin (8 mg/kg) and demonstrated antidepressant behavior in mice" (Ramírez-Rodríguez et al., 2009). "Melatonin also increases neuritogenesis and dendritogenesis with agonist treatment, leading to greater complexity of the dendritic tree" (Ramirez-Rodriguez et al., 2011). "Melatonin has also been shown to improve the irradiation induced decline in adult hippocampal neurogenesis, suggested to occur through its ability to scavenge free radicals" (Manda et al., 2009). "Several studies have implicated the abnormal accumulation of free radicals in neurodegenerative disorders. Free radical scavengers have been shown to protect against cell death" (Reiter, 1992; Miller et al, 1996). "Melatonin is a highly potent free radical scavenger" (Tan et al., 1993; Matuszak et al, 1997).

"Antioxidants are reported to play an essential role in the survival of neuronal cells exposed to various metabolic and oxidative challenges; therefore it would seem likely that they could also influence the production of new cells" (Ott, Gogvadze, Orrenius & Zhivotovsky, 2007). "Though melatonin possesses multiple physiological functions, the antioxidant function is one which has attracted much attention recently" (Koppisetti et al., 2008; Gitto, Pellegrino, Gitto, Barberi & Reiter, 2009). "Melatonin and its metabolites scavenges almost all reactive oxygen and nitrogen species and thereby acts as a powerful neuroprotective agent especially in the age related neurodegenerative disorder (Pohanka, 2011) where oxidative burden is the major culprit" (Tan et al., 2007; Peyrot & Ducrocq, 2008). Kong et al. (2008) investigated the effects of melatonin on the viability and differentiation of NSCs. They found that "melatonin promoted the survival of NSCs derived from rat ventral midbrain. In addition, melatonin-induced NSC differentiation into dopaminergic neurons and reduced astrocyte differentiation (Kong et al., 2008). It also augmented the production of glial cell-line derived neurotrophic factor (GDNF) and brain-derived neurotropic

factor (BDNF) (Zigova et al., 1998). From these data provide supportive evidence for a role of melatonin in regulating the process of neurogenesis"

It is known that endogenous melatonin production diminishes in elderly persons (Reiter, 1992) and that the total antioxidative capacity of serum correlates well with its melatonin levels in humans (Benot et al., 1999). Moreover, melatonin shows beneficial anti-aging effects in rats, preventing lipid peroxidation and other mechanisms related to oxidative stress (Poeggeler 2005; Paredes et al. 2009). Therefore, the age-related decrease of melatonin secretion may play a role in the elevated oxidative damage observed in the elderly population (Reiter, Tan, Mayo, Sainz & Lopez-Burillo, 2002).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Research Design

Experimental design

3.2 Material and Methods

3.2.1 Animals

Female mice (3 months old) will purchase from National Laboratory Animal Center (Mahidol University, Thailand) and will provide with water and food pellets *ad libitum*. All care, surgery, and injury induction will perform in accordance with the Guide for the National Institute of Health (NIH) and Faculty of Medicine, Srinakharinwirot University, Thailand, under supervision of Asst. Prof. Dr. Nopporn Jongkamonwiwat and were approved by the Animal Care and Use Committee at the Faculty of Medicine, Srinakharinwirot University (License number: 11/2555) and School of antiaging and regenerative medicine Mae Fah Lung University, Thailand.

Mice are randomly assignment to four different groups of animals; control group (n=1, 4 slides sample), SCI group (n=1, 4 slides sample), SCI+Melatonin group (n=1, 4 slides sample) and sham group (n=1, 4 slides sample). Each group were kept individual cage.

3.2.2 Sample Size

In this study, the total sample size is 12 calculated by power analysis program. Different mean and SD refer from the research “Alteration of forebrain neurogenesis after cervical spinal cord injury in the adult rat”, (Felix et al., 2012)

Kruskal-Wallis Test - One-Way Design Power Analysis

The formula for the Kruskal-Wallis Test is

$$T = \frac{\frac{12}{N^2+N}\sum_{k=1}^{g}\frac{R_k^2}{n_k} - 3(N+1)}{\left[1 - \frac{\sum_{i=1}^{G}(t_i^3 - t_i)}{N^3 - N}\right]}$$

R_k is the sum of the ranks of the data k^{th} group ($Rk = \Sigma^{nk}_{j=1} r_{kj}$)

G is the number sets of tied ranks

T_i is the number of tied values within set i

$n_1, n_2, \ldots, n_g$ denote the number of subjects in each group

N denote the total sample size of all groups

1. Simulation Summary

Number of Groups	3
Random Number Pool Size	10000
Number of Simulations	500

2. Numeric Results

Table 2.1 Numeric Results

Row	Power	Mean		Target Alpha	Actual Alpha	Std Dev of H1 μ's	Mean of H1 σ's	SD
		Group Sample Size n	Total Sample Size N					
1	0.000	2.0	6	0.050	0.000	0.0	7.9	7.8
2	0.040	4.0	12	0.050	0.060	0.0	7.7	7.8
3	0.044	6.0	18	0.050	0.038	0.0	7.9	7.8
4	0.052	8.0	24	0.050	0.038	0.0	7.8	7.8
5	0.038	10.0	30	0.050	0.058	0.0	7.9	7.8

3. Power and Alpha Confidence Intervals from Simulations

Table 2.2 Sample Size Evaluation

Total Sample Size			Lower Limit of 95% C.I. of Power	Upper Limit of 95% C.I. of Power	Target Alpha	Actual Alpha	Lower Limit of 95% C.I. of Alpha	Upper Limit of 95% C.I. of Alpha	SD
Row	N	Power							
1	6	0.000			0.050	0.000			7.8
2	12	0.040	0.023	0.057	0.050	0.060	0.039	0.081	7.8
3	18	0.044	0.026	0.062	0.050	0.038	0.021	0.055	7.8
4	24	0.052	0.033	0.071	0.050	0.038	0.021	0.055	7.8
5	30	0.038	0.021	0.055	0.050	0.058	0.038	0.078	7.8

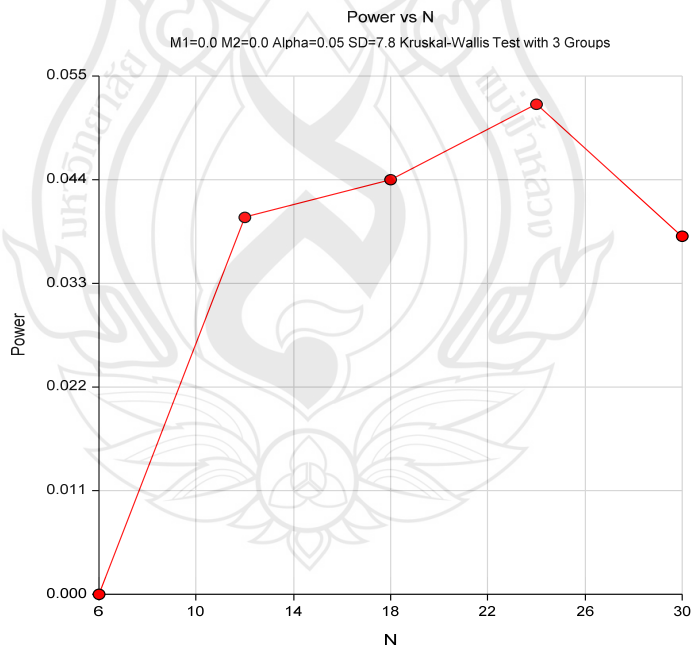


Figure 3.1 Representation of Sample Size Evaluation Graph

3.2.3 Inclusion and Exclusion Criteria

3.2.3.1 Inclusion criteria

1. Female mice 3 months old
2. Mice with complete spinal cord injury, evaluated by the movement of mice without left and right hind limbs movement
3. Uncontrolling bladder injured mice

3.2.3.2 Exclusion criteria

Mice with incomplete spinal cord injury, evaluated by the voluntary movement of mice with left or right hind limbs

3.2.3.3 Spinal cord compression

Dumont forceps number 5 was use to induce spinal cord compression, as described by Krityakiarana et al. (2010). The forceps were used to compress the cord laterally from the sides for 5 sec by ground down to the tips contact. (Krityakiarana et al., 2010).

3.2.3.4 Surgical Procedure

Surgical techniques were performed under aseptic conditions. Mice will be anesthetized followed by posterior incision of the skin. Muscles were retracted and laminectomy will perform at the level of the T12 vertebra. According to the experimental group, Dumont forceps was used to promote spinal cord compression. While in the sham group, spinal cord was free of compression. After surgery, muscle and skin were closed with nylon suture and animals will receive an i.p. injection of 2.5 mL of 0.9% (wt/vol) saline. Mice were placed in a temperature-controlled environment during recovery and testing. Manual bladder expression will perform 3 times a day. Antibiotics and pain-killer are administered after operation for 7 days and 3 days, respectively. The sham operation for SCI mice will perform to investigate the effect of locomotor function. For melatonin treated mice, the melatonin (10 mg/kg) for 14 days will deliver by intra-peritoneal injection 10 min after lesion (Erten et al., 2003; Beni, Kohen, Reiter & Tan, 2004; Reiter, Paredes, Korkmaz, Manchester & Tan, 2008).

3.2.3.5 Immunohistochemical methods

After 14 days the animal was deeply anesthetized with isoflurane and sacrificed by intracardiac perfusion with PBS followed by 4% paraformaldehyde. The brain was removed and soaked in the fixative solution at 4^{o} before histochemistry

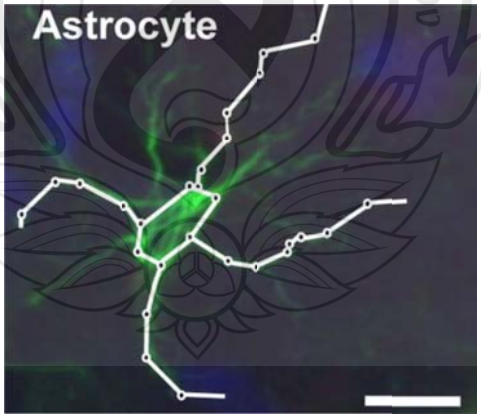
procedures, the brain were frozen at -20^{o}C. Twenty-micrometer serial sections were cut with a cryostat (Leica, Germany).

3.2.4 Immunostaining Protocol

Every 10 slices were randomly selected from each animal. The section were twice dipped for 5 min in PBS, incubate 10% Donkey serum in PBS-B then the sections were incubated with the first antibodies overnight at 4^{o}C. After rinsing in PBS-B for 30 min twice and rinsing in PBS for 30 min later. The sections were incubated in the secondary antibodies for 1 hour in dark box. After rinsing in PBS-B for 30 min twice and rinsing in PBS for 5 min. After staining with 3% DAPI for 5 min, rinsing in PBS for 5 min, they were mounted and seal with nail polish. The sections were examined and taken image by using a fluorescence microscope.

3.2.5 Quantitative Studies

The number of GFAP and Ki-67 immunoreactive glia cells in hippocampus of each section were counted using an Olympus microscope. We counted all GFAP and Ki-67 positive cells in hippocampus. To present the number of immunopositive cells, in the area of hippocampus, they were counted using cell sense software.



Source Cerbai et al. (2012)

Figure 3.2 Schematic Diagram Showing the Method Used to Quantify Astrocytes Brances of Glial Cell

3.3 Statistical Analysis

The values are given as the mean of measurements as well as the standard deviation of the mean. Kruskall wallis test was used for determining the significance of the results with *p*-values less than 0.05 will consider as significant and *p*-values less than 0.01 are considered very significant difference.

3.4 Ethical Consideration

All care, surgery, and injury induction will perform in accordance with the Guide for the National Institute of Health (NIH) and Faculty of Medicine, Srinakharinwirot University, Thailand and approved by the Animal Care and Use Committee at the Faculty of Medicine, Srinakharinwirot University.

3.5 Research Timing

Experimental research was done from November 2013 to March 2015.

CHAPTER 4

RESULTS

The aim of our study was to investigate whether SCI modulates neurogenesis in the adult mice brain. The effects of SCI were investigated in hippocampus area where ongoing adult neurogenesis has been described. Melatonin treatment group consisted in one daily i.p. injection for 14 days. To determine the impact of SCI on neurogenesis, we performed dual immunohistochemical staining for GFAP and Ki67 to label newly generated of glial cells. We evaluated immunoreactivity for GFAP,an astrocyte-specific intermediate filament protein, there were obvious differences observed between control group and treatment group.

GFAP immunoreactive, new glial cells are decreased in melatonin treatment group

Control group, average GFAP intensity, newly glial cells, was measured in hippocampal slices four to six sections per group in the hippocampus generated about 53$\pm$21.2 cells (Table 4.1)

For the study of glial cell proliferation in SCI animal's model, the number of GFAP positive cells were quantified in mice hippocampal sections. In SCI group, GFAP-immunoreactive cells were regularly observed in the dentate gyrus (Figure 4.2). For quantification of newly glial cells, the number of GFAP-immunoreactive cells was determined as shown in figure 10. The number of GFAP-positive cells was significantly increased in SCI group when compared to control group ($p<0.001$). As observed in the SCI group, the number of newly glia cell in hippocampus showed a significantly increasing compared to control, non-injured animals (Table 4.1)

In the melatonin treatment group, GFAP, a marker of glial cells are scattered all over the dentate gyrus in melatonin treatment group. A quantitative analysis revealed a significant increase in the number of newly glial cells when compared to control group ($p<0.001$; Table 4.1) but no significance difference was observed between SCI group.

Melatonin treatment group: SCI group compared to SCI-M group (175.8±71.5 versus 107±67.8) without reachings significance (Table 4.1) thus, melatonin could alter the ability of newly generated cells in the hippocampal of SCI animal's model.

Ki-67 immunoreactive, new glial cells are decreased in melatonin treatment group

Reduced glial cell proliferation in melatonin treatment group. The number of Ki-67 labeling for new glial cell is significantly decreased in melatonin treatment group (16.25 ± 12.2 *p<0.001) whereas 49.8 ±26.4 of Ki-67 positive newly glial cell were counted in SCI group (Table 4.2)

Numerous Ki-67 labeled new glial cells as seen in control group (53.25 ± 42.5) comparing to SCI group (49.8 ±26.4) without reaching significance (Table 4.2)

These studies use Ki-67 to demonstrate newly generated glial cells confirm the reduced gliogenesis in spinal cord injury mice whereas melatonin 10 mg/kg b.w./day for 14 days could alter the dramatic reduction in hippocampal gliogenesis of spinal cord injury mice (Figure 4.7)

a b c

Figure 4.1 Quantitative analysis of glial cell in hippocampus of control group. Characterization of glial cell in hippocampus of mice. Representative fluorescent photographs showing immunoreactivity of (a) DAPI (Blue), (b) GFAP (Red) and (c) Ki-67 (Green)

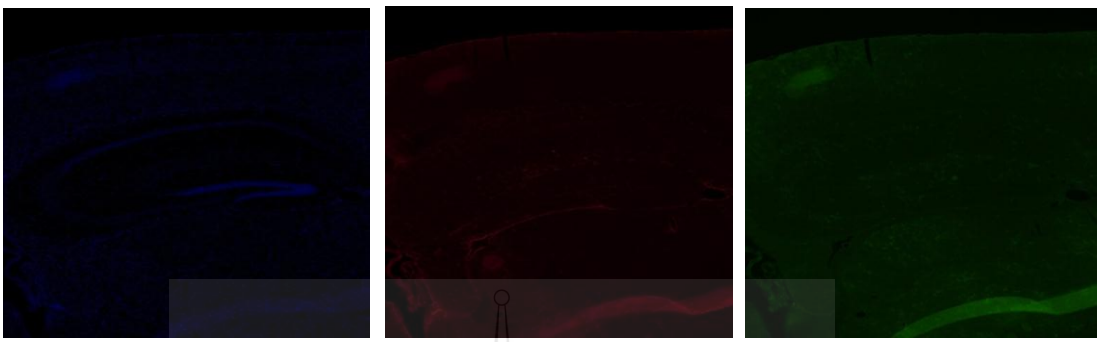
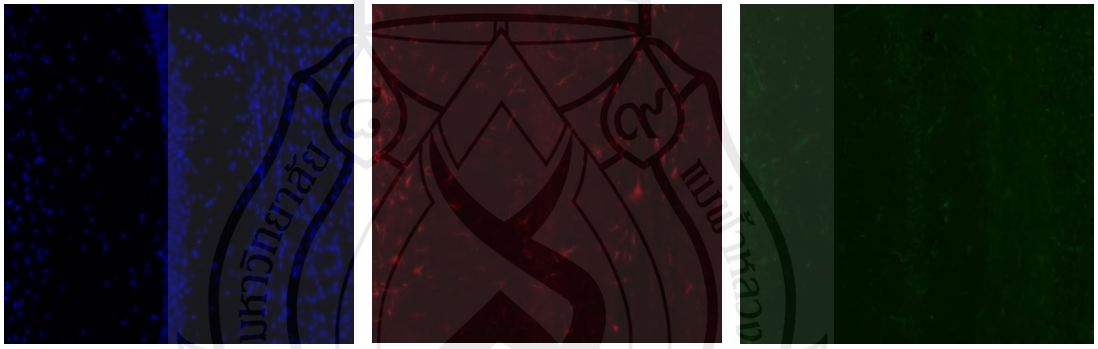
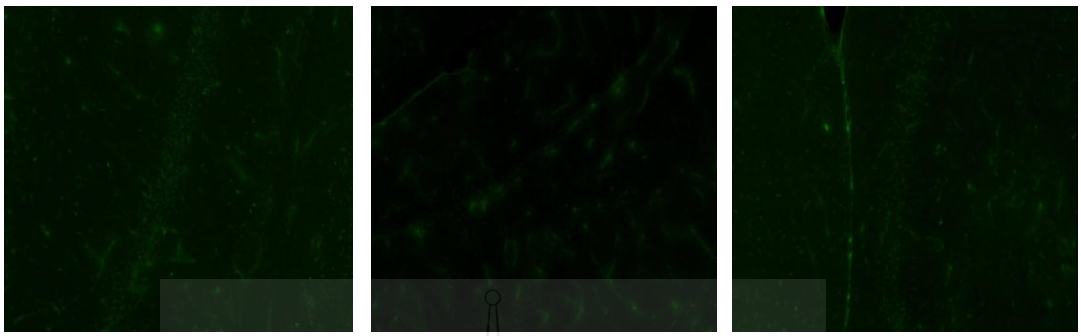


Figure 4.2 Quantitative analysis of glial cell in hippocampus of SCI group. Characterization of glial cell in hippocampus of mice. Representative fluorescent photographs showing immunoreactivity of (a) DAPI (Blue), (b) GFAP (Red) and (c) Ki-67 (Green)



a b c

Figure 4.3 Quantitative analysis of glial cell in hippocampus of SCI-M group. Characterization of glial cell in hippocampus of mice. Representative fluorescent photographs showing immunoreactivity of (a)DAPI (Blue), (b) GFAP (Red) and (c)Ki-67 (Green).



a b c

Figure 4.4 Quantitative analysis of glial cell in hippocampus of mice. Characterization of glial cell in hippocampus of (a) control group, (b) SCI group and (c) SCI-M group. Representative fluorescent photographs showing immunoreactivity of Ki-67 (Green)

a b c

Figure 4.5 Quantitative analysis of glial cell in hippocampus of mice. Characterization of glial cell in hippocampus of (a) control group, (b) SCI group and (c) SCI-M group. Representative fluorescent photographs showing immunoreactivity of GFAP (Red)

Statistical analysis

We use nonpapametric test, Kruskall- Wallis test and post hoc test to determine statistical significant among experimental groups with $p<0.001$, the level considered significant.

Table 4.1 Average GFAP intensity was measured in hippocampal slices, four to six sections per condition. Results are expressed as mean ± SD. (a***) Significantly different with $p < 0.001$ (b) No significantly different ($p = 0.747$) (c***) Significantly different with $p < 0.001$

Groups	**Control**	**SCI**	**SCI-M**	**Kruskall-Wallis test**
Mean ± SD	53 ± 21.2	175.8 ± 71.5	107 ± 67.8	P < 0.001
		a ***	b	
		C***		

Table 4.2 Average Ki-67 intensity was measured in hippocampal slices, four to six sections per condition. Results are expressed as mean ± SD. (a) No significantly different ($p = 0.984$) (b***) Significantly different with $p < 0.001$ (c***) Significantly different with $p < 0.001$

Groups	**Control**	**SCI**	**SCI-M**	**Kruskall-Wallis test**
Mean ± SD	53.25 ± 42.5	49.8 ± 26.4	16.25 ± 12.2	P < 0.001
		a	b***	
		c***		

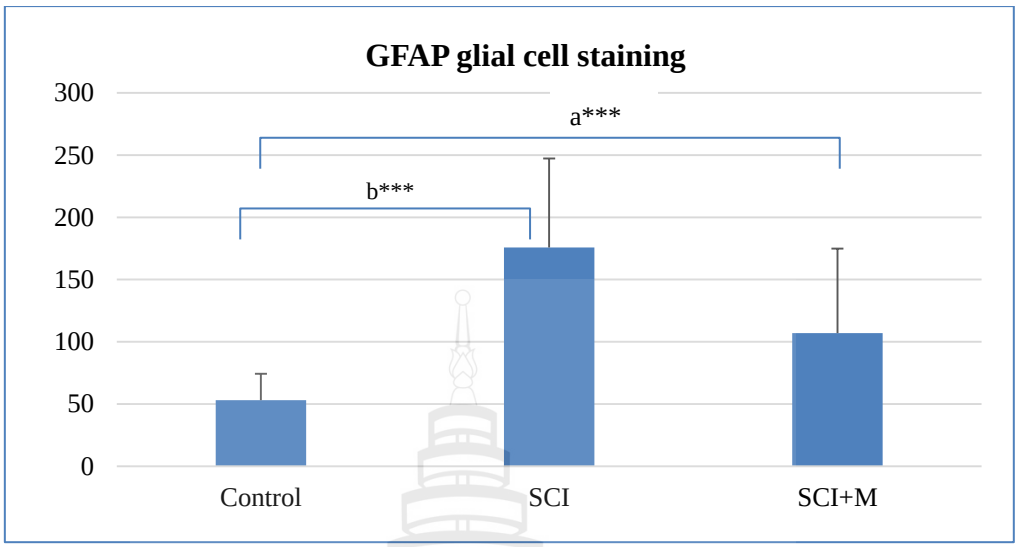


Figure 4.6 GFAP immunoreactivity uptaken in glial cell from hippocampal region after spinal cord injury. Values of GFAP uptaken are expressed as mean±SD of three separated experiments. (a***) Significantly different with $p < 0.001$ (b***) Significantly different with $p < 0.001$

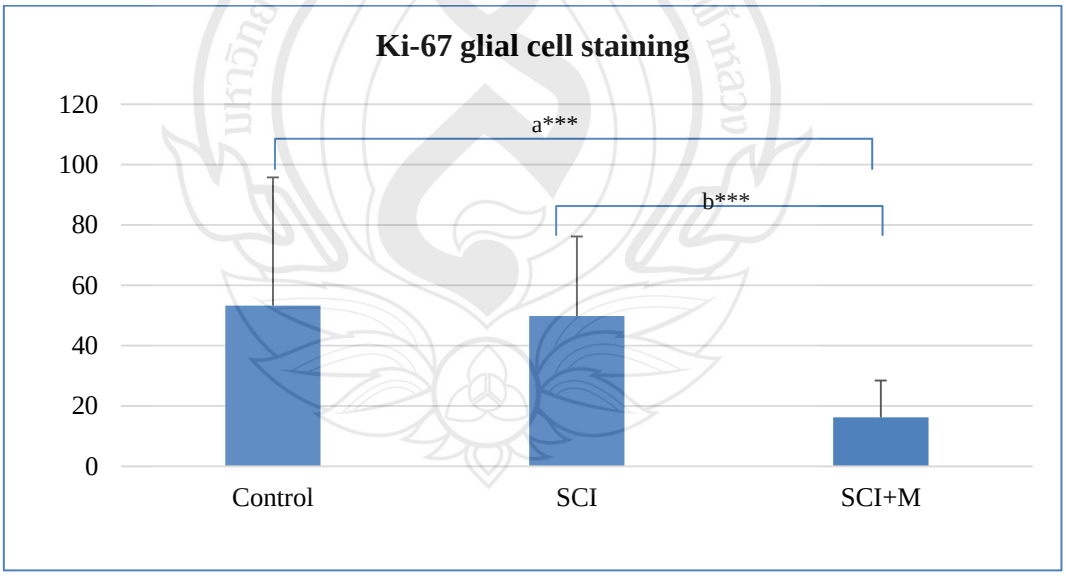


Figure 4.7 Ki-67 immunoreactivity uptaken in glial cell from hippocampal region after spinal cord injury. Values of Ki-67 uptaken are expressed as mean±SD of three separated experiments. (a***) Significantly different with $p < 0.001$ (b***) Significantly different with $p < 0.001$

CHAPTER 5

DISCUSSION

The present study was performed to examine the effect of melatonin (N-acetyl 5 methoxy trytamine) is naturally produced by the pineal gland from serotonin by a process catlysed by enzyme: arylalkylamine-N-acetyltransferase and hydroxyindazole-O-methyltransferase. The synthesis and release of melatonin in normal condition is stimulated by darkness and inhibited by light in response to signals originated in the suprachaiasmatic nucleus (Pandi-Perumal, Srinivasan, Spence & Cardinali, 2007).

In fact, melatonin exerts important antioxidant and anti-inflammatory functions; regulates the expression of multiple genes involved in inflammatory responses and antioxidant defense, and exerts pro- and antiapoptotic effects. Moreover, recent study suggests that melatonin act as a mitochondrial housekeeper. Melatonin able to maintain mitochondrial homeostasis counteracting mitochondrial oxidative stress in aging and neurodegeneration (Escames et al., 2010)

These results were confirmed the anti-inflammatory response and anti-apoptotic effects of melatonin. When melatonin administered intraperitoneally (10 mg/kg b.w./day) for 14 days promotes cell survive like the study of neuroblastoma cells that incubated with Aβ, more than 80% of the neurons died to apoptosis but the presence of melatonin reduced cellular death and DNA damage in a dose-related manner (Escames et al., 2010). The fact that reduction in inflammation were achieved via an anti-inflammatory effect of melatonin induction as seen in our study, the number of GFAP-positive cell was decreased in SCI-M group (107 ± 67.8) comparing with SCI group (175.8 ± 71.5) without reaching significance ($p = 0.747$) even though, it is not possible to claim that melatonin was responsible for the lack of effect so, dose dependent manner of melatonin and more sample size is necessary for future studies to evaluate the anti-inflammatory effects of melatonin on SCI model.

Glial cell pathology in depression and stress

Several cell counting studies in postmortem brain tissue of subjects diagnosed with major depressive disorder (MDD) and or manic-depressive (bipolar) disease (BPD) consistently reported an increase in glial cell density in hippocampal CA subfields and in the granule cell layer of the dentate gyrus (Rajkowska & Miguel-Hidalgo, 2007). Goldman et al explored the relationship between PTSD and MDD in veterans with SCI using the CAPS and the Beck Depression Inventory. Results of this study indicated that 28% of participants who had current PTSD also had current MDD (Nugent et al., 2008)

We can conclude that stresses are generally associated with the proliferation of gliogenesis in the hippocampus of spinal cord injury animals while melatonin can suppressed this process from antioxidant and anti-inflammatory effects; regulates the expression of multiple genes involved in inflammatory responses and antioxidant defense, and exerts pro- and antiapoptotic effects (Escames et al., 2010).

In our study, significantly difference of slightly gliogenesis, represent in Ki-67 labeling, was seen in hippocampus of SCI-M group (16.25 ± 12.2) comparing with SCI group (49.8 ± 26.4) ($p < 0.001$). These results can be confirm the neuroregenerative effect of melatonin do not seem to express in the hippocampus of SCI animal. Moreover, neuroprotective effect may exert around the traumatic lesion of spinal cord as derive from the study of melatonin effect on the functional recovery from SCI animal (Schiaveto-de-Souza, da-Silva, Defino & Del Bel, 2013). Finally, the dose response relationship studies for melatonin and large sample size could promise better results.

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