เมทิเลชันและการแสดงออกของยีน *NR3C1* ในผู้ป่วยโรคซึมเศร้าชาวไทยและผลของยาแก้ซึมเศร้าต่อการแสดงออกของยีน *CRF*

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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METHYLATION AND EXPRESSION OF *NR3C1* GENE IN THAI MAJOR DEPRESSIVE DISORDER PATIENTS AND EFFECTS OF ANTIDEPRESSANTS ON *CRF* GENE EXPRESSION

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มัชฌิมา นันทรัตน์ : เมทิเลชันและการแสดงออกของยีน *NR3C1* ในผู้ป่วยโรคซึมเศร้าชาวไทยและผลของยาแก้ซึมเศร้าต่อการแสดงออกของยีน *CRF* (METHYLATION AND EXPRESSION OF *NR3C1* GENE IN THAI MAJOR DEPRESSIVE DISORDER PATIENTS AND EFFECTS OF ANTIDEPRESSANTS ON *CRF* GENE EXPRESSION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.รัชนีกร ธรรมโชติ, 121 หน้า.

โรคซึมเศร้า มีความเชื่อมโยงกับความผิดปกติของการควบคุมเชิงลบของไฮโปทาลามัส-ต่อมใต้สมอง-ต่อมหมวกไต (เอชพีเอ) ซึ่งสัมพันธ์กับดีเอ็นเอเมทิเลชันในบริเวณโพรโมเตอร์ของยีน *NR3C1* การรักษาโรคซึมเศร้าโดยทั่วไปใช้ยาแก้ซึมเศร้า การศึกษานี้ถูกแบ่งเป็น 2 ส่วน ส่วนแรกมีวัตถุประสงค์เพื่อตรวจสอบการแสดงออกของ glucocorticoid receptor และเมทิเลชันบนโพรโมเตอร์ของยีน *NR3C1* ในผู้ป่วยโรคซึมเศร้าชาวไทย การศึกษานี้ได้ดำเนินการกับกลุ่มผู้ป่วยโรคซึมเศร้า 29 คน (ชาย 9 คน หญิง 20 คน) และกลุ่มคนปกติ 33 คน (ชาย 7 คน หญิง 26 คน) bisulfite pyrosequencing บน CpG จำนวน 7 ตำแหน่ง ที่บริเวณการจับของ NGFI-A ในเอ็กซอน 1_F ของโพรโมเตอร์ของยีน *NR3C1* แสดงระดับเมทิเลชันสูงขึ้นอย่างมีนัยสำคัญทางสถิติที่ CpG ตำแหน่งที่ 7 ในผู้ป่วยโรคซึมเศร้าโดยเฉพาะในเพศหญิง อย่างไรก็ตาม ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติของระดับการแสดงออกของยีน *NR3C1* และคอร์ติซอลระหว่างผู้ป่วยโรคซึมเศร้าและคนปกติ ในอีกทางหนึ่ง การควบคุมเชิงลบของเอชพีเอโดย glucocorticoids แสดงผ่านการยับยั้งกระบวนการถอดรหัสของยีน *CRF* ในไฮโปทาลามัสโดยตรง ดังนั้น ส่วนที่สองของวิทยานิพนธ์นี้มีจุดมุ่งหมายเพื่อศึกษาผลของยาแก้ซึมเศร้าต่อการแสดงออกของยีน *CRF* ผลจากการทำ qRT-PCR แสดงให้เห็นว่า ยาแก้ซึมเศร้าและยารักษาโรคทางจิตเวชน่าจะมีอิทธิพลโดยตรงต่อกระบวนการถอดรหัสของโพรโมเตอร์ของยีน *CRF* โดยการยับยั้งผลของยาต่อการชักนำการแสดงออกของยีน *CRF* ด้วย forskolin การค้นพบเหล่านี้แสดงให้เห็นว่า ไม่เพียงแค่การทำให้เกิดความสมดุลของสารสื่อประสาทเท่านั้น แต่กลไกการทำงานของยาแก้ซึมเศร้าและยารักษาโรคทางจิตเวชน่าจะมีปฏิกิริยาในการควบคุมเชิงลบของเอชพีเอ โดยการลดการแสดงออกของยีน *CRF* ในการตอบสนองต่อความเครียด ดังนั้น ผลของการทำวิทยานิพนธ์นี้ชี้ให้เห็นว่าทั้งยีน *NR3C1* และ *CRF* น่าจะมีบทบาทสำคัญในระบบตอบสนองต่อความเครียดของแกนเอชพีเอ ซึ่งสัมพันธ์กับความผิดปกติทางจิตเวช

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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MATCHIMA NANTHARAT: METHYLATION AND EXPRESSION OF *NR3C1* GENE IN THAI MAJOR DEPRESSIVE DISORDER PATIENTS AND EFFECTS OF ANTIDEPRESSANTS ON *CRF* GENE EXPRESSION. ADVISOR: ASST. PROF. RACHANEEKORN TAMMACHOTE, Ph.D., 121 pp.

Major depressive disorder (MDD) has been associated with hypothalamus-pituitary-adrenal gland (HPA) negative-feedback dysfunction, which is related to DNA methylation in the *NR3C1* promoter region. The most common therapy for MDD is the use of antidepressants. This study is divided into two parts. The first part aims to examine glucocorticoid receptor expression and *NR3C1* promoter methylation in Thai patients with MDD. This study was performed with 29 MDD patients (9 males, 20 females) and 33 normal controls (7 males, 26 females). Bisulfite pyrosequencing on 7 CpG sites at the NGFI-A binding site in the exon 1_F of *NR3C1* promoter region showed significant hypermethylation levels at the CpG7 in MDD patients, especially in female samples. However, no significant differences of *NR3C1* expression level and cortisol between MDD patients and normal controls were observed. On the other hand, regulation of HPA negative-feedback by endogenous glucocorticoids was shown to occur through inhibition of the *CRF* gene transcription directly in hypothalamus. The second part aims to investigate the effects of antidepressants on *CRF* expression *in vitro*. Results from qRT-PCR showed that antidepressants and psychiatric drugs may directly influence the transcriptional activity of *CRF* promoter by inhibitory effects of the drugs on forskolin-induced expression of *CRF* mRNA. These findings revealed that not only neurotransmitters balancing, but the mechanism actions of antidepressants and psychiatric drugs may also act in the HPA negative-feedback regulation by reducing *CRF* expression in response to stress. Taken together, the results from this thesis suggested that both the *NR3C1* and *CRF* genes may play important roles in the HPA axis stress response systems, which were associated to mental disorders.

Field of Study: Biological Sciences Student's Signature .....

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CHAPTER I
INTRODUCTION

Major depressive disorder (MDD) or depression is a common mental disorder affecting more than 350 million people worldwide (World Health Organization, 2012). Depression is a heterogeneous disorder that includes the highly variable emotional symptoms in psychosocial precipitating events such as sadness, feelings of worthlessness, hopelessness, bereavement, anxiety, inappropriate guilt and suicidal thought (Fava and Kendler, 2000; Belmaker and Agam, 2008). Moreover, the effects of mood abnormalities display quite a wide variation in clinical physical symptoms such as slowing of speech and action, appetite and sleep disturbances, loss of interest, vague aches and pains, headache, fatigue, back pain, weight loss or gain (American Psychiatric Association, 2000; Fava and Kendler, 2000). The criteria to diagnose MDD patients just require the physical changes, an obvious mood and psychophysiology, more than 2 weeks and these changes must extremely affect to work, family and personal relationships (Belmaker and Agam, 2008). In fact, depression has multifactor risks and causes that includes biological, psychological, and social factors all play a role in causing depression. Nevertheless, MDD pathological mechanism is not yet elucidated. Previous researches indicated that depression may associate with the stress response in the brain similar to other stress-related disorders (Weaver *et al*., 2004; Oberlander *et al*., 2008; McGowan *et*

al., 2009; Perroud *et al*., 2011). The main system of response to stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, which is regulated by negative feedback mechanism of the endocrine stress response to control cortisol levels (Meaney, 2001; Moser *et al*., 2007). The HPA negative feedback occurred through the activation of glucocorticoid receptor (GR), also known as nuclear receptor subfamily 3, group C, member 1 (NR3C1), by high cortisol levels (Jacobson and Sapolsky, 1991; de Kloet *et al*., 2005). In humans, the GR protein is encoded by *NR3C1* gene on chromosome 5q31-q32 (Hollenberg *et al*., 1985; Francke and Foellmer, 1989). Findings from previous studies on both animal models (Meaney, 2001; Howell and Muglia, 2006) and human (Oberlander *et al*., 2008; McGowan *et al*., 2009; Perroud *et al*., 2011) have shown that the stress response ability is related to the *NR3C1* gene and HPA axis functional reduction in the depression or depression-like conditions and many stress-related behaviors etiology. In addition, inhibited HPA negative-feedback activity is associated with highly circulated cortisol levels in MDD patients (Pariante *et al*., 2004).

Recently, several studies pointed that epigenetic mechanism, which is linked to environmental factors, may influence the *NR3C1* expression. Research in rat models showed that increased DNA methylation of the nerve growth factor-inducible protein A (NGFI-A) binding site located in the *NR3C1* gene exon 1_7 promoter, which is homologous region with exon 1_F in human *NR3C1* (Oberlander *et al*., 2008), was correlated with low maternal care (Weaver *et al*., 2004). This epigenetic process leaded to the decreased

NR3C1 expression and impaired the negative feedback mechanism (de Kloet *et al*., 2005). A few studies supporting this hypothesis have been performed in humans. Oberlander *et al.* (2008) reported that the DNA methylation status of the similar site in human newborn cord blood is sensitive to prenatal maternal mood disorders, and that the higher DNA methylation level was related with increased salivary cortisol levels (Oberlander *et al*., 2008). A study in suicidal victims with a history of childhood abuse showed high *NR3C1* methylation in the hippocampus, which was correlated with the decreased *NR3C1* expression levels (McGowan *et al*., 2009). In addition, Perroud *et al*. (2011) revealed that adults with childhood maltreatment and its severity positively associated with *NR3C1* methylation (Perroud *et al*., 2011). These studies suggested the relationship between traumatic experience in early life, which may develop to mental disorders, and increased *NR3C1* methylation status. However, the exact psychobiological mechanisms of adulthood MDD patients remain unclear. Moreover, the role of DNA methylation status of the human NGFI-A binding site plays in *NR3C1* expression in MDD patients remains to be experimentally proven. We, therefore, undertook this study to examine the association between glucocorticoid receptor expression and methylation status of *NR3C1* gene exon 1F promoter region and adulthood MDD. Moreover, the cortisol levels in blood plasma of MDD patients were also analyzed to correlate with the methylation status.

On the other hand, antidepressants are one of the psychiatric drugs that widely used to relieve or prevent in the clinical therapeutics for depression disorder and some conditions of psychiatric disorders. Since, the imbalance of neurotransmitters in the depression brain is one of in general cause of major depressed mood in patients. Almost all of currently antidepressants were used to increase the amount of circulating neurotransmitters, especially serotonin and norepinephrine, in the gap of synapse by blocking the reuptake transporters of neurotransmitters at the presynaptic neurons. Recently, there are the new antidepressant classes and most popular drugs that available used for depression in the clinical treatment are selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs). These new antidepressant classes often appear the adverse effects in patient fewer than older classes, such as monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs). However, drug responses are not almost immediate yet. In the medication, almost all of antidepressant treatments require long term, two to four weeks, or longer, administration to be effective (Baghai *et al*., 2006), and more than thirty percent of patients are not response with usual doses (Baghai *et al*., 2006; Preskorn, 2011). Recently, the action mechanisms of long-term effects of antidepressant remain poorly uncertain. The focus of many current studies has pointed to the slow onset adaptive changes that regulate gene expression occur within neurons (Stout *et al*., 2002; Budziszewska *et al*., 2002; Conti *et al*., 2004; Basta-Kaim *et al*., 2006), more than acute antidepressant action

on circulating neurotransmitter transportation within the synapse. Researchers believed that long-term changes within neurons may be influenced from other mechanisms of antidepressant effect. Therefore, the mechanisms of therapeutic action and downstream gene-targets partway are extremely needed to understand for the effective clinical treatment of depression disorder.

Interestingly, there is another hypothesis about antidepressants effects are focused on the regulation of gene expression in neurons that play a central role in response to stress (Stout *et al*., 2002; Budziszewska *et al*., 2002). This hypothesis led to put on the corticotropin-releasing factor (*CRF*) gene, a 41 amino acid located on chromosome 8q13.1 (Vale *et al*., 1981), that is one of the important gene plays in the hypothalamic-pituitary-adrenal (HPA) axis regulation induced by stressful situations (Britton *et al*., 1986; Owens and Nemeroff, 1991). In response to stress of HPA axis, *CRF*, produced in the paraventricular nucleus (PVN) within hypothalamus, released the corticotropin-releasing hormone (CRH) via specific G protein-coupled CRF receptor type 1 (CRF_1 receptors) to stimulate the secretion of adrenocorticotrophic hormone (ACTH) from an anterior pituitary (Whitnall, 1993; Suda *et al*., 2004). The anterior pituitary mediates its effects to stimulate an adrenal grand to secrete the steroid stress hormone, cortisol or glucocorticoids, to many parts of the body during the stress (Whitnall, 1993). Repression by endogenous glucocorticoids was shown to occur through inhibition of the *CRF* gene transcription directly in hypothalamus, as a HPA negative-feedback regulation to inhibit

the release of cortisol in response to stress (Itoi et al., 1987; Karagianni and Tsawdaroglou, 1994; Malkoski and Dorin, 1999). However, findings supporting the downstream signaling partway regulation of *CRF* systems and HPA axis activity are remains unclear.

Although there are some researchers reported that chronic antidepressant treatment in rat and mice rather reduce the sensitivity of *CRF* neurons to stress (Brady *et al*., 1992; Stout *et al*., 2002; Conti *et al*., 2004) and some antidepressants can inhibit the human *CRF* promoter activity in neuro-2a neuroblastoma cells (Budziszewska *et al*., 2002), the mechanism of antidepressant action on *CRF* promoter activity in the regulation of hypothalamic *CRF* expression is poorly understood yet. In 2008, Kageyama *et al*. used mutagenesis technique of some region on *CRF* promoter to demonstrate the regulation of the *CRF* gene transcription in rat hypothalamic 4B cells (Kageyama *et al*., 2008). They found that glucocorticoids-dependent repression of cAMP-stimulated *CRF* promoter activity may mediate by both negative glucocorticoid regulatory element (nGRE) and serum-response element (SRE) regions on the *CRF* promoter (Kageyama *et al*., 2008). This is interesting to investigate the molecular mechanisms involved in the direct effects of antidepressants on human *CRF* promoter interactions in particular hypothalamus.

To prove the hypothesis that the action of antidepressants may involve in reduces the hypothalamic *CRF* expression in stress response. We, therefore, determine the efficacy of antidepressant treatment both to prevent and reduce the stress on forskolin-induced *CRF* mRNA levels. Moreover, in order to confirm whether antidepressants may

directly affect *CRF* gene promoter activity, the transient-transfected hypothalamus cell lines with the constructs of human *CRF* promoter-luciferase were generated. We also evaluated the action of antidepressants on the human *CRF* promoter both to prevent and reduce the forskolin-induced luciferase mRNA expression.

Research objectives

1. To examine glucocorticoid receptor expression and *NR3C1* promoter methylation in Thai patients with major depressive disorder.
2. To investigate the effects of antidepressants on *CRF* expression and *CRF* promoter activity *in vitro*.

CHAPTER II
LITERATURE REVIEW

2.1 Major Depressive Disorder

Major depressive disorder (MDD), usually known as depression, is an abnormal mood symptom that one of a common psychiatric disorder. The observation in 2012 by World Health Organization (WHO) reported that more than 350 million people worldwide have depression symptoms (World Health Organization, 2012). Depression is a heterogeneous disorder that includes the highly variable emotional symptoms in psychosocial precipitating events such as sadness, feelings of worthlessness, hopelessness, bereavement, anxiety, inappropriate guilt and suicidal thought (Fava and Kendler, 2000; Belmaker and Agam, 2008). Moreover, the effects of mood abnormalities display quite a wide variation in clinical physical symptoms such as slowing of speech and action, appetite and sleep disturbances, loss of interest, vague aches and pains, headache, fatigue, back pain, weight loss or gain (American Psychiatric Association, 2000; Fava and Kendler, 2000). The criteria to diagnose MDD patients just require the physical changes, an obvious mood and psychophysiology, more than 2 weeks and these changes must extremely affect to work, family and personal relationships (Belmaker and Agam, 2008). In fact, depression has multifactor risks and causes that includes biological, psychological and stressful life events factors all play a role in causing depression (Paykel

2003; Belmaker and Agam, 2008). There are some evidences that depression and other psychiatric disorders may be caused by the abnormal overactivation of the hypothalamic-pituitary-adrenal (HPA) axis regulation, which was one of the main factor of stress-related disorders as in depression patients (McEwen *et al*., 1997; de Kloet *et al*., 1998; Weaver *et al*., 2004). The overactive HPA system results in an effect of increased cortisol levels in the body, suggesting disturbances of the neuroendocrine regulation system that play a role in stress-related symptoms, including MDD and other psychiatric disorders (Weaver *et al*., 2004). On the other hand, the neurotransmitters, such as dopamine (DA), norepinephrine (NE) and serotonin (5-HT), imbalance in the brain is another one of important factor involved in the cause of psychiatric disorders, including major depression (Nutt, 2008; Krishnan and Nestler, 2008). Nevertheless, MDD pathological mechanism is not yet elucidated.

2.2 Biological mechanisms involved in MDD and psychiatric disorders

2.2.1 Hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamic–pituitary–adrenal (HPA) axis is the regulation of neuroendocrine system consists of three components: the paraventricular nucleus (PVN) of hypothalamus, the anterior lobe of the pituitary gland and the cortex of adrenal glands (Jacobson and Sapolsky, 1991; Kino and Chrousos, 2004b; Nader *et al*., 2010). A mainly part of the common mechanism for interactions between HPA axis systems is the

neuroendocrine regulation that response to stress. In stress response period, corticotropin-releasing hormones (CRH) or arginine vasopressin (AVP) are produced by the hypothalamus when the body has high stress, and release them into the anterior lobe of the pituitary gland to stimulate adrenocorticotropic hormones (ACTH) secretion (Chrousos, 2009). Circulating ACTH then rapidly stimulates the adrenal cortex to produce and secret glucocorticoid hormones (also known as cortisol in humans and corticosterone in rodents) (Chrousos, 2009). High levels of secreted glucocorticoids (cortisol) was regulated by negative feedback mechanism to suppress both CRH and ACTH secretions from the hypothalamus and the pituitary gland, which result to control glucocorticoid stress hormones (Jacobson and Sapolsky, 1991; Meaney, 2001; Chrousos, 2009) (Figure 2.1).

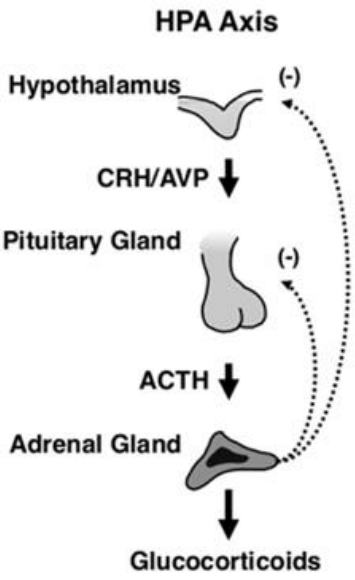

Figure 2.1 Organization of the hypothalamic–pituitary–adrenal (HPA) axis (Kino, 2015).

Reports from many previous researchers hypothesized the association of depression and the mechanism of the stress response in the brain that may be similar to other stress-related disorders (Weaver *et al*., 2004; Oberlander *et al*., 2008; McGowan *et al*., 2009; Perroud *et al*., 2011). The stress response has evolved as a highly adaptive reaction that ensures survival when an organism was confronted with physiological or psychological challenge. The main mechanism of stress response in the brain was mediated by the HPA neuroendocrine regulation, which was controlled by negative feedback mechanism to control stress hormone (cortisol) levels (Jacobson and Sapolsky, 1991; Meaney, 2001; Moser *et al*., 2007). The negative feedback mechanism of HPA axis occurred via the initiation of high levels of cortisol and other glucocorticoids activating the glucocorticoid receptor (GR), also known as nuclear receptor subfamily 3, group C, member 1 (NR3C1) (Jacobson and Sapolsky, 1991; de Kloet *et al*., 2005).

2.2.2 Glucocorticoid receptor (GR)

In humans, the GR protein, or NR3C1, was encoded by *glucocorticoid receptor* (*GR*) or *NR3C1* gene. (Hollenberg *et al*., 1985). The GR protein, or NR3C1, was expressed in almost every cell, their primary mechanism of action is the regulation of gene transcription controlling the development, metabolism and immune response (Rhen and Cidlowski, 2005; Lu *et al*., 2006). In the absence of glucocorticoids, GR-protein complexes resides in the cytosol including several heat shock proteins (HSP), such as

HSP90, 70, 23 and the protein FKBP52 (FK506-binding protein 52) (Kumar and Thompson, 1999; 2005; Pratt *et al*., 2006; Kino, 2015). When glucocorticoids that were secreted by adrenal glands diffuse into cell membrane, GR releases HSPs complexes to bind with glucocorticoid and translocates into the nucleus (Pratt *et al*., 2006; Egeland *et al*., 2015; Kino, 2015). In the nucleus, Glucocorticoid-receptor complex directly binds with the specific binding site, usually called glucocorticoid response elements (GREs) to activate their transcription and other transcription factors regulation (Kino, 2015). GREs binding site was located in the promoter region of glucocorticoid-responsive genes. After glucocorticoid-receptor complex binding with GREs, there are many transcriptional cofactors and the RNA polymerase II complex were attracted to stimulate their transcriptional activity (Egeland *et al*., 2015; Kino, 2015). After the transcriptional regulation of glucocorticoid-responsive genes, GR returns to reform with their ligand protein complexes into the cytosol (Egeland *et al*., 2015; Kino, 2015) (Figure 2.2).

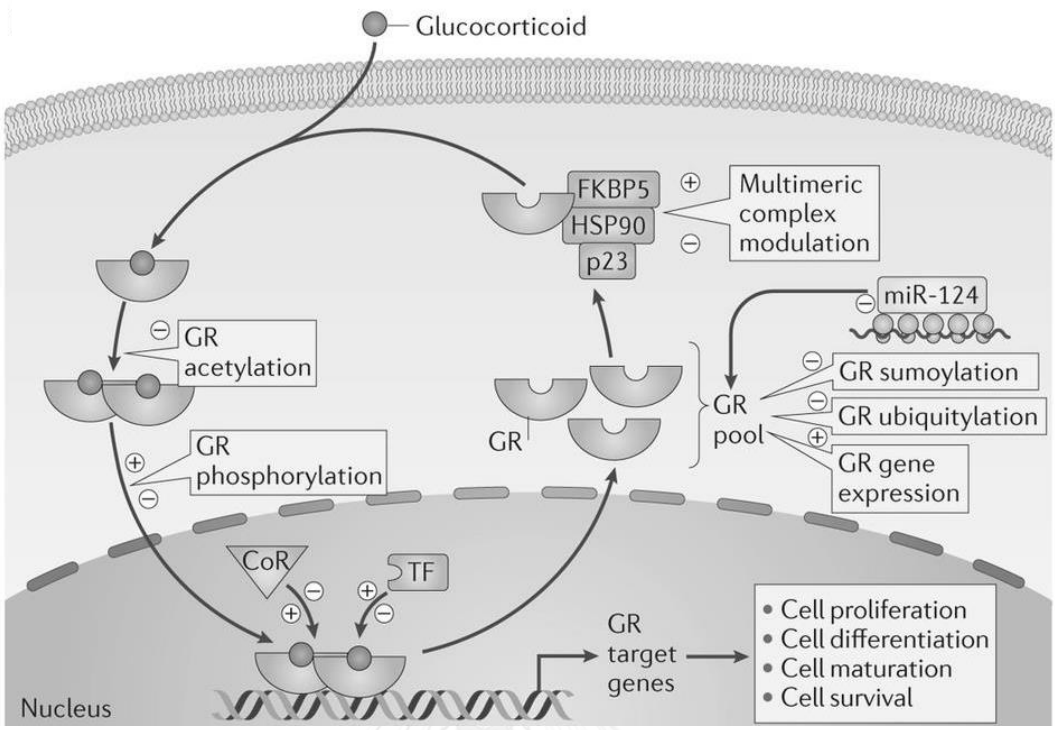

Figure 2.2 Intracellular circulation of the glucocorticoid receptor (GR) (Egeland *et al*., 2015).

2.2.3 Epigenetic mechanism

Epigenetics is the heritable trait changes study in gene expression that occur without changing the DNA sequences and these may or may not be hereditable (Ledford, 2008). The possible through means such as DNA methylation and histone modification (usually know as histone methylation and acetylation) as shown in Figure 2.3, and all of these are reversible (Carey, 2011). Recently, many studies found that these modification processes are caused by external or environmental factors involved in genes on and off regulation (Moore, 2015). Several environmental factors such as exposure to

heavy metals (arsenic, nickel) and cigarette smoke, and dietary factors such as vitamin and folate deficiencies have been linked to abnormal changes in epigenetic pathways (Dolinoy *et al*., 2006; Wade, 2006). These epigenetic processes may occur through during the cell cycle (Bird, 2007). Chandler (2007) suggested these processes mostly occur only within one individual lifetime. Nevertheless, some epigenetic mechanisms can be transferred by a sperm or egg cell in fertilization process to the next generation (Chandler, 2007). Recently, epigenetic changes studies, especially DNA methylation, have been observed in different stages of cancer progression (Nan *et al*., 2005; Pogribny *et al*., 2006; Perry *et al*., 2010; Sharma *et al*., 2010; Choong and Tsafnat, 2012), in the process of aging (Munoz-Najar and Sedivy, 2011; Brunet and Berger, 2014), and in other human diseases such as HIV latency (Hakre *et al*., 2011), asthma (Martino and Prescott, 2011), allergies (Kuriakose and Miller, 2010), metabolic diseases (Lillycrop *et al*., 2007; Lillycrop, 2011) and cardiovascular diseases (Bertram *et al*., 2008).

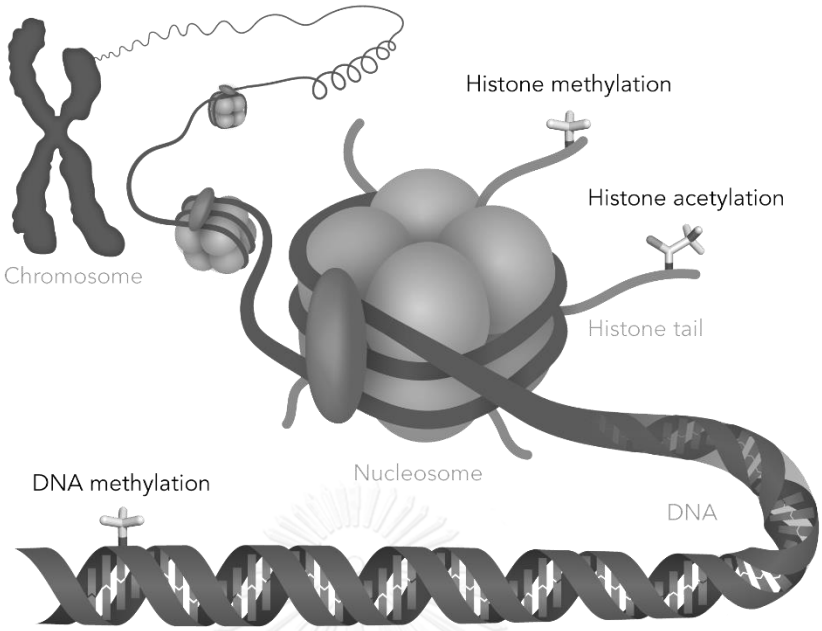

Figure 2.3 The most important epigenetic marks including DNA methylation, histone methylation and acetylation. (source: https://www.horizondiscovery.com/cell-lines/all-products/explore-by-your-research-area/epigenetics)

2.2.3.1 DNA methylation

DNA methylation, one of the important mechanism of epigenetics, is a chemical process modification affecting CpG dinucleotides by which methyl groups (Ch_3) are added on cytosine bases to modify the function of the DNA (Laird and Jaenisch, 1996). Addition process of methyl (CH_3) group was catalyzed by DNA methyltransferases enzymes (DNMTs), which takes place by CH_3 via S-adenosyl-L-methionine (SAM) at the carbon 5 of the cytosine ring of 5' to 3'oriented CpG sites to be 5-methylcytosine (Singal and Ginder, 1999) (see Figure 2.4). Recent studies used genome-wide evolutionary

analysis to study eukaryotic DNA methylation, and reported that the DNA methylation process has been found in almost all of eukaryotic organisms including algae, fungi, plants, invertebrates and vertebrates (Zemach, 2010)

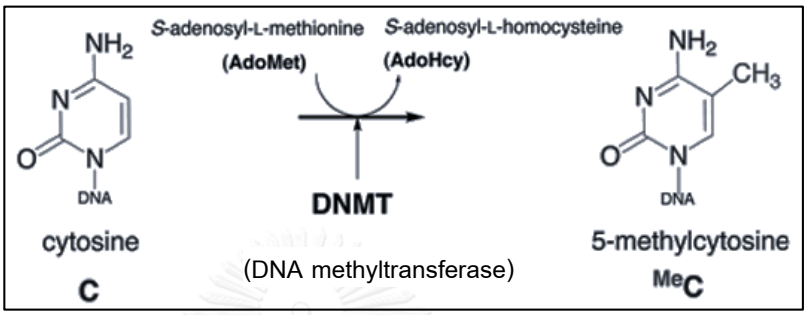

Figure 2.4 Addition process of methyl (CH_3) group onto Cytosine.
(source: http://atlasgeneticsoncology.org/Deep/DNAMethylationID20127.html)

Normally, gene promoter regions are the area that mostly found high frequency of CpG islands (Gardiner-Garden and Frommer, 1987); therefore, the methylated DNA modification is an essential regulatory mechanism in gene expression by interfering the binding of transcription factors (TF) to the promote region (Barter, 2012) (Figure 2.5). When methyl groups were located on CpG island in a gene promoter region, this DNA modification typically acts to repress gene transcription. DNA methylation is mechanism for suppressing (or silencing) gene transcription by preventing transcription factors and thus RNA polymerase activities accessing a gene promoter region, which is required for transcribing DNA into RNA (Ideraabdullah *et al*., 2008; Barter, 2012).

Moreover, the essential of DNA methylation also involved in normal control of gene expression for normal development processes such as X-chromosome inactivation and genomic imprinting.

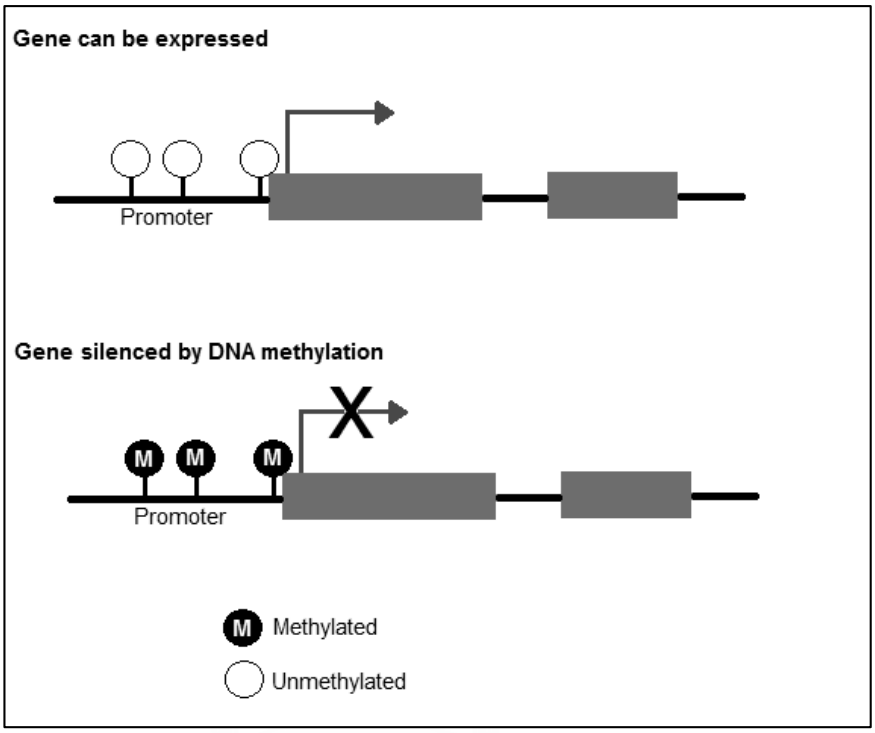

Figure 2.5 Addition of methyl groups to CpG dinucleotides that located within gene promoter region interferes the transcription factor to bind with promoter, which one is the mechanism of DNA methylation for suppressing (or silencing) gene transcription by (source: http://www.epibeat.com/what-is-epigenetics/).

2.3 Candidate genes involved in depression and psychiatric disorders.

2.3.1 *NR3C1* gene

NR3C1 gene was located on chromosome 5q31-q32 (Hollenberg *et al*., 1985; Francke and Foellmer, 1989) (Figure 2.6), and contains 17 exons, nine of them are non-coding exons located at the proximal *NR3C1* gene promoter region (Palma-Gudiel *et al*., 2015). Palma-Gudiel *et al*., (2015) recently reported structure of *NR3C1* gene (Figure 2.7). There are eight coding exons (exon 2 - 9) and nine non-coding first exons (exon 1_A – 1_J). Moreover, CpG sites located at non-coding exons 1_D – 1_H estimating 3 Kb along the proximal 5' untranslated *NR3C1* gene promoter region as shown in Figure 2.7 (Palma-Gudiel *et al*., 2015). These currently reviews also showed the complete extended sequences of CpG island that located at seven non-coding first exons (exons 1_D – 1_H) as shown in Figure 2.8 reported by Palma-Gudiel *et al*. (2015). Furthermore, they also indicated the nerve growth factor-inducible protein A (NGFI-A) transcription factor binding site regions that were located in exon 1_F (shown in dashed boxes in Figure 2.8) (Palma-Gudiel *et al*., 2015). The 7 CpG dinucleotides in our study were evaluated in CpG 33 – 39 in Figure 2.8, which were also located in exon 1_F promoter region. These previous findings found that the NGFI-A transcription factor binding site placed in exon 1F, especially the CpG 37 (shown by red color in Figure 2.8) was widely used to report in both animal and human studies (Oberlander *et al.,* 2008; McGowan *et al*., 2009; Palma-Gudiel *et al*., 2015; Nantharat *et al*., 2015).

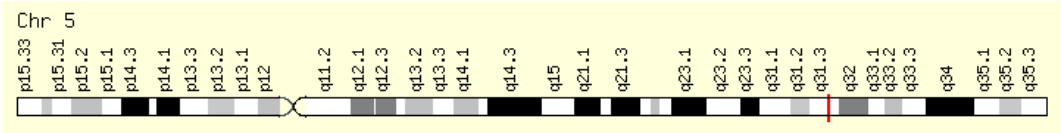

Figure 2.6 The location of *NR3C1* gene (red bar) on chromosome 5.

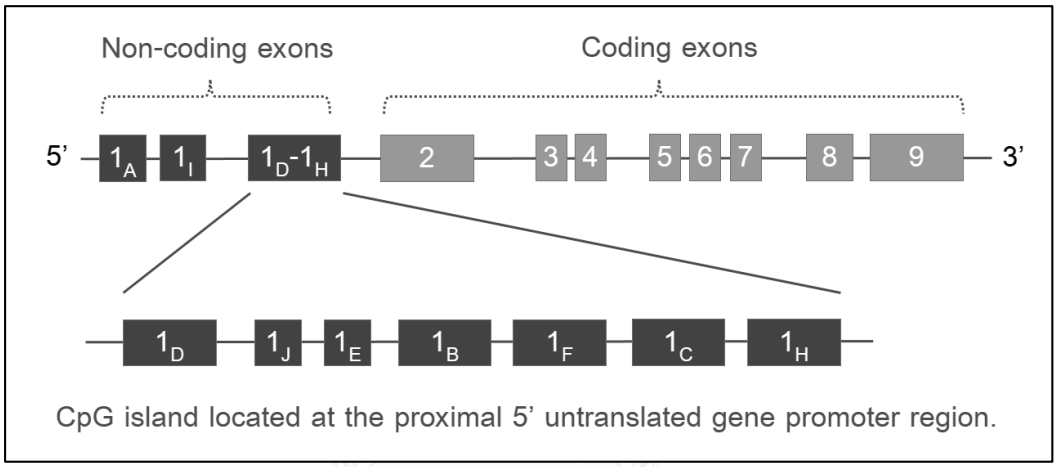

Figure 2.7 *NR3C1* gene structure reported by Palma-Gudiel *et al*., (2015)

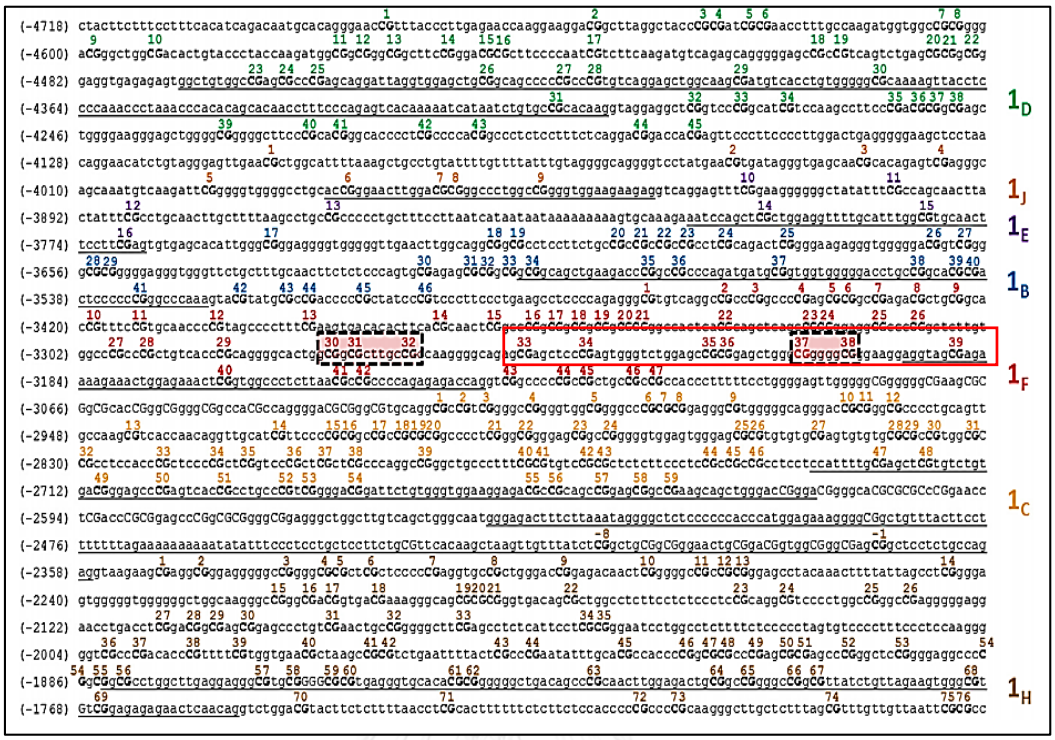

Figure 2.8 Extended sequences of the CpG island located within non-coding exons 1_D – 1_H of the proximal *NR3C1* gene promoter region reported by Palma-Gudiel *et al*., (2015). The CpG dinucleotides were shown in capital letters by separating with the difference color of the group numeration of each exons. Dashed boxes refer to the NGFI-A transcription factor binding site regions as placed in exon 1_F (Palma-Gudiel *et al*., 2015). The 7 CpG dinucleotides in our study showed in red box.

Previously, findings in animal studies have shown that the stress response ability in the depression-like condition models is associated with the *NR3C1* gene and HPA axis inhibitions (Meaney, 2001; Howell and Muglia, 2006). These findings supported the studies in the repression of the *NR3C1* gene and HPA axis in depression and other stress-related disorders in human (Oberlander *et al*., 2008; McGowan *et al*., 2009; Perroud *et al*., 2011; Nantharat *et al*., 2015). In addition, HPA negative-feedback dysfunction is related with high levels of cortisol in major depression patients (Pariante *et al*., 2004). The GR (NR3C1) is therefore believed to be more important in the regulation of the response to stress when endogenous levels of glucocorticoids are high (McEwen *et al*., 1997; de Kloet *et al*., 1998). Because patients with major depression exhibit impaired HPA negative feedback in the context of elevated circulating levels of cortisol (Pariante *et al*., 2004), a number of studies have considered the possibility that the expression or function of *NR3C1*, or both, are reduced in depressed patients and other psychiatric disorders (Gormley *et al*., 1985; Yehuda *et al*., 2002; Calfa *et al*., 2003).

Recent studies have also become increasingly important to identify psychobiological mechanisms (Singer, 2001), linking environmental influences to changes at the molecular level that predispose, or sustain disease processes. Epigenetic mechanisms, such as DNA methylation and changes in chromatin structure, have been implicated as a means by which environmental factors, such as maternal behavior and early care (Weaver *et al*., 2004), can influence gene expression and are thought to

produce long-term health consequences (Meaney and Szyf, 2005). In 2004, Weaver *et al*. found that low maternal care behaviors in rats related to highly DNA methylation at the binding site of the nerve growth factor-inducible protein A (NGFI-A) transcription factor located in the *NR3C1* gene exon 1_7 promoter (Weaver *et al*., 2004). This previous finding supported the correlation between influence of DNA methylation and depressive behaviors. According to study in animal models, several recent studies tried to describe association between DNA methylation, which is linked to environmental factors, and related psychobiological disorders in human such as depression (Oberlander *et al*., 2008) and bipolar disorders (Kaminsky *et al*., 2012). de Kloet *et al*. (2005) also mentioned that the process of increased DNA methylation may result in the inhibition of *NR3C1* gene expression and HPA negative feedback mechanism (de Kloet *et al*., 2005).

To date, there are a few studies supporting this hypothesis have been performed in humans (Oberlander *et al*., 2008; McGowan *et al*., 2009; Perroud *et al*., 2011). In 2008, Oberlander *et al.* found that the DNA methylation status at the exon 1_F of *NR3C1* gene in newborn cord blood was sensitive to maternal mood disorders. Moreover, the increased DNA methylation was correlated with high levels of salivary cortisol (Oberlander *et al*., 2008). This evidence support a role for epigenetic processes, especially DNA methylation, in early environmental programming of HPA function (Weaver *et al*., 2004). Studies of the human GR (*NR3C1*) promoter region (Breslin *et al*., 2001; Turner *et al*., 2006) identified a possible analogous human NGFI-A binding site (Turner *et*

al., 2006) and methylation status in post mortem brains (Moser *et al.,* 2007; McGowan *et al*., 2009) have been also reported. According to study the methylation status in newborn cord blood, McGowan *et al*. (2009) tried to use the specific brain tissues to analyze the relevance of DNA methylation status and early environmental adverse events. Their results revealed that the hippocampus of suicidal victims with a history of childhood abuse showed the increased methylation status at the exon 1_F of *NR3C1* gene, which was related to the decreased *NR3C1* expression levels (McGowan *et al*., 2009). Morever, adults with childhood maltreatment also associated with *NR3C1* methylation (Perroud *et al*., 2011). These findings indicated that traumatic experience in early adverse events associated with high levels of *NR3C1* methylation status, and may develop to mental disorders. It remains unclear, however, about the factor that triggers changes in methylation, and the timing when the different tissues are most susceptible to epigenetic programming. Moreover, the exact psychobiological mechanisms of adulthood MDD patients, and the role of DNA methylation of the NGFI-A binding site, which was placed in exon 1_F of *NR3C1* gene promoter region, plays in *NR3C1* expression in depression patients stills to be experimentally proven.

2.3.2 *CRF* gene

On the other hand, there are some reports that the *corticotropin-releasing factor* (*CRF*) gene (Figure 2.9), located on chromosome 8q13.1, plays a central role in controlling the HPA axis during stressful periods (Vale *et al.,* 1981). This gene encodes a member of the corticotropin-releasing factor family, also known as corticotropin-releasing hormones (CRH) that were released by the PVN of the hypothalamus in response to stress. During the stress period, CRF (CRH) activates HPA axis, acting at CRF1 receptor on the anterior pituitary gland to stimulate the release of ACTH. (Chrousos, 2009).

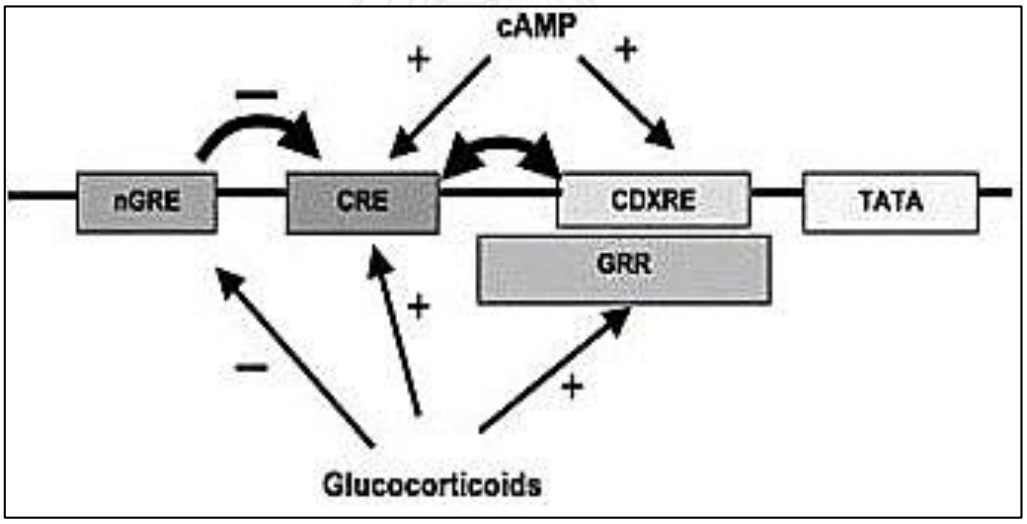

Figure 2.9 General structure of the human *CRF* gene promoter (Ni and Nicholson, 2006).

Increased CRH production has been observed to be associated with Alzheimer's disease and depression (Raadsheer et al., 1995). There are many previous findings found that repression by endogenous glucocorticoids was shown to occur through inhibition of the *CRF* gene transcription directly in hypothalamus (Karagianni and Tsawdaroglou, 1994; Malkoski and Dorin, 1999). The available evidence suggests that CRF has been reported to have an involvement in the development of anxiety related and mood disorders (Arborelius *et al*., 1999). Studies have found that abnormality of *CRF* expression in stress responsively, which referred to HPA axis dysregulation, resulted in the onset of anxiety-like behaviors and depression (Arborelius *et al*., 1999; Reul and Holsboer, 2002). Moreover, increased cerebrospinal fluid (CSF) *CRF* concentrations in human have been repeatedly observed in major depression patients (Nemeroff *et al*., 1984; Banki *et al*., 1987; Hartline *et al*., 1996) and suicide victims (Arato *et al*., 1989). Many studies found that treatment with antidepressants reduces CSF *CRF* concentrations (De Bellis *et al*., 1993; Veith *et al*., 1993; Heuser *et al*., 1998). Interestingly, there is evidence that antidepressants exert effects on *CRF* systems.

There are many previous studies pointed to psychiatric disorders may directly associate with abnormality of the HPA negative-feedback regulation (Mitchell, 1998; McGowan *et al*., 2009; Perroud *et al*., 2011). Another important point to the mechanism partway of *CRF* gene, which play a central role in the negative-feedback mechanism of HPA (Owens and Nemeroff, 1991; Pelleymounter *et al*., 2000; Stout *et al*.,

2002), may potentially response for mental disorders in mediating both the HPA stress-response regulation and the neurotransmitter circulation in the brain (Vermetten and Bremner, 2002a). Some researchers tried to find about the interaction between *CRF* gene expression and neuroendocrine systems within the direct synaptic neurons, especially dopamine-*CRF* interaction (Swerdlow *et al*., 1986; Toufexis *et al*., 2004; Meloni *et al*., 2006).

2.4 Neurotransmitters related with psychiatric disorders

There are the three main monoamine neurotransmitters in the brain, including serotonin (5-HT), norepinephrine (NE) and dopamine (DA), specific symptoms of MDD and other psychiatric disorders (Nutt, 2008) shown in Figure 2.10. Specific symptoms were associated with the increase or decrease of specific neurotransmitters in the synaptic gab between neurons in the brain (*Carlson*, *2005*), and subsequently specific antidepressant drugs could target symptom-specific neurotransmitters (Linde *et al*., 2015). In 2008, Nutt (2008) has been indicated the association of symptoms-specific neurotransmitters and depression, and other psychiatric disorders. The results revealed that reduced 5-HT is related to anxiety, obsessions and compulsions (Nutt, 2008). In addition, dysfunctional NE neurotransmitter activity is implicated with decreased alertness, low energy, problems of inattention, concentration and cognitive ability, while

dopamine (DA) deficiency is associated with problems of motivation, pleasure, and reward (Nutt, 2008).

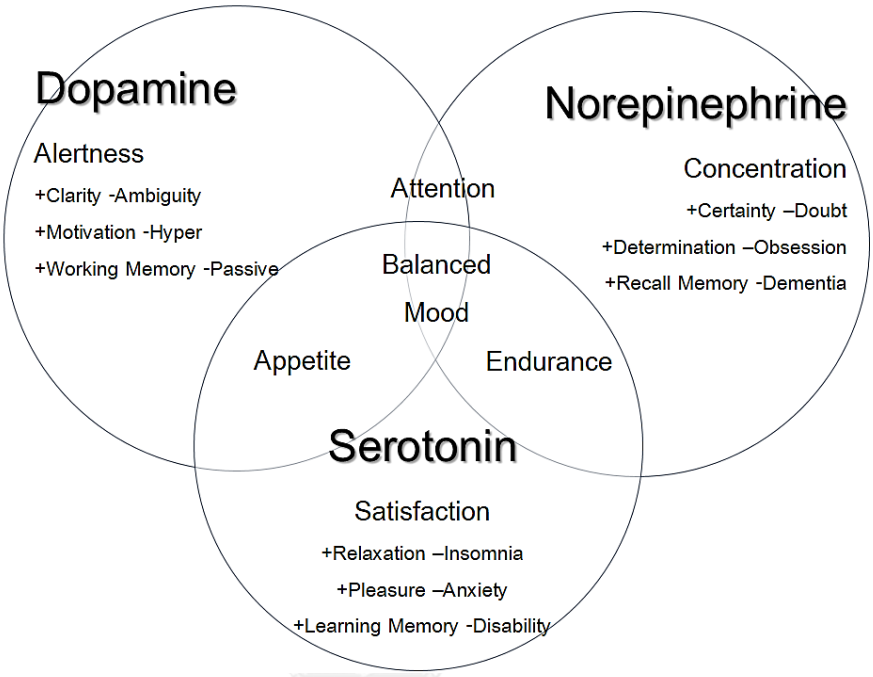

Figure 2.10 Three main neurotransmitters correlated with symptoms of MDD and other psychiatric disorders.

Although the underlying pathophysiology of depression has not been clearly defined, there are evidences suggest imbalances in serotonin (5-HT), norepinephrine (NE), and dopamine (DA) neurotransmitters levels in the synaptic gab in the brain (Belmaker and Agam, 2008; Krishnan and Nestler, 2008). There are some observers hypothesized that decreased serotonin activity may allow depression systems to act in unusual behaviors (Shah *et al*., 1999; Barlow and Durand, 2005). The observation of this efficacy led to the monoamine hypothesis of depression, which postulates that the deficit

of certain neurotransmitters is responsible for the corresponding features of depression (Barlow and Durand, 2005). According to this hypothesis, depression arises when low serotonin levels and also promotes low levels of norepinephrine (Shah *et al*., 1999). Evidence also suggests that depression may involve in the imbalance of circulating neuroendocrine response to stress which results in an effect similar to an overactive HPA axis (Monteleone, 2001).

2.5 Clinical treatment for depression and psychiatric disorders

2.5.1 Antidepressants

Antidepressants are drugs used for the treatment of MDD and other psychiatric disorders, including anxiety, dysthymia, obsessive compulsive disorder (OCD) and, in some conditions, such as dysmenorrhea, migraine, addiction and attention-deficit hyperactivity disorder (ADHD). Many antidepressants designed to increase serotonin (5-hydroxytryptamine, 5-HT) monoamine neurotransmitter levels in the synapse, and serotonin may enhanced to regulate other neurotransmitter systems (*Carlson*, *2005*). The most important classes of antidepressants consisted of the selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), reversible monoamine oxidase A inhibitors (rMAO-A inhibitors), tetracyclic antidepressants (TeCAs) and noradrenergic and specific serotonergic antidepressants (NaSSAs) (Linde *et al*., 2015).

However, the currently available of therapeutic antidepressants commonly used in clinical treatment are two classes that are SSRIs and SNRIs classes because of lower adverse effects compared with other classes (Linde *et al*., 2015). The most popular drugs were used to relieve MDD disorders in the clinical treatment in Thailand hospitals such as fluoxetine (Prozac), venlafaxine (Effexor) and sertraline (Zoloft).

(1) Fluoxetine

Fluoxetine, also known by trade names is Prozac, is a SSRI antidepressant class (Benfield et al., 1986). It is usually used for acute and maintenance treatment of MDD, and other conditions of OCD, panic and premenstrual dysphoric disorders (Asberg *et al*., 1986; Benfield *et al*., 1986; Sommi *et al*., 1987). The common side effects of fluoxetine are trouble sleeping, loss of appetite, dry mouth, rash, and abnormal dreams (Wernicke, 1985; Asberg *et al*., 1986). Its mechanism of action is not entirely clear but presumed to be linked to potentiation of serotonin neurotransmitter activity in the *central nervous system* (CNS) resulting from its inhibition of CNS neuronal reuptake of serotonin (5-HT) in the brain (Lemberger *et al*., 1985; Stark *et al*., 1985; Benfield *et al*., 1986; Sommi *et al*., 1987) (Figure 2.11).

(2) Venlafaxine

Venlafaxine, brand names is Effexor, is a SNRI antidepressant class (Muth *et al*., 1986; Yardley *et al*., 1990). The action of this drug class increases the concentrations of the neurotransmitters both serotonin and norepinephrine in the body and the brain (Goeringer *et al*., 2001). This drug was licensed for the maintenance treatment of MDD, and other psychiatric conditions such as anxiety, ADHD, post-traumatic stress disorder (PTSD), panic disorder and social phobia (Pae *et al*., 2007; Cipriani *et al*., 2009; Ghanizadeh *et al*., 2013; Rossi, 2013). The rate of lethal outcomes for suicidal overdoses of venlafaxine is lower than for the other drug classes (White *et al*., 2008). The very common adverse effects seen in people taking venlafaxine including headache, nausea, insomnia, dizziness, somnolence, asthenia, dry mouth and sweating (Ghanizadeh *et al*., 2013; Rossi, 2013). The venlafaxine mechanism of action works by blocking the transporter reuptake neurotransmitters both serotonin and norepinephrine (Goeringer *et al*., 2001) as shown in Figure 2.11. Moreover, high doses of venlafaxine also weakly inhibits the reuptake of dopamine neurotransmitter (Wellington and Perry, 2001). However, an exactly action remains to be proven.

(3) Sertraline

Sertraline, or trade names is Zoloft, is one of SSRI antidepressant class. This drug was primarily used for MDD patient in adult as well as OCD, panic disorder and anxiety, in both adults and children (Turner *et al*., 2008; Brunton *et al*., 2010; Sanchez *et al*., 2014). Its types of adverse events are usually similar to other SSRIs, including diarrhea, nausea, trembling, sexual dysfunction and weight gain (Taylor *et al*., 2012; Brayfield, 2013; Sanchez *et al*., 2014). The action of sertraline mechanism is primarily to inhibit the serotonergic neurotransmitter reuptake in the synapse with a binding affinity towards the serotonin transporter (Meyer *et al*., 2004) (see in Figure 2.11). Some studies found that sertraline also weakly inhibited the dopamine neurotransmitter reuptake (Owens *et al*., 2002; Mohapatra, 2013). It remains; however, unclear an exactly mechanism of this drug action.

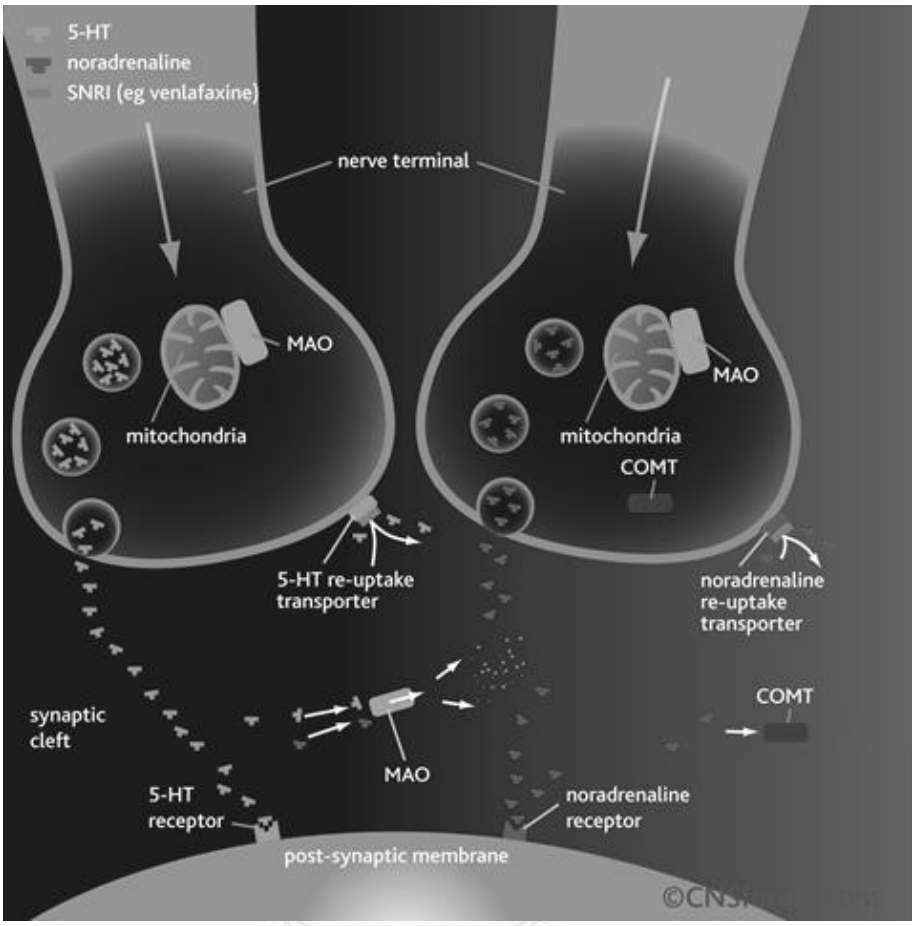

Figure 2.11 Actions of SSRIs and SNRIs antidepressants.

(source: http://pharmacologycorner.com/differences-between-tricyclic-antidepressants-and-selective-serotonin-norepinephrine-reuptake-inhibitors-mechanism-of-action/)

In general, SSRIs and SNRIs work by inhibiting the reuptake transporters of specific neurotransmitters to increase the amount of their neurotransmitters circulating, especially serotonin (5-HT) and norepinephrine (NE), in the synaptic gap. However, there are reports that many antidepressant treatments require two to eight weeks administration to be effective (Baghai *et al*., 2006), and more than thirty percent of patients do not response well with usual doses (Baghai *et al*., 2006; Preskorn, 2011). The action mechanism of long-term effects of antidepressants has not been elucidated. Researchers had demonstrated that long-term changes within neurons may be influenced by other mechanisms of antidepressant effect (Stout *et al*., 2002; Budziszewska *et al*., 2002; Conti *et al*., 2004; Basta-Kaim *et al*., 2006). Therefore, information regarding mechanisms of therapeutic action and downstream target genes are needed to administer the effective clinical treatment for depression disorder.

There are many reports indicated that depression may relate to the hypothalamic-pituitary-adrenal (HPA) axis stress response the brain (Meaney, 2001; Pariante and Miller, 2001; Weaver *et al*., 2004; Oberlander *et al*., 2008). Some researches on antidepressants effects are focused on the association with gene expression in neurons that play a central role in the HPA axis (Stout *et al*., 2002; Budziszewska *et al*., 2002). One of the genes in focus is the corticotropin-releasing factor (*CRF*) gene, a gene expressed in the paraventricular nucleus (PVN) within the hypothalamus (Vale *et al*., 1981). *CRF* expression is activated by stressful situations, and it is one of the important

genes in the HPA axis negative-feedback regulation (Britton *et al*., 1986; Owens and Nemeroff, 1991). In HPA negative feedback, repression of the HPA axis by cortisol (endogenous glucocorticoids) occurs directly via inhibition of the *CRF* gene transcription in hypothalamus to inhibit the release of cortisol in response to stress (Itoi *et al*., 1987; Karagianni and Tsawdaroglou, 1994; Malkoski and Dorin, 1999).

Researches in animal models showed that the *CRF* neurons sensitivity can be reduced by chronic antidepressant treatment (Brady *et al*., 1992; Stout *et al*., 2002; Conti *et al*., 2004). This is interesting to examine the molecular mechanisms associated with the direct effects of antidepressants on *CRF* promoter interactions in a hypothalamus cell line. To date, findings supporting the regulation of *CRF* systems and HPA axis activity by antidepressant treatment are remains unclear. Almost all of currently drugs, which used to alleviate the symptoms of depression, will be used to inhibit the transport of transmitter back into the presynaptic terminal. However, drug effects on transporters are almost immediate yet antidepressant action is not noticeable for several weeks. And, the therapeutic mechanisms of action remain poorly understood (Stout *et al*., 2002; Conti *et al*., 2004).

2.5.2 Psychiatric drugs

Psychiatric drugs are typically used to treat patients suffered from mental disorders such as depression, obsessive compulsive disorder (OCD), schizophrenia, anxiety, bipolar disorder and attention deficit-hyperactivity disorder (ADHD) (Schatzberg, 2000; Stahl, 2008). Many types of psychiatric drugs are used to treat psychiatric disorders. Diazepam (Riss, 2008), risperidone (Hamilton, 2015) and haloperidol (Stevens, 2004) are among popular therapeutic drugs.

(1) Diazepam

Diazepam, or originally trade names is Valium, is mainly used to treat a range of psychiatric symptoms including panic attacks, insomnia, anxiety and acute alcohol withdrawal symptom (Calcaterra and Barrow, 2014). The mechanism of action of diazepam is positive allosteric stimulator of the gamma-aminobutyric acid (GABA) type A (GABAA) receptors. Binding of the drug to the GABAA receptors enhances binding of GABA neurotransmitter, which increased the chloride ions (Cl^-) influx into cytosol through the chloride channel (Riss *et al*., 2008) (Figure 2.12). Consequently, resting and threshold abilities of neurons were differently increased. There are some previous studies have revealed that diazepam may involve in HPA axis activity reduction in stressful periods (Lakic *et al*., 1986; Pivac and Pericic, 1993), although drug influences seem to raise the

activity of basal HPA axis in animal models under some experimental conditions (Vargas *et al*., 2001).

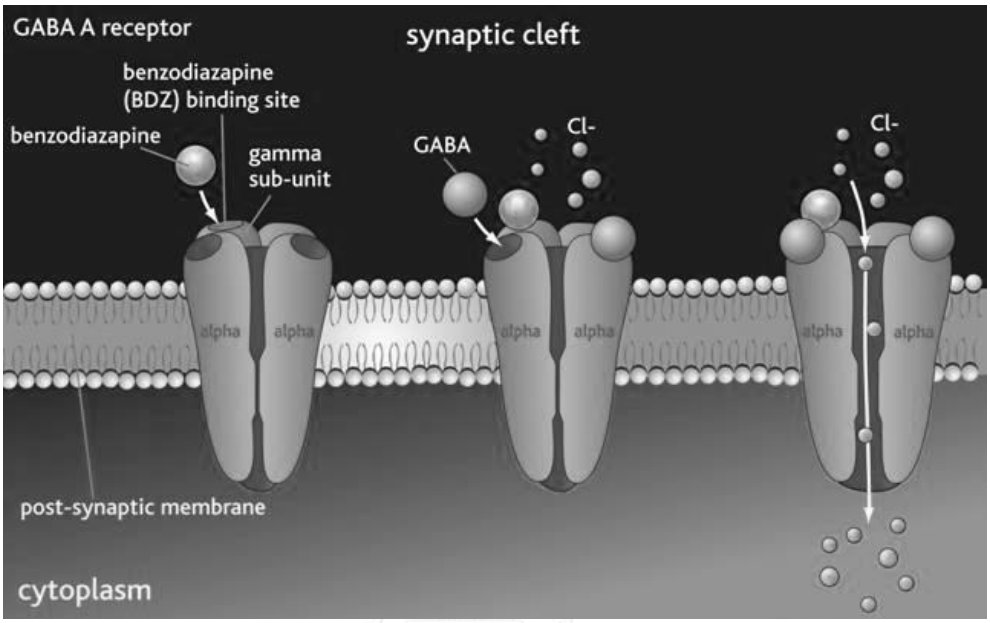

Figure 2.12 Pharmacological action of diazepam enhances the GABA receptor. (source: http://intranet.tdmu.edu.te.ua/data/kafedra/internal/pharmakologia/)

(2) Risperidone

Risperidone, also known by trade name is Risperdal, is an atypical antipsychotic medication that is commonly used to treat in various psychotic disorders including schizophrenia, bipolar disorder and irritability/aggression in autistic patients (Leucht *et al*., 2013; Muralidharan *et al*., 2013; Sharma and Shaw, 2012; Kirino, 2014). The common side effects in patients who take this drug are trouble of movement and seeing, sleepiness, constipation and weight gain (Hasnain *et al*., 2012; Hamilton, 2015).

The main action of risperidone is an antagonist of the dopamine receptors including D_1, and D_2 receptors to decrease dopamine neurotransmitter (Brunton *et al*., 2010). Moreover, this drug also shows antagonistic actions at the serotonin (5-HT_2), alpha α_1 and α_2 adrenergic and Histamine (H_1) receptors (Brunton *et al*., 2010; Hecht and Landy, 2012) as shown in Figure 2.13.

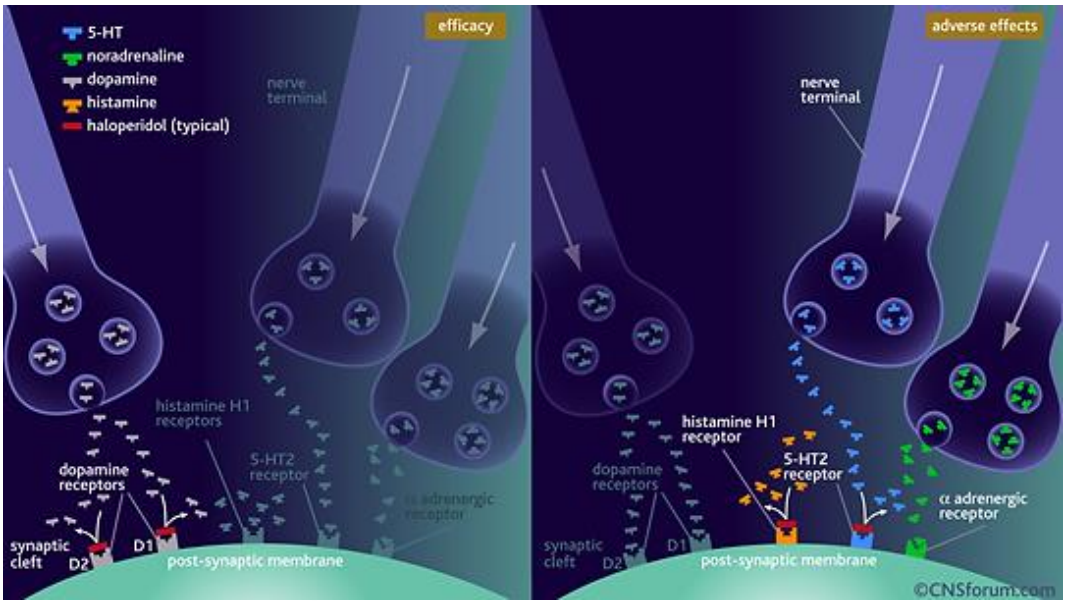

Figure 2.13 Pharmacological action of risperidone and haloperidol drugs is dopamine antagonist. (source: https://www.cnsforum.com/educationalresources/imagebank/drug_neuroleptic/drug)

(3) Haloperidol

Haloperidol, the trade name is Haldol, is a typical antipsychotic drug that is normally used in the treatment of various symptoms of psychotic disorders, such as schizophrenia, acute psychosis, mania, delirium, and severe anxiety (Brayfield, 2013). The main action of haloperidol pharmacology is the dopamine (D_2) receptor antagonist (Schotte *et al*., 1993; Seeman and Tallerico, 1998; Brayfield, 2013) (see in Figure 2.13). Schotte *et al*. (1993) indicated that haloperidol also acts with alpha α_1 and serotonin (5-HT_2) receptors; moreover, affinity for histamine (H_1) and muscarinic (M_1) acetylcholine receptors can be slightly found (Schotte *et al*., 1993).

Reports from previous researches of the interaction of risperidone and haloperidol treatments in a life-long *CRF* overproduction (*CRF*-OE) transgenic mouse model found that both drugs are able to reverse the startle reactivity and inhibit the prepulse in the transgenic *CRF*-OE mouse (Dirks *et al*., 2003). The result of *CRF* system abnormalities may associate with neuropsychiatric disorders patients, especially schizophrenia, OCD, and possibly post-traumatic stress disorder (PTSD) (Braff *et al*, 1992; 2001; Bremner *et al*., 1997; Grillon *et al*., 1998). Furthermore, Meloni *et al*. (2006) has been reported the effectiveness of the selective dopamine receptor antagonists on *CRF*-enhanced startle might have anxiety-like effects (Meloni *et al*., 2006).

On the other hand, there are a few studies have demonstrated that another possibility of antidepressants and psychiatric drug actions may alternate affect

within neurons (Vargas *et al*., 2001; Budziszewska *et al*., 2002; Dirks *et al*., 2003; Park *et al*., 2011). These hypothesizes indicated that the mechanism of drug action on the downstream regulation of *CRF* mRNA levels in HPA negative-feedback mechanism in stressful period that is of great interest. In 2002, Budziszewska *et al*. tried to find about the relevance of some antidepressant role and psychiatric drugs on the activity of HPA negative-feedback regulation that may involve through directly *CRF* gene promoter activity in Neuro-2A neuroblastoma cells (Budziszewska *et al*., 2002). Interestingly, a site-directed mutagenesis in the *CRF* promoter demonstrated that the glucocorticoids signaling pathway repressed the cAMP-stimulated *CRF* promoter activity. This phenomenon may be mediated by both the negative glucocorticoid regulatory element (nGRE) and serum response element (SRE) regions on the *CRF* promoter (Kageyama *et al*., 2008) as shown in Figure 2.14.

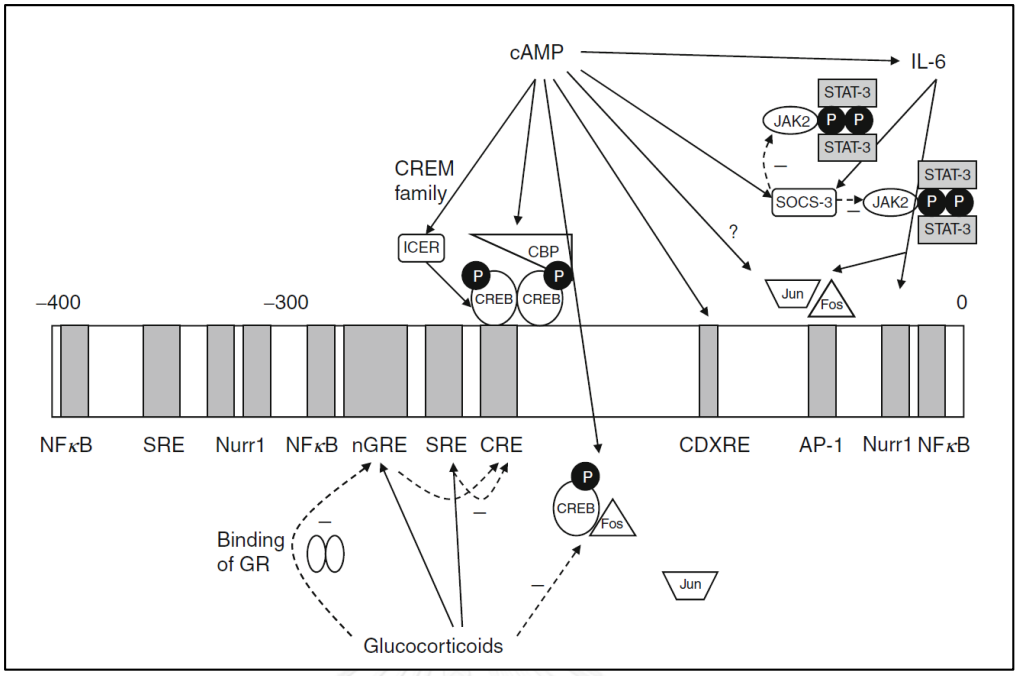

Figure 2.14 The glucocorticoid signaling pathway in the *CRF* promoter (Kageyama and Suda, 2010).

However, there has been no report on the drug action on the direct *CRF* promoter regions. Therefore, the understanding of the exact mechanisms of downstream target genes of antidepressants and other psychiatric drugs action on *CRF* promoter activity are needed to identify for further developing the therapeutic drug efficiency in medical treatment for depression and other psychiatric disorder patients.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Participants

All participants of this research were recruited from the hospitals around Thailand, including (I) the northern part: Suanprung Hospital and Maharaj Nakorn Chiang Mai Hospital; (II) northeastern part: Loei Rajanagarindra Psychiatric Hospital; (III) the central part: Ramathibodi Hospital, Srithanya Hospital, and Prasat Neurological Institute; (IV) the southern part: Songklanagarind Hospital. A total of 62 subjects comprised of 29 MDD patients (9 males/ 20 females with average age of 48.63 ± 8.43 years old/ 48.00 ± 12.08 years old, respectively), and 33 normal individuals (7 males/ 26 females with average age 41.14 ± 7.71 years old/ 42.92 ± 7.82 years old, respectively). All MDD subjects were diagnosed by psychiatrist and have not been treated with an antidepressant, and normal controls were evaluated using Thai language version of the Mini-International Neuropsychiatric Interview (Thai MINI) to estimate the phenotype of participants, who were identified as normal persons with no sign or symptom of depression.

The ethical approval request form, including Thai version protocol, patient information sheet and informed consent form had been approved by The Ethical Review

Committee for Research in Human Subjects (Mental Health and Psychiatry), Ministry of Public Health, Thailand.

3.1.2 Psychiatric medications

In this study, selected drugs were chosen from the most popular drugs that were used to relieve psychiatric disorders in the clinical treatment in Thailand hospitals. For antidepressants, Prozac (fluoxetine), Zoloft (sertraline) and Effexor (venlafaxine), three representatives of the selective serotonin reuptake inhibitors (SSRIs), fluoxetine and sertraline, and serotonin–norepinephrine reuptake inhibitors (SNRIs), venlafaxine, antidepressant classes, were used in this experiment. On the other hand, three representatives of the other types of psychiatric drugs that were used in this experiment are Diazepam (antianxiety drug), risperidone and haloperidol (antipsychotic drugs). The final concentration of each psychiatric drugs used in this study are based on the reference range of the blood or plasma drug concentrations in persons receiving the drug therapeutically affects that have been reported: 200 ng/ml for fluoxetine hydrochloride and venlafaxine hydrochloride and 100 ng/ml for sertraline hydrochloride (Mauri *et al*., 2002; Baumann *et al*., 2004; Wille *et al*., 2008). 0.5 mcg/ml for diazepam, 50 ng/ml for risperidone, and 20 ng/ml for haloperidol (Reidenberg *et al*., 1978; Moulin *et al*., 1982; Ereshefsky *et al*., 1984). Moreover, ten μM forskolin was used as a stress reagent, and 100 nM dexamethasone was used as a positive control to suppress *CRF* expression

(Kageyama *et al.,* 2008). All reagents were purchased from Sigma-Aldrich Pte Ltd, Singapore.

3.1.3 Hypothalamus cell lines

The embryonic mouse hypothalamus cell line N6 (mHypoE-N6), which immortalized from mouse embryonic hypothalamic primary cultures by retroviral transfer of SV40 T-Ag, was purchased from CELLutions Biosystems Inc. (Cedarlane laboratories, Canada).

3.2 Methods

3.2.1 Methylation and expression studies

3.2.1.1 Sample collection and preparation

Peripheral blood samples were obtained from all 62 participants in the morning (average time 10.13±0.37 a.m.) and kept in EDTA tube (purple top) containing EDTA potassium salt additive as an anticoagulant. All blood samples were immediately centrifuged at 1200 rpm for 10 minutes to separate plasma serum, Buffy coat and red blood cells. Cortisol analysis was performed in plasma serum. Genomic DNA and total RNA were extracted from Buffy coat using the illustra™ blood genomic Prep Mini Spin Kit (GE Healthcare, UK) and the QIAamp® RNA Blood Mini Kit (Qiagen, Germany), respectively according to the manufacturer's instructions. The extracted genomic DNA

was then stored in a -20°C freezer, and the extracted total RNA was then long-term stored in a -80°C freezer until used.

3.2.1.2 Bisulfite pyrosequencing DNA methylation analysis

Genomic DNA was sent to EpigenDx Inc. (www.epigendx.com) to analyze the DNA methylation status according to standard procedures. The human long interspersed nuclear element-1 (LINE-1) was used to estimate the global DNA methylation or whole-genome methylation status as previous described (Yang *et al.*, 2004). It was mostly used as internal control to study methylation status in human. In parallel, the genomic DNA of the same subjects was used to analyze the methylation status in the exon 1_F promoter region of *NR3C1* gene.

Bisulfite pyrosequencing procedure consists of two main approaches that are sodium bisulfite treatment and pyrosequencing. Firstly, sodium bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen, USA) was used to convert the unmethylated cytosines (C) in genomic DNA according to the manufacturer's instructions. Unmethylated cytosines (C) in DNA sequences were converted to uracils (U) and then changed to thymines (T) after PCR performing. The bisulfite converted DNA was stored at -20°C until used. Subsequently, pyrosequencing method was used to analyze DNA methylation status using the PyroMark LINE-1 kit (Biotage-Qiagen, USA) according to the manufacturer's instructions. The universal forward and reverse PCR primers for

amplification of a region in the LINE-1 gene were contained in the PyroMark kit. The PCR primers for the exon 1_F promoter region of *NR3C1* gene, as well as pyrosequencing primers were amplified using the following Oberlander *et al*. (2008)'s primers: sense, 5'-GAG TGG GTT TGG AGT-3' and antisense, 5'-AGA AAA GAA ATT GGA GAA ATT-3'. The amplification conditions consisted of an initial denaturation (95°C for 15 minutes), followed by 45 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 30 seconds), extension (72°C for 30 seconds), and a final extension of 10 minutes at 72°C. After amplification, 20 µl of PCR product was used to analyze pyrosequencing in the next step.

The PCR products were purified and sequenced using a PyroMark MD System (Biotage-Qiagen, USA) to analyze the DNA methylation status. In-house methylation samples, which exhibited low and high methylation status, were used as controls of the methylation analysis. The methylation percentage at each CpG sites of both global and *NR3C1* gene-specific methylation analyses were used the PyroMark Q MD software (Biotage-Qiagen, USA).

A portion of the exon 1_F promoter region of *NR3C1* gene containing 7 CpG sites (Figure 3.1) was analyzed with CpGs 5 and 6 representing the NGFI-A binding site that regulates gene transcription. All 7 CpG sites correspond to CpGs 33-39, which was described by McGowan *et al*. (2009) and are located upstream to the CpGs 1-5 reported by Oberlander *et al*. (2008). The average methylation percentage of all CpG sites was calculated and used to analyze the methylation status of each sample.

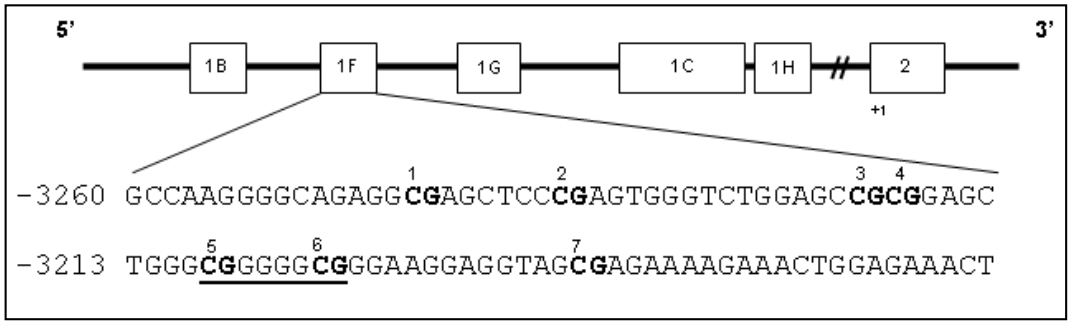

Figure 3.1 The schematic representation of the human *NR3C1* gene exon 1_F promoter region methylation from nucleotide -3260 to -3167 (the numbering is relative to the translational start site considered as +1). The diagram is based on the previously characterized 5'end of the human *NR3C1* gene promoter region (Oberlander *et al.*, 2008; McGowan *et al.*, 2009), which contains multiple first exons. The 7 CpG dinucleotides in exon 1_F analyzed by bisulfite pyrosequencing are in bold fonts. The NGFI-A transcription factor binding site including the CpG5 and CpG6 is underlined.

3.2.1.3 Quantitative RT-PCR analysis of NR3C1 expression

Extracted total RNA was reverse transcribed using the iScript™ Reverse Transcription Supermix (Bio-Rad, USA) according to the manufacturer's protocol. The RT reaction master mix contained 5X iScript™ Reverse Transcription Supermix, 1 µg – 1 pg total RNA, and nuclease-free water. The RT condition consisted of priming (25°C for 5 minutes), reverse transcription (42°C for 30 minutes), and RT inactivation (85°C for 5 minutes). Real-time PCR was performed to quantitate the different level of mRNA expression between MDD patients (N = 29) and control subjects (N = 33). Real-time PCR was carried out in triplicate using the 2X SYBR Green PCR Mastermix (QuantiTect® SYBR®

Green PCR Kit, Qiagen, Germany) following the manufacturer's instructions. *NR3C1* primers were the following sequences: sense, 5'-CTC CTC AAC AGC AAC AAC AG-3' and antisense, 5'-CAA TCA TTC CTT CCA GCA CAT AG-3'. Glyceraldehyde-3-phosephate dehydrogenase (*GAPDH*) was used as an internal control and was amplified using primers described by McGowan *et al*. (2009): sense, 5'-GAA GGT GAA GGT CGG AGT C-3' and antisense, 5'-GAA GAT GGT GAT GGG ATT TC-3'. A commercial primer set was used as a positive control to evaluate the total amount of human *NR3C1* gene expression (GR_{total}) (McGowan *et al*., 2009) using RT^2 qPCR primer assay (product ID. QIAPPH02652A, Qiagen). A PCR reaction master mix contained 2X SYBR Green PCR Mastermix, 10 μM of the sense and antisense primers, and the cDNA. Subsequently, the qPCR condition consisted of an initial activation cycle step at 95°C (15 minutes), followed by 35 cycles of denaturation step at 95°C (30 seconds), annealing step at 58°C (30 seconds), and elongation step at 72°C (30 seconds). Each fluorescence detecting was obtained at the end of each elongation cycle. Then, PCR product specificity was confirmed by a single melting curve following program: 75 °C up to 90°C with a temperature transition rate of 0.2°C/second.

The relative gene expression of *NR3C1* was established from the Ct values. To calculate the relative changes in *NR3C1* gene expression data used the $2^{-\Delta\Delta Ct}$ method that modified from Pfaffl (2001)'s model for quantification in real-time PCR: $\Delta\Delta Ct = \Delta Ct(case) - \Delta Ct(control)$, when $\Delta Ct(case)$ is the difference between

Ct(case$_{NR3C1}$) and Ct(case$_{GAPDH}$), ΔCt(control) is the difference between Ct(control$_{NR3C1}$) and Ct(control $_{GAPDH}$).

3.2.1.4 Cortisol level analysis

A cortisol test was done to measure the level of the hormone cortisol in the blood serum, which was taken in the morning (average time 10.13±0.37 a.m.). Plasma serum, which was immediately separated from blood sample centrifugation at 1200 rpm for 10 minutes, was analysed cortisol level to examine the functional implications which correlated to *NR3C1* methylation status and expression between case (MDD) and control. All serum samples were sent to the PCT Laboratory Services Co., Ltd (Thailand) which provides assay cortisol level analyses service using the electrochemiluminescence immunoassay (ECLIA) method. The ECLIA used microplate luminometers to quantitatively measure antigen in serum samples, and luminometer to detect the light. Briefly, a biotinylated monoclonal cortisol-specific antibody and a monoclonal cortisol-specific antibody were labeled with a ruthenium complex and incubated. After incubated, the sandwich complex was formed with cortisol carrying a biotinylated and a ruthenylated antibody. The streptavidin-coated microparticles were added to reaction complex mixture. The complex was bound to the solid phase via interaction of biotin and streptavidin. The microparticles were magnetically captured onto the surface of the electrode, whereas unbound substances were then removed.

Application of a voltage to the electrode was then induced chemiluminescent emission light which was measured by a photomultiplier (Roche Diagnostics International Ltd, 2013). The average cortisol level of cases and controls was calculated and used to analyse the difference of cortisol level between case and control.

3.2.1.5 Statistical analysis

DNA methylation statuses, relative gene expression values, and cortisol levels between MDD patients and controls were compared using the independent (unpaired) *t*-test. $P \leq 0.05$ was considered statistically significant.

3.2.2 *In vitro* study of antidepressants effect

3.2.2.1 Hypothalamus cell culture

The embryonic mouse hypothalamus cell lines were cultured in 1X Dulbecco's modified eagle's medium (DMEM) (Gibco BRL) containing 25 mM glucose and 1% penicillin/streptomycin supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and maintained at 37 ^{o}C with 5% CO_2. The cells were grown in monolayer culture, attached to the tissue culture flask for 3-5 days or 80% confluence. Cells were plated into 6-well tissue culture plate at 10^6 cells/well and incubated at 37 ^{o}C with 5% CO_2 overnight before testing each experiment.

3.2.2.2 Human CRF-promoter luciferase reporter construction

Human DNA was extracted from peripheral blood using the illustra™ blood genomic Prep Mini Spin Kit (GE Healthcare, UK) according to the manufacturer's instructions. Human *CRF* promoter constructs were prepared as described by Budziszewska *et al*. (2002) and Kageyama *et al*. (2008). A 787 bp restriction fragment containing the human *CRF* promoter (-663 to +124 relative to the proximal transcription start point) was obtained by PCR (Budziszewska *et al*., 2002). PCR primers are the following sequences: sense; 5' CGC GGT ACC GAG AGA CGT CTC CGG GGG C 3' underlined as a *Kpn*I cutting site, and antisense; 5' GCG AGA TCT GGC TCA TAA CTC CTT TAT GTG CTT GC 3' underlined as a *Bgl*II cutting site (Budziszewska *et al*., 2002). The PCR products were confirmed by 1% agarose gel electrophoresis in 1x TBE buffer as a running buffer and sequencing. Normally, the electrophoresis was operated at 100 voltages for 30 minutes. The melted gel was contained with 0.5 mg/ml ethidium bromide solution, and 100 bp DNA ladder marker was used as a standard DNA marker. The loading sample commonly composed of 5 µl of the PCR products and 2 µl of a loading dye. PCR bands were then visualised and photographed under UV light with a gel documentation (Syngene, UK).

This DNA fragment was used to generate the *CRF*-promoter-driven luciferase reporter construct, *CRF*-663luc or *CRF*/pGL4.15[*luc2P*/Hygro], by two-step cloning method (Kageyama *et al*., 2008). Briefly, the DNA fragment was cloned into

pGEM-T Easy vector (Promega, Madison, WI), then digested with *Kpn*I and *Bgl*II, and sub cloned into *Kpn*I and *Bgl*II cloning sites of the pGL4.15[*luc2P*/Hygro] expression vector (Promega, Madison, WI) (Figure 3.2).

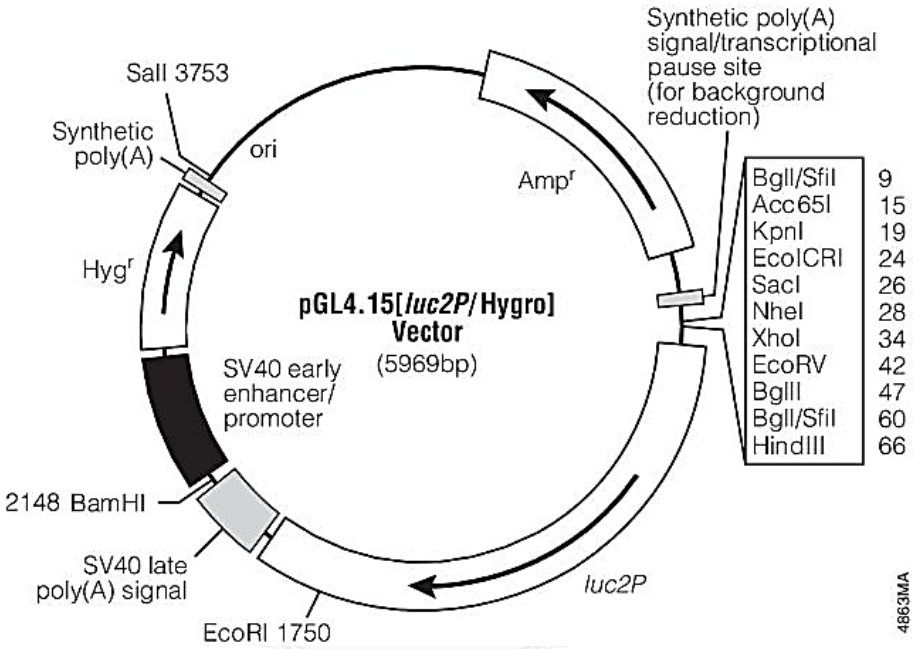

Figure 3.2 pGL4.15[*luc2P*/Hygro] expression vector circle map.

(source: https://embed.widencdn.net/img/promega/f1iovk56rb/640px/4863MA.jpeg?u=7fvzhm)

3.2.2.3 Transient transfection

Briefly, cells were plated at the density of 10^6 cells/well into the 6-well tissue culture plate for 1 day prior to transfection to achieve at 60-70% final confluence and grow in DMEM medium with 10% FBS. Transient transfections were performed in 3 replicates of 6 wells/plate for each treatment and each reaction was repeated minimum of 3 times (N=3). Transfections were achieved using the FuGENE HD transfection reagent (Promega, Madison, WI), according to the manufacturer's instructions. FuGENE HD was used 3 µl to 1 µg plasmid DNA in each well. For each well, 1 ml total volume of DNA- FuGENE HD complex that was diluted with Opti-MEM I serum-free medium (Gibco BRL) was added to the cells that removed growth medium. Incubate the cells at 37°C in a 5% CO_2 incubator for 24 hours post-transfection before treating the cells with antidepressants for transgene expression assay. Twenty-four hours following transfection, cells were washed with 1XPBS and incubated overnight in DMEM medium with 10% FBS before each treating experiment was then performed in the next day.

3.2.2.4 Drug treatments

3.2.2.4.1 Drug effect on *CRF* gene expression

Two experiments were performed to analyze the effects of psychiatric drugs on endogenous *CRF* gene expression: reducing stress and preventing the cells from stress. To analyze the effects of the drugs on stress reduction, mHypoE-N6 untransfected cells were grown in 6-well/plate (60-70% confluence), incubated with DMEM containing 10 μM forskolin for 2 h (Kageyama *et al.,* 2008), then incubating the cells in each well for 30 min with DMEM containing 100 nM dexamethasone (Kageyama *et al.,* 2008), 200 ng/ml fluoxetine hydrochloride, 200 ng/ml venlafaxine hydrochloride, and 100 ng/ml sertraline hydrochloride, respectively. In another experiment, mHypoE-N6 cells in each well were pre-incubated for 30 min in DMEM supplemented with antagonist reagents, 100 nM dexamethasone (Kageyama *et al.,* 2008) and each drugs (200 ng/ml fluoxetine, 200 ng/ml venlafaxine, and 100 ng/ml sertraline), then the cells were incubated with DMEM containing stress reagent (10 μM forskolin for 2 h (Kageyama *et al.,* 2008)) for the experiment of preventing the cells from the stress.

On the other hand, the additional experiments of analyzing the effects of the other psychiatric drug types (Diazepam, risperidone and haloperidol) on *CRF* gene expression were also performed. Two sup-experiments of reducing stress and preventing the cells from stress were rearranged as well as antidepressants experiments. To analyze the effects of the drugs on stress reduction, untransfected cells were

incubated with DMEM containing 10 μM forskolin for 2 h (Kageyama *et al.,* 2008), and incubated with DMEM containing either 100 nM dexamethasone (Kageyama *et al.,* 2008), 0.5 mcg/mL diazepam, 50 ng/mL risperidone, or 20 ng/mL haloperidol for 30 min. To analyze the effects of the drugs on stress protection, cells were pre-incubated in DMEM supplemented with 100 nM dexamethasone (Kageyama *et al.,* 2008) and each drug (0.5 mcg/mL diazepam, 50 ng/mL risperidone, and 20 ng/mL haloperidol) for 30 min, then subsequently incubated with DMEM containing 10 μM forskolin for 2 h (Kageyama *et al.,* 2008). All experiments were carried out in triplicate.

3.2.2.4.2 Antidepressants effect on *CRF* promoter activity

In this assay, we also separated two sup-experiments to test the antidepressants efficacy on *CRF* promoter activity in transfected hypothalamus cell lines for both reducing the stress in the cells and preventing the cells from the stress. Following the stress reducing experiment, post-transfected cells with the pGL4.15[*luc2P*/Hygro] plasmid in each well of the 6-well/plate were incubated for 2 h in DMEM with added 10 μM forskolin stress reagent (Kageyama *et al.,* 2008), then the cells were treated for 30 min with DMEM containing 100 nM dexamethasone (Kageyama *et al.,* 2008), 200 ng/ml fluoxetine, 200 ng/ml venlafaxine, and 100 ng/ml sertraline, respectively. The second experiment of preventing the cells from the stress, post-transfected cells in each well were pre-incubated for 30 min in DMEM with 100 nM dexamethasone

(Kageyama *et al.,* 2008), 200 ng/ml fluoxetine, 200 ng/ml venlafaxine, and 100 ng/ml sertraline, respectively. Then, the cells in each well were incubated with DEME containing 10 μM forskolin stress reagent for 2 h (Kageyama *et al.,* 2008). Untransfected cells were used as control. All experiments were carried out in triplicate.

3.2.2.5 Quantitative RT-PCR analysis

Total RNA from the post-harvested treated hypothalamus cell lines in all experiments were extracted using the RNeasy plus Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted cellular RNA was reverse transcribed using the Sensiscript Reverse Transcription Kit (Qiagen). Real-time PCR was performed to quantitate the different level of *CRF* mRNA expression in untransfected cells, and luciferase mRNA level on *CRF* promoter activity in transfected cells. Real-time PCR was carried out in triplicate using the SsoFast™ EvaGreen® supermix (Bio-Rad) following the manufacturer's instructions. For *CRF* gene expression, mouse *CRF* primers are the following sequences: sense, 5'-CAG GAA ACT GAT GGA GAT TAT CG-3' and antisense, 5'-AGA AAT TAA GCA TGG GCA ATA CA-3'. Mouse *GAPDH* was used as an internal control and was amplified using primer sequences: sense, 5'-AAC TTT GGC ATT GTG GAA GG-3' and antisense, 5'-ACA CAT TGG GGG TAG GAA CA-3'. For *CRF* promoter activity, luciferase primers are the following sequences: sense, 5'-GCT CAG CAA GGA GGT AGG TG-3' and antisense, 5'-TCT TAC CGG TGT CCA AGT CC-3'. In addition, the

normalized internal control was also used mouse *GAPDH* primers. The qPCR condition consisted of an initial activation cycle step at 95°C (20 sec), followed by 40 cycles of denaturation step at 95°C (3 sec), annealing step at 58°C (20 sec), and elongation step at 72°C (20 sec). The relative gene expression of both *CRF* and luciferase was calculated using the $2^{-\Delta\Delta_{CT}}$ method (Pfaffl, 2001).

3.2.2.6 Statistical analysis

Relative gene expression values between groups of treated cells with forskolin-induced *CRF* expression and *CRF* promoter activity were compared with each antagonistic antidepressant treatment using the independent (unpaired) *t*-test. $P \leq 0.05$ was considered statistically significant.

CHAPTER IV
RESULTS

4.1 Methylation and expression studies

4.1.1 Methylation status of *NR3C1* in Thai MDD patients

Methylation statuses at each of the 7 CpG sites in the *NR3C1* gene exon 1_F promoter region at a predicted NGFI-A binding site in blood samples of Thai MDD patients and normal controls are shown in Figure 4.1 and 4.2. The global DNA methylation was represented by the LINE-1 methylation status at four sites. The percentages of LINE-1 methylation status of MDD patients that were comparable with normal controls showed no significant difference ($p > 0.05$; Table 4.1 and Figure 4.1). Whereas, the results of methylation status at each the 7 CpG dinucleotides between MDD patients and normal controls showed significant difference at the CpG7 ($p < 0.05$) (Figure 4.2A). The average of methylation status percentages at 7 CpG sites within a predicted NGFI-A binding site of the exon 1_F *NR3C1* promoter region shown in Table 4.2. Interestingly, when the samples were separated into male and female groups to quantify the level of methylation in each group, the results also showed significant difference between female MDD patients and normal females at the CpG7 ($p < 0.05$; Figure 4.2B). However, there was no significant difference of methylation level between male groups (Figure 4.2C).

Table 4.1 The average of LINE-1 methylation status percentages at four sites.

	CpG1	CpG2	CpG3	CpG4
MDD (mean ± SEM)	82.36	81.54	75.94	73.79
Normal (mean ± SEM)	82.23	81.49	75.95	73.40
p-value	0.81	0.86	0.97	0.47

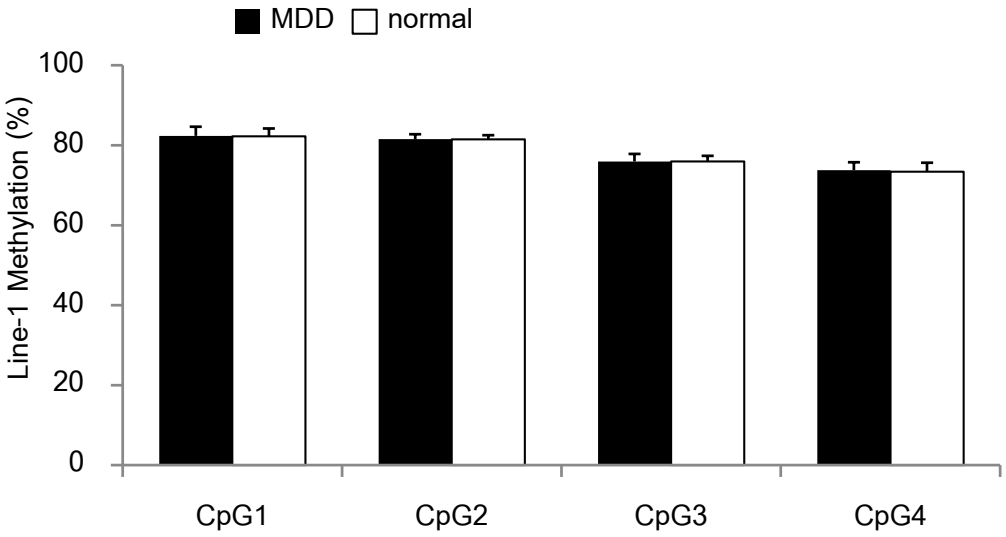

Figure 4.1 The percentages of LINE-1 methylation status (mean ± SEM) at 4 CpG sites in Thai MDD patients (n=29) and normal controls (n=33).

Table 4.2 The average of methylation status percentages at 7 CpG sites within a predicted NGFI-A binding site of the exon 1_F *NR3C1* promoter region.

	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
Overall							
MDD	0.47	0.33	0.00	0.25	0.41	0.00	1.42
normal	0.58	0.57	0.19	0.26	0.63	0.00	0.85
p-value	0.59	0.43	0.22	0.98	0.37	0.00	**0.05***
Female							
MDD	0.54	0.29	0.00	0.18	0.41	0.00	1.18
normal	0.32	0.25	0.05	0.17	0.40	0.00	0.73
p-value	0.22	0.77	0.17	0.93	0.94	0.00	**0.05***
Male							
MDD	0.28	0.43	0.00	0.43	0.42	0.00	2.02
normal	1.57	1.75	0.71	0.57	1.47	0.00	1.29
p-value	0.12	0.33	0.36	0.77	0.27	0.00	0.43

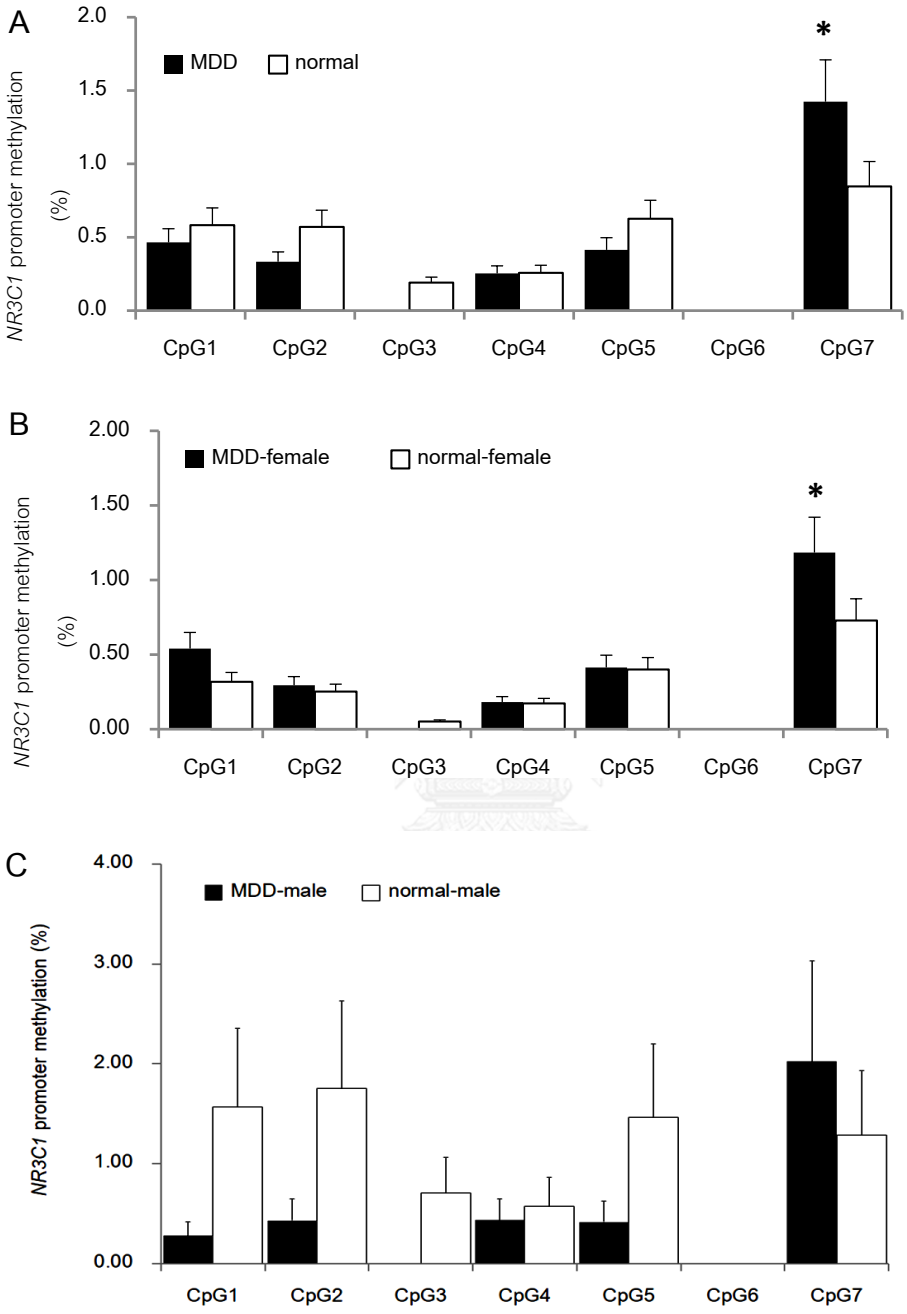

Figure 4.2 The percentages of methylation status (mean ± SEM) of 7 CpG sites within a predicted NGFI-A binding site of the exon 1_F *NR3C1* promoter region in Thai MDD patients, (A) overall samples (n=29) and normal controls (n=33), (B) female group sample; MDD (n=20) and normal (n=26), (C) male group sample; MDD (n=9) and normal (n=7). * indicates $p < 0.05$.

4.1.2 Expression of *NR3C1* in Thai MDD patients

The expression levels of *NR3C1* were analyzed by real-time PCR in blood cDNA. The results showed no significant difference in expression levels between samples from MDD patients and normal subjects ($p > 0.05$; Figure 4.3. No significant difference was observed when the samples were separated by gender ($p > 0.05$; Figure 4.3).

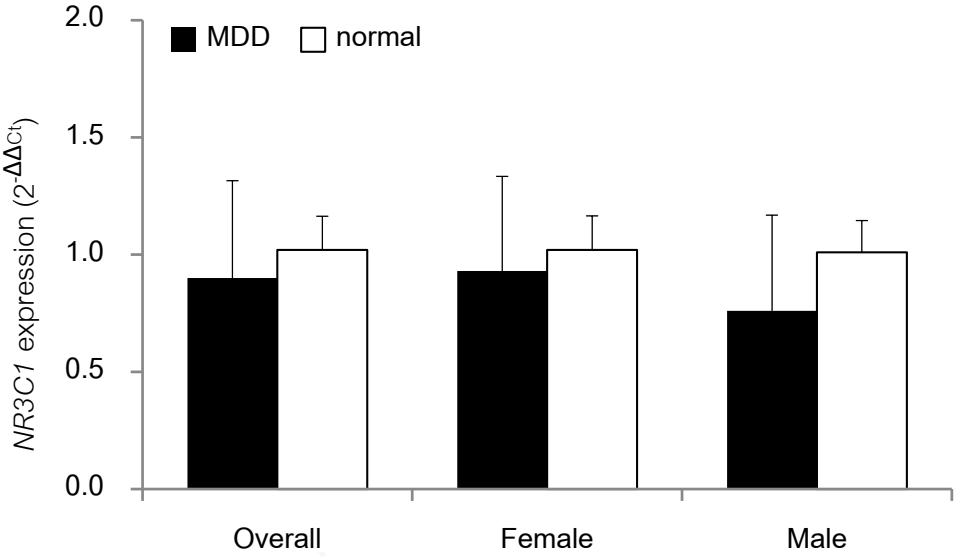

Figure 4.3 *NR3C1* mRNA expression ratios between MDD patients and normal controls. The overall samples contain 33 MDD patients and 12 control subjects. Female samples contain 23 MDD patients and 10 controls. Male samples contain 10 MDD patients and 2 controls.

4.1.3 Cortisol levels in Thai MDD patients

The cortisol level in the blood plasma of MDD patients and control subjects were analyzed to examine the functional implications of the *NR3C1* hypermethylation status. The results showed no significant difference in the cortisol level between the MDD patients and normal subjects ($p > 0.05$; Figure 4.4). The cortisol concentrations of all samples showed rather low levels. The highest, 14.38 µg/dL, and lowest, 4.42 µg/dL, cortisol levels were observed in a depressed female and a normal female, respectively. (Reference normal range: 8.7-22.4 µg/dL; Peeters *et al*., 2003).

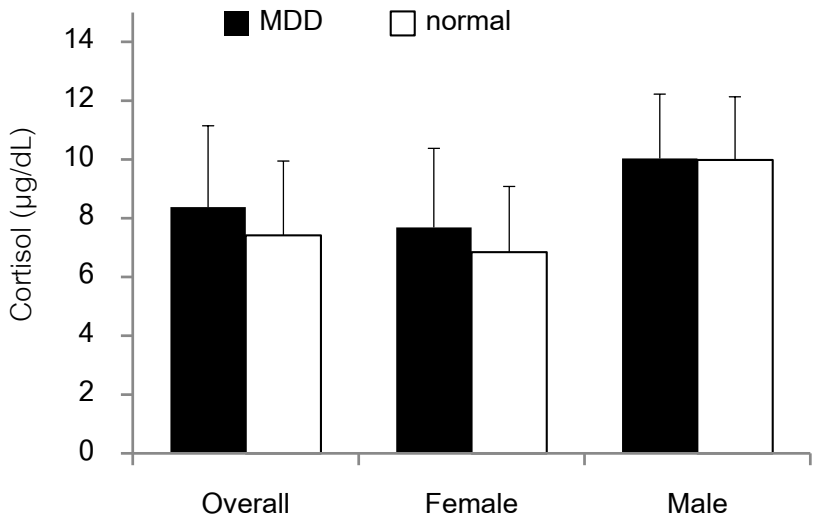

Figure 4.4 Cortisol level in blood plasma between Thai MDD patients (n=18) and normal controls (n=12). The overall samples containing n=18 MDD patients and n=12 control subjects, female group-sample was separated from overall samples containing n=12 MDD patients and n=10 controls, and male group-sample containing n=6 MDD and n=2 controls.

4.2 *In vitro* study of antidepressants effect

4.2.1 Time-dependent effects analysis

Time-dependent effects of forskolin, the stress-inducing reagent, on *CRF* gene expression levels showed that the effect of forskolin can induce *CRF* expression since 30 min compared with cells that were treated with medium alone. The incubation time of the highest *CRF* expression after incubated with forskolin was 2 h (Figure 4.5). Therefore, 2 h was the time-dependent effects of forskolin that was used in this study. On the other hand, time-dependent effects of dexamethasone and antidepressants on *CRF* inhibition were also tested (Figures 4.6 and 4.7, respectively). The results showed that dexamethasone, which was used as positive control, can suppress *CRF* gene expression at the RNA level since 30 min until 4 h (Figure 4.6). In addition, the effect of all 3 representative antidepressants (fluoxetine, venlafaxine and sertraline) also showed *CRF* mRNA level suppressing since 30 min until 4 h (Figures 4.7A – 4.7C). Therefore, 30 min was the incubation time that was used as the time-dependent effects of dexamethasone and all drugs on *CRF* gene expression study.

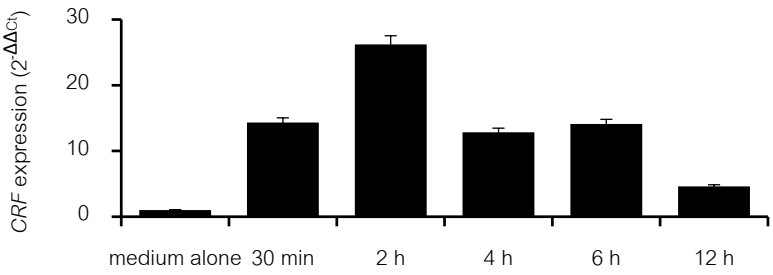

Figure 4.5 Time-dependent effects of forskolin on *CRF* gene expression in hypothalamus cell lines. Cells were treated with DMEM medium containing 10 μM forskolin for 30 min, 2 h, 4 h, 6 h and 12 h, respectively. Expression of *CRF* mRNA was indicated by qPCR.

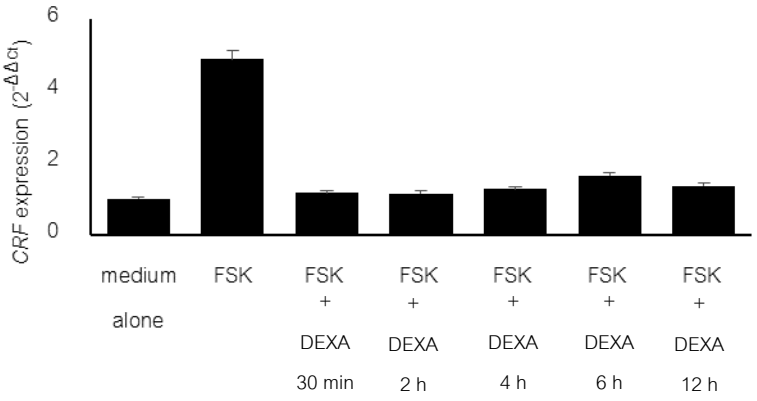

Figure 4.6 Time-dependent effects of dexamethasone on *CRF* gene expression in hypothalamus cell lines. Cells were treated with DMEM medium containing 10 μM forskolin (FSK) for 2 h prior to treat with 100 nM dexamethasone (DEXA). The incubation time in each condition was vary from 30 min, 2 h, 4 h, 6 h and 12 h, respectively. Expression of *CRF* mRNA was indicated by qPCR.

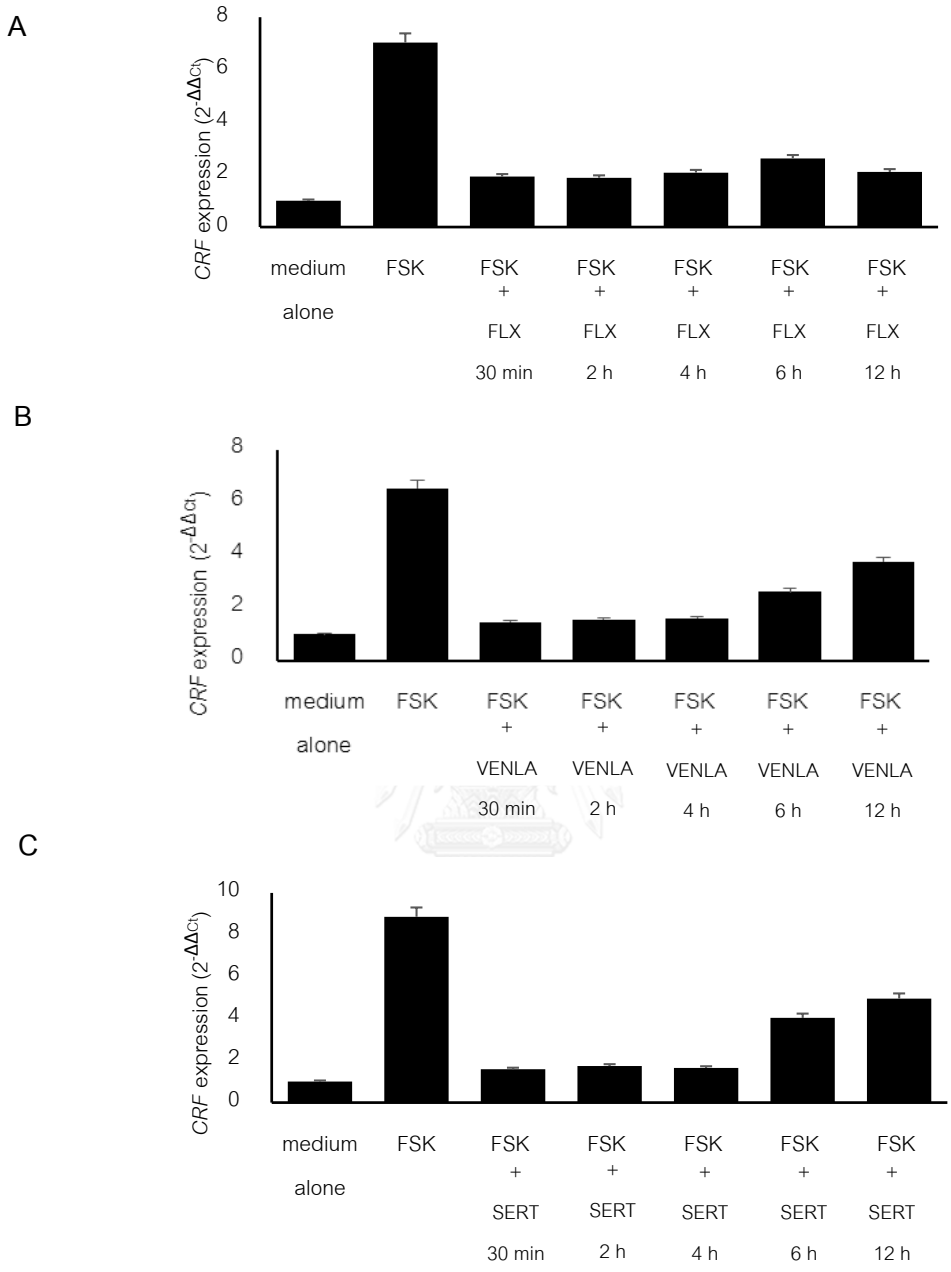

Figure 4.7 Time-dependent effects of three representative antidepressants on *CRF* gene expression in hypothalamus cell lines. Cells were treated with DMEM medium containing 10 μM forskolin (FSK) for 2 h prior to treat with 200 ng/ml fluoxetine (FLX) (A), 200 ng/ml venlafaxine (VENLA) (B), and 100 ng/ml sertraline (SERT) (C). The incubation time in each condition was vary from 30 min, 2 h, 4 h, 6 h and 12 h, respectively. Expression of *CRF* mRNA was indicated by qPCR.

4.2.2 Drug effect on *CRF* gene expression

The effects of each selected antidepressants on *CRF* gene expression in untransfected hypothalamus cell lines were shown in Figure 4.8. Cells treated with medium alone and medium with forskolin were used as controls. Dexamethasone was used as positive control. The results showed that all of antidepressants tend to suppress *CRF* gene expression at the RNA level under stress both before and after the cells were incubated with forskolin, the stress reagent (Figures 4.8A and 4.8B, respectively). When cells were treated with the antidepressants prior to forskolin, fluoxetine (a SSRI) significantly reduced *CRF* mRNA levels ($p < 0.01$) (Figure 4.8A). Interestingly, venlafaxine can significant decrease the stress in the cells by suppressing forskolin-induced *CRF* mRNA level compared with the cells that were treated with forskolin alone ($p < 0.05$), whereas no significantly suppress by fluoxetine and sertraline (Figure 4.8B). In addition, fluoxetine can strong significant protect the cells from stress compared with forskolin alone ($p < 0.01$) by reducing *CRF* mRNA expression that was induced by forskolin (Figure 4.8A). Although sertraline and venlafaxine can also protect the cells from stress, their efficacy were not significantly suppressed forskolin-induced *CRF* expression compared forskolin alone that showed in Figure 4.8A.

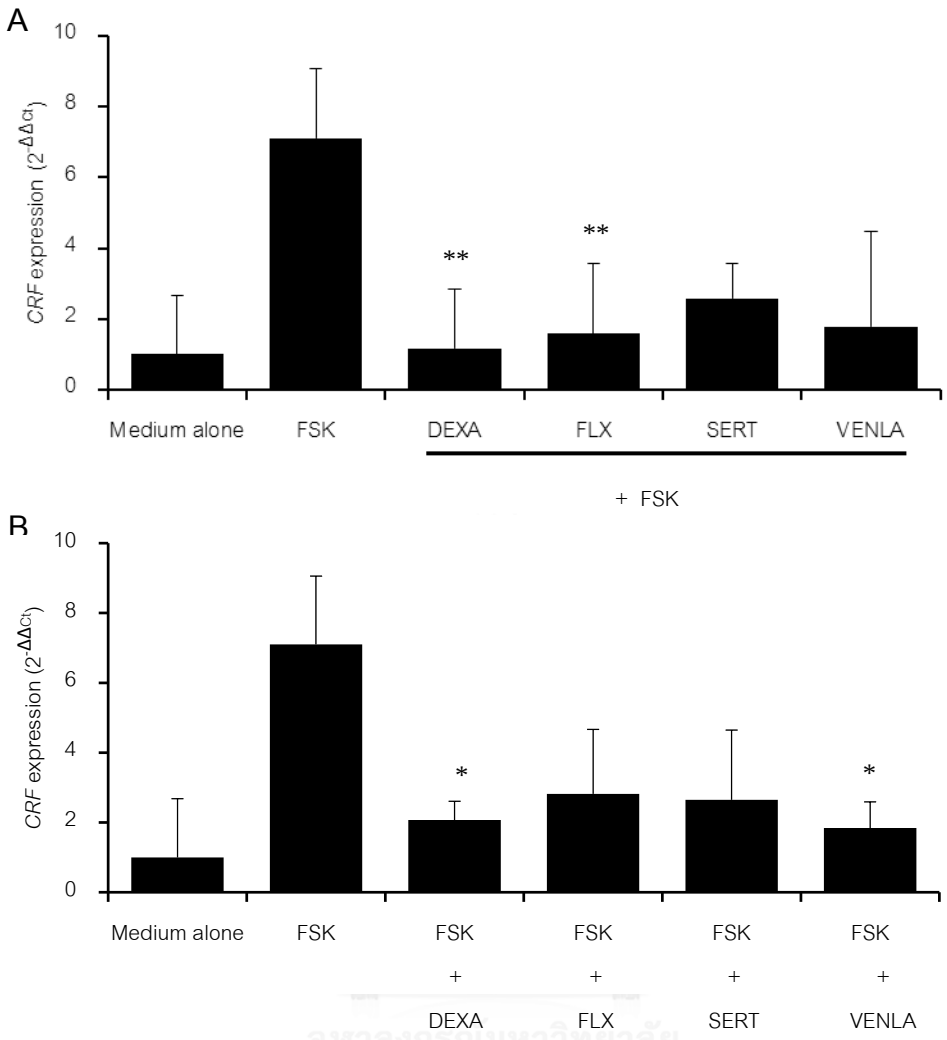

Figure 4.8 Effect of dexamethasone (DEXA) and three antidepressants, fluoxetine (FLX), sertraline (SERT), and venlafaxine (VENLA), on forskolin (FSK)-induced corticotrophin-releasing factor (*CRF*) gene expression in hypothalamus cell lines. (A) DEXA and antidepressants affected to protect the cells from stress. Expression of *CRF* mRNA was also indicated by qPCR. $^{**}p<0.01$ (compared with the cells were treated with FSK alone condition). (B) DEXA and antidepressants affected to reduce the stress from the cells. Expression of *CRF* mRNA was analyzed by quantitative real time PCR. $^{*}p<0.05$ (compared with the cells were treated with FSK alone condition).

On the other hand, the additional studies of the effects of other psychiatric drug types (Diazepam, risperidone and haloperidol) on *CRF* gene expression in hypothalamus cell lines were shown in Figures 4.9A and 4.9B. Cells were treated with medium alone and medium with forskolin were used as controls. Dexamethasone was used as a positive control. Results showed that all of psychiatric drugs tend to suppress *CRF* gene expression at the RNA level under stress both before and after the cells were incubated with forskolin, the stress reagent (Figures 4.9A and 4.9B, respectively). When cells were treated with psychiatric drugs prior to forskolin, only risperidone significantly reduced *CRF* mRNA levels ($p < 0.05$) (Figure 4.9A). Although diazepam and haloperidol tend to also protect the cells from stress, their efficacy was not significant. On the other hand, all of drugs significantly decreased the *CRF* expression in the cells compared with cells treated with forskolin alone, when cells were treated with drugs after forskolin ($p < 0.05$) (Figure 4.9A).

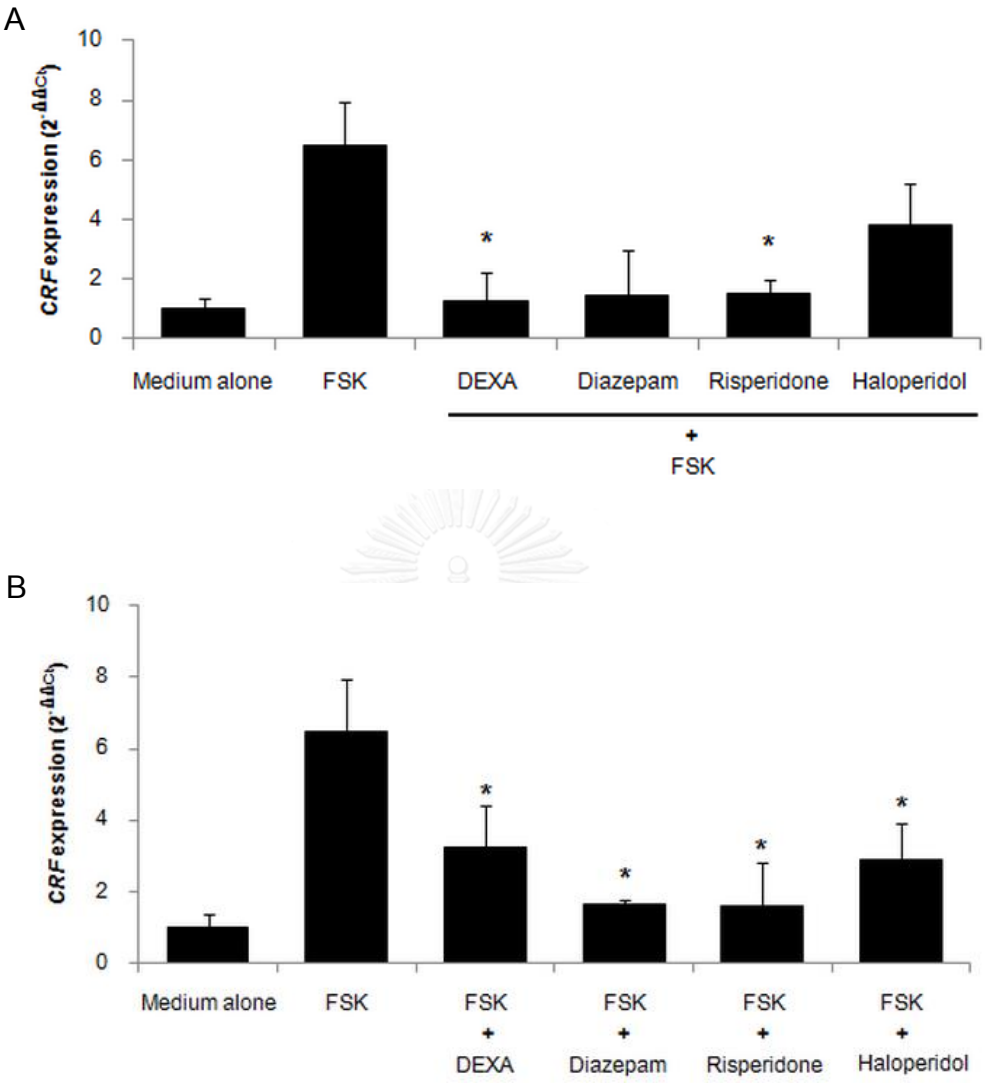

Figure 4.9 Effects of dexamethasone (DEXA) and three selected psychiatric drugs, diazepam, risperidone, and haloperidol, on *CRF* gene expression in hypothalamus cell lines before (A) and after (B) incubation with forskolin (FSK). The *CRF* expression levels were compared with *GAPDH*. *$p < 0.05$.

4.2.3 Drug effect on *CRF* promoter activity

4.2.3.1 Human *CRF* promoter amplification

The human CRF promoter was amplified by PCR on extracted human DNA. The sizes of the PCR products were estimated to be approximately 787 basepairs (bp) (-663 to +124 relative to the proximal transcription start point) (lanes 1-2 in Figure 4.10).

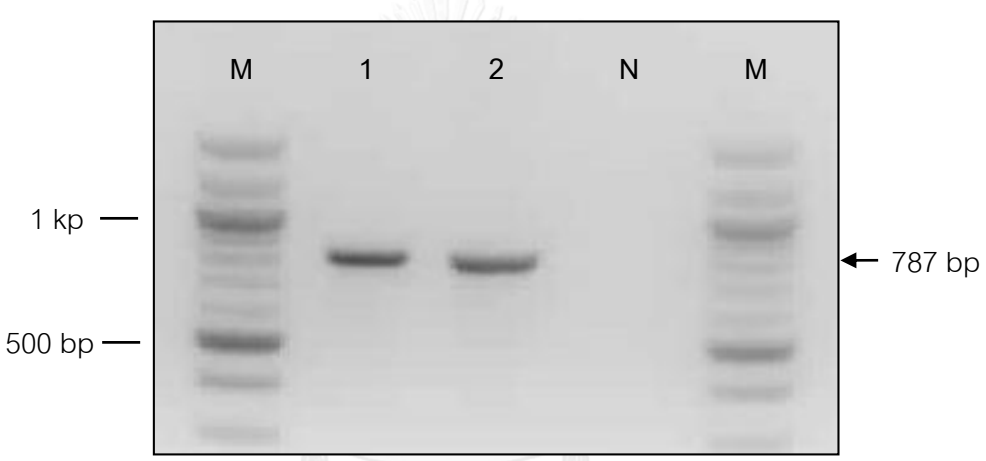

Figure 4.10 PCR product of human *CRF* promoter amplified with Budziszewska *et al*., (2002)'s primer-pairs was about 787 bp (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-2 = human *CRF* promoter fragments from DNA sample no.1 and no.2, respectively, N = negative control).

4.2.3.2 Human *CRF*-promoter luciferase reporter constructs

The human *CRF*-promoter fragment was used to generate the *CRF*-promoter-driven *luciferase* reporter construct by two-step cloning method. Firstly, the human *CRF*-promoter fragment into pGEM-T Easy vector was ligated by T4 DNA ligase. The product size of the recombinant plasmid (*CRF*/pGEM-T Easy) was estimated to be approximately 3802 bp (lanes 1-4 in Figure 4.11).

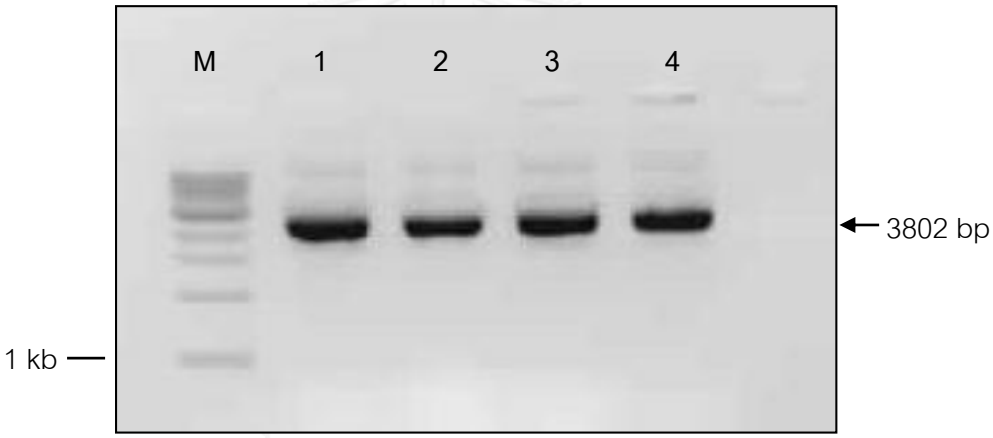

Figure 4.11 Product size of the recombinant plasmid (*CRF*/pGEM-T Easy) extraction was about 3802 bp (787+3015) (Lane M = 1 kb DNA marker, no. 1-4 = the *CRF*/pGEM-T Easy recombinant plasmid extracted from colony no.1–4, respectively).

To confirm that the recombinant plasmid (*CRF*/pGEM-T Easy) was ligated with the human *CRF*-promoter fragment, PCR amplification using the *CRF*/pGEM-T Easy recombinant plasmid as a template was performed. The product size of the PCR products was consistent with that of *CRF*-promoter fragment as about 787 bp shown in lanes 1-3 in Figure 4.12.

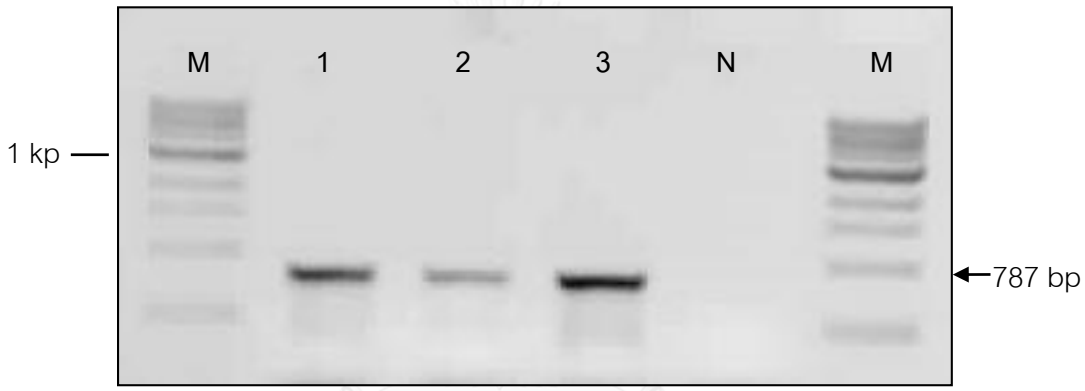

Figure 4.12 Product size of the *CRF*-promoter insertion fragment into the recombinant plasmid (*CRF*/pGEM-T Easy) was about 787 bp (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-3 = the *CRF*-promoter insertion fragment amplified from *CRF*/pGEM-T Easy recombinant plasmid no.1 – 3, respectively, N = negative control).

Next, the human *CRF*-promoter recombinant plasmid (*CRF*/pGEM-T Easy) was digested with *Kpn*I and *Bgl*II restriction enzymes. The results of digested products are shown in Figure 4.13 (lanes 1-2). The human *CRF*-promoter digested fragment was about 787 bp, as expected.

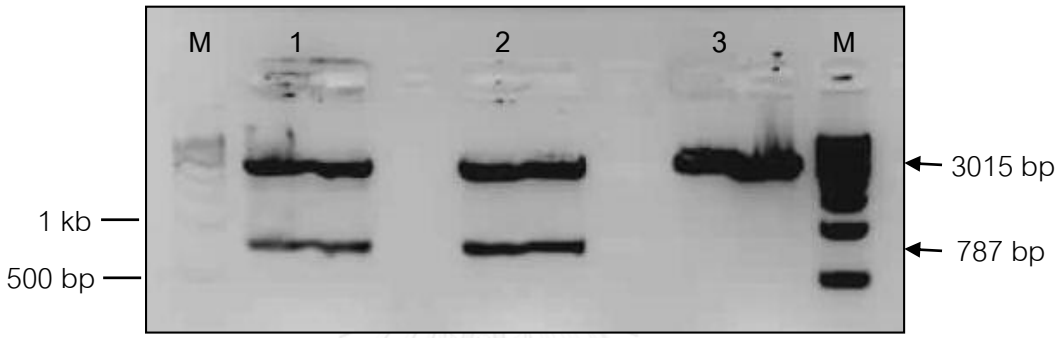

Figure 4.13 The product of human *CRF*-promoter fragment digested from the recombinant plasmid (*CRF*/pGEM-T Easy) using *Kpn*I and *Bgl*II restriction enzymes. The human *CRF*-promoter fragment was the lower band as about 787 bp (Lane M = 1.5 kb + 100 bp DNA marker, no. 1-2 = the lower band was the *CRF*-promoter fragment that was successfully digested).

After that, the human *CRF*-promoter fragment was cut out and extracted from the gel. The purified human *CRF*-promoter fragment was sub-cloned into *Kpn*I and *Bgl*II cloning sites of the pGL4.15[*luc2P*/Hygro] expression vector. In addition, PCR was performed to confirm the recombinant plasmid construct (*CRF*/pGL4.15[*luc2P*/Hygro]) that was ligated with the human *CRF*-promoter fragment using the recombinant plasmid as PCR template. Next, the human *CRF*-promoter *luciferase* reporter construct (*CRF*/pGL4.15[*luc2P*/Hygro]) was transfected into the mHypoE-N6 hypothalamus cell using FuGENE HD transfection reagent.

4.2.3.3 Effect of antidepressants on *CRF* promoter activity

The effects of each selected antidepressants on *CRF* promoter activity in transfected hypothalamus cell lines with plasmid containing *luciferase* gene under the control of the human *CRF* gene promoter (pGL4.15[*luc2P*/Hygro]) were shown in Figure 4.14. Although all three antidepressants seemed to be able to suppress *luciferase* mRNA expression induced by forskolin both in reducing the stress from the cells (Figure 4.14A) and in protecting the cells from the stress (Figure 4.14B), no significant differences in *luciferase* expression of any drug was found.

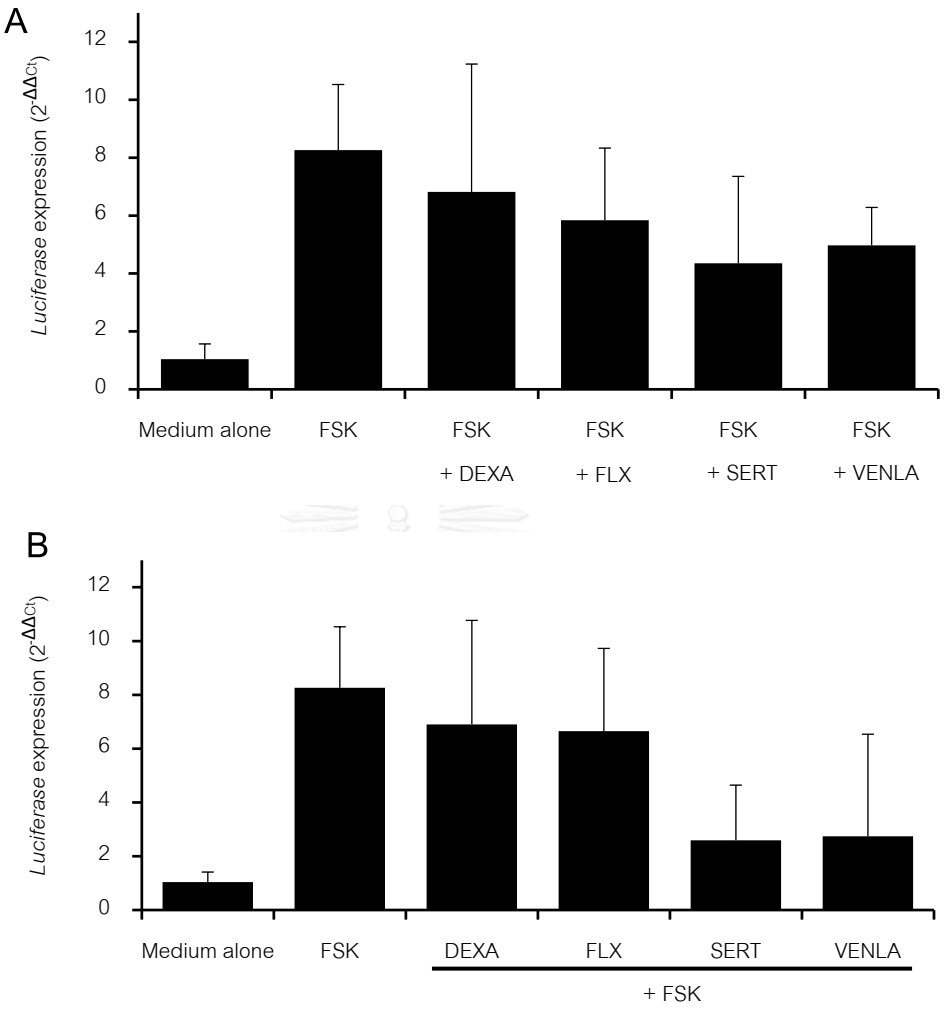

Figure 4.14 Effect of dexamethasone (DEXA) and three antidepressants, fluoxetine (FLX), sertraline (SERT), and venlafaxine (VENLA), on forskolin (FSK)-induced corticotrophin-releasing factor (*CRF*) promoter activity in hypothalamus cell lines. Dexamethasone and antidepressants affected to reduce the stress from the cells (A) and to protect the cells from stress (B). Expression of *luciferase* mRNA level was analyzed by quantitative real time PCR.

CHAPTER V
DISCUSSIONS

5.1 Methylation and expression studies

Methylation at a potential NGFI-A transcription factor binding site of *NR3C1* gene promoter has been correlated with controlling the HPA negative-feedback function in response to stress. According to our bisulfate-pyrosequencing results in blood samples, from 7 CpG dinucleotides in the exon 1_F of *NR3C1* promoter region, the significant difference ($p < 0.05$) in mean methylation status between MDD patients and normal controls was observed at the CpG7 (Figure 4.2A). The results support the previous findings of the association between DNA methylation and stress-related disorders reported by Oberlander *et al.* (2008) and McGowan *et al*. (2009). Interestingly, our methylation results in human blood samples also show significantly higher methylation level at CpG7 in female MDD patients compared with female normal subjects ($p < 0.05$; Figure 4.2B). Our results of *NR3C1* hypermethylation, therefore, suggested that the hypermethylation status at this specific site may positively relate to depression disorder in Thai MDD patients, especially in female patients. Similarly, a recent study found that an epigenetic risk factor of the *ADCYAP1R1* gene, which was associated with an estrogen stress response element, was more impacted on the later development of posttraumatic stress disorder (PTSD) in women than men (Ressler *et al*., 2011). There are many reports

from previous revealed the regulation of circulating hormones, especially estrogen, that mainly affected the rate of depression increasing in women (Formanek and Gurian, 1987; Ressler *et al*., 2011). Lack of estrogen hormone, such as menopausal symptoms in women, indicated the rates of MDD in women increased from 39% to 55% (Formanek and Gurian, 1987). Therefore, the possibility of the higher rates of depression in women can be occurred at menopausal stages because women feel they become elderly and infertile, which effected from estrogen hormone changing. Furthermore, the other hormonal regulations in women life-time many more extremely changes than men, including menstruation, contraceptive drugs, pregnancy, childbirth, postpartum period and menopause (Formanek and Gurian, 1987; Denmark and Paludi, 1993). Although there are many reasonable possibilities given to explain the higher rates of depression symptoms in women, a few studies about the influence of DNA methylation to develop depression disorder. Moreover, it remained unclearly evident as to why women were more susceptible to depression than men. However, these our hypermethylation findings in *NR3C1* gene promoter may confirm previous results that indicated the higher rate of stress-related mood disorders in women compared with men. It has furthermore been suggested that clinical investigations of PTSD and depression should focus on sex differences in the epigenetic regulation of HPA-axis activity in response to stress and adverse events (Raabe and Spengler, 2013).

In this finding, hypermethylation status at CpG7 in exon 1_F of *NR3C1* gene promoter region in Thai MDD patients has not yet reported and found in previous findings. However, our result of hypermethylation at CpG7 in blood samples of MDD patients was not the same CpG site as previous reported by McGowan *et al*. (2009), who found high methylation status at a potential NGFI-A transcription factor binding site of *NR3C1* gene promoter in brain samples of suicide victims that have the history of childhood abuses (McGowan *et al*., 2009). As the limitation of target tissues, such as the brain samples, in psychiatric disorders cannot be biopsied from alive patients, peripheral blood samples may be further essential biomarker for predicting psychosis symptoms (Le-Niculescu *et al*., 2013; Song *et al*., 2014). Currently, there are some researchers tried to find psychosis biomarkers using peripheral blood samples (Le-Niculescu *et al*., 2013). Le-Niculescu *et al*. (2013) used a Convergent Functional Genomics (CFG) approach to validate and identify the gene candidates of relevance to some psychiatric disorders, including bipolar and schizophrenia disorders. They indicated the *SAT1*, *PTEN*, *MARCKS* and *MAP3K3* may as top biomarkers findings in blood samples that may use to predict bipolar and some mental disorders (Le-Niculescu *et al*., 2013). Furthermore, Song *et al*. (2014) indicated the DNA methylation in exon 1 region of the *brain-derived neurotrophic factor* (*BDNF*) gene promoter from the saliva of depression patients. They mentioned that DNA methylation status in *BDNF* gene may hopefully use as a biomarker for clinical predicting depression patients (Song *et al*., 2014). However, there are a few researches of blood

biomarkers for predicting MDD, especially pointed out the DNA methylation approach. Therefore, our findings was in accordance with previous demonstrates, suggesting that altered DNA methylation status may be used as a biomarker of depression in clinical diagnosing (Song *et al*., 2014).

Based on the methylation analysis results in this study, we hypothesized that DNA methylation in the exon 1_F of the *NR3C1* gene promoter region may decrease *NR3C1* mRNA expression, thereby influencing the negative feedback mechanism in the HPA axis (de Kloet *et al*., 2005; McGowan *et al*., 2009); however, no significant differences in *NR3C1* mRNA expression levels were observed between the MDD patients and control subjects. Although there was a trend of lower *NR3C1* expression in MDD patients compared with controls, these apparent differences were not significant, possibly due to the low levels of *NR3C1* expression in the blood (Turner *et al*., 2006) and that such small changes in gene expression cannot be detected by this method. Another explanation is that some CpGs reflect the gene expression level better than others in the promoter region of the same gene (Everhard *et al*., 2009).

In this study, the cortisol levels in blood plasma of all participants were investigated as they have been associated with some stress-related disorders and abnormalities in the HPA stress response activity (Young *et al*., 2001; Pariante *et al*., 2004; Oberlander *et al*., 2008). Young *et al*. (2001) demonstrated that 80% of examined MDD patients had lower serum cortisol levels than the standard level. On the other hand, no

obvious difference in serum cortisol levels was observed between depressed male and healthy male subjects (Posener *et al*., 2004). Our findings reported here showed no significant differences in the cortisol levels in MDD patients compared with normal subjects, either in the whole sample set or separated by gender. Cortisol levels in all MDD samples were rather low, which is consistent with the low levels of serum cortisol reported for depression patients when blood samples were collected in the morning. The cortisol levels were observed to be higher in the afternoon (Burke *et al*., 2005).

5.2 *In vitro* study of antidepressants effect

5.2.1 Drug effect on *CRF* gene expression in untransfected cells

In this *in vitro* study, we have preliminary demonstrated the effects of antidepressants and others selected psychiatric drugs on endogenous *CRF* gene expression in the untransfected hypothalamus cell lines both before and after the cells were incubated with the stress reagent. Effects of each selected antidepressants and psychiatric drugs on *CRF* gene expression in hypothalamus cell lines are shown in Figures 4.8 and 4.9, respectively. Cells were treated with medium alone and medium with forskolin, which were used as controls. Dexamethasone was used as a positive control. The results showed that all of drugs tend to suppress *CRF* gene expression at the RNA level under stress both before and after the cells were incubated with forskolin, the stress reagent (Figures 4.8 and 4.9, respectively).

When cells were treated with drugs prior to forskolin, fluoxetine (a SSRI antidepressant) and risperidone (an antipsychotic drug) significantly reduced *CRF* mRNA levels ($p < 0.01$; Figure 4.8A, $p < 0.05$; Figure 4.9A, respectively). Although others selected drugs tend to also protect the cells from stress, theirs efficacies were not significant. These our results suggested that all of selected drugs used in this study tend to prevent cells from stress, the differences were statistically significant in fluoxetine (a SSRI antidepressant) and risperidone (antipsychotic drug) efficacies. Interestingly, when cells were treated with forskolin prior to the drugs, venlafaxine (a SNRI antidepressant) and all of psychiatric drugs tested significantly suppressed *CRF* mRNA expression (Figures 4.8B and 4.9B, respectively). These results indicated that all of selected drugs used in this study tend to decrease stress from the cells, the differences were statistically significant in venlafaxine (a SNRI antidepressant) and all of three selected psychiatric drugs efficacies.

Therefore, it is possible that these antidepressants and psychiatric drugs actions may involve the HPA axis negative feedback mechanism through *CRF* gene expression, as reported in earlier studies performed in Neuro-2A neuroblastoma and AtT-20 pituitary cell lines (Budziszewska *et al*., 2002; 2004). Currently, there are many researches indicated that antidepressants and psychiatric drugs may already stimulate therapeutic effects via through the HPA axis regulation (Budziszewska *et al*., 2002; 2004; Keller *et al*., 2006; Park *et al*., 2011). The first consideration in animal models was

investigated in 2006, Keller *et al*. indicated the effect of imipramine, a tricyclic antidepressant (TCA), to decrease *CRF* that was related to a reversal of anxiety and depression in rats (Keller et al., 2006). Based on this analysis, other studies of depression and psychiatric disorders was conducted (Howland, 2013).

Our preliminary results are consistent with the previous findings about the effect of some antipsychotic drugs administration on the immobilization-stress-induced *CRF* mRNA expression in the PVN of rat's hypothalamus (Park *et al*., 2011). Park *et al*. (2011) found that haloperidol increased *CRF* expression under basal conditions; however, chronic ziprasidone administration prevented *CRF* mRNA expression that was stimulated by the immobilization-stress (Park *et al*., 2011). They also suggested that ziprasidone may regulate *CRF* expression induced by immobilization-stress (Park *et al*., 2011).

Recent studies have identified the abnormal symptoms in psychotic disorder patients that associated between unusual *CRF* expressions and psychiatric drugs action, including hyperactive *CRF* activity, high concentrations of *CRF* in the CNS and abnormal *CRF* receptor expression (Keller *et al*., 2006). However, the exact mechanism of antidepressants and psychiatric drugs involved in hypothalamic *CRF* activity should be further identified.

5.2.2 Drug effect on *CRF* promoter activity

We subsequently analyze the effects of antidepressants on human *CRF* promoter activity. Although results showed that antidepressants tend to inhibit the *luciferase* mRNA expression in hypothalamus cell lines both before and after the cells were incubated with the stress reagent, theirs efficacies were not statistically significant (Figure 4.14). These results indicated that the regulatory mechanism of human *CRF* gene transcription may relate with antidepressants actions to inhibit the forskolin-induced *CRF* promoter activity. Moreover, it revealed that antidepressants mechanism may associate with the HPA axis negative feedback regulation induced by stressful periods, not only their another effect on inhibition of the neurotransmitters reuptake in the synaptic gap in the brain.

Our results supported the previous findings about the first studies of psychotropic drugs effect on *CRF* promoter regulation in Neuro-2A neuoblastoma and AtT-20 pituitary cell lines that have reported that the basal mechanism of *CRF* gene promoter activity may implicate with the direct repression of the chloramphenicol acetyltransferase (CAT) enzyme activity by some antidepressants (Budziszewska *et al*., 2002 and 2004). Although Neuro-2A neuoblastoma (neuron-like cells) and AtT-20 pituitary cell lines are the popular cells that used to studies the neuronal physiological mechanism model of regulatory pathway of *CRF* gene (Seasholtz *et al*., 1988; Guardiola-Diaz *et al*., 1994; Itoi *et al*., 1996; Budziszewska *et al*., 2002 and 2004), little studies in the directly

PVN of hypothalamus neuronal cell lines (Kasckow *et al*., 2003; Kageyama *et al.,* 2008). In this study, we used the embryonic mouse hypothalamus cell lines N6 (mHypoE-N6), which contained *CRF* gene expression marker, transfected with human *CRF* promoter-luciferase constructs as a model system. Moreover, these cells also expresses the variety of neuronal markers that associated with *CRF*, HPA axis activity, and depression pathology such as corticotrophin-releasing factor receptor 1 and 2 (*CRF*R1 and R2) genes, glucocorticoid receptor (*GR*) gene, and brain-derived neurotrophic factor (*BDNF*) gene. Likewise, the recent research used the rat hypothalamic 4B cell lines to investigate the CRE association of regulatory elements on *CRF* promoter after forskolin-induced cAMP/protein kinase A (PKA) inhibition by dexamethasone, a glucocorticoids-dependent repressor (Kageyama *et al.,* 2008).

There are previous studies demonstrated that the cyclic adenosine monophosphate (cAMP) response element (CRE) region may play an important role in the basal upstream regulatory pathway of *CRF* gene (Seasholtz *et al*., 1988; Guardiola-Diaz *et al*., 1994; Itoi *et al*., 1996). In addition, some reports also suggested that *CRF* gene transcription was induced by forskolin via cAMP-stimulated CRE region on *CRF* promoter. In contrast, forskolin-induced *CRF* transcription was repressed by dexamethasone via some regions on *CRF* promoter (Kageyama *et al.,* 2008). However, the obvious downregulation mechanism pathway of antidepressants on *CRF* promoter activity should be identified. Furthermore, the lack of clearly downregulated mechanism of drug action

on particular *CRF* promoter regulatory elements and attenuated HPA axis activity was still poorly understand.

There are evidences showed the possible of upregulated and downregulated mechanisms of *CRF* gene expressions that played a central role in HPA axis mechanism response to stress stimuli. In upregulation mechanism, signaling from cAMP/PKA/p38 MAPK (mitogen-activated protein kinases) may mediate via CRE-binding protein (CREB) to bind with CRE region on *CRF* promoter (Hansen *et al*., 1999; Morikawa *et al*., 2004; Kageyama *et al.,* 2008). In addition, the downregulation pathway of *CRF* expression, which involved with HPA axis negative feedback mechanism, can be implied that glucocorticoids-dependent repression may cooperatively induce with both nGRE and SRE regions on *CRF* promoter to suppress cAMP-simulated *CRF* gene transcription (Kageyama *et al.,* 2008). All in all, our positive results, consequently, about the regulation pathway of antidepressants action, which was affected on *CRF* promoter activity by suppressing *luciferase* mRNA level, may associate with the downregulation mechanism of *CRF* gene expression that was previous indicated (Kageyama *et al.,* 2008). It is important to note that, the particular *CRF* promoter regions that associate with antidepressant inhibition cAMP-induced *CRF* promoter activity should be further investigated. Moreover, many drugs need to develop the efficiency of therapeutic action via *CRF* mechanisms other than neurotransmitters circulation of the brain and nervous system.

CHAPTER VI
CONCLUSIONS

In *NR3C1* methylation and expression studies, the relationships between *NR3C1* promoter methylation, *NR3C1* gene expression and plasma cortisol levels in MDD patients were assessed with aim of gaining insight into gene-related causes of MDD. Hypermethylation was identified in the 7th CpG site of exon 1_F in the *NR3C1* gene promoter region in Thai MDD patients. No significant differences in *NR3C1* mRNA expression and plasma cortisol levels were observed between the Thai MDD patients and the normal Thai control subjects. The findings reported here provide insight into the etiology and pathophysiology of MDD and others stress-related disorders. They may contribute elementary information necessary to early diagnose MDD patients and others stress-related disorders. Moreover, blood samples may be applied to use to determine methylation status of *NR3C1* gene in depression patients, which were used as blood biomarkers for predicting MDD in clinical diagnosing in the future.

The second part of the studies of antidepressants and psychiatric drugs effect on *CRF* expression in hypothalamus, we have analyzed the downregulated antidepressants effects on forskolin-induced *CRF* gene expression and *CRF* promoter activity in hypothalamus cell lines. We found fluoxetine (a SSRI antidepressant) and risperidone (antipsychotic drug) can significant protect the cells from stress. Moreover, venlafaxine (a

SNRI antidepressant) and all of three selected psychiatric drugs, diazepam, risperidone and haloperidol, significantly affect to reduce the stress in the cells. Furthermore, *CRF* promoter transcription was also regulated by antidepressant drugs to suppress cAMP-stimulated luciferase mRNA level. These findings suggested that not only neurotransmitters reuptake inhibitor, the mechanism actions of antidepressants may also act in HPA negative-feedback control by regulating *CRF* promoter transcription and reducing mRNA expression in response to stress. Knowledge gained from these findings may contribute towards understanding of molecular mechanism of HPA axis negative feedback pathway response to stressful periods. Moreover, they may be used to further elucidate the mechanism of antidepressant actions, and design a novel potential therapeutic approach for treating MDD and others stress-related disorders in clinical treatment in the future.

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APPENDIX

APPENDIX

Appendix 1: The results of methylation percentages from bisulfite-pyrosequencing analysis of human LINE-1 methylation status

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4
1	M	33	MDD	84.4	83.6	74.6	75.3
2	M	43	MDD	83.7	81.5	77.6	74.8
3	M	59	MDD	82.5	82.6	78.7	75.6
4	M	52	MDD	83.4	81.9	76.2	75.8
5	M	58	MDD	83.8	81.9	78.5	77.2
6	M	49	MDD	78.4	80.8	78.0	74.8
7	M	45	MDD	78.6	81.3	75.8	72.7
8	M	52	MDD	81.9	81.8	75.4	73.6
9	M	46	MDD	83.9	83.32	77.12	75.62
10	F	41	MDD	84.9	84.3	76.1	75.8
11	F	41	MDD	80.7	80.7	75.5	72.2
12	F	45	MDD	82.9	81.4	75.5	73.5
13	F	39	MDD	82.2	81.6	72.4	74.0
14	F	45	MDD	83.3	81.0	75.2	72.6
15	F	57	MDD	84.4	80.7	74.2	71.8
16	F	37	MDD	82.3	81.7	77.8	73.5
17	F	38	MDD	82.5	80.8	76.3	71.6
18	F	38	MDD	84.3	81.3	78.8	75.3
19	F	46	MDD	84.4	80.6	76.6	74.3
20	F	45	MDD	85.7	82.1	76.7	75.7
21	F	42	MDD	79.3	81.1	73.2	70.3
22	F	45	MDD	81.4	81.8	74.1	71.8
23	F	54	MDD	77.3	80.4	72.9	69.7
24	F	66	MDD	81.2	78.9	76.8	73.0

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4
25	F	65	MDD	81.7	79.8	74.9	74.3
26	F	60	MDD	81.7	81.6	74.1	73.2
27	F	29	MDD	78.0	80.4	75.0	70.2
28	F	75	MDD	83.9	81.8	74.3	75.6
29	F	48	MDD	85.8	84.3	80.2	76.2
30	M	36	normal	83.8	82.9	78.2	75.9
31	M	35	normal	83.1	83.8	78.2	73.8
32	M	45	normal	83.1	81.6	77.6	77.3
33	M	31	normal	84.5	81.3	77.2	72.9
34	M	47	normal	78.9	81.8	76.0	72.5
35	M	41	normal	80.5	81.6	75.5	74.5
36	M	53	normal	83.8	84.8	79.2	80.0
37	F	23	normal	81.0	81.2	74.2	72.7
38	F	35	normal	80.8	82.2	75.1	73.5
39	F	40	normal	80.1	81.7	73.6	73.1
40	F	43	normal	82.3	82.3	76.1	75.1
41	F	36	normal	82.6	81.0	74.9	73.4
42	F	42	normal	85.4	81.6	75.1	73.5
43	F	48	normal	80.3	81.2	74.3	73.1
44	F	47	normal	84.4	80.8	78.0	74.7
45	F	48	normal	80.6	80.9	76.2	67.3
46	F	36	normal	82.7	82.8	75.1	73.3
47	F	46	normal	86.2	82.4	77.4	75.4
48	F	57	normal	79.9	80.9	76.8	71.1
49	F	57	normal	78.9	80.7	74.2	72.5
50	F	40	normal	82.4	80.4	75.1	72.7
51	F	31	normal	80.6	81.3	76.5	69.4

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4
52	F	57	normal	78.9	80.2	75.9	71.9
53	F	43	normal	81.6	81.3	77.1	71.7
54	F	41	normal	83.0	80.5	74.5	72.7
55	F	35	normal	85.7	82.0	75.4	75.3
56	F	48	normal	83.2	80.8	77.8	75.8
57	F	44	normal	80.2	80.6	74.4	72.7
58	F	40	normal	83.0	80.7	74.5	73.7
59	F	43	normal	82.5	81.1	76.4	74.2
60	F	49	normal	83.0	80.1	75.1	70.9
61	F	44	normal	83.8	80.8	75.9	72.9
62	F	43	normal	82.6	81.9	75.3	72.9

Appendix 2: The results of methylation percentages from bisulfite-pyrosequencing analysis of human *NR3C1* gene promoter methylation status

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7
1	M	33	MDD	0.0	0.0	0.0	0.0	1.4	0.0	1.4
2	M	43	MDD	0.0	0.0	0.0	0.0	0.0	0.0	6.7
3	M	59	MDD	0.0	1.0	0.0	1.3	0.0	0.0	1.4
4	M	52	MDD	0.0	1.6	0.0	0.0	0.0	0.0	2.7
5	M	58	MDD	0.6	0.0	0.0	0.0	0.7	0.0	1.1
6	M	49	MDD	1.7	0.0	0.0	0.0	1.3	0.0	1.8
7	M	45	MDD	0.0	0.0	0.0	1.2	0.0	0.0	0.0
8	M	52	MDD	0.0	0.0	0.0	1.1	0.0	0.0	0.0
9	M	46	MDD	0.0	0.8	0.0	1.1	0.0	0.0	1.2
10	F	41	MDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11	F	41	MDD	1.0	0.0	0.0	1.1	0.0	0.0	2.1
12	F	45	MDD	0.0	0.0	0.0	0.0	0.0	0.0	1.6

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7
13	F	39	MDD	0.0	0.0	0.0	0.0	0.0	0.0	1.8
14	F	45	MDD	1.3	1.0	0.0	0.0	1.0	0.0	1.9
15	F	57	MDD	1.0	0.0	0.0	0.0	0.0	0.0	1.7
16	F	37	MDD	1.6	1.4	0.0	0.0	1.0	0.0	1.6
17	F	38	MDD	0.0	0.0	0.0	0.8	0.0	0.0	1.7
18	F	38	MDD	0.0	1.0	0.0	0.0	0.0	0.0	1.1
19	F	46	MDD	2.0	1.1	0.0	0.0	1.3	0.0	2.0
20	F	45	MDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
21	F	42	MDD	1.6	0.0	0.0	0.0	0.0	0.0	2.0
22	F	45	MDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	F	54	MDD	1.0	1.0	0.0	0.0	1.2	0.0	1.4
24	F	66	MDD	0.0	0.0	0.0	1.1	1.5	0.0	0.0
25	F	65	MDD	0.0	0.0	0.0	0.0	0.0	0.0	1.5
26	F	60	MDD	0.0	0.0	0.0	0.0	0.9	0.0	1.1
27	F	29	MDD	0.6	0.0	0.0	0.6	0.0	0.0	1.3
28	F	75	MDD	0.7	0.5	0.0	0.0	1.4	0.0	1.0
29	F	48	MDD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	M	36	Control	5.3	3.9	0.0	0.0	1.5	0.0	2.9
31	M	35	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	M	45	Control	1.1	0.0	0.0	0.0	1.4	0.0	0.0
33	M	31	Control	1.1	0.0	0.0	0.0	0.0	0.0	1.3
34	M	47	Control	0.0	0.0	0.0	1.2	0.0	0.0	0.0
35	M	41	Control	1.1	0.0	0.0	0.0	1.1	0.0	1.2
36	M	53	Control	2.4	8.4	5.0	2.8	6.4	0.0	3.7
37	F	23	Control	0.0	0.0	0.0	0.0	1.3	0.0	2.0
38	F	35	Control	0.0	0.0	0.0	1.2	1.1	0.0	1.3
39	F	40	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0

No.	Sex	Age	Diag	CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7
40	F	43	Control	0.0	0.9	0.0	0.0	0.0	0.0	1.3
41	F	36	Control	0.8	0.6	0.0	0.0	0.9	0.0	0.8
42	F	42	Control	1.5	1.2	0.8	0.8	1.0	0.0	0.0
43	F	48	Control	0.9	0.0	0.0	0.0	0.7	0.0	0.0
44	F	47	Control	0.0	0.0	0.0	0.0	0.9	0.0	1.0
45	F	48	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
46	F	36	Control	0.6	0.0	0.0	0.7	0.7	0.0	0.8
47	F	46	Control	0.6	0.6	0.0	0.7	0.0	0.0	0.0
48	F	57	Control	1.0	1.6	0.0	1.1	0.0	0.0	0.0
49	F	57	Control	0.0	0.0	0.0	0.0	0.0	0.0	1.6
50	F	40	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
51	F	31	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
52	F	57	Control	1.1	0.0	0.0	0.0	0.0	0.0	1.7
53	F	43	Control	0.0	0.0	0.0	0.0	0.0	0.0	1.7
54	F	41	Control	1.0	0.0	0.0	0.0	1.3	0.0	1.2
55	F	35	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
56	F	48	Control	0.8	0.8	0.6	0.0	0.0	0.0	0.8
57	F	44	Control	0.0	0.0	0.0	0.0	1.2	0.0	1.0
58	F	40	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
59	F	43	Control	0.0	0.0	0.0	0.0	1.2	0.0	1.8
60	F	49	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
61	F	44	Control	0.0	0.9	0.0	0.0	0.0	0.0	1.9
62	F	43	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
In-house Methylation Control				0.0	6.0	1.2	0.0	2.8	2.7	1.8
In-house Methylation Control				60.5	60.4	60.9	55.5	60.4	61.4	59.5
No template negative control										

Appendix 3: Real-time PCR results of *NR3C1* expression.

No.	sex	diag	Ct (GAPDH) mean	Ct (GR1F) mean	**Δ**Ct	**ΔΔ**Ct	$2^{-\Delta\Delta Ct}$
1	M	MDD	17.317	20.92	3.60	0.50	0.71
2	M	MDD	17.833	21.31	3.47	0.37	0.77
3	M	MDD	12.420	16.75	4.33	1.23	0.43
4	M	MDD	13.220	16.77	3.55	0.45	0.73
5	M	MDD	16.49	20.43	3.94	0.84	0.56
6	M	MDD	14.58	18.56	3.98	0.88	0.54
7	M	MDD	22.23	24.43	2.19	-0.91	1.88
8	M	MDD	17.06	20.42	3.37	0.27	0.83
9	M	MDD	16.29	18.69	2.40	-0.70	1.62
10	M	MDD	15.70	18.71	3.01	-0.09	1.06
11	F	MDD	17.23	20.44	3.21	-0.27	1.21
12	F	MDD	15.64	19.06	3.41	-0.07	1.05
13	F	MDD	16.05	19.76	3.71	0.23	0.85
14	F	MDD	15.19	19.14	3.94	0.46	0.73
15	F	MDD	15.13	18.57	3.44	-0.04	1.03
16	F	MDD	15.74	19.30	3.56	0.08	0.95
17	F	MDD	15.87	19.06	3.19	-0.29	1.23
18	F	MDD	14.09	17.50	3.41	-0.07	1.05
19	F	MDD	15.05	18.59	3.53	0.05	0.96
20	F	MDD	15.73	19.21	3.48	0.00	1.00
21	F	MDD	15.62	17.85	2.23	-1.25	2.38
22	F	MDD	15.40	19.27	3.87	0.39	0.76
23	F	MDD	23.393	26.40	3.01	-0.47	1.39
24	F	MDD	20.993	24.00	3.00	-0.48	1.39
25	F	MDD	18.160	22.10	3.94	0.46	0.73
26	F	MDD	14.230	17.61	3.38	-0.10	1.07

No.	sex	diag	Ct (GAPDH) mean	Ct (GR1F) mean	**Δ**Ct	**ΔΔ**Ct	$2^{-\Delta\Delta Ct}$
27	F	MDD	16.793	19.61	2.81	-0.67	1.59
28	F	MDD	16.257	20.43	4.18	0.70	0.62
29	F	MDD	13.170	17.20	4.03	0.55	0.68
30	F	MDD	19.833	22.80	2.96	-0.52	1.43
31	F	MDD	15.403	19.50	4.10	0.62	0.65
32	F	MDD	15.290	19.08	3.79	0.31	0.81
33	F	MDD	16.043	20.71	4.67	1.19	0.44
34	M	control	16.37	19.67	3.30	0.20	0.87
35	M	control	14.52	17.43	2.91	-0.19	1.14
36	F	control	15.38	18.64	3.25	-0.11	1.08
37	F	control	15.81	19.34	3.53	0.17	0.89
38	F	control	16.20	19.77	3.57	0.21	0.87
39	F	control	16.14	19.29	3.15	-0.21	1.16
40	F	control	16.29	19.66	3.37	0.01	0.99
41	F	control	16.32	19.36	3.04	-0.32	1.25
42	F	control	15.25	18.79	3.55	0.19	0.88
43	F	control	15.09	18.74	3.65	0.29	0.82
44	F	control	14.48	18.10	3.62	0.26	0.84
45	F	control	14.27	18.33	4.06	0.70	0.61

Appendix 4: Real-time PCR result of *CRF* expression in untransfected hypothalamus cell lines suppressed by antidepressants.

Untransfected hypothalamus cell lines (antidepressants)					
Condition	Ct(GAPDH) mean	Ct(CRF) mean	**Δ**Ct	**ΔΔ**Ct	$2^{\wedge-\Delta\Delta Ct}$
Medium alone	14.06	24.44	10.38	0.00	1.00
Dexamethasone	15.28	25.66	10.39	0.01	1.00
Forskolin	16.00	23.55	7.55	-2.83	7.10
Dexamethasone + Forskolin	13.63	24.37	10.74	0.36	1.17
Fluoxetine + Forskolin	13.06	23.52	10.46	0.09	1.59
Venlafaxine + Forskolin	12.92	22.45	9.54	-0.84	1.79
Sertraline + Forskolin	16.28	25.47	9.19	-1.19	2.59
Forskolin + Dexamethasone	14.22	23.62	9.40	-0.98	2.07
Forskolin + Fluoxetine	13.86	23.46	9.60	-0.78	2.82
Forskolin + Venlafaxine	13.71	23.33	9.63	-0.75	1.84
Forskolin + Sertraline	16.44	26.06	9.61	-0.76	2.65

Appendix 5: Real-time PCR result of *CRF* expression in untransfected hypothalamus cell lines suppressed by psychiatric drugs.

Untransfected hypothalamus cell lines (psychiatric drugs)					
Condition	Ct(GAPDH) mean	Ct(CRF) mean	**Δ**Ct	**ΔΔ**Ct	$2^{-\Delta\Delta Ct}$
Medium alone	13.90	24.37	10.47	0.00	1.00
Dexamethasone	15.51	25.63	10.13	-0.25	1.20
Forskolin	16.05	23.53	7.52	-2.82	7.08
Dexamethasone + Forskolin	13.51	24.48	10.97	0.59	1.11
Diazepam + Forskolin	20.81	29.19	8.38	-1.99	3.98
Risperidone + Forskolin	21.24	29.77	8.53	-1.85	3.61
Haloperidol + Forskolin	21.57	30.10	8.51	-1.87	3.66
Forskolin + Dexamethasone	13.88	23.95	10.07	-0.31	1.24
Forskolin + Diazepam	21.13	29.72	8.59	-1.79	3.45
Forskolin + Risperidone	21.17	30.35	9.17	-1.20	2.30
Forskolin + Haloperidol	21.58	29.77	8.20	-2.18	4.53

Appendix 6: Real-time PCR result of *CRF* promoter activity in transfected hypothalamus cell lines suppressed by antidepressants.

Transfected hypothalamus cell lines					
Condition	Ct(GAPDH) mean	Ct(CRF) mean	**Δ**Ct	**ΔΔ**Ct	$2^{\wedge-\Delta\Delta Ct}$
Medium alone	17.11	20.74	3.63	0.00	1.00
Dexamethasone	18.92	20.71	1.79	-1.84	3.58
Forskolin	20.04	21.68	1.63	-2.00	8.35
Dexamethasone + Forskolin	19.93	20.77	0.84	-2.79	6.90
Fluoxetine + Forskolin	20.08	20.98	0.90	-2.73	6.65
Venlafaxine + Forskolin	18.34	20.52	2.18	-1.45	2.74
Sertraline + Forskolin	18.56	20.82	2.26	-1.37	2.59
Forskolin + Dexamethasone	20.28	21.14	0.86	-2.77	6.82
Forskolin + Fluoxetine	21.36	21.42	0.06	-2.55	5.84
Forskolin + Venlafaxine	18.69	20.00	1.32	-2.31	4.97
Forskolin + Sertraline	19.59	21.10	1.51	-2.12	4.35

VITA

Miss Matchima Nantharat was born on January 15th, 1984 in Phatthalung Province, Thailand. She finished her secondary school level from Phatthalung School, Phatthalung Province, in 2002. In Bachelor's degree, She got the scholarship from the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand; SAST), and received Bachelor's degree of Science in Biology from Department of Biology, Faculty of Science, Prince of Songkla University, Songkla, in 2006. She has taken master research grants from Thailand Research Fund (TRF) and Thailand Tobacco Monopoly, Ministry of Finance, and received Master's degree of Science in the program of Biotechnology, Chulalongkorn University, since 2007. In Ph.D., she was a Ph.D. student in the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand; SAST), and studied in Biological Science Program since 2015. Nowadays, she is a government official at Ministry of Science and Technology.

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