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Transcriptional Regulation of 5-HT1A A Receptor Gene ; Freud-2
a Novel Repressor of 5-HT1A A Receptor Gene

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Transcriptional regulation of 5-HT_{1A} receptor gene; Freud-2 a novel repressor of 5-HT_{1A} receptor gene

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This thesis is submitted as a partial fulfilment of the PhD program in Neuroscience

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Abstract

The serotonin 1A receptor (5-HT_{1A}) is expressed presynaptically on raphe neurons and works as an inhibitory autoreceptor. It is also expressed postsynaptically in limbic and cortical areas involved in mood and emotional regulation. Alteration in expression and function of 5-HT_{1A} receptors is believed play an important role in etiopathology and treatment of mood disorders such as major depression.

5-HT_{1A} receptor gene regulation was characterized. We have reported a dual repressor element that binds two complexes in non expressing 5-HT_{1A} receptor cells (Freud-1/CC2D1B), but the identity of the second protein was unknown. We identified a mouse Freud-1 homologue (Freud-2/CC2D1B) that binds to the dual repressor element on the 5-HT_{1A} promoter. We show that Freud-2 specifically reduces transcriptional activity of the post synaptic 5-HT_{1A} receptor gene. Knocking down Freud-2 by specific siRNA unregulates expression of 5-HT_{1A}.

In addition, we extended our studies to identify and examine the human Freud-2. We show repressor function of Freud-2 in different human cell lines. Further studies on human normal post mortem brains using a specific anti-Freud-2 antibody reveal that Freud-2 is highly expressed in the nucleus of postsynaptic neurons in prefrontal cortex and hippocampus region in human and distributed in grey matter of these regions.

We conclude that Freud-2 has a potential role in modulating mood and emotional response by altering the expression of postsynaptic 5-HT_{1A}.

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List of abbreviations

5HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
5-HT1A	5-hydroxytryptamine-1A receptor
5-HTT	5-hydroxytryptamine transporter
5-HTTLPR	5-hydroxytryptamine transporter linked polymorphic region
8-OH-DPAT	8 hydroxy-2-(di-n-propylaminotetralin)
AC	adenylyl cyclase
ACTH	adrenocorticotropin hormone
BDNF	brain-derived neurotrophic factor
Bp	base pair
CAM	calcium/calmadulin
CAMK	calcium/calmadulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CC2D1B	Coiled-coil C2 domain-1B
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CREB	cAMP response element binding protein
CRF	corticotrophin-releasing factor
CSF	cerebrospinal fluid
CTD	C-terminal repeat domain

Ctrl	control
DA	dopamine
dCTP	deoxycytosine triphosphate
DG	dentate gyrus
DMI	desipramine
DNA	deoxyribonucleic acid
DRE	dual repressor element
DRN	dorsal raphe nucleus
DSM IV	diagnostic and statistical manual IV
DTT	dithiothreitol
E	embryonic
ECT	electroconvulsive therapy
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol-bis tetra acetic acid
EMSA	electro mobility shift assay
ER	estrogens receptor
FLX	fluoxetine
FRE	five prime repressor element
Freud-2	five prime repressor element under dual repression protein
GABA	gamma amino butyric acid
GFAP	glial fibrillary acidic protein
GM	grey matter
GPCR	G-protein coupled receptor

GR	glucocorticoid receptor
GRK	G-protein coupled receptor kinase
GST	glutathione S transferase
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
HEK	human embryonic kidney cells
HLH	helix-loop-helix
HPA	hypothalamus-pituitary-adrenal
HSF	heat shock factor
INR	initiator
KCl	potassium chloride
KDa	kilodalton
LC	locus ceuroleus
LTP	long term potentiation
MAOI	monoamine oxidase inhibitor
MAPK	mitogen-activated protein kinase
MAZ	Myc-associated zinc finger protein
MDD	major depression disorder
MnR	median raphe nucleus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
Nacl	sodium chloride
NE	norepinephrine

NFκB	nuclear factor κB
NG-108	neuroglioma-108 cells
NGF	nerve growth factor
NRI	norepinephrine reuptake inhibitor
NRSF	neuronal restrictive silencing element
NT	neurotrophin
NUDR	nuclear DEAF-1 related protein
PBS	phosphate buffer saline
pCMV-βgal	plasmid cytomegalovirus promoter/βgalactoside reporter gene
PCPA	p-chlororphenylalanine
PCR	polymerase chain reaction
PDE	phosphodiesterase-4
PFC	prefrontal cortex
PIC	pre-initiation complex
PKA	protein kinase A
PKC	protein kinase C
POMC	pro-opiomelacortin
PS	prenatal stress
PVN	paraventricular nucleus
REST	repressor element silencing transcription factor
RT	room temperature
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Ser	serine

SERT	serotonin transporter
SiRNA	stealth interference ribunucleotic acid
SSRI	selective serotonin reuptake inhibitor
TBP	transcription binding protein
TCA	tricyclic antidepressant
TFIIB	transcription factor IIB
TFIID	transcription factor IID
TFIIE	transcription factor IIE
TFIIF	transcription factor IIF
TPH	tryptophan hydroxylase
TrK	tyrosine kinase
TTX	tetrodotoxin
VTA	ventral tegmental area

Dedication

I would like to dedicate my thesis to my wife, Mojdeh, for her abundant support, for her patience and understanding, and for her love, to my children, Mohammad, Hamed, and Ehsun, for making every thing worthwhile.

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CHAPTER I- INTRODUCTION

I-1 Major depressive disorder (MDD)

One of the goals of this thesis has been to identify novel transcription regulators that could be implicated in depression and anxiety. Thus a rationale for identification of genes involved in depression and for the strategy taken is presented.

I.1.1 Prevalence and diagnosis of depression

Depression and anxiety disorders are chronic, recurrent, and life threatening forms of mental illness that affect 20% of the general population (Fava and Kendler, 2000; Manji et al., 2001; Nestler et al., 2002a; Berton and Nestler, 2006). Major depression disorder (MDD) is twice as common in women as in men (Fava and Kendler, 2000). Since the 1960's, depression has been diagnosed as "major depression" based on the criteria set by the Fourth Diagnostic and Statistical Manual (DSMIV, 2000) (Table-1).

These criteria (summarized in Table-1) indicate that a diagnosis of major depression is based on symptoms, but as yet there is no objective diagnostic test such as serum chemistry or organ biopsy to make the diagnosis of depression.

I.1.2 Etiopathophysiology

The etiology of depression is unknown. Several factors are implicated in the induction of mood disorders such as neurotransmitter dysfunction (serotonin, noradrenalin, and dopamine), genetic factors, HPA (hypothalamus-pituitary-adrenal) axis dysregulation, and immune system imbalance. Although these factors may play roles in different aspects of depression, not all depressed patients have detectable malfunctions in these biogenic factors. Recently, a new term has been suggested for the above mentioned factors, i.e., vulnerability or sensitivity (Nestler et al., 2002a; Nemeroff and Vale, 2005).

Depressed mood
Irritability
Low self esteem
Feeling of hopelessness, worthlessness, and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain
Insomnia or hypersomnia
Low energy, fatigue, or increased agitation
Decreased interest in pleasurable stimuli (e.g., sex, food, social interaction)
Recurrent thought of death and suicide

Table-1: Diagnostic criteria of major depressive disorder

Major depressive disorder is a clinical disorder that is characterized by five of the above symptoms accompanied by at least one of the "depressed mood or decreased interest in pleasurable stimuli" at the time of diagnosis. These symptoms should persistent continuously for at least two weeks.

Genetic factors

The heritability of major depression in most twin studies varies between 31-42% (Sullivan et al., 2000). One of the most studied neurotransmitter systems in MDD is serotonin (5-HT). It has been suggested that alterations in 5-HT levels may be heritable and that alterations in genes that regulate this system could constitute important heritable vulnerability factors for mental disorders. Below are discussed some examples of serotonin-regulatory genes for which functional genetic polymorphisms have been identified and associated with mood disorders.

Serotonin transporter (5-HTT) polymorphism

The 5-HTT is expressed on serotonin neurons and transports serotonin from the synaptic cleft into the neurons where it can be degraded or stored in vesicles for later release. Studies have identified two 5-HTTLPR (5-HTT linked polymorphic region) alleles (Collier et al., 1996; Heils et al., 1996). A short form (S) 5-HTT encodes a 484 amino acid protein while the long form (L) encodes 528 amino acids. Further studies revealed that the L- variant is more active than the S- variant, resulting in higher serotonin transporter expression and function.A post mortem brain study showed that S/S and S/L genotype are associated with 40% less 5-HTT expression than L/L genotype (Mann et al., 2000).

An association study has revealed that 5-HTT polymorphism accounts for 3 to 4 % of total variance and 7 to 9% of inherited variance in anxiety-related personality (Lesch et al., 1996). Furthermore, subsequent studies have also revealed an association of 5-HTT short (S) allele in dissocial alcoholic personality (Sander et al., 1998), and with

neuroticism in personality disorders (Greenberg et al., 2000; Jacob et al., 2004), as well as an increased vulnerability to depression in children and young adults with short form (S) allele expression (Caspi et al., 2003; Kaufman et al., 2004), particularly in combination with environmental factors such as early lifetime stress or maltreatment.

Studies examining association of depression with 5-HTT polymorphism studies are not consistent. Some studies reported association between S-allele and depression (Collier et al., 1996; Lesch and Mossner, 1998; Neumeister et al., 2002; Joiner et al., 2003), while others found no association (Ohara et al., 1998; Mann et al., 2000; Minov et al., 2001). Although the 5-HTT LPR is the most studied polymorphism in association with depression, this association remains controversial. Recent studies were reported the exitence of low frequency allele variant, L_G, whose function equivalent to S allele(Hu et al., 2006; Zalsman et al., 2006; Steiger et al., 2007) (Hu et al., 2006; Zalsman et al., 2006; Steiger et al., 2007). So, 5-HTTLPR has triple allele, S and L_G representing low functional variant and L_A shows higher functiona.

5-HT1A receptor gene polymorphism

5-HT1A receptors are expressed presynaptically as autoreceptors in serotonin neurons of the raphe nuclei (especially dorsal raphe nucleus, DRN) and post synaptically in other brain regions that receive serotonergic innervation. A recent study identified a common polymorphism in the human 5-HT1A promoter region (Wu and Comings, 1999), and its association with suicide and depression was examineed (Lemonde et al., 2003).

The 5-HT1A polymorphism is a C/G polymorphism located -1019 bp upstream of translation initiation site in 5-HT1A receptor gene. It has been shown that the G (-1019) allele reduced repression of presynaptic 5-HT1A receptor gene expression, resulting in

increased expression of 5-HT1A autoreceptors, which is predicted to reduce serotonin transmission and predispose individuals to depression and suicide. Association of the 5-HT1A polymorphism is not specific for depression, as an association was also reported in panic disorder (Neumeister et al., 2004a) suggesting that this polymorphism may act as a vulnerability factor in mood disorders.

Gender

Epidemiological studies have demonstrated that the prevalence of depression is higher in females than in males (Piccinelli and Wilkinson, 2000). This difference arises around puberty when women enter their reproductive life. It has been shown that female rats have higher 5-HT activity in dorsal raphe (DR) nuclei, brainstem, and limbic areas (Carlsson and Carlsson, 1988; Dominguez et al., 2003), as well as increased synthesis and turnover of 5-HT (Haleem et al., 1990). In humans, levels of 5-HT metabolites in CSF are higher in females than in males (Young et al., 1980; Agren et al., 1986), suggesting that females have higher basal activity of the 5-HT system than males.

Estrogen modulates several neurotransmitter systems, such as 5-HT and dopamine (Osterlund and Hurd, 2001), via ER-β and ER-α receptors (Ostlund et al., 2003). It has been shown that ER-β knockout mice have significantly lower levels of serotonin and dopamine in several brain regions, including hippocampus (Imwalle et al., 2005). Taken together, gender is one of the key genetic factors that may influence the vulnerability of an individual to depression, although the exact mechanisms involved remain unclear.

I.2 Pharmacotherapy

The etiology of depressive disorder is still unknown. Indeed, much of what we know about depression is based on antidepressant action in targeting the symptoms of this

disease. For almost half century, theories about the mechanisms of action of drugs used to treat mood disorders have focused primarily on their actions to inhibit neurotransmitter reuptake (e.g., serotonin reuptake inhibitors, norepinephrine reuptake inhibitors) or inhibition of neurotransmitter metabolism (monoamine oxidase inhibitors [MAOI] (Coyle and Duman, 2003). However, some treatments such as lithium, valproic acid and electroconvulsive therapy (ECT), have very indirect or widespread effects on synaptic neurotransmission (Coyle and Duman, 2003). Recently, investigators have begun to examine the effect of antidepressant drugs on intracellular signaling pathways that could regulate synaptic transmission (Manji et al., 2001; Coyle and Duman, 2003; Ji et al., 2005). Alterations in neurochemical signaling are thought to play an important role in the pathophysiology of mood disorders (Duman, 2002). The treatment of depression was revolutionized with the serendipitous discovery of effective antidepressants about a half-century ago. The tricyclic antidepressants (e.g., imipramine) arose from antihistamine research, and early monoamine oxidase inhibitors were discovered from actions of anti-tubercular drugs (Nemeroff and Owens, 2002; Nestler et al., 2002b). Current antidepressants act on neurotransmitter systems through: (1) inhibition of monoamine transporter protein, (2) inhibition of monoamine oxidase, or (3) blockade of pre- or postsynaptic receptors that modulate monoamine signaling (Reid and Stewart, 2001; Nemeroff and Owens, 2002; To et al., 2005). Although most antidepressants exert their initial effects by increasing the intrasynaptic level of serotonin and/or norepinephrine, their therapeutic effects are observed only after chronic administration (3-5 weeks after initiation of treatment) (Manji et al., 2001; Nemeroff and Owens, 2002). This observation has led to the hypothesis that although dysfunction of monoaminergic

systems may likely mediate some aspects of major depression disorders (MDD), they are not the final common pathway of antidepressant action (Nibuya et al., 1996; Duman et al., 1997; Manji et al., 2001). Another explanation for the action of antidepressants is that there is a common intracellular mechanism beyond the alterations of levels of 5-HT and NE and their receptors (Nibuya et al., 1996). In this case, alterations in 5-HT and NE release may lead to regulation of post synaptic receptors and their signaling pathways. One post-synaptic receptor mediated signally pathway which could be regulated by 5-HT or NE in response to different antidepressant treatments is the cAMP pathway (Nibuya et al., 1996).

I.2.1 Role of cAMP cascade and CREB in antidepressants treatment

A role for the cAMP cascade in the chronic action of various antidepressant treatments is supported by studies demonstrating that chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in the rodent hippocampus (Nibuya et al., 1996; Thome et al., 2000; Nakagawa et al., 2002; Tardito et al., 2006; Sairanen et al., 2007). In addition, it has been shown that the cAMP signaling pathway regulates phosphodiesterase4 (PDE4) gene expression via CREB in primary cortical neurons (D'Sa et al., 2002). PDE4 is highly expressed in brain and breaks down cAMP to terminate signaling through cAMP. Moreover, PDE4 expression is regulated by chronic administration of antidepressants such as fluoxetine, a selective serotonin reuptake inhibitor (D'Sa et al., 2002). Additional evidence for a role of CREB activation in antidepressant action comes from further studies, which showed that viral-mediated over expression of CREB in hippocampal DG (dentate gyrus) produced an antidepressant-like effect in behavioral tests (Chen et al., 2001a; Sairanen et al., 2007).

Activation of CREB is triggered by phosphorylation at a specific serine residue (Ser133) (Lonze and Ginty, 2002; Blendy, 2006; Tardito et al., 2006), which is mediated by different signal transduction pathways. One of best known mediators is the cAMP signaling cascade. Activation of adenylyl cyclase (AC), which is modulated through signaling via G-protein coupled receptors (GPCR's), increases levels of cAMP, leading to activation of cAMP-dependent protein kinase A (PKA) in most cells (Lonze and Ginty, 2002; Blendy, 2006). Upon binding cAMP to the regulatory subunit of PKA, the catalytic subunit dissociate and enter the nucleus, and phosphorylates nuclear substrates such as CREB and CREB family members (Lonze and Ginty, 2002; Blendy, 2006; Tardito et al., 2006). Other signaling pathways that could activate CREB consist of Ca^{2+}-calmodulin-dependent kinase, protein kinase C (Duman, 2002), MAPK2, and members of the (RSK) and MSK families of protein kinases (Tardito et al., 2006). Regulation of CREB is involved in development and maturation of nervous system (Lonze and Ginty, 2002; Tardito et al., 2006).

In 1996, Nibuya and colleagues found that chronic but not acute administration of different classes of antidepressant compounds such as fluoxetine, a selective serotonin reuptake inhibitor (SSRI) or tricyclic antidepressants imipramine and desipramine, up-regulated the expression of CREB mRNA in rat hippocampus (Nibuya et al., 1996). In contrast, a recent study in the rat using the same time course and similar doses of drugs, showed that CREB protein level in hippocampus was not altered by desipramine and fluoxetine treatment, but both drugs increased CREB phosphorylation in frontal cortex (Laifenfeld et al., 2005). An important distinction between these two studies is the time at which tissues were examined after the last drug administration and the region of brain

that was examined. Consistent with the Nibuya's results, another study has demonstrated that after chronic administration of imipramine, the level of phospho- CREB was increased in both medial prefrontal cortex and hippocampus (Sairanen et al., 2007). Further study revealed that activation of the cAMP cascade by injection of rolipram, an inhibitor of cAMP breakdown, increased the proliferation of newborn cells in adult mouse hippocampus, which was accompanied by activation of CREB phosphorylation in dentate gyrus (Nakagawa et al., 2002).

CREB expression was also investigated following chronic treatment with fluoxetine (FLX) or desipramine (DMI) in mice with impaired glucocorticoid receptor function (a depression model). Whereas in wild type mice, both drugs increased CREB expression in hippocampus but not in cerebral cortex, in transgenic mice FLX increased expression in hippocampus and both drugs did so in cerebral cortex (Blom et al., 2002). A transgenic mouse line has been generated that expresses a gene containing tandem CRE sequence in front of a LacZ reporter gene. CREB activity was measured by LacZ expression. The activity at the CRE is increased after chronic tranylcypromine (monoamine oxidase inhibitor) in the cortex, hippocampus, hypothalamus, and amygdala whereas fluoxetine increased CRE activity in the cortex, amygdala and hypothalamus (Thome et al., 2000). Unlike the above-mentioned results, over-expression of CREB in the basolateral amygdala produced pro-depressive-like responses in the learned helplessness model and enhanced fear conditioning (Wallace et al., 2004). In a loss of function paradigm, CREB knockout mice were chronically treated with desipramine or fluoxetine. Both wild type and CREB-deficient mice responded similarly to DMI and FLX in the forced swim test and tail suspension test. However, up-regulation of BDNF, amolecular target of CREB,

was abolished in the CREB-deficient mice after chronic administration of DMI (Conti et al., 2002). Thus although CREB activation is implicated in antidepressant action, the precise role of CREB depends on the type of antidepressant, and the behavioral paradigm studied.

Post-mortem studies have revealed abnormal CREB expression in depressed patients. In a recent study, total CREB protein, CREB mRNA, and CRE-DNA binding using the gel shift assay of post-mortem brain from teenage suicide victims were assessed. CRE-DNA binding, the protein expression of CREB and CREB mRNA were significantly decreased in the PFC (prefrontal cortex) of teenage victims compared to controls. However, there was no significant difference in CRE-DNA binding or the protein and mRNA expression of CREB in the hippocampus of teenage suicide victims (Pandey et al., 2007). In another post-mortem study, it has been demonstrated that immuonoreactivity of CREB and phosphorylated CREB were significantly decreased in the orbitofrontal cortex of depressed subjects (Yamada et al., 2003).

Overall, the above-mentioned results support the hypothesis that upregulation of CREB is one of the target proteins in antidepressant action, although the exact mechanism of CREB action is still unknown. These data also suggest that dysregulation of CREB may play a role in the pathophysiology of mood disorders.

I.2.2 Brain-derived neurotrophic factor (BDNF) and mood disorders

The mammalian neurotrophin family members consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), as well as neurotrophin-4/5 (NT-4/5). They control a variety of important neural activities such as neuronal survival, long-term synaptic plasticity, and cellular differentiation during brain

development (Reid and Stewart, 2001; Lessmann et al., 2003). Neurotrophins bind with high affinity to the Trk tyrosine receptor kinase family (Huang and Reichardt, 2003; Durany and Thome, 2004). Trk receptors comprise a family of three subtypes, each of which can be activated by one or more of the four neurotrophins. The Trk family of receptor tyrosine kinases derives its name from the proto-oncogene tropomyosin-related kinase (Trk), which consists of the first seven of eight exons of non muscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase later identified as an NGF receptor (Huang and Reichardt, 2003).

Several lines of evidence show that stress reduces BDNF expression, while antidepressant treatments increase BDNF expression in animal models of depression (Kempermann and Kronenberg, 2003; Duman, 2004; Dranovsky and Hen, 2006; Duman and Monteggia, 2006). Stress is used as a model to study alterations of brain structure and function because mood disorders often result from acute or chronic actions of stress. Initial studies examined the influence of acute immobilization stress and showed a significant decrease in BDNF mRNA expression in the dentate gyrus, CA3, CA1 pyramidal cell layers of hippocampus (Duman, 2004; Duman and Monteggia, 2006). Further studies have found decreased BDNF expression in response to other types of stress such as unpredictable foot shock, social isolation, social defeat, forced swim test, and maternal deprivation (Duman, 2004; Duman and Monteggia, 2006). Moreover, it has been shown that psychosocial stress can decrease neurogenesis via activation of hypothalamus-pituitary-adrenal axis (Dranovsky and Hen, 2006).

One of the first lines of evidence suggesting the involvement of BDNF in the treatment of depression was the finding that local infusion of BDNF in the midbrain has antidepressant

effects in two behavioral models of depression, the forced swim and learned-helplessness paradigms (Siuciak et al., 1997; Shirayama et al., 2002). In addition, it has been reported that chronic ECT as well as antidepressants drugs from different classes such as SSRIs, tricyclic antidepressants, and MAOI, enhance and prolong the expression of BDNF and TrkB mRNA in treated animals (Nibuya et al., 1995). In the hippocampus of animals treated with different classes of antidepressant drugs, BDNF mRNA expression was increased only after chronic treatment (21 days).

Further studies provide additional evidence, showing an enhancement of BDNF mRNA expression upon chronic co-administration of imipramine with rolipram, a PDE-4 inhibitor (Fujimaki et al., 2000). Induction of BDNF mRNA in response to this repeated co-administration paradigm occurred in the dentate gyrus, CA1, and CA3 pyramidal cell layers of hippocampus. Consistent with these results, another study found that in the learned-helplessness model, co-administration of imipramine and rolipram almost completely eliminated escape failures (Itoh et al., 2004). In other studies, induction of different exons of BDNF expression after ECS and antidepressant treatment was examined (Dias et al., 2003). Chronic ECS induced BDNF exons I, II, and IV mRNAs, but did not influence exon III. In contrast, chronic treatment with tranylcypromine and desipramine increased exon II and exon III BDNF mRNAs in hippocampal and cortical subfields. Similar to the above-mentioned results of upregulation of BDNF mRNA after antidepressant treatment, Altar et al, measured BDNF protein using ELISA in adult male rats following ECS or antidepressant treatments (Altar et al., 2003). They showed an increase in BDNF protein expression 10 days after ECS treatment in parietal cortex, hippocampus, frontal cortex, as well as septum. In contrast, an increase in BDNF protein

in the frontal cortex and neostriatum was observed only after 3 weeks tranylcypromine treatment, and no increase in BDNF protein was observed following administration of desipramine and fluoxetine.

To address the role of BDNF in learning and memory, conditional BDNF knockout mice were used. In mice in which the BDNF gene was deleted in the forebrain region, a deficit in hippocampal-dependent learning and long-term potentiation was observed (Monteggia et al., 2004). It has also been demonstrated that the loss of forebrain BDNF attenuates the action of antidepressants in the forced swim test, suggesting the involvement of forebrain BDNF in antidepressant efficacy (Monteggia et al., 2004). In a recent study, the expression of five BDNF splice variant mRNAs (I-V) in chronic social defeat in mice was examined (Tsankova et al., 2006). Experiments showed that defeat stress induces histone methylation of BDNF promoters II and IV that correlated with a long-lasting down-regulation of BDNF transcripts II and IV. Chronic treatment with imipramine increased histone acetylation of these promoters and reversed the down-regulation of BDNF transcripts (Tsankova et al., 2006). Thus chronic stress reduces expession of BDNF and is correlated with impaired learning and memory and can be reversed by chronic antidepressant treatment.

BDNF induces its effects via activation of the TrkB receptor. Further studies have focused on BDNF-TrkB signaling in depression and its role in antidepressant treatment. One way to show the role of the TrkB receptor in depression and its treatment is over-expression or deletion of this receptor in animal models. Transgenic mice that over-express a truncated form of TrkB (TrkB.T1), a dominant negative mutant, showed resistance to the effects of antidepressant treatment in the forced swim test (Saarelainen et

al., 2003). This indicates that normal TrkB signaling is required for antidepressant actions on depressive behavior. In this model, phosphorylation of CREB was increased in the prefrontal cortex by the administration of antidepressants. The CREB response was also reduced in the truncated TrkB-over expressing mice (Saarelainen et al., 2003; Hashimoto et al., 2004). In contrast to results for the TrkB.T1 over-expressing mice, over expression of full length TrkB (TrkB.TK$^+$) in mouse brain showed increased TrkB activity in brain tissue (Koponen et al., 2005) and these mice showed an antidepressant-like increase in latency to immobility in the forced swim test, similar to that observed upon fluoxetine treatment of wild-type mice. However, FLX had no further effect on the swimming behavior of the TrkB.TK$^+$ mice. These results suggest that TrkB contribute antidepressant action on depressive behaviors in mice (Koponen et al., 2005).

Studies in human subjects provide additional insight regarding the regulation of BDNF and TrkB by antidepressant drugs. A post-mortem study showed an increase in BDNF expression in dentate gyrus, hilus and supragranular regions in antidepressant-treated subjects compared with untreated subjects (Chen et al., 2001b). Furthermore, studies of prefrontal cortex Brodman area 9 and hippocampus obtained from suicide victims, revealed that BDNF and TrkB mRNA are significantly reduced in these regions compared to non-suicide control brains (Dwivedi et al., 2003). However, these changes were observed in all suicide subjects and were unrelated to psychiatric disease, post mortem interval, age, sex, or pH of the brain. In addition, it was reported that first and multiple-episode depressed patients perform poorly on various acute recall tests involving hippocampal function, and that multiple-episode depressed subjects displayed reductions

in hippocampal volume (MacQueen et al., 2003), which may involve reduced BDNF expression.

Recently researchers examined the effect of chronic antidepressant treatment on serum BDNF levels in depressed patients (Aydemir et al., 2005). They reported that serum BDNF was significantly lower in depressed patients than in control subjects. However, in the depressed patients, 12-week antidepressant treatment resulted in serum BDNF levels that were significantly increased compared to baseline before treatment (Aydemir et al., 2005). In contrast, a study of neural stem cell proliferation failed to show any reduction in stem cell proliferation in post-mortem brains of depressed patients compared to non-depressed controls (Reif et al., 2006). It is well accepted that BDNF plays an important role in synapse formation (Ji et al., 2005). Moreover, BDNF plays a role in axonal branching, dendritic growth, and activity-dependent refinement of synapses. In a recent study it has been shown that cAMP regulates BDNF function in mature hippocampal neurons by modulating the signaling and trafficking of its receptor TrkB (Ji et al., 2005). Taken together, the above-mentioned results suggest that the activation by antidepressant treatments of downstream transcription factors, such as CREB, increase BDNF-TrkB expression in limbic regions resulting in neuronal sprouting, increased neurogenesis, and synaptic plasticity, contributing to their behavioral effects in models of depression (Coyle and Duman, 2003).

I.3 Hypothalamus-Pituitary-Adrenal (HPA) axis

One of the potent environmental factors to induce depression is stress. Stress increases the likelihood of depressive episodes (Connor and Leonard, 1998). The human body reacts to stress by increased activity of the HPA, which controls the release of glucocorticoids.

I.3.1 Physiology of HPA

The HPA originates from neurons that contain corticotrophin-releasing factor (CRF), a 41-amino acid peptide that is localized in paraventricular nucleus of hypothalamus. CRF stimulates the release of corticotrophin (ACTH) from the pituitary into the bloodstream, leading to synthesis and release of glucocorticoids from the adrenal cortex (Antoni et al., 1983). CRF immunoreactivity is also detected in the locus coeruleus, parabrachial nucleus, raphe nuclei and numerous other sites(Heim and Nemeroff, 1999). CRF modulates target cells via two type of receptors, CRF1 and CRF2, both of which are G-protein coupled receptors (Chalmers et al., 1996). CRF1 receptors are highly expressed in pituitary gland, also in cortical and subcortical regions of the brain. CRF2 receptors are expressed peripherally in heart and testes, as well as in brain regions such as the septum, hypothalamus, and dorsal raphe nuclei.

I.3.2 Stress responses of HPA

Stress increases the synthesis and release of CRF into the portal blood system that carries CRF to the pituitary gland. Activation of CRF receptors in the pituitary gland results in stimulation of pro-opiomelanocortin (POMC) synthesis and release of ACTH (adrenocorticotropin hormone), β-endorphin. ACTH in turn stimulates the synthesis and release of glucocorticoids from the adrenal cortex which regulate the body's response to stress. Glucocorticoids negatively control the activity of the HPA through a variety feedback mechanisms (Dallman et al., 1987).

Glucocorticoids exert their effects through mineralocorticoid (MR) and glucocorticoid receptors (GR). MR has a higher affinity for glucocorticoids than GR and is highly expressed in the hippocampus, where it modulates circadian regulation of the HPA. The

low affinity GR is expressed throughout the brain and pituitary gland, and is thought to regulate the HPA in stressful events (De Kloet et al., 1998). Upon activation, glucocorticoid receptors translocate to the nucleus and regulate gene expression.

In addition to regulation of glucocorticoids, CRF mediates autonomic, immune, and behavioral responses. It has been reported that direct injection of CRF into the CNS, increased autonomic responses by enhancing the release of catecholamine, leading to increased heart rate and mean arterial pressure (Dunn and Berridge, 1990). Behavioral effects of central administration of CRF include decreases in reproductive behavior, food intake, feeding behavior, and increased locomotor activity in a familiar environment. These behavioral phenotypes are very similar to what has been observed in depressed patients. Moreover, direct CNS injection of CRF induced some anxiety-like behaviors in animals such as suppression of exploratory behavior in a new environment and facilitated fear conditioning, enhancing shock-induced freezing response (Dunn and Berridge, 1990; Owens and Nemeroff, 1991). Indeed, injection of CRF-receptor antagonists or CRF antisense blocked the above mentioned anxiogenic responses (Dunn and Berridge, 1990; Skutella et al., 1994).

The amygdala is a key regulator of emotional and fear responses. It has been reported that stress increases CRF expression in the amygdala, and direct injection of CRF into the amygdala reduced exploration in the open field test and increased fear conditioning (Liang and Lee, 1988). CRF-expressing neurons in amygdala project to the locus coeruleus, and synapse with noradrenergic neurons (Van Bockstaele et al., 1996). It has been demonstrated that increased CRF concentration in the locus coeruleus enhances

tyrosine hydroxylase activity (Melia and Duman, 1991), and induces anxiety-like behavior (Butler et al., 1990).

Increased CRF and cortisol also modulate the serotonergic system by increasing tryptophan availability and by stimulation of TPH activity (Davis et al., 1995; Maccari et al., 2003). Corticosterone also modulates the function of both presynaptic and postsynaptic 5-HT_{1A} receptors. 8-OH-DPAT, a selective 5-HT_{1A} agonist, produces a hypothermic response in mice, which is thought to involve an action at presynaptic 5-HT_{1A} autoreceptors. This effect was attenuated by administration of corticosterone (Young et al., 1994), suggesting that corticosterone reduces presynaptic 5-HT_{1A} function to increase 5-HT release. Moreover, chronic treatment with corticosterone reduced 5-HT1A mRNA levels in the DG of hippocampus, which was correlated with the plasma level of corticosterone (Meijer and de Kloet, 1994).

On the other hand, serotonin can modulate the HPA by increasing the levels of corticotrophin-releasing factor (CRF) (van Praag, 2004). The above-mentioned evidence supports the idea that stress increases HPA activity, which in turn down regulates presynaptic 5-HT_{1A} receptor function, resulting in enhanced 5-HT neurotransmission in the brain. Enhancement in serotonin transmission positively regulates the HPA resulting in excess secretion of glucocorticoids.

It has been reported that excessive glucocorticoids can potentiate excitotoxicity in hippocampal neurons, which leads to dendritic atrophy, inhibition of neurogenesis in DG, and hippocampal atrophy (Watanabe et al., 1992; Gould et al., 1997; Sapolsky, 2000; Malberg and Duman, 2003; de Kloet et al., 2005a). Many of these changes can be prevented by antidepressants. In addition, dysregulation of corticosteroid receptors (GR,

MR) may be mediated by the increase in 5-HT release after exposure to stress (Semont et al., 1999; Lai et al., 2003; Robertson et al., 2005). Based on the above-mentioned evidence it has been proposed that an alteration in inhibitory glucocorticoid feedback mechanisms may be a marker for vulnerability to depression. In particular, it was shown that the dexamethasone failed to suppress the HPA activity in first-degree relatives of depressed patients (Modell et al., 1998).

I.3.3 Early life stress and depression

Several lines of evidence have shown that prenatal and early life stress experiences may confer increased vulnerability to depression and anxiety disorder in adulthood (Heim and Nemeroff, 2001; Seckl, 2001). An animal model of prenatal stress (PS) was developed and is characterized by dysregulation of the HPA and sleep disturbances that are similar to what has been observed in depressed patients (Morley-Fletcher et al., 2003). This model responded vigorously to stress by long-lasting corticosterone secretion and increased immobility time in the forced swim test. Chronic antidepressant treatment reduced the immobility behavior of the PS animals. Moreover, rat PS offspring also showed increased response to stress (Kofman, 2002; Maccari et al., 2003; Morley-Fletcher et al., 2003). In addition, PS offspring in adulthood showed low central 5-HT activity, HPA impairment, and anhedonic behaviors (Hayashi et al., 1998), similar to those seen in depressed patients. The postnatal effects of prenatal stress (PS) were explained by the hypotheses that high maternal glucocorticoids may influence fetal brain development, down-regulate glucocorticoid receptors in the hippocampus and impair the development of the serotonin system (Maccari et al., 2003; Morley-Fletcher et al., 2003; Huizink et al., 2004). Hypercortisolism in the fetus results in a higher level of 5-HT

during embryonic development. Higher plasma levels of serotonin which can pass through blood brain barrier (BBB) during development may negatively regulate 5-HT terminal development, reducing serotonergic terminals in adulthood (Whitaker-Azmitia, 2005). Studies in man have also showed that the incidence of depression is higher in children prenatally exposed to the 1976 earthquake trauma in China (Watson et al., 1999). Stressful early life experiences are also important factors contributing to psychiatric disorders. In rodents early life stress leads to dysregulation of serotonergic function and permanently alters HPA responsiveness to stress (Heim et al., 2004; de Kloet et al., 2005a; de Kloet et al., 2005b). Parental behaviors can influence the development of the offspring. Animal studies have shown that offspring of high licking and grooming mothers showed fewer fearful responses, enhanced cognitive performance in spatial learning test, and reduced HPA activity (Liu et al., 1997; Francis et al., 1999). Furthermore, the effect of caring mothers on their offspring is associated with increased expression of the GR gene. In caring mothers,GR gene expression is increased in hippocampus (Weaver et al., 2004). On the other hand, separation of pups from their mothers for 3 hr daily resulted in increased HPA activity and increased emotional responses to brief stressors in adulthood (Ladd et al., 2004). These behavioral responses were accompanied by higher CRF mRNA expression in the central amygdala and PVN and reduced expression of GR in cortical region.

Investigators have studied the effect of early life stress on 5-HT transmission in hippocampus in mid-life (van Riel et al., 2004). They separated 3-day-old rats from their mothers for 24 hr and studied the functional response to 5-HT and 5-HT_{1A} receptor

mRNA expression at 3 months of age. Hippocampal responses to 5-HT_{1A} receptor were attenuated in rats deprived from their mothers.

Fewer studies were done on the effect of early life stress in humans compared to rodents. Studies on sexually abused children revealed blunted ACTH responses to CRF (Kling et al., 1994). Another study reported that children suffering ongoing abuse showed higher ACTH responses to CRF, whereas cortisol levels were normal (Kaufman et al., 1997). Consistent with previous reports, another study found that adult survivors of childhood abuse (with or without current major depression) showed significantly higher ACTH responses compared to control (Heim and Nemeroff, 1999). Moreover, maternal factors also play an important role in resilencing the HPA in preschoolers and in cognitive development of infants exposed to early life stress (Essex et al., 2002; Tu et al., 2007). Taken together, several lines of evidence indicate that stress is an important risk factor for mood disorders. To summarize some key points, patients with depressive disorders show hyperactivity of HPA, and hypercortisolemia that can affect anxiety and induce cognitive impairment, as well as altering the serotonin system in a similar way to that seen in depression. Antidepressants increase MR and GR in limbic structures to reverse HPA hyperactivity. In addition, early life stress increases vulnerability to HPA hyperactivity and depressive behavior in later life. Intracerebral injection of CRF induces anxiety- and depression-like behaviors, while CRF1 antagonists show anxiolytic and antidepressant activity (Bale and Vale, 2004; de Kloet et al., 2005a; Keck et al., 2005; Kehne, 2007). Unfortunately the hepatotoxicity of these drugs precludes their use (Bosker et al., 2004).

I.4 Role of noradrenergic system in depression

Depression is a very complex disorder; no single brain area or neurotransmitter system is responsible for induction of the multiple behavioral manifestations. Behavioral symptoms of depression result from overlapping actions of dysregulation of at least three main neurotransmitter systems: NE, 5-HT, and DA. Serotonin and norepinephrine play important roles in etiopathology of major depression disorders. The role of NE system in mood disorders and interconnection with serotonin system is discussed in this section.

I.4.1 Adrenergic system in the brain

Noradrenergic (norepinephrine or NE) neurons are located in the locus coeruleus, which is adjacent to the fourth ventricle of the pontine brainstem. Noradrenergic neurons project to many brain regions including thalamus, hypothalamus, hippocampus, amygdala, pre-frontal cortex, and dorsal raphe nucleus (Kimble and Kaufman, 2004). The locus coeruleus is responsible for the baseline tone of cortical arousal and associated with increased alertness, acute stress, and physiological arousal in the presence of stressful stimuli (Berridge and Waterhouse, 2003). Norepinephrine, via binding to different receptor classes (α1, α2, and β) modulates target neurons. In the neocortex, the β-receptor is coupled to Gs-proteins, while α1 couples to Gq and α2 couples to Gi/Go-proteins (Berridge and Waterhouse, 2003). Currently multiple subtypes of these receptors have been identified β (β1-β3), α1 (a, b, d), α2 (A-D) (Jones and Palacios, 1991; Bylund et al., 1992). Multiple peptides also co-localize with NE in LC neurons, such as vasopressin, somatostatin, neuropeptide Y, enkephalin, CRF, and galanin (Olpe and Steinmann, 1991; Berridge and Waterhouse, 2003). NE neurons fire action potentials at a low, regular rate

(Jacobs et al., 1991) and their firing rate is controlled by α2-autoreceptors expressed on their soma (Blier, 2001a).

I.4.2 Therapeutic action of NRIs

Norepinephrine reuptake inhibitors (NRIs), such as desipramine and reboxetine, block the NE-transporter and increase extracellular NE, which in turn activates α2-adrenergic receptors to reduce firing activity of the NE neurons (Szabo et al., 2000; Wong et al., 2000). Studies in rats show that sustained administration of desipramine (10 mg/kg for 2 days) reduces the firing activity of locus coeruleus (Szabo et al., 2000), an effect that is likely mediated by recurrent activation of α2-adrenergic autoreceptors. In another study reboxetine showed the same effect on LC firing as desipramine at a lower dose (2.5 mg/kg), suggesting that reboxetine is more potent to inhibit the norepinephrine transporter (Wong et al., 2000). In addition to effects on 5-HT, the anxiolytic activity of SSRIs (selective serotonin reuptake inhibitors) could involve reducing NE transmitter release. It has been shown that chronic treatment of rats with paroxetine reduces the firing activity of LC neurons (Szabo et al., 2000). Moreover, escitalopram (SSRI) after 2 days administration also reduced NE neuronal firing (Dremencov et al., 2007). This effect was blocked by co-administration of 5-HT synthesis inhibitor p-chlorphenylalanine (PCPA), suggesting that the serotonergic system modulates NE neuronal activity. On the other hand, NE neurons also modulate firing of serotonergic neurons (Blier, 2001b). For example, systemic injection of mirtazapine (α2-adrenoreceptor antagonist) transiently enhanced the firing activity of 5-HT neurons in the rat (Haddjeri et al., 1996).

Two non-selective antidepressant classes are among the most effective, namely monoamine oxidase inhibitor (MAOI) and tricyclic antidepressant (TCA). MAOI's block

the degradation of 5-HT, NE, DA in the nerve terminal, while TCA's block 5-HT and NE reuptake. Furthermore studies reveal that chronic administration of NRIs increased the concentration of NE in several brain regions including the hippocampus and prefrontal cortex (Invernizzi et al., 2001; Parini et al., 2005) and induced inhibition of NE action in the hippocampus via activation of α1- and α2-adrenoreceptors and the desensitization of β-receptors. Chronic injection of desipramine also increases extracellular 5-HT concentration in rat hippocampus (Yoshioka et al., 1995). NRIs are good adjuvant to SSRIs in the case of SSRI-resistant depressed patients (Tremblay and Blier, 2006) and the combination of fluoxetine (SSRI) and desipramine (NRI) increased remission rate in depressed patients (Nelson et al., 2004). Combination therapy has led to the production of dual reuptake inhibitors such as venlafaxine, clomipramine, or duloxetine. Studies on venlafaxine and duloxetine revealed that they are potent NE-transporter blockers, as well inhibiting 5-HT reuptake (Beique et al., 2000; Vincent et al., 2004).

Taken together, NRIs can induce antidepressant effects alone or increase chance of remission in combination with SSRIs. One of the important issues in treatment of depressive disorder is achievement of the remission state. Only one third of depressed patients achieve remission in the first course of antidepressant treatment with an adequate dose (Tremblay and Blier, 2006), supporting the advantages of using dual blockers rather than single blocker therapy.

I.5 Serotonin and its role in psychiatric disorders

Serotonin was discovered in 1868 when it was shown that serum of clotted blood contains a factor which led to vasoconstriction, and its chemical structure was published in 1948 by Rapport (Rapport et al., 1948). Thereafter, more studies revealed that serotonin or 5-

hydroxytryptamine (5-HT) also functions as a neurotransmitter that participates in a wide variety of biological processes.

I.5.1 Serotonin synthesis and location of serotonergic neurons

Serotonin is synthesized from the essential amino acid L-tryptophan, which is hydroxylated by tryptophan hydroxylase (TPH) to produce 5-hydroxytryptophan (5-HTP). 5-hydroxytryptophan is then decarboxylated by aromatic amino acid decarboxylase (AADC) to produce 5-hydroxytryptamine (5-HT). 5-HT is metabolized by monoamine oxidase (MAO) to 5-hydroxyindole acetic acid (5-HIAA) (Fig-1). TPH is the rate-limiting enzyme in synthesis of serotonin. Serotonin is then packaged and stored in synaptic vesicles for exocytotic release. Following release, serotonin neurotransmission is terminated by reuptake via serotonin transporter into pre-synaptic neuronal terminal where it is degraded by MAO enzyme or repackaged into vesicles. Serotonin in the pineal gland is metabolized to melatonin by specific enzymes and then secreted into blood flow. Melatonin plays an important role in sleep cycling. Serotonergic neurons are located in the raphe nuclei of the brainstem. Serotonergic fibers project from the raphe nuclei to several brain regions; including cortex, hippocampus, cerebellum, and midbrain, as well as to the spinal cord. 5-HT-containing cell bodies are subdivided into nine groups, B1-B9 (Dahlstrom and Fuxe, 1964; Hensler, 2006). Most of these subgroups reside in the raphe nuclei; however, not all of the cell bodies in the raphe nuclei are serotonergic, and some serotonergic neurons are found outside of the raphe nuclei (Descarries et al., 1982; Kohler and Steinbusch, 1982; Molliver, 1987; Tork, 1990). The B6-B7 nuclei form the dorsal raphe nuclei, the B8 group is the median raphe nucleus and the B1-B4 clusters are located

more caudally and send projections to the median raphe. Interconnection between dorsal raphe and median raphe have also been reported (Mosko et al., 1977).

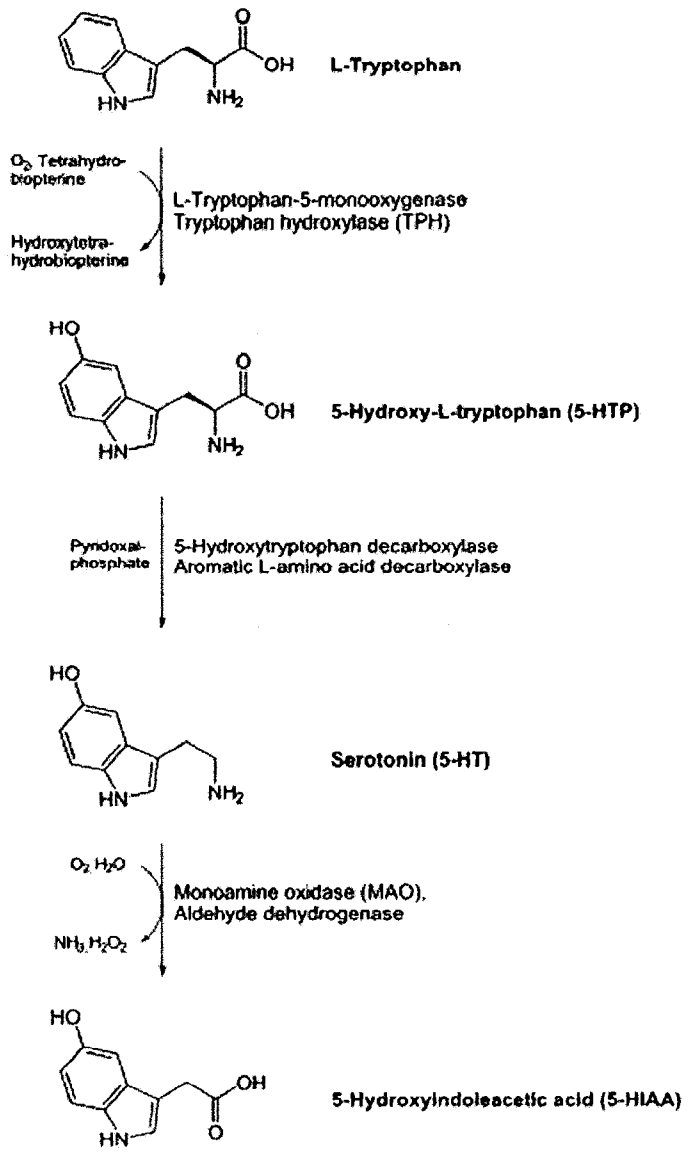

Fig- I-1: Synthesis and metabolism of serotonin-Serotonin is synthesized by hydroxylation of L-tryptophan by TPH to produce 5-hydroxytryptophan (5-HTP), which is decarboxylated by AADC to produce 5-hydroxytryptamine (5-HT). 5-HT is metabolized by monoamine oxidase (MAO) to 5-hydroxyindole acetic acid (5-HIAA).

Moreover, synaptic formation occurs within dorsal raphe nucleus, suggesting local interconnection between serotonergic neurons within the dorsal raphe nuclei (Descarries et al., 1982; Kapadia et al., 1985; Chazal and Ralston, 1987). Such intra-raphe innervation may explain how autoreceptors that are located on soma and dendrites of serotonergic neurons are activated by 5-HT released from nerve terminals and regulate the firing of 5-HT neurons. Dorsal raphe(DR) and median raphe(MnR) respond differently to somatodendritic autoreceptors and they have different electrophysiological properties (Kirby et al., 2003; Beck et al., 2004). For example, in DR, serotonergic and non-serotonergic neurons responded to 5-HT_{1A} agonist by reducing neuronal firing, while in MnR only serotonergic neurons responded to 5-HT1A agonist, suggesting 5-HT_{1A} has roles as an auto- and heteroreceptor in DR, but only has the autoreceptor function in MnR.

In addition, axonal fibers that originate from neuronal cell bodies in DR form "D" fibers or a basket axon system. They are thick and non-varicose, forming short and thin branches and have large round buttons at the end. In contrast, axonal projections arising from MnR form "M" fibers or the thin varicose system. They are diffuse, branch profusely and have small fusiform buttons at their terminals (Kosofsky and Molliver, 1987). Indeed, the axonal distribution and 5-HTT expression differ between axons arising from DR and MnR. It has been shown that most parts of nucleus accumbens are innervated by "D" fibers that express 5-HTT, but shell part of nucleus accumbens receives "M" fibers that lack 5-HTT (Brown and Molliver, 2000), suggesting that serotonergic terminals supplying nucleus accumbens may respond differently to antidepressants.

DR fibers project to forebrain, subcortical regions such as ventral tegmental area (VTA), substantia nigra, amygdala, some nuclei in the thalamus and hypothalamus (Molliver, 1987; Vertes, 1991). The prefrontal cortex and ventral part of hippocampus are innervated by DR fibers while the dorsal part of hippocampus is innervated by MnR fibers (Molliver, 1987). Dorsal raphe nuclei 5-HT neurons are modulated by noradrenergic fibers (Mundey et al., 1994; Szabo and Blier, 2001a, b; Pudovkina et al., 2002). This excitatory effect is mediated directly via α1-adrenogenic receptors on 5-HT neurons and indirectly through α2-adrenoreceptor inhibiting inhibitory interneurons (GABA) that modulate 5-HTergic neurons.

I.5.2 Serotonergic receptors and regulation of 5-HT release

There are 16 different subtypes or isoforms of 5-HT receptors (Naughton et al., 2000). They classified based on operational, structural, and transduction properties.

5-HT1 receptors

5-HT1 receptors include 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors. All belong to the G-protein coupled receptor family (GPCR) containing seven predicted transmembrane domains, and are encoded by intronless genes consisting of 365-422 amino acids. They are negatively linked to adenylyl cyclase via coupling to Gi/Go proteins. 5-HT_{1A} receptors are expressed presynaptically on the soma and dendrites of serotonergic neurons and negatively regulate the firing of neuronal cells (Fig-2).

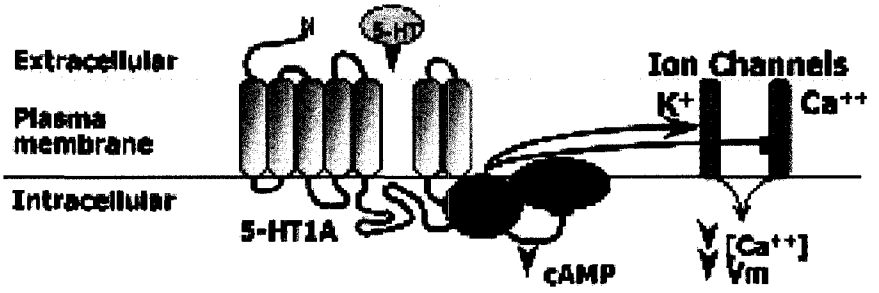

Fig I-2- 5-HT1A receptor structure- The 5-HT_{1A} receptor belongs to G-protein coupled receptor family. It consists of seven predicted transmembrane domains, and three intracellular loops. The 5-HT_{1A} receptor is coupled to Gi/Go proteins which negatively regulate the activity of adenylyl cyclase (AC), activate G-protein regulated potassium channels and inhibit voltage-gated calcium channels that results in hyperpolarization of the neuronal cell.

It also expressed postsynaptically in the hippocampus, amygdala, septum, and cortical limbic areas. There is substantial amino acid similarity between 5-HT_{1A} receptors and adrenergic receptors, which may explain why some adrenergic agents such as pindolol, propanolol can bind to 5-HT_{1A} receptors (Julius, 1991).

5-HT2 receptors

5-HT2 receptor family consists of three members: 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors. All are Gq-coupled receptors (Gq/G11) that positively regulate phosphoinoside metabolism (Boess and Martin, 1994). 5-HT2 receptors are expressed peripherally and centrally. IN the CNS they are expressed centrally postsynaptically on non-serotonergic neurons in the neocortex, olfactory nuclei, and basal ganglia (Staner et al., 1992). It has been reported that they suppressed the release of glutamate, dopamine, and noradrenalin, as well as regulating slow-wave sleep.

5-HT3 receptors

The 5-HT3 receptor is a ligand-gated non-selective Na^+/K^+ ion channel, which mediates fast synaptic transmission (Maricq et al., 1991). The 5-HT3 receptors modulate acetylcholine release and the activity of GABAergic neurons (Zifa and Fillion, 1992). 5-HT3 antagonists are able to suppress nausea and vomiting induced by chemotherapy or radiotherapy (Naughton et al., 2000).

5-HT4, 5, 6, 7 receptors

All of these receptors are coupled to Gs protein and increase intracellular cAMP by activating adenylyl cyclase (AC). 5-HT4 receptors are expressed in substantia nigra, basal ganglia, and nucleus accumbens as well as GABAergic neurons (Patel et al., 1995). Recently it has been reported that 5-HT4 agonists reduce immobility in the forced

swimming test and three days treatment was enough to reduce hyperlocomotion in olfactory bulbectomized rodents (a model of depression) and showed antidepressant-like effects (Lucas et al., 2007). The 5-HT6 receptors are highly expressed in limbic and cortical brain regions in rodents and are proposed to play roles in psychiatric disorders in man, as some antipsychotic drugs such as clomipramine, clozapine, olanzapine act as 5-HT6 antagonists (Monsma et al., 1993; Hoyer et al., 1994). Although microdialysis of 5-HT6 antagonists did not change the basal level of DA, NE, or 5-HT in the striatum, frontal cortex, dorsal hippocampus, or nucleus accumbens, it increased glutamate levels in both frontal cortex and dorsal hippocampus, indicating a possible therapeutic role in the treatment of cognitive and memory dysfunction (Dawson et al., 2001). 5-HT7 receptors also couple to Gs-proteins and positively regulate AC activity. They are regulated by the level of glucocorticoids, which may link them to the stress response and depression (Yau et al., 1997).

I.5.3 Regulation of 5-HT release

The amount of extracellular release of 5-HT is regulated by calcium, 5-HTT, 5-HT_{1A}, and 5-HT_{1B} receptors. It has been reported that the basal level of 5-HT in the raphe area is calcium dependent (Hery et al., 1982). It was also shown increased potassium concentration in raphe area enhanced release of 5-HT and this effect was blocked by tetrodotoxin (TTX) (Hery et al., 1982). However, the TTX dependency of 5-HT release was not consistent in different experiments; Some studies reported sensitivity of 5-HT release to tetrodotoxin (Bosker et al., 1994) and some reported insensitivity to TTX (Adell et al., 1993; Matos et al., 1996). The action of released 5-HT is terminated by reuptake into the presynaptic nerve terminal via the 5-HT transporter or SERT.

Therefore, blockade of the action of SERT by SSRIs leads to increased levels of extracellular 5-HT.

Acute administration of SSRI increases the 5-HT level in the raphe nuclei (Bel and Artigas, 1992; Gartside et al., 1995; Malagie et al., 1995; Hervas and Artigas, 1998), which activates 5-HT_{1A} autoreceptors and reduces the firing activity of serotonergic neurons (Artigas et al., 1996). However, enhancement in 5-HT neurotransmission in postsynaptic regions was observed only after chronic treatment with SSRIs, corresponding to the timing required for therapeutic effects of SSRIs (Bel and Artigas, 1992; Rutter et al., 1994; Invernizzi et al., 1995; Hervas et al., 2001). Moreover, lack of 5-HTT in knockout mice did not alter basal firing rate of DR neurons, but desensitized somatodendritic 5-HT_{1A} in DR (Mannoury la Cour et al., 2001). These data indicate that blockade or deletion of 5-HTT leads to desensitization or down-regulation of 5-HT_{1A} autoreceptors, and loss of autoreceptor-mediated inhibition of firing. In contrast, Gobbi and his colleagues showed enhancement of 5-HT neuronal firing in 5-HTT -/- mice following administration of 5-HT_{1A} antagonist (Gobbi et al., 2001).

Another factor that modulates the amount of extracellular 5-HT is the 5-HT_{1A} autoreceptor. In the DR nuclei 5-HT_{1A} receptors are expressed on soma and dendrites, and function as autoreceptors to reduce 5-HT neuronal cell firing (Aghajanian et al., 1990; Riad et al., 2000). 5-HT_{1A} receptors are also expressed in postsynaptic areas such as the hippocampus, frontal cortex, and other parts of limbic system and exert inhibitory effects on target cells (Sprouse and Aghajanian, 1988; Dong et al., 1998). Chronic administration of 5-HT_{1A} agonist or SSRIs desensitized presynaptic 5-HT_{1A} receptors but not postsynaptic 5-HT_{1A} receptors (Blier and de Montigny, 1987; Blier et al., 1987).

The other regulators of extracellular 5-HT are the 5-HT1B/D autoreceptors. 5-HT1B/D autoreceptors are located at the serotonergic nerve terminal and regulate release of 5-HT neurotransmitter (Sari et al., 1999). 5-HT1B/D receptor knockout mice have aggressive behaviors (Olivier and van Oorschot, 2005), although these effects may depend primarily on the median raphe (MnR)-5-HT system. Consistent with this, 5-HT1B/D agonists like sumatriptan inhibit 5-HT release at dorsal hippocampus which is innervated by the MnR-5-HT system (Schlicker et al., 1989; Pineyro et al., 1995).

I.5.4 Role of 5-HT system in mood disorders

Dysfunction of the serotonergic system predisposes individuals to develop mood disorders. For example, in patients suffering from seasonal affective disorder and in remission from depression, acute tryptophan depletion induced relapse (Neumeister et al., 1998), which was reversed by light therapy in seasonal affective disorder. Moreover, 5-HT_{1A} knockout mice exhibit reduced exploratory activity, increased fear-related behaviors, and decreased immobility time in the forced swim test (Ramboz et al., 1998). Indeed behavioral studies of 5-HT_{1A} and 5-HT_{1B} knockout mice show that 5-HT_{1B} knockout displayed less anxiety, but became more aggressive and reactive compared to wild-type control mice. In contrast, 5-HT_{1A} knockout mice showed less aggression and reactive behavior, but more anxiety-like behavior (Zhuang et al., 1999). These behavioral effects of knocking out 5-HT_{1A} or 5-HT_{1B} receptor genes may differ by gender of the animals (Jones and Lucki, 2005). For example, female 5-HT_{1B} knockout showed higher reduction in immobility than male 5-HT_{1B} knockout on the tail suspension test (TST). Moreover, autoradiography of 5-HT_{1A} and 5-HT_{1B} serotonergic receptors and serotonin transporter (5-HTT) demonstrated down-regulation of 5-HTT expression in several brain

regions and hyper-innervation of amygdalohippocampal nuclei, and ventral hippocampus of 5-HT_{1B} knockout mice, which may explain their aggressive behaviors (Ase et al., 2001).

More supporting evidence for the role of the serotonin system in mood disorders comes from post-mortem and imaging studies of human brain. An increase in 5-HT2 receptors in frontal cortex of depressed patients postmortem brain section has been reported (Yates et al., 1990), consistent with previous studies showing higher levels of post synaptic 5-HT2 binding in major affective disorders (McKeith et al., 1987). In contrast position emission tomography (PET) studies in depressed patients did not show any significant difference between patients and control in cortical 5-HT2 receptor expression (Attar-Levy et al., 1999), similar to another report of unchanged 5-HT2 receptor binding in depressed patients (Cheetham et al., 1988; Lowther et al., 1994). Further studies of 5-HT2 receptor expression did find changes in the frontal cortex, but not in hippocampal regions (Rosel et al., 2000; Mintun et al., 2004). Studies of the role of other serotonergic receptors revealed that 5-HT_{1A} binding potential is decreased in PET imaging studies in depressed patients, prominently in midbrain raphe (Drevets et al., 1999; Meltzer et al., 2004). It was also reported that 5-HT_{1A} receptor binding is reduced in limbic and neocortical regions (Drevets et al., 2000b). Moreover, there is a reduction in 5-HT_{1A} binding in cortical areas of recovered depressed subjects, but not in raphe (Bhagwagar et al., 2004).

Exposure of expectant mothers to stressful conditions such as physical or psychological stress might affect the serotonin system of the offspring. Recent immunostaining studies revealed that 5-HT_{1A} immunoreactivity is reduced in the ventral hippocampus of male rat offspring following prenatal stress, but not in the dorsal hippocampus, which participates

in learning and memory (Van den Hove et al., 2006). Exposure of 2-, 3-, or 10-12 week-old rats to foot shock induced disinhibition of long-term potentiation (LTP) in response to 5-HT_{1A} agonist tandospirone (Matsumoto et al., 2005). Alterations in 5-HT_{1A} receptor mediated synaptic plasticity may be responsible for the attenuation of freezing behavior seen in those rats, suggesting that 5-HT_{1A} receptors play an important role in the regulation of emotional responses during postnatal development. Consistent with immunostaining studies in rats, studies in depressed monkeys revealed that 5-HT_{1A} binding potential (BP) in PET imaging study was reduced. Reduction in 5-HT_{1A} binding potential in the amygdala and hippocampus was related to monkeys' aggressive behaviors (Shively et al., 2006).

Additional studies in man have demonstrated more roles for 5-HT_{1A} receptors in other mood disorders. PET imaging studies on unmedicated panic patients revealed reduction in binding potential of 5-HT_{1A} receptors in anterior and posterior cingulate cortices, as well in the raphe nuclei (Neumeister et al., 2004a). It has also reported that 5-HT_{1A} receptor binding levels are also changed in anorexia nervosa (Bailer et al., 2005). Recent studies on depressed patients showed that 5-HT_{1A} binding potential might be a predictor tool to evaluate the antidepressant response, as it was shown that higher 5-HT_{1A} binding potential indicates a poorer response to antidepressant therapy (Parsey et al., 2006).

I.6 Gene transcription

Gene expression is crucial to dictate cellular function. Expression of a particular gene involves a number of different processes. Transcription of a gene starts with the synthesis of a complementary RNA from a DNA template. The primary transcript is modified in

the nucleus to become messenger RNA (mRNA), which is then transported to the cytoplasm, where it binds to the ribosome and is translated to protein.

I.6.1 General Mechanisms

Expression of a gene requires the transcription of the DNA strand, which is template strand or antisense strand into the complementary sense RNA by RNA polymerase II. RNA polymerases III and I catalyze the synthesis of ribosomal RNA. The whole transcription process starts with binding of RNA polymerase II to the transcription initiation complex at the promoter, a specific DNA sequence that signals the initiation of transcription. The most common promoter element is the TATA box with the TATAAA consensus sequence, which is located -35 to -20 bp upstream of initiation. There are two other well characterized promoter elements that can direct RNA polymerase II to initiate transcription on a specific gene:1) Initiator (INR), and 2) promoter proximal element, CAAT and GC box, which are located within 200 bp of transcriptional start site. However, this process requires assembly of transcription factors (TF).

Thus, the initiation of transcription starts with assembly of the pre-initiation complex (PIC) (Fig-3). TFIID forms from transcription binding protein (TBP) and TBP associated factor (TAF), which recruits TFIIB. TFIIB makes a bridge between TFIID and RNA polymerase II and is joined by TFIIF. Then they recruit TFIIE and TFIIH. The C-terminal repeat domain (CTD) of polymerase II, which contains many proline, serine, threonine residues, is necessary for this step. The CTD dictates which factors associate with RNA polymerase II (Phatnani and Greenleaf, 2006). Formation of an open complex prior to assembly of PIC at the promoter is required for transcriptional initiation. TFIIE, and TFIIF are required to unwind double stranded DNA into a single stranded DNA in an

ATP-dependent process (Goodrich and Tjian, 1994; Holstege et al., 1996; Kim et al., 2000). Elongation is the next step. Before elongation starts the PIC is partially disassembled, some TFs remain at the promoter and function as a scaffold for the next transcription initiation complex formation (Zawel et al., 1995; Yudkovsky et al., 2000). This stage called promoter clearance which is facilitated by TFIIH.

The next round of initiation starts after promoter clearance and is a faster process (Orphanides and Reinberg, 2000). Clearance of the promoter is accompanied by phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. Phosphorylation of CTD dictates the transcriptional site and recruits proteins that are required for elongation (Orphanides and Reinberg, 2002). For example, CTD phosphorylation at Ser5 is associated with 5' transcription, while the amount of phosphorylation at Ser2 is increased towards the 3'-end of gene.

The above-mentioned factors affect transcription independent of chromatin. The basic unit of chromatin is composed of ~ 147 bp of DNA wrapped around a single histone octamer. The histone contains two copies of histones proteins H2A, H2B, H3, and H4. Histones are small basic proteins composed of a globular domain and histone tail (N-terminus). Histone modifications such as acetylation, methylation, and phosphorylation have important effects on gene transcription (Jenuwein and Allis, 2001). It is clear now that histone modification in concert with elongation of transcription modulates gene transcription (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

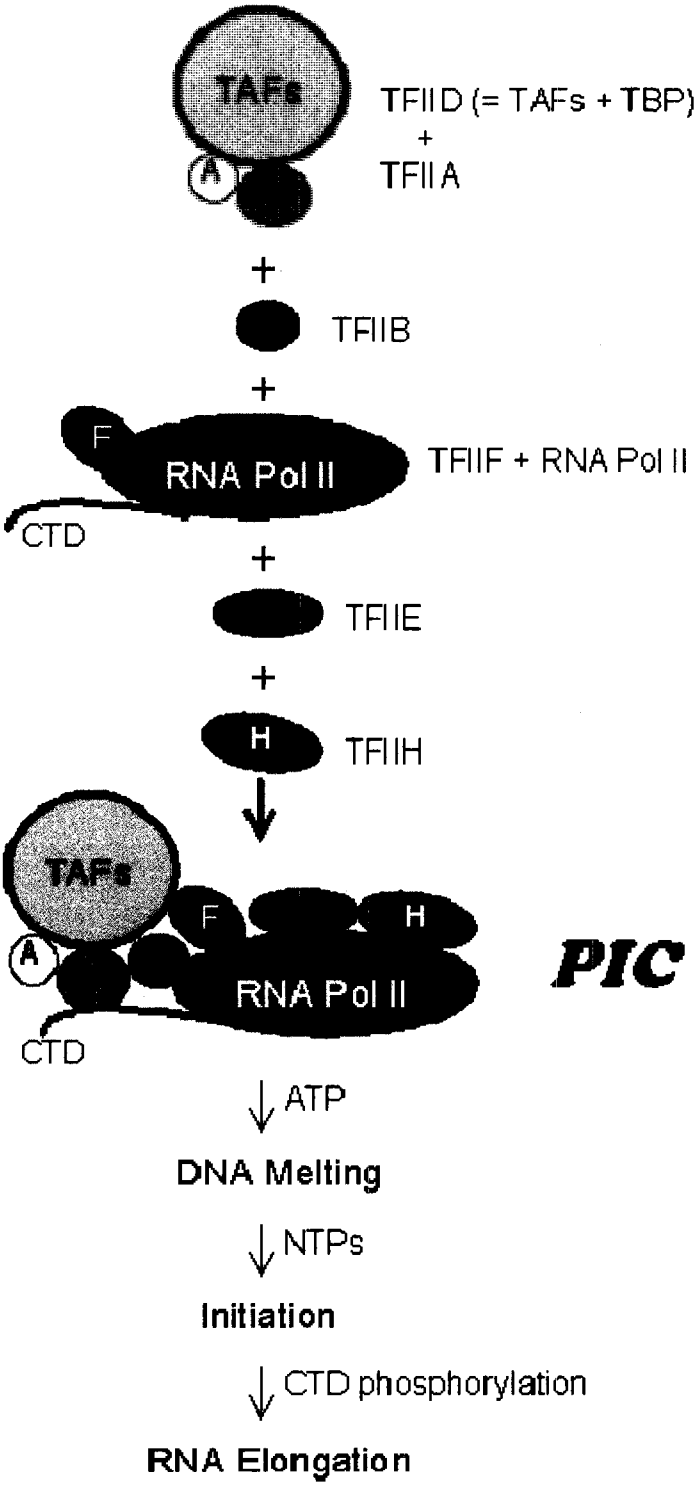
TAFs
TFIID (= TAFs + TBP)
+
TFIIA
+
TFIIB
+
F
RNA Pol II
TFIIF + RNA Pol II
CTD
+
TFIIE
+
H
TFIIH
TAFs
F
H
A
RNA Pol II
PIC
CTD
ATP
DNA Melting
NTPs
Initiation
CTD phosphorylation
RNA Elongation

Fig-I-3

FigI-3-Assembly of the pre-initiation complex (PIC) - TFIID via TBP binds to the promoter at the TATA box and then recruits TFIIB. RNA polymerase II and TFIIF are bound together and then recruited by TFIIB to the initiation complex. Finally RNA polymerase II recruits TFIIE, which in turn recruits TFIIH to complete the formation of PIC on the promoter.

After elongation the primary RNA transcript is capped by the capping enzyme, which adds 7-methylguanylate to the 5' end. The capping process is stimulated by phosphorylation of the CTD on Ser5 (Ho and Shuman, 1999; Moteki and Price, 2002). The primary transcript also undergoes splicing via the splicing machinery which is highly regulated by the CTD of RNA polymerase II as it was shown that capping and splicing processes were inhibited by truncated form of CTD (McCracken et al., 1997). Final trimming of the primary transcript is carried out by 3'-end cleavage and polyadenylation. This process is also associated with CTD function, as it was reported that loss of Ser2 CTD phosphorylation causes defects in 3'-end processing (Ahn et al., 2004; Ni et al., 2004).

In particular, histone modification modulates recruitment of co-activators or co-repressors, as observed for heat-shock factor-1 (HSF1) recruitment of SW1/ SNF factors to activate transcription on HSP70 gene (Brown et al., 1996). Modifications of histones, such as ADP-ribosylation, acetylation, methylation, ubiquitination, and phosphorylation, occur at the N-terminus (Vaquero et al., 2003). Histone acetylation is important for active transcription status and breaks down inter-nucleosome bonds and destabilizes chromatin structure, allowing for access to transcriptional activators.

I.6.2 Transcription regulation

Regulatory proteins (repressors and enhancers) bind to specific DNA element upstream or downstream of a specific gene promoter and allow the individual genes to be turned on or off specifically. Transcriptional regulators have DNA binding motifs which allow them to bind to the DNA. One of the first DNA-binding motifs to be recognized was the Helix-

Turn-Helix. It is constructed from two α-helices connected by short extended chain of amino acids. The two helices are held in a fixed angle which allows one α helix to lie in the wide (major) groove of DNA, while the other lies at an angle across DNA, the latest part (N-terminal) help the former (recognition helix) to lodge into major groove of DNA. The zinc finger proteins contain a zinc finger in which two Cys and two His bind to the central zinc and make a bridge between one end of the helix and one end of the β sheet (Lee, 1989). Leucine zipper motif consists of a stretch of amino acids with a leucine residue in every seventh position. Two α helical DNA-binding domain dimerize through their helical leucine zipper to form a Y-shape structure. Helix-Loop-Helix (HLH) motif makes of a short α helix which connected to a larger α helix by a loop. The flexibility of the loop allows the protein to dimerize with another protein (homodimerization or heterodimerization).

Transcription enhancers promote the transcriptional activity by acting directly on general transcriptional machinery or by changing the chromatin structure. Upon binding to their DNA elements, transcription activators attract and position the general transcription factors and RNA polymerase. They also can modify local chromatin structure by recruiting remodeling enzymes such as HATs (histone acetyl transferases) which increase accessibility of the DNA and facilitate assembly of the general transcription factors and RNA polymerase. In contrast to the transcription enhancers, transcription repressors act through different mechanisms. They can compete with gene activator for binding to the same DNA sequence, or mask the enhancer element, or may directly interact with the general transcription factor assembly. Transcriptional repressors are able to remodel

chromatin structure by recruiting histone deacetyl transferases (HDACs) to prevent accessibility of DNA to general transcription factors and RNA polymerase.

I.6.3 5-HT_{1A} receptor transcriptional regulation

5-HT_{1A} receptors play key roles in the serotonergic system. They control firing of 5-HTergic neuronal cells as an autoreceptor, and mediate serotonin response in several target brain structures such as hippocampus, frontal cortex, and other areas of the limbic system. Understanding the mechanisms that regulate the expression of 5-HT_{1A} receptors will provide more insight to better understand the mechanisms of antidepressant action and the role of environmental factors on 5-HT_{1A} receptor expression.

Studies on the 5'-flanking region of 5-HT_{1A} gene revealed DNA motifs for binding of MAZ1/SP1, and NFκB transcription factors that drive transcription initiation from a TATA-less CG-rich promoter and enhance transcription of the human and mouse 5-HT_{1A} receptor genes (Parks and Shenk, 1996b). Further studies of the rat 5-HT_{1A} gene showed a single transcriptional initiation site that is located -967 bp upstream of translational start site. Transcription of the rat 5-HT_{1A} promoter is driven via a TATA box (Storring et al., 1999). Moreover, 5-HT_{1A} transcriptional activity is negatively regulated by a repressor region which is located at -1590 /-1519 bp from the translational start site (Ou et al., 2000b). Further studies identified a 31-bp dual repressor element (DRE) in that segment which strongly inhibits transcriptional activity of the 5-HT_{1A} gene in either neuronal or non-neuronal cell lines. Incubation of rat raphe RN46A cells nuclear extract with 31-bp probe showed a single protein-DNA complex formation which is blocked upon mutation in the first 14-bp segment, but not the adjacent 12-bp element (Ou et al., 2000b),

suggesting that in presynaptic 5-HT_{1A}-expressing cells the 14-bp element is the main site for protein-DNA interaction. However, incubation of L6 myoblast nuclear extract with 31-bp DRE probe revealed two DNA-protein complexes that were competed with 12+14 bp cold probe. Studies of transcriptional activity using 5-HT_{1A} promoter-luciferase constructs showed that mutation of the 14-bp element was sufficient to completely de-repress transcription in RN46A raphe cells, but in L6 myoblast cells, mutation of both 14- and 12-bp sites was required. These studies indicate that the protein that binds to the 14-bp segment is sufficient to repress the 5-HT_{1A} receptor gene in presynaptic 5-HT_{1A} expressing cells, while in other cells at least two proteins are involved.

More recent studies identified Freud-1, a novel transcription factor, as the repressor of 5-HT_{1A} receptor in neuronal cells that binds to the 14-bp segment (Ou et al., 2003b). Freud-1/CC2D1A contains four DM-14 domains of unknown function, a helix-loop-helix domain and a protein kinase C C2 domain (Rogaeva, 2007). Studies have shown that Freud-1 binds to the DRE in the 5-HT_{1A} promoter and D2 receptor genes and represses their transcriptional activity to reduce their expression levels (Rogaeva, 2007; Rogaeva, 2007). Deletion of the Freud-1 binding sites derepressed the activity of either 5-HT_{1A} or D2 receptor genes. Further more studies demonstrated that Freud-1 is a basal repressor of transcription that is negatively regulated by calcium (Rogaeva, 2007). Phosphorylation of Freud-1 via calcium dependent calmodulin kinase reduces interaction of Freud-1 with its binding site (Ou et al., 2003b). Indeed, another study showed that truncated form of Freud-1 lacking the C-terminal domains (one DM-14, helix-loop-helix, and C2 domain) is linked with non-syndromic mental retardation implicating Freud-1 in nervous system

development (Basel-Vanagaite, 2006). However, the second DRE-binding protein has not yet been identified, and this was the aim of this thesis.

The existence of an RE-1 element close to the DRE also inhibited transcription of the 5-HT_{1A} receptor gene in non-neuronal cells, providing a protein-DNA interaction site for pan-neuronal repression by the silencer REST/NRSF (Schoenherr and Anderson, 1995; Lemonde et al., 2004a). The REST protein is expressed in nonneuronal cells, and prevents the inappropriate expression in these cells of several neuronal genes, such as the 5-HT_{1A} receptor. However, REST is not highly expressed in brain, hence is not likely to regulate the expression of 5-HT_{1A} receptors in brain.

I.6.4 other regulators of the 5-HT_{1A} receptor

The 5-HT1A receptor gene is a primary target for regulation by corticosteroids. It has been shown that corticosterone down-regulates expression of the 5-HT_{1A} receptor in the hippocampus (Zhong and Ciaranello, 1995; Meijer et al., 2000). Thus adrenalectomy increased hippocampal 5-HT_{1A} mRNA expression and chronic stress as well corticosterone administration decreased 5-HT_{1A} RNA expression (Mendelson and McEwen, 1991; Meijer and de Kloet, 1994; Flugge, 1995; Meijer et al., 1997; Lopez et al., 1998). This effect is mediated via mineralocorticoid (MR) receptor or heterodimerization of MR and GR on nMRE/ GRE elements (Meijer et al., 2000; Ou et al., 2001). It has also been demonstrated that glucocorticoids may interfere with the action of transcriptional activator factors such as AP1 and NF-κB and prevent them from activating gene expression (Jonat et al., 1990; Yang-Yen et al., 1990; Ray and Prefontaine, 1994).

Most antidepressants such as SSRIs, MAOI, and TCA are proposed to increase the activity of serotonin neurotransmission. Inhibition of the 5-HT transporter by the SSRIs is sufficient to induce therapeutic effects in treatment of anxiety, bulimia, depression and other forms of mood disorders (Pineyro and Blier, 1999; Coyle and Duman, 2003). SSRIs crossed the blood brain barrier and block 5-HTT resulting in immediate increases in 5-HT level. To produce clinical relief, chronic treatment with SSRIs for at least 2-3 weeks is required. This delayed response is proposed to be enough to down-regulate the 5-HT_{1A} autoreceptor in the raphe (Albert et al., 1996; Pineyro and Blier, 1999), and restore the firing rate of serotonergic neurons in raphe (Stahl, 1998; Hjorth et al., 2000). In line with this hypothesis 5-HTT -/- mice displayed desensitization of pre but not postsynaptic 5-HT1A receptors without changing G-protein couplin(Li et al., 2000; Mannoury la Cour et al., 2001). Thus, desensitization of 5-HT_{1A} receptor is also another important regulator of the available amount of 5-HT_{1A} on cell surface.

Desensitization is a process through which response to stimuli reduces after repeated stimulation by agonist. Upon activation of 5-HT_{1A} receptors by agonist (serotonin), receptor uncoupling happens within seconds. Uncoupling blocks receptor signaling via receptor phosphorylation by G-protein coupled receptor kinase (GRK), and PKA as well as PKC (Albert and Lemonde, 2004). This process is followed by removal of the receptor from the cell surface through clathrin-coated vesicle formation (Shenoy and Lefkowitz, 2003). The internalized receptor may undergo ubiquitinylation, which leads to degradation of the receptor in lysosomes. Internalized receptors can also recycle to the membrane following its dephosphorylation upon removal of agonist (Albert and Lemonde, 2004) (Fig-4).

Depression is twice more common in women than men, indicating a sex difference exists in the vulnerability of an individual to depression. Ovarectomized female rats receiving estrogen alone or in combination with progesterone showed tissues-specific changes in 5-HT_{1A} mRNA expression (Birzniece et al., 2001). Two weeks treatment with Estrogen alone after reduced 5-HT_{1A} mRNA in the dentate gyrus, whereas the combination of estradiol with progesterone increased 5-HT_{1A} mRNA in CA1 and CA2 subregion of dorsal hippocampus. The estrogen/progesterone combination also decreased presynaptic 5-HT_{1A} mRNA in ventrolateral part of dorsal raphe, suggesting that ovarian hormones regulate presynaptic 5-HT_{1A} autoreceptor expression and may affect their signaling pathways (Osterlund et al., 2000; Birzniece et al., 2001; Mize and Alper, 2002).

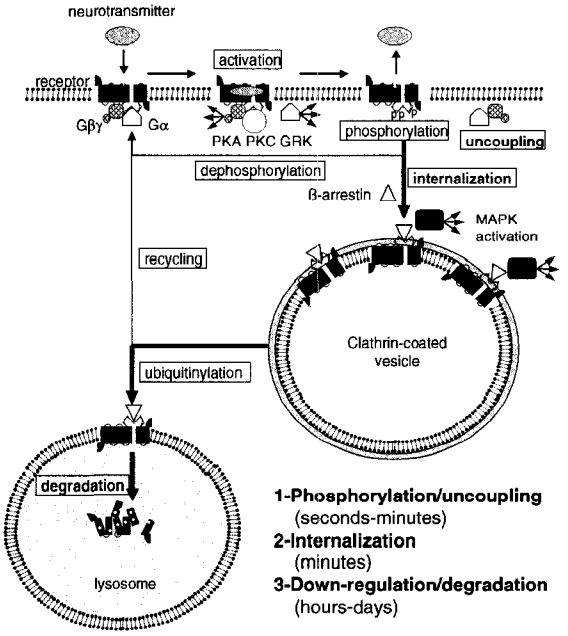
neurotransmitter
receptor
activation
Gβγ
Gα
PKA PKC GRK
phosphorylation
uncoupling
dephosphorylation
internalization
ß-arrestin
MAPK
activation
recycling
Clathrin-coated
vesicle
ubiquitinylation
degradation
lysosome
1-Phosphorylation/uncoupling
(seconds-minutes)
2-Internalization
(minutes)
3-Down-regulation/degradation
(hours-days)

Fig-I-4

Fig I-4- Desensitization of 5-HT_{1A} receptor- Activation of 5-HT_{1A} receptor triggers activation of intracellular signaling pathway and protein kinases. Phosphorylation of 5-HT_{1A} receptor by protein kinases such as PKA or PKC provides a binding site for β-arrestin and facilitates internalization of receptor to clathrin-coated vesicles. Internalized receptor may trigger a new signaling cascade by recruiting Src/ MAPK. Dephosphorylation of the internalized receptor sends the receptor back to the cell surface whereas; ubiquitinylation facilitates degradation of the internalized receptor by lysosomes.

Hypothesis and Approach

As discussed above, transcriptional regulation of the 5-HT_{1A} receptor gene is a key site for regulation of the entire serotonergic system since the 5-HT_{1A} receptor is expressed both as an autoreceptor to regulate serotonin neurons, and is highly expressed post-synaptically, especially in the limbic system. Several mouse models in combination with imaging and binding studies in humans implicate dysregulation of the 5-HT_{1A} receptor gene in mood disorders such as depression, anxiety, etc.

We have previously detected two DNA-protein complexes in nuclear extract of non-neuronal rat L6 cells that bind to the 31-bp-DRE. The novel repressor Freud-1 was identified in a yeast one-hybrid assay using the DRE, and shown to bind to the 5'-region including the first 14-bp segment within the 31-bp-DRE and to mediate repression of 5-HT_{1A} transcription in presynaptic raphe and neuronal cells. I hypothesized that a second protein, which binds adjacent to the 14-bp segment, represses the 5-HT_{1A} receptor gene in post-synaptic 5-HT_{1A}-expressing cells as well as in 5-HT_{1A} non-expressing cells like L6 myoblasts. The main aim of my thesis is to identify this transcription factor. After several unsuccessful yeast one-hybrid screens using the DRE, I discovered by Genbank screening a second isoform of Freud-1, which is encoded by separate gene. I therefore hypothesized that this second variant, Freud-2, regulates the 5-HT_{1A} receptor gene at the DRE, and may constitute the second protein that binds the DRE.

Chapter II- Freud-2/CC2D1B mediates dual repression of the 5-HT1A receptor gene

Freud-2/CC2D1B mediates dual repression of the 5-HT1A receptor gene.

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II-1Abstract

In the brain, the serotonin (5-HT)-1A receptor is a key presynaptic autoreceptor that regulates the activity of serotonergic neurons, and is also widely expressed as a post-synaptic receptor to mediate antidepressant action. The 5-HT1A receptor gene is strongly repressed by a dual repressor element (DRE), which is recognized by two proteins: the mental retardation gene Freud-1/CC2D1A and another unknown protein. We report characterization of mouse Freud-2/CC2D1B as the complementary repressor of the 5-HT1A DRE. Freud-2 is homologous to Freud-1 and contains conserved DM-14, HLH domain, and a conserved C2 domain. Mouse Freud-2 binds to the 31-bp-DRE of the rat 5-HT1A receptor gene at a site that is adjacent and partially overlapping to Freud-1 site. By supershift assay, Freud-2-DRE complexes were identified in nuclear extracts. Freud-2 mRNA and protein are expressed widely in brain and peripheral tissues. Freud-2 repressed transcriptional activity of 5-HT1A promoter-reporter constructs in post-synaptic cell models such as NG108-15-15 cells, but had no regulatory effects in presynaptic 5-HT1A-positive cells such as serotonergic rat RN46A cells. Knockdown of Freud-2 using a specific SiRNA reduced endogenous Freud-2 protein levels and derepressed the transcriptional activity of the 5-HT1A promoter. Taken together, these data show that Freud-2 is the second component that with Freud-1 mediates dual repression of the 5-HT1A receptor gene at the DRE. While Freud-1 regulates both pre- and post-synaptically expressed 5-HT1A receptors, Freud-2 is more selective to regulate post-synaptic 5-HT1A receptor expression.

II-2 Introduction

The serotonin (5-HT) system originates from neurons of the midbrain raphe nuclei that project widely throughout the brain (Törk, 1990) and regulate the development of anxiety, aggression and stress responses (Gordon and Hen, 2004; Lesch, 2005), and is implicated in feeding behavior, sleep disorders, and emotional function (Jacobs and Azmitia, 1992). The 5-HT1A receptor is expressed presynaptically on serotonergic raphe neurons as an autoreceptor (Sotelo et al., 1990; Riad et al., 2000) that plays a key role in a negative feedback pathway to regulate the activity of the entire serotonin system (Pineyro and Blier, 1999). The 5-HT1A receptor is also expressed post-synaptically in hippocampus, septum, hypothalamus, cortex (Albert et al., 1990; Pompeiano et al., 1992). Mice lacking 5-HT1A receptors display increased anxiety and fear behaviors, altered sleep patterns and reduced behavioral and neurogenic responses to antidepressants (Boutrel et al., 2002; Gross et al., 2002a; Santarelli et al., 2003a; Toth, 2003; Tsetsenis et al., 2007). The anxiety phenotype can be rescued by early postnatal expression of 5-HT1A receptors in the forebrain (Gross et al., 2002a), suggesting that the level of expression of 5-HT1A receptors mediates early developmental synaptogenesis that set the anxiety phenotype in the adult (Faber and Haring, 1999; Gross and Hen, 2004; Scott and Deneris, 2005; Whitaker-Azmitia, 2005; Alexandre et al., 2006).

In order to elucidate the mechanisms that regulate 5-HT1A receptor expression in pre- and post-synaptic neurons, we have characterized its promoter and transcriptional regulators (Albert and Lemonde, 2004). We have reported that expression of the rat 5-HT1A receptor gene is negatively regulated by a strong repressor element, the 31-bp dual repressor element (DRE) which resides between -1590/-1519 bp upstream of translational

start site (Ou et al., 2000b). Using the DRE as target sequence in yeast one-hybrid cloning, we identified the mental retardation gene Freud-1/CC2D1A (Ou et al., 2003a; Rogaeva et al., 2007a), which binds to the 5' 14-bp segment (FRE) within the 5-HT1A DRE and represses the 5-HT1A promoter in raphe RN46A cells. However in L6 myoblasts and other cell lines, a second protein binds to the 3' 12-bp (TRE) portion of the DRE to mediate dual repression (Ou et al., 2000a). Mutations in the 14-bp (FRE) eliminated binding of Freud-1 and de-repressed the 5-HT1A expression in raphe cells; but in L6 myoblast cells, mutation of both FRE and TRE was required to eliminate repression of 5-HT1A promoter activity. Thus, although Freud-1 regulates the basal expression of 5-HT1A receptors in raphe RN46A cells, a second unknown repressor mediates dual complementary repression of the 5-HT1A receptor gene in other cell types.

In the present study we identify the novel transcription factor Freud-2/CC2D1B, a Freud-1 homologue, as the second component that binds to the 5-HT1A DRE. Interestingly, unlike Freud-1, Freud-2 represses post-synaptic 5-HT1A receptors, but not pre-synaptic 5-HT1A receptor expression.

II-3 Materials and Methods

PCR and Plasmids. A 2.5 kb fragment of mouse Freud-2 cDNA was amplified from an NIH-3T3 cDNA library (Clontech) using specific primers:
Forward; 5'-CCGCTCGAGCGGCAGGCCCCAGGCTCCAGGACC-3';
Reverse; 5'-CCGGAATTCCGGATGCCAGGGCCAAGACCTCG-3'. PCR products were gel purified, subcloned in pGEMT-Easy vector (Promega, Madison, WI). Freud-2 expression plasmids were created by subcloning the coding sequence of mouse Freud-2

from pGEMT-Easy vector to EcoRI/XhoI site in either pcDNA3 (Invitrogen, Burlington, Ontario, Canada) or pGEX-4T-1 (Amersham Bioscience). All constructs were verified by DNA sequencing analysis.

Expression of Freud-2 protein. Transcription/translation of recombinant Freud-2 was done using EcoProT7 system (Novagen). Briefly, the desired amount of Freud-2 expression vector and vector alone as a control were combined with the EcoPro extract, methionine and water based on the manufacturer's protocol and incubated 60 min at 37°C. Expression of Freud-2 was tested by Western blot assay using specific peptide antibody against the Freud-2 protein.

Cell culture and transient transfection. L6 myoblast and NG108-15 cells were cultured and transfected as previously described (Ou et al., 2000a). Briefly, cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) contained 10% fetal calf serum at 37°C in 5% CO_2. The medium was replaced 12 hr before transfection and cells (except NG108-15) were transiently transfected by calcium phosphate coprecipitation (Charest et al., 1993) using 20 μg/plate of luciferase constructs and 10 μg/plate pCMVβgal. NG108-15 cells were transfected by Lipofectamine2000 reagent (Invitrogen) at 50-60% confluency in Primaria 6-well plates (Falcon, Franklin Lakes, NJ) with 1.5 μg plasmid/well. RN46A cells were cultured as previously described (Ou et al., 2000b). RN46A cells were transfected with 1:1.5 ratio of plasmid: Lipofectamine2000 reagent (Invitrogen) using 7.5-10 μg/plate of luciferase plasmid and equal amount of protein expression vector or empty vector with 2μg/plate pCMVβgal.

Luciferase and β-galactosidase assay. The luciferase reporter plasmid constructs have been described previously (Ou et al., 2000b). For reporter assays, triplicate samples after

48 h of transfection were washed 3 times with cold PBS and extracted with 150 µl of reporter lysis buffer (Promega). Supernatants were collected, assayed for luciferase activity using Spectramax M2 (Molecular Devices) and was measured by Softmax Pro 4.8. Activities were obtained from at least three independent experiments in which transfections were performed in triplicate and corrected for transfection efficiency by calculating the ratio of luciferase/β-galactosidase activity and normalized to vector-transfected extracts. Data are presented as mean ± SEM. Statistical significance was evaluated using two tailed unpaired *t* test.

Nuclear extracts and electrophoresis mobility shift assay (EMSA)

Nuclear proteins were extracted from L6 cells as previously described (Lemonde et al., 2004b). For EMSA, sense and antisense oligonucleotides of the 5' or 3' rat DRE with CC/GG 3'-overhang were hybridized and labelled with [α-^{32}P]-dCTP using Klenow fragment DNA polymerase (Ou et al., 2000b). Labelled probe was incubated with L6 protein (60 µg/reaction or in vitro transcribed mouse Freud-2 or vector as control, with or without competitor DNA in 25µl reaction containing gel shift DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2mM EGTA, 100mM KCl, 5% glycerol, and 2mM DTT, pH 7.9) and 2µg poly (d (I-C)) at room temperature.

Unlabelled-double-stranded 31-bp DRE as well as 12-bp and 14-bp segments of 31-bp-DRE (Table-1) used as a competitor. For supershift assay, polyclonal rabbit anti-Freud-2 antibody was purified using Montage antibody purification PROSEP-G spin column (Millipore). 3 µl of purified serum antibody against C-terminal of Freud-2 (CDGRKPTGGKLF) was used in 25 µl reaction and incubated 20 min at 37 °C. [^{32}P]-labelled 31-DRE probe (60,000-100,000) was added and incubated for more 20 min at

room temperature. The DNA/protein complexes were separated on a 5% polyacrylamide gel at 4°C, gel dried and exposed to film overnight at 80°C with an intensifying screen.

SiRNA.transfection.

Stealth SiRNA targeting human Freud-2 [CC2D1BHSS153336] (5'-cccugcagcagaggcugaacaagua-3') and stealth RNAi negative control duplexes (Invitrogen) were purchased. NG108-15 cells were transfected using Lipofectamine2000 (Invitrogen) with a final SiRNA concentration of 100 nM. Transfection efficiency control was performed with Block-iT^{TM} fluorescent oligo (Invitrogen). For luciferase assay, 5 μl specific Freud-2-SiRNA (CC2D1B-SiRNA) or RNAi negative control (CG scrambled) were co-transfected with 1.5μg of rat 5-HT1A luciferase construct (5-HT1A) in NG108-15 cells and incubated for 72 hr. Luciferase activity was normalized to that β-galactosidase and normalized to control. All data are presented as the mean ± SEM of at least three independent experiments.

Northern blot and Western blot analyses.

Mouse Multiple Tissues Northern blot (MTN) was purchased (Clontech Laboratories, Inc). It was probed with 800 bp mouse Freud-2 using Strip EZ DNA kit (Ambion). Northern blot assay was done as described previously (Mao et al., 2004). For Western blot analysis tissues were dissected from male C57BL6 mouse and homogenized in homogenization buffer (10mM Tris, 150mM $NaCl_2$, 2mM $MgCl_2$, 1 mM protease inhibitor) on ice. Homogenized tissues were filtered and centrifuged at 4°C for 5 min at 200g followed by adding 2% SDS and 1% NP-40. Samples were sonicated on ice (3 times, PW 3, 10 sec, and 10 sec off) and centrifuged at 10,000g for 10 min at 4 °C. The supernatant was transferred to new tubes and centrifuged at 10,000g for 15 min at 4°C. Supernatants were transferred to new tubes and kept at -80°C. 60 μg of extracts were loaded on 8% SDS-gel, electrophoresed and blotted onto Nitrocellulose membrane. The membrane was incubated in 5% Western blocking reagent (Roche) in 1xTBS at 4°C over night, and then incubated with streptavidin 1:5000 for 10 min at room temperature, washed 3x in wash solution (0.1% Tween in PBS) incubated with biotin 1:1000, 10 min at room temperature, and washed 3x in wash solution. The blot was incubated with 1:2000 C-HF2 serum polyclonal antibodies over night at 4°C. Next day after 3x wash it was incubated with biotinylated peptide 1:6000 30 min at room temperature. After 3x wash with wash solution, the membrane was incubated with 1:10,000 HRP-streptavidin as a secondary antibody for 30 min at room temperature, washed and the reactive bands were visualized using the ECL-kit (Amersham) after exposure to film.

II-4 Results

II-4.1 Freud-2: Molecular cloning and domains

To identify the second protein that binds the 5-HT1A DRE, we repeated yeast one hybrid screening but did not identify any new positive clones. However, screening of the Genbank database for Freud-1 homologues using Blast2 sequence search engine identified the Freud-2 protein (Fig.1). The Freud-2 cDNA encodes an 812-aa protein. Freud-2 contains a number of conserved domains such as four DM-14 domains, a helix-loop-helix (HLH) domain, and a protein kinase C conserved domain (C2) (Fig.1). Mouse Freud-2 has 50% amino acid identity to mouse long form Freud-1 and 80% amino acid identity to human Freud-2, with highest similarity within the conserved domains. The C2 domain (protein kinase C conserved region 2 (CalB), mediates calcium-dependent lipid binding of PKC, phospolipases, and synaptotagmin and protein-protein interactions (Nalefski and Falke, 1996; Sondermann and Kuriyan, 2005). The DM-14 is characteristic of the Freud family, but its function is not yet clear (Rogaeva et al., 2007a). Freud-2 also contains a novel Lck/Src tyrosine kinase site (Y620), and a strong PKC site (S787) which is not present in Freud-1 protein.

31-bp DRE	5'-	CGGCATAAGCAAGCCCTTATTGCACAGAGCT-
14-bp	5'-	GGCATAAGCAAGCC-
12-bp	5'-	GCCCTTATTGCA-

Table-1

Table-1 DNA Sequence of probe and cold competitors

Shown are oligonucleotides of the rat 31-bp-DRE probe, 14-bp, and 12-bp segments that used as competitors in an electomobility shift assay (EMSA).

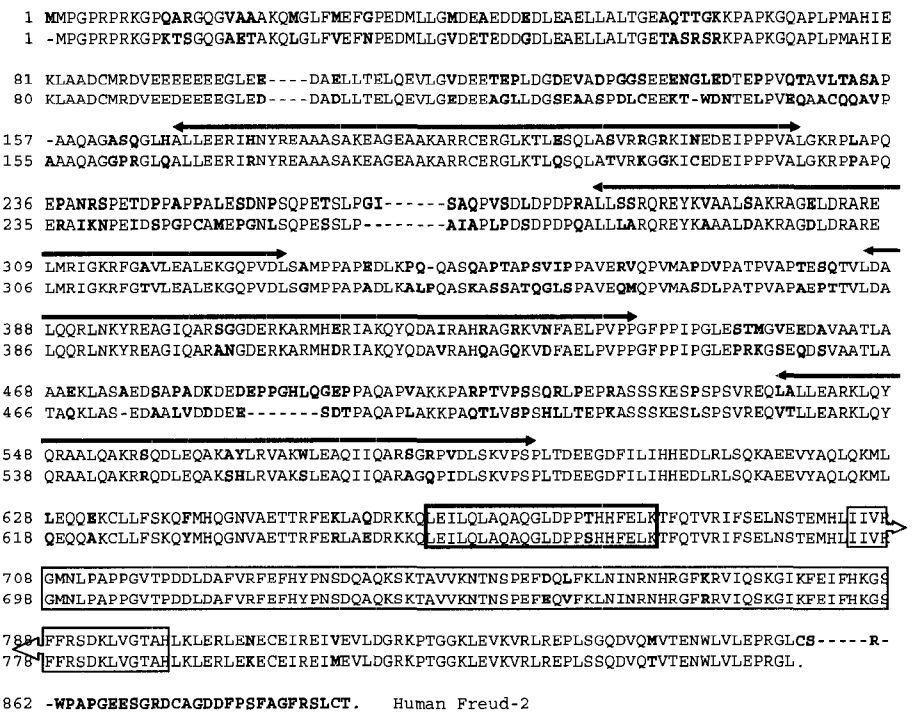

Fig. II-1

Fig.II- 1. Alignment of human and mouse Freud-2

Shown is the amino acid alignment of human (upper sequence) and mouse (lower sequence) Freud-2, with mismatched amino acids in bold. Red solid lines represent the DM-14 domain and the boxes show the conserved HLH and C2 domains. Domains were identified by NCBI blast (http;//www.ncbi.nlm.nih.gov/BLAST) conserved domain alignment. The HLH domain was identified by HELIXTURNHELIX.

II-4.2 Freud-2 binding to the rat 5-HT1A 31-bp DRE

To examine the binding of Freud-2 to 5-HT1A 31-bp DRE, we incubated *in vitro* transcriptional translated GST-Freud-2 cDNA or GST vector control with labeled DRE (Fig.2a). A single specific protein-DNA complex was detected with GST-Freud-2 but not GST, which was competed by unlabelled DRE oligonucleotides (lane 2, 3). This complex was also competed by either 50- or 100-fold molar excess of unlabelled 12-bp (TRE) oligonucleotides (lanes 4, 5) but not the 14-bp FRE primers (lanes 6, 7), suggesting that Freud-2 specifically binds to 12-bp (TRE) portion of 5-HT1A-DRE. Anti-GST antibody did not shift the band in this assay. We further examined whether Freud-2 is the second protein that can bind to 5-HT1A DRE in non-neuronal cells. Nuclear extract from L6 myoblasts was incubated with labeled 5-HT1A-DRE in the presence or absence of specific Freud-2 antibody in a band shift assay (Fig.2b). Two bands were detected. As reported previously Freud-1 formed the lower protein-DNA complex (Ou et al., 2000a; Ou et al., 2003a). In the presence of the specific anti-Freud-2 antibody only the upper band was super-shifted, indicating that Freud-2 is the second protein that binds to 5-HT1A 31-bp DRE, recognizing the 12-bp TRE segment.

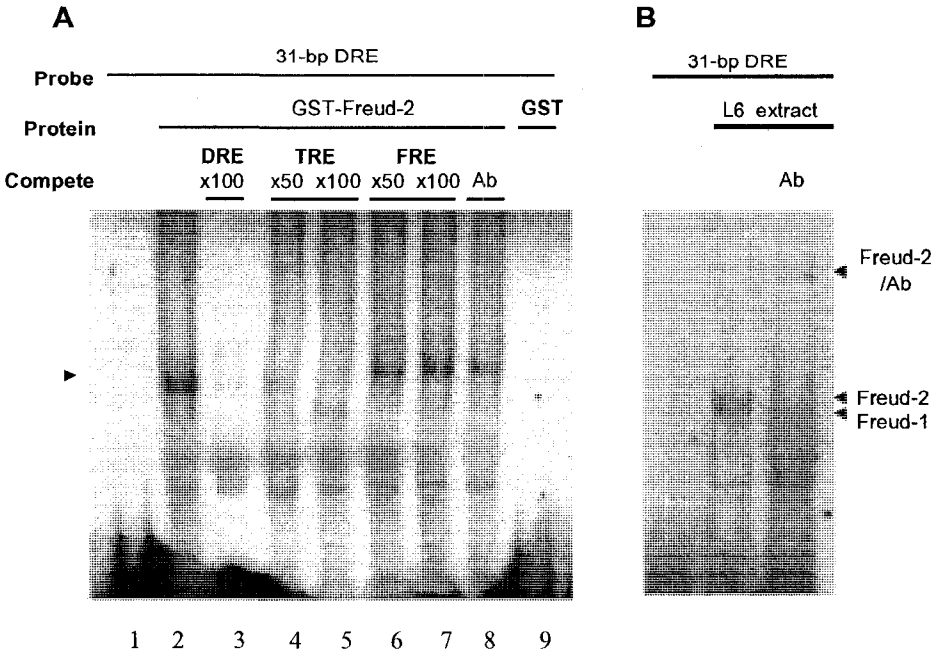
A
Probe
31-bp DRE
Protein
GST-Freud-2
GST
Compete
DRE
x100
TRE
x50 x100
FRE
x50 x100
Ab
1 2 3 4 5 6 7 8 9
B
31-bp DRE
L6 extract
Ab
Freud-2
/Ab
Freud-2
Freud-1

Fig. II-2

Fig.II-2 Specific binding of Freud-2 to the 5-HT1A-TRE.

A) Direct binding of recombinant Freud-2 to the 5-HT1A DRE. [^{32}P]-labeled 5-HT1A-DRE (31-bp DRE) as probe was incubated with in vitro transcribed/translated GST-Freud-2 or GST vector as control. A single specific band (arrowhead) was detected with GST-Freud-2. Binding of Freud-2 to 31-DRE was abolished in the presence of 50- or 100- molar excess of unlabelled DRE or 12-bp TRE (3' portion of 5-HT1A-DRE) but not 14-bp FRE (5'-portion of DRE). B) Freud-2 is the second DRE-protein complex in L6 cells. L6 nuclear extracts were incubated with [^{32}P]-labeled 31-bp 5-HT1A DRE oligonucleotides and two protein-DNA complexes were observed: the lower one represents Freud-1 (shown previously) and the upper complex contains Freud-2 (arrowhead) as shown by specific supershift of that complex after incubation with specific antibody against Freud-2 (Freud-2/Ab arrowhead).

II-4.3 Freud-2 protein and mRNA distribution

To address the distribution of Freud-2 in tissues, we examined the RNA and protein expression profile of Freud-2 using Northern blot assay (Fig. 3A) and Western blot analysis (Fig. 3B). Freud-2 mRNA expression profile was examined by Northern blot assay (Fig. 4). Freud-2 mRNA was ubiquitously expressed in rat brain and peripheral tissues, with highest levels in testes and kidney (Fig. 3A). Freud-2 protein was detected using a rabbit polyclonal antibody developed against C-terminal of Freud-2 that does not cross-react with Freud-1 (data not shown). Freud-2 protein was detected as a 120-kDa species in all brain regions examined, including hippocampus, hypothalamus, as well as in the midbrain (Fig.3B). A low molecular weight species of 70-kDa was also detected mainly in hippocampus. These species are consistent with long and short isoforms of Freud-2 protein predicted from cDNA species that differ by use of alternative translation initiation sites, analogous to isoforms identified for Freud-1 (Rogaeva and Albert, 2007). Taken together, the pattern of Freud-2 RNA and protein distribution shows a central and peripheral distribution in mouse, consistent with a general role for Freud-2 in repressing 5-HT1A receptors in both neuronal and non-neuronal cells.

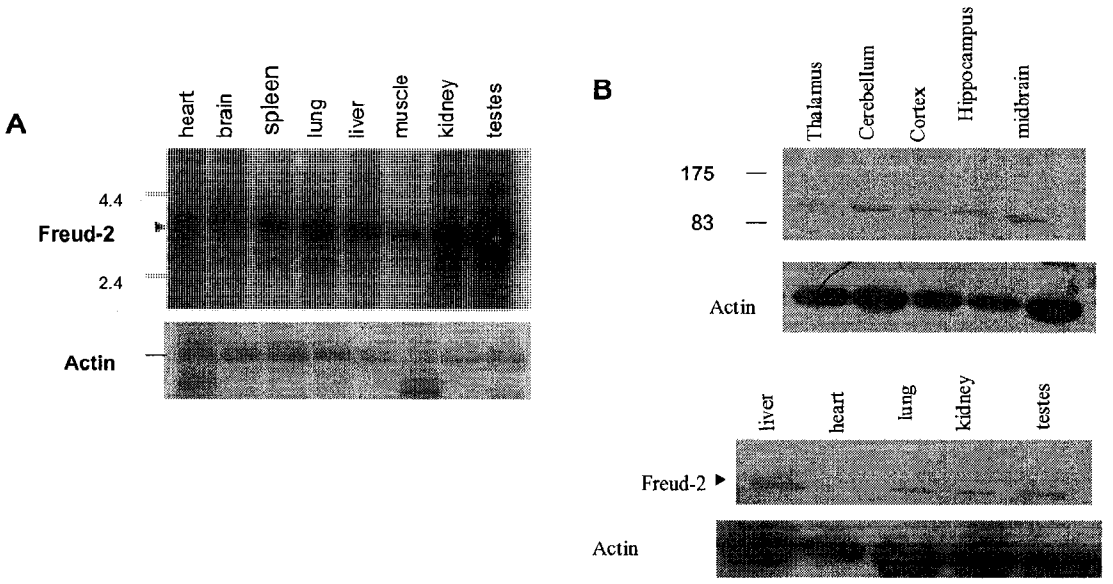
A
heart
brain
spleen
lung
liver
muscle
kidney
testes
4.4
Freud-2
2.4
Actin
B
Thalamus
Cerebellum
Cortex
Hippocampus
midbrain
175
83
Actin
liver
heart
lung
kidney
testes
Freud-2
Actin

Fig.II-3

Fig. II-3. Tissue distribution of Freud2 RNA and protein expression

A) Freud-2 mRNA expressions in rat tissues. RNA prepared from indicated rat tissues was used in Northern blot analysis and hybridized to the mouse Freud-2 cDNA probe. An arrow indicates Freud-2 RNA hybridization, which migrated with approximate size of 4-kb. The blot was reprobed with beta-actin cDNA as a loading control. B) Freud-2 protein expression in mouse brain. Various region of mouse brain were homogenized and subjected to Western blot analysis. Freud-2 protein expression (arrow head) was detected by specific anti-Freud-2 antibody.

II-4.4 Freud-2 repression of post-synaptic 5-HT1A receptor expression

Transcriptional activity of Freud-2 was compared in a 5-HT1A-positive presynaptic model cell line (RN46A), post-synaptic 5-HT1A receptor expressing model cells (NG108-15), and non-neuronal 5-HT1A-negative L6 myoblasts (Storring et al., 1999; Ou et al., 2000a; Czesak et al., 2006). L6 cells were cotransfected with Freud-2 or vector and 5-HT1A promoter-luciferase reporter constructs containing 2300-bp of upstream sequence flanking the rat 5-HT1A receptor initation of translation and including the DRE (-1550/-1519) (Fig. 4). These reporter constructs included the wild-type sequence (5-HT1A) or the corresponding 5-HT1A-DRE mutants m1, m2, and m3, which lack the FRE site, TRE site, or both FRE and TRE sites (Ou et al., 2000a). Freud-2 significantly repressed the activity of the wild-type 5-HT1A construct in L6 cells compared to control. Mutation in either 14-bp FRE that recognizes Freud-1 or of the 12-bp TRE segment of 5-HT1A DRE did not block Freud-2-induced repression (Fig.5). However, mutation of both FRE and TRE (m3 construct) resulted in strong de-repression of the basal activity of the 5-HT1A promoter, similar to the de-repression observed previously upon deletion of the entire DRE (Ou et al., 2000b). These results are consistent with our previous data (Ou et al., 2000b), suggesting that in non-expressing 5-HT1A receptor cells existence of either Freud-2 or Freud-1 could suppress the transcription of the 5-HT1A receptor gene. The lack of effect of the m2 mutant on Freud-2 repression suggests that additional DNA sequences participate in Freud-2 binding and repression.

We further investigated Freud-2 activity in 5-HT1A-positive NG108-15 neuroblastoma x glioma hybrid cells (post-synaptic model) or raphe RN46A serotonergic cells (presynaptic model). Freud-2 significantly repressed the activity of 5-HT1A

promoter construct in NG108-15(Fig. 5), and as observed in L6 cells, Freud-2 induced repression was not blocked in the m2 mutant of the TRE site. Similarly, the m3 mutant de-repressed basal 5-HT1A promoter activity, and Freud-2 repression was blocked and even enhanced 5-HT1A activity. These data suggest that Freud-2 may bind to the TRE and partial sequence in the FRE that overlaps with the Freud-1 site. In RN46A cells, Freud-2 lacked repressor activity at 5-HT1A reporter construct. There was a strong basal repression of the 5-HT1A promoter (mediated by Freud-1) that was derepressed by mutation of FRE (not shown) or both FRE and TRE (m3), but not TRE alone (m2) in these presynaptic 5-HT1A expressing cells (Fig. 6). These data suggest that Freud-2 is more active in repressing 5-HT1A transcriptional activity in post-synaptic 5-HT1A expressing cells, consistent with our previous data showing that in RN46A cells Freud-1-DRE complexes are detected, but not Freud-2-DRE complexes (Ou et al., 2000b).

To further address the role of endogenous Freud-2 to regulate 5-HT1A transcription, we knocked down the expression of Freud-2 by using specific SiRNA (CC2D1B) against human Freud-2. First we examined the effect of different amounts and types of SiRNA in L6 cells (Fig. 7A). Maximal depletion was obtained using 5 ul of siRNA Si-36, resulting in a 30% reduction in Freud-2 protein. We next co-transfected NG108-15 cells with the 5-HT1A promoter construct and SiRNA-CC2D1B (Si-36) scrambled Si-CG as a negative control, or without SiRNA (Fig. 7B). In the presence of CC2D1B-SiRNA, 5-HT1A promoter activity was significantly enhanced compared to control, indicating that reduction of Freud-2 protein levels de-represses the 5-HT1A promoter. Taken together these experiments have shown that Freud-2 is a transcription

factor which negatively regulates the expression of the 5-HT1A receptor gene, particularly in post-synaptic 5-HT1A expressing cells.

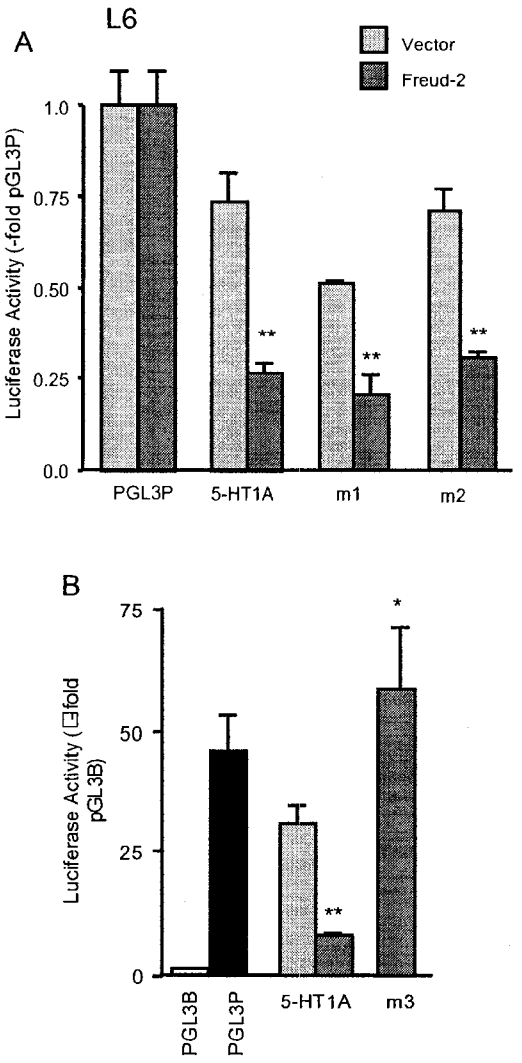

Fig II-4

Fig.II- 4. Repression of the rat 5-HT1A receptor gene by Freud-2 protein in L6 myoblast cells.

A, B) Freud-2 repression at the 5-HT1A DRE. The DRE-containing -2300 bp rat 5-HT1A promoter luciferase reporter pGL3B construct (5-HT1A), or inactivating mutations of the 14-bp FRE (m1), 12-bp TRE (m2), or double mutant of FRE and TRE (m3) were transiently transfected in L6 myoblast cells in the present of vector (pcDNA3) or Freud-2, relative luciferase activity was measured and normalized to pGL3P (P) SV40 positive control (A) or luciferase vector pGL3B (B). Freud-2 significantly repressed the transcriptional activity of 5-HT1A receptor gene, and this suppression was only eliminated in the present of FRE+TRE-mutant (m3). Variation in transfection efficiency was corrected by cotransfection of a β-galactosidase plasmid with each construct. Data represent the mean ± SEM of three independent experiments. $^{**}P < 0.005$ compared with vector-transfected and $^{*}P < 0.05$ (m3 vs. 5-HT1A) by *t*-test.

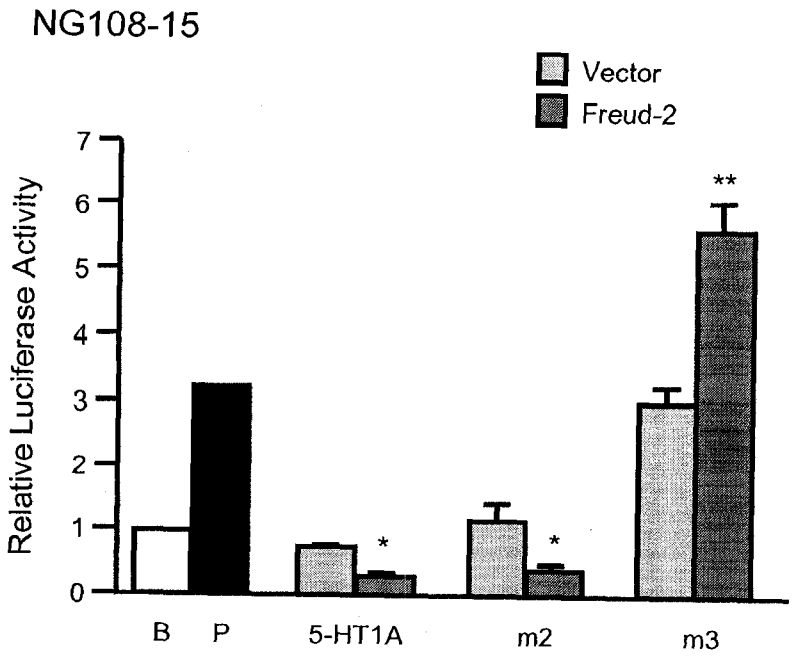
NG108-15
Vector
Freud-2
Relative Luciferase Activity
0
1
2
3
4
5
6
7
*
*
**
B
P
5-HT1A
m2
m3

Fig-II.5

Fig.II- 5. Repression of 5-HT1A receptor gene by Freud-2 in NG-108 neuroglioma cell line.

The 2300-bp 5-HT1A promoter luciferase construct (5-HT1A), m2 (TRE mutant) and m3 (double FRE/TRE mutant) were transiently transfected with vector (pcDNA3) or Freud-2 in NG108-15 cells. Luciferase activity was normalized to pGL3B (luciferase vector). Freud-2 repressed the 5-HT1A and m2 promoter constructs. Note that the 5-HT1A receptor promoter was derepressed in the m3 double mutant and that Freud-2 further de-repressed its activity. Data represent the mean ± SEM of three independent experiments; $^{**}P < 0.005$, $^{*}P < 0.05$ compared with vector-transfected by *t*-test.

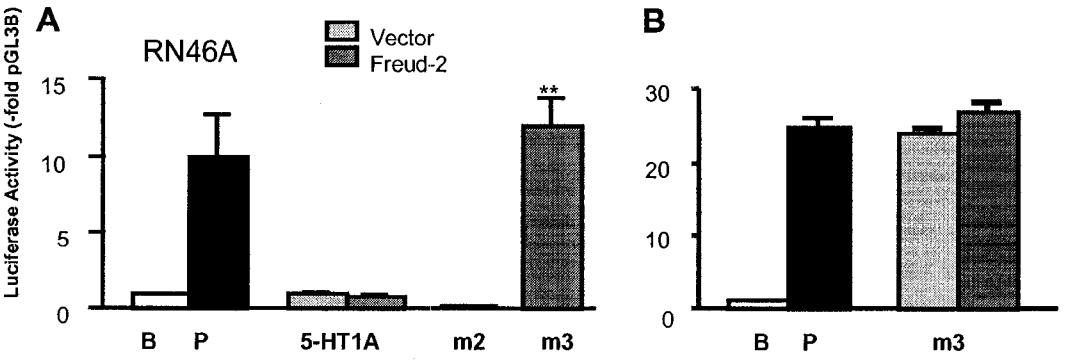
A
RN46A
Vector
Freud-2
Luciferase Activity (-fold pGL3B)
15
10
5
0
**
B
P
5-HT1A
m2
m3
B
30
20
10
0
B
P
m3

Fig.II-6

Fig.II- 6. Lack of Freud-2 repression in RN46A raphe cells.

The 2300-bp 5-HT1A, m2 (TRE mutant), and m3 (double FRE/TRE mutant) reporter constructs were transfected in RN46A raphe cells with vector or Freud-2 expression plasmids. Luciferase activity was normalized to pGL3B. Freud-2 did not show any activity in raphe RN46A cells. 5-HT1A receptor gene was completely de-repressed in the present of 31-DRE double mutation. Data represent the mean ± SEM of three independent experiments.

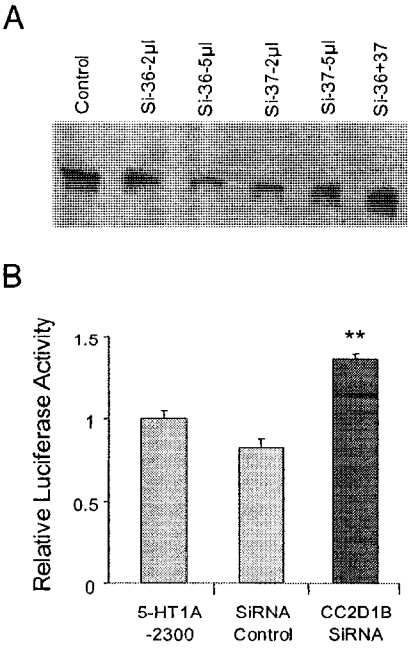
A
Control
Si-36-2μl
Si-36-5μl
Si-37-2μl
Si-37-5μl
Si-36+37
B
Relative Luciferase Activity
1.5
1
0.5
0
**
5-HT1A -2300
SiRNA Control
CC2D1B SiRNA

Fig.II- 7

Fig.II- 7. Freud-2 depletion by SiRNA derepresses the 5-HT1A promoter in NG-108 neuroglioma cells.

A) Depletion of Freud-2 by SiRNA. Two different specific human Freud-2 SiRNAs (36-37) were examined in L6 myoblast cells. Cells were transfected with CG-scrambled SiRNA (control) or Freud-2 SiRNA (36-37) or combination of both SiRNAs (36+37) with 2μl or 5μl of each, and the expression of Freud-2 was detected using a specific anti-Freud-2 antibody in Western blot assay. 1.5 μg rat -2300 5-HT1A luciferase construct was transfected alone (5-HT1A) or co-transfected with 5 μl of specific Freud-2 SiRNA36 (CC2D1B-SiRNA) or CG-scrambled SiRNA (control) in NG-108 cells and incubated for 72 hr. Luciferase activity is expressed relative to 5-HT1A reporter alone. Data represent the mean ± SEM of three independent experiments. ** $P< 0.005$ in comparison to control (5-HT1A).

II-5 Discussion

5-HT1A receptors play key roles in serotonergic signaling as autoreceptors in dorsal raphe nuclei (Sotelo et al., 1990; Riad et al., 2000) and as post-synaptic receptors present on pyramidal and interneurons of cortex (Aznar et al., 2003; Palchaudhuri and Flugge, 2005) as well as in the septum, hippocampus, and hypothalamus (Albert et al., 1990; Pompeiano et al., 1992). Recent studies of 5-HT1A gene knockout or transgenic animals clearly indicate a role for regulation of 5-HT1A receptor expression in the etiology and treatment of anxiety and depression phenotypes in mice. Knockout of the 5-HT1A receptor gene results in an anxiety phenotype in several mouse strain backgrounds (Toth, 2003), while transgenic over expression of the receptor results in reduced anxiety behavior (Kusserow et al., 2004). Rescue of the anxiety phenotype in mice required early post-natal induction of forebrain 5-HT1A receptor expression, while rescue of receptor expression after post-natal day 21 failed to rescue the anxiety phenotype (Gross et al., 2002a). These studies indicate that the level of expression of 5-HT1A receptors, particularly during development, is a key determinant of the anxiety phenotype in mice. Hence we have addressed the specific DNA elements and transcription factors that regulate 5-HT1A receptor expression.

II-5.1 Freud-2/CC2D1B: a novel repressor of post synaptic 5-HT1A receptor gene

We have used a 5'-deletion approach to map the 5-HT1A promoter, and identified the DRE as a strong, conserved repressor element that when deleted leads to 10-fold induction of 5-HT1A promoter activity in neuronal and non-neuronal cells (Storring et al., 1999; Ou et al., 2000a). Freud-1 was shown to bind to and regulate 5-HT1A receptor

expression at the 5' portion of the DRE (FRE) in a variety of cells, including in 5-HT1A-expressing RN46A cells, a presynaptic model. In this study we identified Freud-2/CC2D1B as a repressor of 5-HT1A receptor transcription via binding to the 3' portion of the 5-HT1A-DRE (TRE). Although Freud-2 specifically bound to the TRE (Fig. 2), mutational inactivation of the TRE (Ou et al., 2000a) did not affect Freud-2 repression, but the combined mutation of both FRE and TRE did. This could indicate that Freud-2 binding includes FRE sequence, or that the presence of Freud-1 bound to the FRE stabilizes Freud-2 binding. However, in L6 cell extracts that contain both Freud-1 and Freud-2, we observed distinct Freud-1-DRE (Ou et al., 2003a) and Freud-2-DRE (Fig. 2B) complexes, with no evidence for a third complex containing both. Thus Freud-2 appears to recognize the TRE and a part of the FRE to repress the activity of the 5-HT1A promoter.

Freud-2 repressed the 5-HT1A receptor gene in either 5-HT1A receptor-negative L6 cells or post-synaptic 5-HT1A-expressing NG108-15 cells, but not in presynaptic raphe RN46A cells (Fig. 4-6). We previously found that Freud-1 represses pre-synaptic 5-HT1A expression in RN46A cells, and that specific depletion of Freud-1 using transfection of antisense Freud-1 cDNA de-repressed the 5-HT1A promoter to increase 5-HT1A receptor expression in RN46A cells but not L6 cells (Ou et al., 2003a). Thus Freud-1 is a primary determinant of basal 5-HT1A receptor expression in RN46A cells, while Freud-2 is inactive in these cells. In L6 cells, both Freud-1 and Freud-2 repressed the 5-HT1A promoter. In post-synaptic 5-HT1A-positive NG108-15 cells, Freud-2 appear to play an important role in regulating basal 5-HT1A receptor expression, since depletion of Freud-2 using siRNA de-repressed the 5-HT1A promoter to a modest extent.

The limited de-repression in Freud-2 SiRNA treated cells may reflect repression by Freud-1 and by the remaining Freud-2 protein. Thus, Freud-2 has preferential activity in post-synaptic 5-HT1A receptor-expressing cell models, consistent with our finding that Freud-2 RNA is not expressed in human dorsal raphe nucleus as visualized by immunohistochemistry and Western blot of post mortem brain slices (Hadjighassem et al., manuscript submitted). By contrast, Freud-1 was expressed in serotonergic raphe neurons colocalized with 5-HT and 5-HT1A receptor immunostaining (Ou et al., 2003a).

Freud-2 RNA and protein was detected in a variety of tissues, indicating a ubiquitous role for Freud-2 in repression of 5-HT1A receptors in neuronal and non-neuronal tissues. Similarly, Freud-1 is also widely expressed. Thus, the combination of Freud-1 and Freud-2 provides a redundant dual mechanism for regulating the expression of 5-HT1A receptors. In 5-HT1A receptor negative tissue these repressors may play an important role in silencing the receptor, in concert with REST/NRSF, which also represses the 5-HT1A promoter at a DNA element located immediately 3' of the DRE (Lemonde et al., 2004a). In 5-HT1A positive neurons, both Freud-1 and Freud-2 repress, but Freud-2 is more active at post-synaptic cells, while Freud-1 is more active at presynaptic cells. The presence of two different protein isoforms of Freud-2 is not unexpected; for Freud-1 we demonstrated that both short and long isoforms are functional repressors of the 5-HT1A receptor gene (Ou et al., 2003a; Rogaeva and Albert, 2007). The short form starts at a downstream ATG site, and lacks two DM-14 domains, but retains the critical C-terminal HLH and C2 domains. Freud-2 short form also retains these C-terminal domains, and is likely functional, although the major isoform observed was the long isoform.

II-5.2 Potential roles of Freud-2 in vivo.

As a homologue of Freud-1, Freud-2 complements Freud-1 action to repress the 5-HT1A promoter via an adjacent, partly overlapping DNA element within the DRE, as described above. The strong conservation of these proteins in critical domains (Fig. 1) suggests conserved roles as transcriptional regulators of common gene targets. In particular, the C2 domain of Freud-1 is essential to mediate its transcriptional repression and participates in DNA interactions (Ou et al., 2003a), and is highly conserved in Freud-2 (Fig. 1). We recently found that Freud-1 is a strong repressor of the dopamine-D2 receptor gene and recognizes a highly conserved DRE in the second intron of this gene (Rogaeva et al., 2007b). The highest conservation of the D2 DRE was in the FRE region, but it is possible that Freud-2 may also regulate this gene, although this remains to be tested.

The recent linkage of a deletion mutation of the Freud-1/CC2D1A gene locus with non-syndromic mental retardation (NSMR) indicates a role for Freud-1 in cognitive development (Basel-Vanagaite et al., 2006), which may be shared by Freud-2. The NSMR Freud-1 mutant lacks the C-terminal portion of the protein, including the HLH and C2 domains that are implicated in Freud-1 transcriptional repression, and is thus predicted to lack repressor activity. This would be predicted to result in de-repression of 5-HT1A and dopamine-D2 receptor genes. Similarly, a mutation in Freud-2 might also result in derepression of the 5-HT1A receptor gene particularly in cortical or hippocampal neurons, which could contribute to mental retardation.

As discussed above, alterations that increase 5-HT1A receptor expression result in reduced anxiety, but also impaired cognitive ability in the Morris water maze (Bert et al.,

2005). Thus the loss of repression of 5-HT1A receptors due to mutation or reduced activity of Freud-1 or Freud-2 could contribute to mental retardation. Oppositely, genetically deletion of 5-HT1A receptors in mouse caused increases in fear responses and anxiety behaviors that were rescued by expression of post-synaptic 5-HT1A receptors in the forebrain (Gross et al., 2002a; Klemenhagen et al., 2006). Activation of Freud-2 would selectively reduce expression of post-synaptic 5-HT1A receptors, and may contribute to setting anxiety response, while inactivation of Freud-2 may produce anti-anxiety effects. Further studies will require revealing the roles of Freud-2 in memory formation and fearing conditioning behaviors.

Taken together, in this study we demonstrate that Freud-2/CC2D1B functions as a novel repressor selectively for post-synaptic 5-HT1A expression, which binds to the 5-HT1A-DRE at a site adjacent to and partly overlapping the Freud-1 site. Freud-2 is expressed in brain regions such as hippocampus, hypothalamus, cerebellum as well as peripheral tissues, and preferentially regulates 5-HT1A receptor expression in these regions compared to Freud-1 which preferentially regulates presynaptic 5-HT1A receptor expression. Thus these transcription factors provide complementary regulation of the 5-HT1A receptor gene.

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Chapter III- Human Freud-2/CC2D1B2: a novel repressor of post-synaptic 5-HT1A receptor expression

Human Freud-2/CC2D1B: a novel repressor of post-synaptic 5-HT1A receptor expression.

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Authors' contribution:

Mahmoud R. Hadjighassem conducted the research presented in this paper as well as preparation of manuscript. Human brain studies were performed by Bernadetta Szewczyk, and Craig Stockmeier. Their works were supervised by Mark C. Austin. Anti-Freud-2 antibody was provided by Mahmoud R. Hadjighassem for conducting human post mortem brain examinations. All work and editing was supervised by Paul R. Albert.

III.1 Abstract

The serotonin-1A receptor (5-HT1A) functions as an inhibitory autoreceptor on raphe neurons, and is expressed post-synaptically in limbic and cortical areas involved in mood and emotion. Alterations in expression or activity of 5-HT1A receptors have been implicated in mood disorders, such as major depression and anxiety. To identify mechanisms that determine 5-HT1A receptor expression, we have characterized 5-HT1A receptor gene regulatory regions. We previously identified a dual repressor element that binds a single complex in raphe cells (Freud-1/CC2D1A), but the identity of the second protein in post-synaptic cells was unknown. Here we identify a new Freud-1 homologue (Freud-2/CC2D1B) with 50% amino acid identity. Freud-2 RNA was present in brain and peripheral tissues, and Freud-2 protein was present in nuclear fractions of brain tissue. Freud-2 was enriched human hippocampus and prefrontal cortex, but weakly expressed in dorsal raphe nucleus, and was co-localized with 5-HT1A receptors and neuronal and glial markers. Recombinant hFreud-2 protein bound specifically to 5' or 3' human DRE. Human Freud-2 showed strong repressor activity at the human 5-HT1A or heterologous promoter in neuronal (SK-N-SH) and non-neuronal (HEK293) cell lines, indicating that hFreud-2 acts as a repressor in post-synaptic 5-HT1A receptor-positive cells. Furthermore siRNA knockdown of endogenous hFreud-2 expression de-repressed 5-HT1A promoter activity in either neuronal or non-neuronal cell lines or increased levels of 5-HT1A receptor protein in post-synaptic models of 5-HT1A-expressing cells. We conclude that human Freud-2 binds to the 5-HT1A DRE and represses the human 5-HT1A gene to regulate its expression in post-synaptic neurons.

III.2 Introduction

The 5-HT1A receptor is expressed presynaptically as an autoreceptor in raphe nuclei and postsynaptically in the limbic system including lateral septum, hippocampus, amygdala, and entorhinal cortex (Albert et al., 1990; Pompeiano et al., 1992) and is implicated in regulation of the serotonin system and control of mood and emotion. Reductions in 5-HT1A receptor expression or activity are observed in patients with anxiety, major depression or suicide victims (Albert and Lemonde, 2004; Pitchot et al., 2005; Sullivan et al., 2005; Lanzenberger et al., 2007). Down regulation of postsynaptic 5-HT1A receptors in the hippocampus and prefrontal cortex is implicated in schizophrenia, major depression, and type I bipolar disorder (Lopez-Figueroa et al., 2004; Gray et al., 2006). Genetic rescue studies indicate that early post-natal restoration of forebrain 5-HT1A receptors restores normal anxiety-like behaviour in 5-HT1A-/- mice (Gross et al., 2002a). Moreover, postsynaptic 5-HT1A receptors are a potential target of antidepressant drugs (Santarelli et al., 2003a), implicating the level of expression of postsynaptic 5-HT1A receptors in the pathophysiology and treatment of mood disorders.

In order to identify the mechanisms of transcriptional regulation of the 5-HT1A receptor gene, we and others have investigated the 5-HT1A promoter region. The 5-HT1A proximal promoter contains a series of GC-rich MAZ/Sp1-binding sequences that drive strong expression in all cell types, which is silenced by upstream repressor elements (Parks and Shenk, 1996a; Storring et al., 1999). In the rat 5-HT1A promoter we identified a 31-bp dual repressor element (DRE) that is located between -1555/-1524 bp from the translation initiation codon that strongly silences the promoter (Ou et al., 2000a). The human 5-HT1A receptor gene contains two tandem imperfect repeats of the DRE (-

1624 to -1570 bp) with 71% nucleotide identity to the rat 5-HT1A DRE and displaying similar silencer activity (Lemonde et al., 2004a; Rogaeva and Albert, 2007). The DRE is composed of a 5' 14-bp element (FRE) and adjacent 3'-element (TRE). In post-synaptic 5-HT1A-expressing neuronal cells or 5-HT1A-negative cells, two protein complexes bound to the DRE, and deletion of the entire DRE was required to de-repress the gene. However, in raphe cells a single complex bound the DRE and mutation of the FRE blocked this complex and completely de-repressed the 5-HT1A promoter. By yeast one-hybrid screen we identified a novel transcription factor named Freud-1 (FRE Under Dual repression binding protein-1)/CC2D1A (Coiled-coil/C2-Domain-1A) that interacts with FRE and represses the 5-HT1A promoter in raphe RN46A cells (Ou et al., 2003a). However the identity of the second protein complex that binds to the 5-HT1A-TRE has remained unknown.

In this study we report a new Freud-1 homologue, Freud-2/CC2D1B, which binds to the 5-HT1A-DRE at distinct sites that overlap with the Freud-1 site. Freud-2 negatively regulates 5-HT1A receptor gene transcription via the DRE, and depletion of Freud-2 increased 5-HT1A transcription and receptor expression in a post-synaptic 5-HT1A cell model. Freud-2 staining was enriched in hippocampus and prefrontal cortex, but weak in dorsal raphe nucleus. These data indicate that unlike Freud-1, Freud-2 functions primarily in regulation of post-synaptic 5-HT1A receptors.

III.3 Material and Methods

PCR and Plasmids. A 2.6-kb fragment of human Freud-2 cDNA including the complete coding sequence was amplified using specific primers 5-CCGGAATTCC GGATGCCAGG GCCAAGACCT CG-3', 5'-CCGCTCGAGC GGCAGGCCCC GAGGCTCCAG GACC-3' from a human brain cDNA library (Clontech). PCR products were gel purified, subcloned in pGEMT-easy vector (Promega, Madison, WI), and sequenced using ABI/PRISM automated system. Freud-2 expression plasmids were created by subcloning of coding sequence of human Freud-2 from pGEMT-easy vector to EcoRI/XhoI site in either pcDNA3 (Invitrogen, Burlington, Ontario, Canada) or pGEX-4T-1 (Amersham Bioscience). All constructs were verified by DNA sequence analysis. Human 5-HT1A receptor promoter construct contains 5' and 3'- DRE were as described previously (Lemonde et al., 2004a).

Freud-2 protein expression. Escherichia coli BL21cells were transformed with pGEX-4T-1-Freud-2 to express GST-Freud-2 or with pGEX-4T-1 vector as a control, and grown in 250 ml LBA medium at 37°C with shaking to OD_{600}=0.6, induced with 0.1 M isopropyl-β-D-thiogalactopyranoside and grown to OD_{600}=1.5-3 . Bacterial cultures were pelleted (3000xg, 4°C, 20 min) and recombinant proteins purified from pellets using B-PER GST Fusion Protein Purification Kit (Pierce, IL,USA) following manufacturer's protocol. Purified proteins were stored at -80°C.

Cell culture and transfection. Human embryonic kidney (HEK) 293 cells and SK-N-SH human neuroblastoma cells were cultured and transfected as described previously (Ou et

al., 2000a; Lemonde et al., 2004a; Czesak et al., 2006). HEK293 cells were transfected by calcium phosphate co-precipitation as described previously (Charest et al., 1993), with 20μg luciferase reporter construct and 5 μg pCMVβgal per 10-cm plate. For co-transfections, 10 μg of luciferase expressing vector and indicated amounts of protein-expressing constructs were used, keeping the total DNA at 25 μg using an empty vector, with 0.1 μg pCMVβgal to normalize transfection efficiency. All plasmids were purified by maxiprep kit (Sigma), quantified spectrophotometrically, and verified by ethidium bromide staining. SK-N-SH cells were subcultured into 6 well plates and transfected at 50-60% confluency with 1:1.5 ratio of plasmid:lipofectamine 2000 reagent (Invitrogen) using 7.5-10 μg/plate of luciferase plasmid and equal amount of Freud-2 expression construct or empty vector with 2μg/plate pCMVβgal. For reporter assays, triplicate samples after 48h of transfection were washed x3 with cold 1xPBS and extracted with 150μl of reporter lysis buffer (Promega). Supernatants were collected, assayed for luciferase activity using Spectramax M2 (Molecular Devices) luminometer and analyzed using Softmax Pro 4.8 software. Activities were obtained from at least three independent experiments in which triplicate transfection were performed and corrected for transfection efficiency by calculating the ratio of luciferase/βgalactosidase activity and normalized to vector-transfected extracts. Data are presented as mean ± SEM. Statistical significant was evaluated using two tailed unpaired *t* test.

Electrophoretic mobility shift assay. Sense and antisense oligonucleotides of the 5' or 3' human DRE with CC/GG 3'-overhang were hybridized and labelled with [α-^{32}P]-dCTP using klenow fragment DNA polymerase (Ou et al., 2000a). Purified recombinant GST-

human Freud-2 protein or GST protein as a negative control was incubated with or without competitor DNA in 25μl reaction containing gel shift DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2mM EGTA, 100 mM KCl, 5% glycerol, and 2 mM DTT, pH 7.9) and 2 μg poly(d(I-C)) at room temperature. Unlabeled double-stranded 5'-DRE, 3'-DRE or 19-bp or 12-bp segments of 3'-DRE (Table III-1) were used as a competitor. For supershift assay 2 μl of purified (Pierce) polyclonal rabbit antibody (Cedarlane, Hornby,ON) against a C-terminal peptide of human Freud-2 (CDGRKPTGGKLF) was used in 25 μl reaction and incubated 20 min at 37°C. ^{32}P-labeled probe (60,000-100,000 cpm/sample) was added and the samples were incubated for more 20 min at room temperature. The DNA/protein complexes were separated on a 5% polyacrylamide gel at 4°C dried and exposed to film overnight at -80°C with an intensifying screen.

Table III-1

Sequence Name (Location)	DNA sequence
5'-DRE (-1624/-1598)	**AGATGGCACTCTAAAACATTTGCCAGA**
17-bp 5′(-1624/-1608)	**AGATGGCACTCTAAAAC**
16-bp 5′ (-1613/-1598)	**TAAAACATTTGCCAGA**
3'-DRE (-1597/-1565)	**AGGTGGCGACATAAAACCTCATTGCTTAGAACT**
19-bp 3′ (-1597/-1578)	**AGGTGGCGACATAAAACCT**
12-bp 3′ (-1578/-1566)	**CATTGCTTAGAA**

Table III-1- 5-HT1A DRE primers for competition assay.

The human 5-HT1A 5' and 3' DRE nucleotide sequences and location (relative to translation initiation site) of forward primers used for competition assays are aligned. The minimal consensus sequence in common among primers that competed for Freud-2 binding is underlined.

Northern blot analysis. Human brain MTN Blot and 12-lane MTN Blot (Clontech) were probed with 900-bp human Freud-2 cDNA fragment using Strip EZ DNA kit (Ambion). 50 µl of 250,000 cpm/µl purified probe was incubated with human brain MTN Blot membranes in 5 ml Ultrahyb buffer overnight at 42°C. Membranes were washed twice with 2x SCC/0.1% SDS 10 min at 42°C followed by 0.1%SCC, 0.1% SDS for 30 min at 65°C. Membranes were exposed to film overnight at -80°C with intensifier screen.

Western blot analysis. Immunolabeling of Freud-2 was determined in tissue punches from human PFC, hippocampus and dorsal raphe nuclei. Tissues were homogenized in buffer A containing 10mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 1 mM DTT, protease inhibitor cocktail and 0.1% Igepal CA-630) and centrifuged for 1 min. The supernatant was discarded and the pellet was resuspended in buffer C (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM each of DTT, EDTA, EGTA; and protease inhibitor cocktail), incubated on shaker for 15 min. and centrifuged at 11,000xg for 5 min. The supernatant containing the nuclear fraction was used in Western blot. 30 or 40 ug of protein were resolved on 12.5% SDS polyacrylamide gel and blotted on nitrocellulose membrane. The blots were incubated overnight at 4°C with affinity-purified primary rabbit anti-Freud-2 polyclonal antibody or preimmune serum (1:5000) followed by washing and incubation with secondary horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Biosciences, Buckinghamshire, England). After incubation, blots were washed with PBS and developed using enhanced chemiluminescence detection (ECL; Perkin-Elmer Life Sciences Inc., Boston, MA) and exposed to film. Nuclear protein of SK-N-SH cells were extracted as previously described (Lemonde et al., 2004a).

60 μg of extracts were separated by SDS-PAGE on an 8% polyacrylamide gel. Polyclonal rabbit anti-Freud-2 antibody was purified using Montage antibody purification PROSEP-G spin column (Millipore).

Immunohistochemistry and immunofluorescence. For immunohistochemistry, frozen 30 μm sections of human post-mortem PFC, hippocampus and raphe nuclei tissue were fixed in 4% paraformaldehyde (in 0.05M phosphate-buffered saline, PBS) for 1h at RT, preincubated in 5% normal horse serum in PBS for 30 min and then incubated for 24h at 4°C in the same solution containing rabbit anti-Freud-2 polyclonal antibody (1:500). Sections were washed in PBS and incubated for 4h at room temperature in biotinylated horse anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) in PBS buffer. After incubation, the sections were processed using the Vectastain ABC immunoperoxidase kit (Vector, Burlingame, CA) for 24h at 4°C. Antibody distribution was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.05%, Sigma, St. Louis, MO, USA). For immunofluorescence, frozen 20-μm sections of human post-mortem brain tissue were subjected to dual immunofluorescence. Sections were incubated overnight with rabbit polyclonal anti-Freud-2 antibody (1:500) and mouse monoclonal anti-GFAP (1:1000) or mouse monoclonal anti-NeuN (1:1000) diluted in the incubation solution. After three washes in 0.1M Tris-HCl buffer (pH 7.6), sections were incubated for 90 min with a mixture of goat anti-mouse antibody conjugated with the fluorochrome Cy2 (Jackson Immunochemicals; 1:200) and goat anti-rabbit antibody conjugated with the fluorochrome Cy5. After incubation, the sections were washed again and cover-slipped and viewed using fluorescence microscopy.

siRNA.transfection. Stealth siRNA targeting hFreud-2 [CC2D1BHSS153336] (5'-cccugcagcagaggcugaacaagua-3') and stealth RNAi negative control duplexes were purchased from InVitrogen. HEK cells were transfected using lipofectamine 2000 (Invitrogen) with a final siRNA concentration of 100 nM. Transfection efficiency control was performed with Block-iT^{TM} fluorescent oligo (Invitrogen). For luciferase assay, 5 µl specific Freud-2-SiRNA or RNAi negative controls was co-transfected with 1.5 µg of human 5-HT1A luciferase construct (h5-HT1A) in HEK cells and incubated for 48-72 hr and assayed as described. To test the effect of endogenous Freud-2 on 5-HT1A receptor expression, Freud-2 SiRNA or negative control was transfected into HEK cells using Lipofectamine 2000 (Invitrogen) and incubated for 72 hr. Total protein was extracted as previously described (Lemonde et al., 2004a). 60 µg of total protein separated by SDS-PAGE on a 8% polyacrylamide gel. Immunoblotting was performed as described previously (Ou et al., 2003a). Rabbit 5-HT1A polyclonal antibody (Cedarlane laboratory) was used at a dilution of 1:1000 followed by a 1:2000 dilution of the secondary horseradish peroxidase (HRP)-linked rabbit antibody (Amersham). The reactive bands were visualized by chemiluminescence using the ECL-kit (Amersham) after exposure to film (Kodak). Staining for β-actin (Santa Cruz Biotechnology) served as a control for equal loading of samples.

III.4 Results

III.4.1 Molecular cloning of human Freud-2

To identify Freud-1 homologues, we screened the GenBank database and identified Freud-2/CC2D1B, distinct gene located on chromosome 1p32(NP_115825) that encodes an 858-aa protein with 50% overall amino acid identity to hFreud-1 (Fig.III 1). Freud-2 contains highest conservation in a number of known domains such as four DM14 domains which extend from amino acids 170-237, 270-332, 385-433, 537-594; predicted helix-loop-helix (648-673); and a conserved protein kinase C (PKC) conserved region (C2 domain; 705-799). The DM-14 domain is highly conserved among species of Freud/CC2D1 family, although its function is not known. The helix-loop-helix domain mediates protein interaction and DNA binding, while in Freud-1 the C2 domain was important for DNA binding and essential for its repressor activity. The conservation of important functional domains suggested that like Freud-1, Freud-2 may also bind to DNA and repress transcription.

Fig. 1

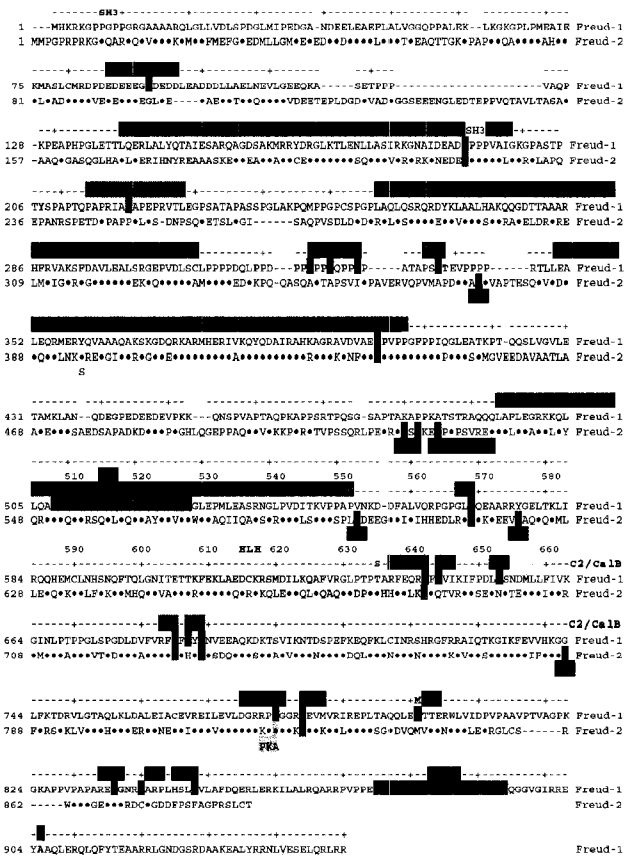

Fig.III.1. Amino acid alignment of human Freud-1 and Freud-2.

Alignment of the Ensembl database predicted amino acid sequences of human Freud-1 (CC2D1A, ENSG00000132024) and Freud-2 (CC2D1B, ENSG00000154222) was performed using the ClustalW analysis in the DNAStar MegAlign program, with identical (•) or differing residues shown. Predicted structural motifs in Freud-2 are illustrated. The conserved DM-14 and C2/CalB domains were identified by NCBI blast (http://www.ncbi.nlm.nih.gov/BLAST/) conserved domain alignment (Marchler-Bauer et al., 2003). Consensus phosphorylation or interaction sites were identified by Scansite (scansite.mit.edu) search (Obenauer et al., 2003); the HLH domain was identified by HELIXTURNHELIX (http://www.bioweb.pasteur.fr/docs/EMBOSS/helixturnhelix.html).

III.4.2 Freud-2 RNA and protein expression

To verify the expression of Freud-2 *in vivo,* we first examined the tissue distribution of Freud-2 mRNA by Northern blot analysis of human tissues (Fig-III-2). A single 3.9-kb mRNA was highly expressed in several brain regions and in peripheral tissues including skeletal muscle, kidney, and liver and at low levels in heart, intestine and lung tissue. Freud-2 protein expression was determined using a specific antibody generated against full-length Freud-2, which does not cross-react with Freud-1 (data not shown). By Western blot analysis of nuclear extracts from human postmortem brain tissue, a major specific 130-kDa species corresponding to Freud-2 protein was observed in PFC and hippocampus and weakly in dorsal raphe nuclei by anti-Freud-2, but not preimmune serum, (Fig.III-3). Specific staining for lower molecular mass proteins that may represent short isoforms of Freud-2 was also observed. As observed for Freud-1, the apparent molecular weight of the major Freud-2 isoform was larger than the predicted molecular weight of 89-kDa (Rogaeva and Albert, 2007), which may be due to post-translational modification and secondary structure. The presence of Freud-2 in the nuclear fraction of these brain regions is similar to that observed for Freud-1 and consistent with a possible role as a transcription factor.

Freud-2 protein was further localized by immunohistochemistry using anti-Freud-2 (Fig.III-4). Consistent with Western blot data, Freud-2 immuonoreactivity was also enriched in the grey matter (GM) of the entorhinal cortex and hippocampus, but weakly

detectable in the dorsal raphe (DR) nucleus, while no staining was observed using preimmune serum (Fig.III-4). By immunofluorescence, Freud-2 immunoreactivity strongly detected in cells of the human prefrontal cortex (PFC) with a primarily nuclear localization, and was colocalized with both astrocyte (GFAP) and neuronal (NeuN) markers (Fig.III-5). Thus Freud-2 displays a predominant distribution in cells the prefrontal cortex and hippocampus compared to presynaptic serotonergic raphe nuclei, and is expressed in the nuclei of subsets of neurons and glia.

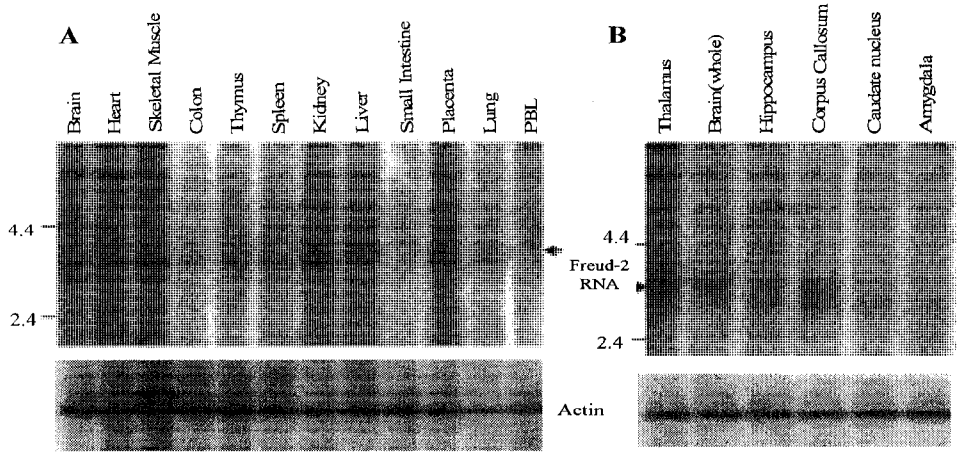
A
Brain
Heart
Skeletal Muscle
Colon
Thymus
Spleen
Kidney
Liver
Small Intestine
Placenta
Lung
PBL
4.4
2.4
Actin
B
Thalamus
Brain(whole)
Hippocampus
Corpus Callosum
Caudate nucleus
Amygdala
4.4
Freud-2
RNA
2.4

Fig. 2

Fig.III-2. Tissue distribution of human Freud-2 RNA.

RNA prepared from the indicated human tissues (Clontech) (A) or brain regions (B) was hybridized to labeled human Freud-2 cDNA for Northern blot analysis. A major Freud-2 RNA species of approximately 3.5-kb was identified (arrowhead) in most tissues; molecular size markers are shown. Below, the blots were reprobed with labeled beta-actin cDNA to control for RNA loading.

Figure 3.

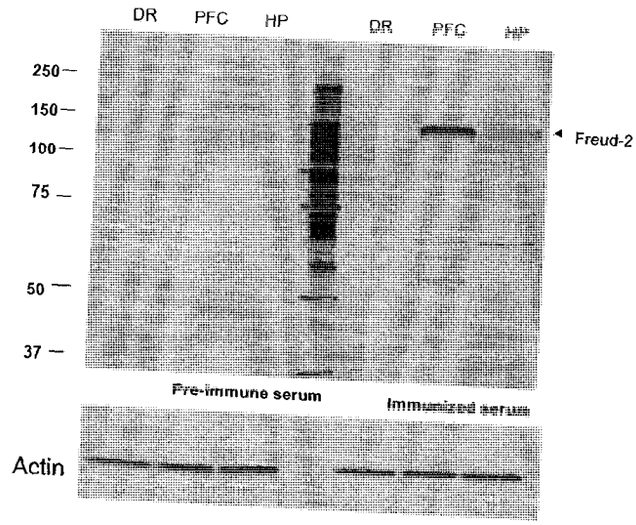
DR
PFC
HP
DR
PFC
HP
250
150
100
75
50
37
Freud-2
Pre-immune serum
Immunized serum
Actin

Fig.III-3. Freud-2 protein is enriched in human prefrontal cortex and hippocampus, but not raphe. Nuclear fractions from human prefrontal cortex (PFC), dorsal raphe (DR) and hippocampus (HP) were isolated and probed using anti-Freud-2 antiserum (immunized) or preimmune serum as negative control. A single major 130-kDa Freud-2 protein species was identified as a doublet. Blots were reprobed for beta-actin as loading control.

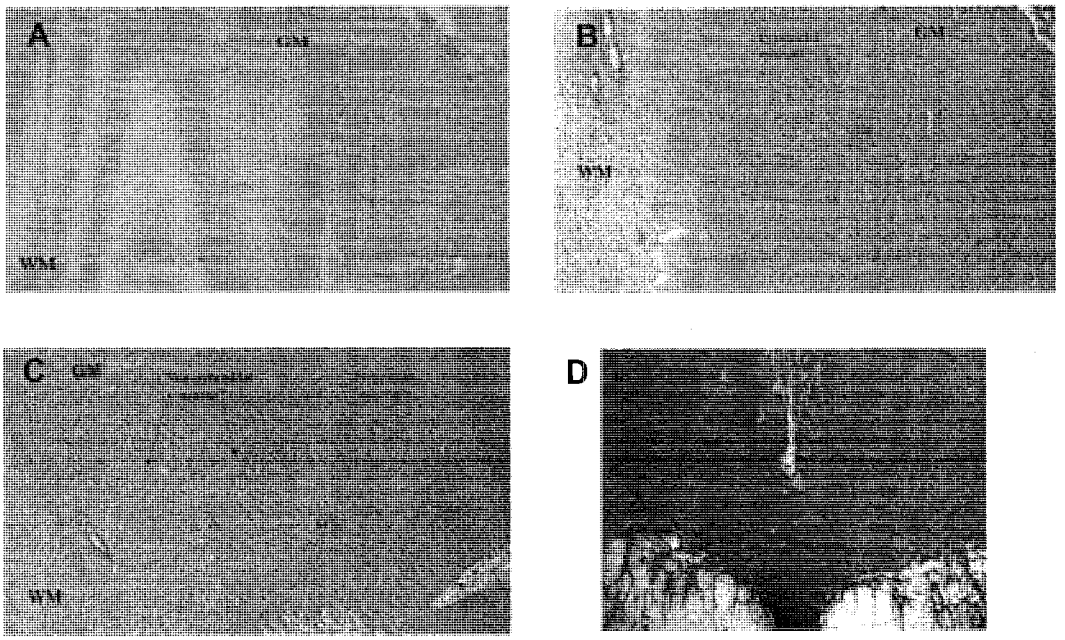

Fig.III- 4

Fig.III-4. Distribution of Freud-2 immunoreactivity in human PFC, hippocampus and dorsal raphe nuclei.

Frozen sections of post-mortem human PFC, hippocampus and dorsal raphe nuclei (DR) were incubated with anti-Freud-2 antibody and processed for immunohistochemistry. Freud-2 is enriched in GM (grey matter) of entorhinal cortex (C) and hippocampus (B), but sparsely staining in dorsal raphe nuclei (DR) (D). Pre-immune serum was used as a negative control (A).

Fig. 5

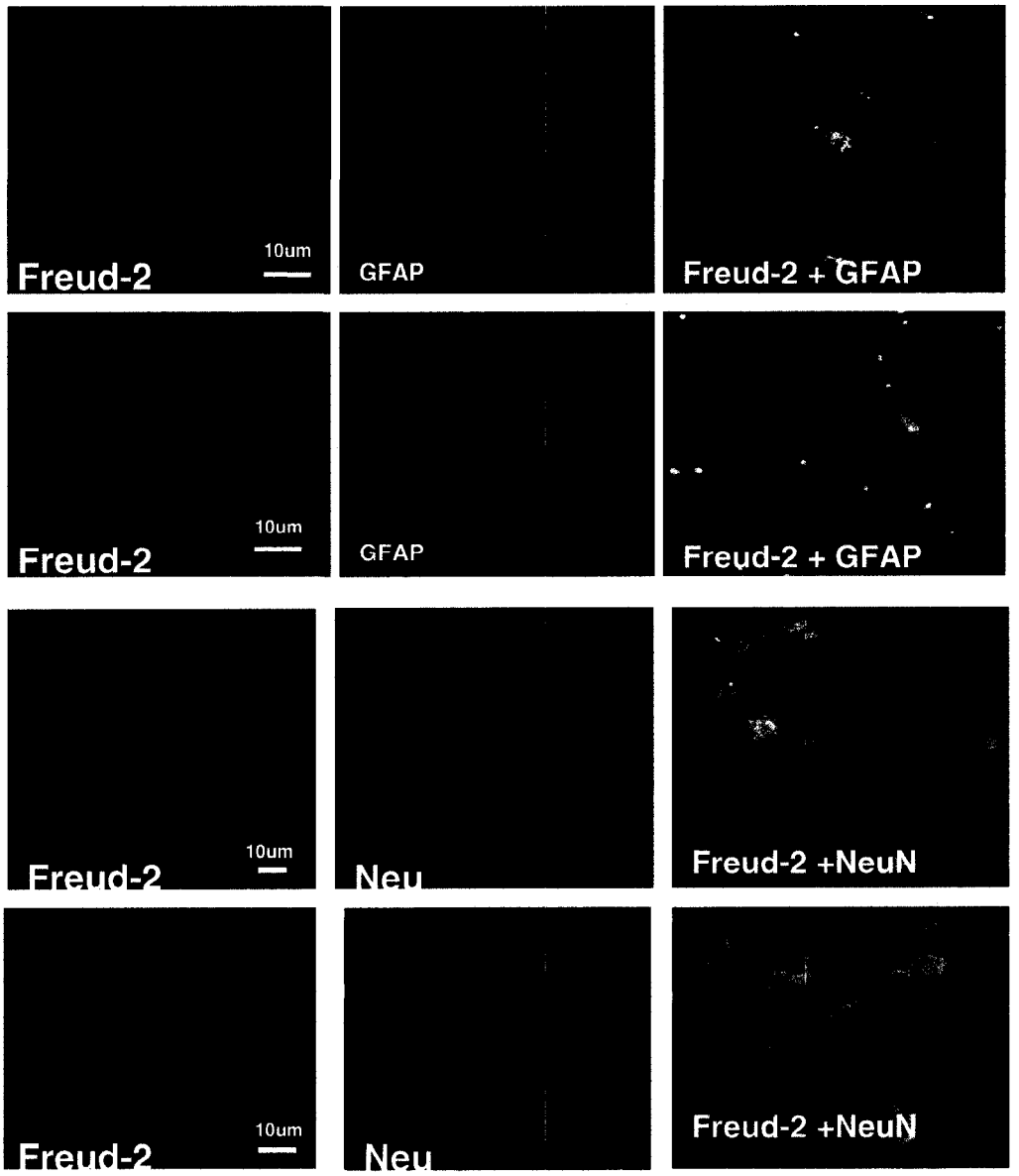
Freud-2
10um
GFAP
Freud-2 + GFAP
Freud-2
10um
GFAP
Freud-2 + GFAP
Freud-2
10um
Neu
Freud-2 +NeuN
Freud-2
10um
Neu
Freud-2 +NeuN

Fig.III- 5. Colocalization of Freud-2 with glial and neuronal markers in human PFC. Brain sections from human post-mortem prefrontal cortex were probed using anti-Freud-2, GFAP (glial) or NeuN (neuronal) antibodies and processed for immunofluorescence. Freud-2 was colocalized with GFAP and NeuN indicating its presence in the nuclei of glial and neuronal cells.

III.4.3 Freud-2 binding to human 5-HT1A DRE

We hypothesized that Freud-2 may bind to the 5-HT1A DRE based on the amino acid similarity between Freud-1 and Freud-2 (Fig. 1) and the partially overlapping sequences of the 5-HT1A DREs (Ou et al., 2000a; Lemonde et al., 2004a). To examine Freud-2 binding to the DRE, EMSA was done using labeled 5-HT1A DREs incubated with purified recombinant GST-Freud-2 fusion protein (Fig. 6). GST-Freud-2, but not GST alone, bound to labeled 5'- or 3'-DRE as a single complex, which was competed by unlabelled DRE oligonucleotides indicating that Freud-2 protein binds specifically to both 5' and 3' 5-HT1A DRE elements. To localize the site within the DRE that Freud-2 recognizes, competition EMSA was done in which GST-Freud-2 was incubated with labeled 3' or 5' 5-HT1A-DRE and competed with unlabelled segments of the DRE (Table I, Fig. 7). A single 3'-DRE-Freud-2 complex was detected, which was competed as effectively with the 19-bp oligonucleotides as with complete 3'-DRE, but was not competed with the adjacent 12-bp portion (Fig. 7A), indicating that Freud-2 binds specifically to the 5' half of the 3'DRE (Table-1). A polyclonal antibody raised against Freud-2 C-terminal peptide (Anti-cF2) super-shifted the protein-DNA complex (upper arrowhead), while antibody alone did not form a complex with the probe (Fig. 7A). These results confirm the presence of Freud-2 in the complex. Analysis of Freud-2 interactions with the 5-HT1A 5'-DRE revealed that both 16- and 17-bp primers competed for Freud-2 binding to the 5' DRE (Fig. 7B). Alignment of these sequences in Table I reveals that recombinant Freud-2 binds specifically to a minimal consensus sequence of 5'-TAAAAC-3', conserved between 5' and 3' DREs, and competing oligonucleotides.

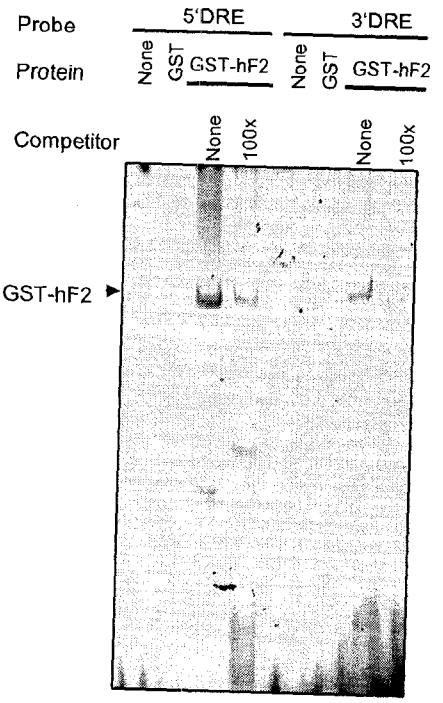
Probe
5'DRE
3'DRE
Protein
None
GST
GST-hF2
None
GST
GST-hF2
Competitor
None
100x
None
100x
GST-hF2

Fig.III- 6

Fig.III-6. Specific binding of Freud-2 to human DRE sequences.

Electrophoretic mobility shift assay (EMSA) was done using bacterially expressed purified recombinant GST-Freud-2 fusion protein (GST-hF2) or GST alone with labelled 5'or 3'DRE from human 5-HT1A promoter. For competition, unlabelled 5'or 3'DRE (cold) were used at 100-fold molar excess. A single band (arrow) was detected which was competed with excess unlabeled 5' or 3' DRE, indicating that Freud-2 protein binds both 5' and 3' DRE from human 5-HT1A promoter.

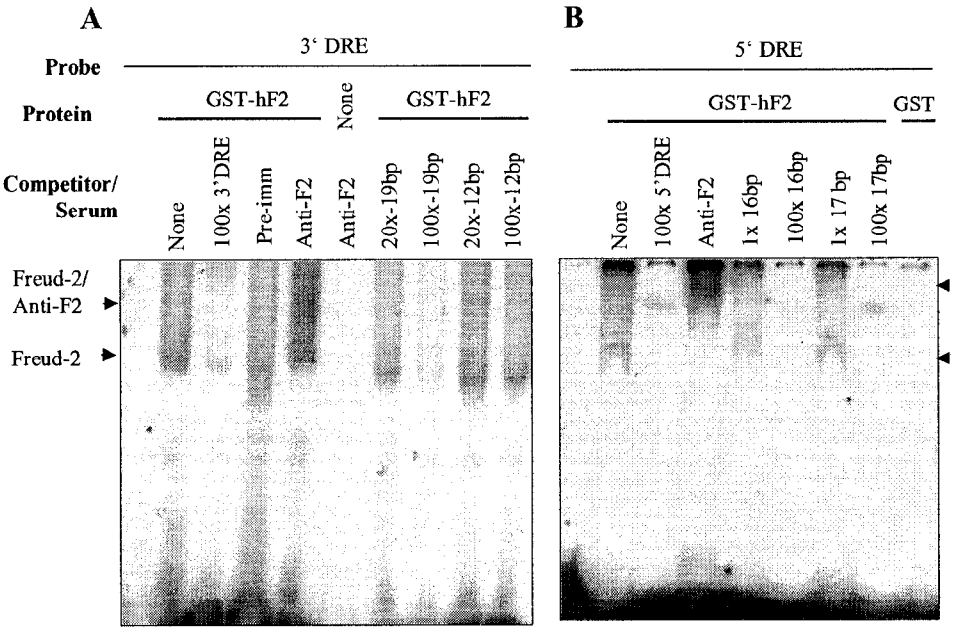
A
B
Probe
3' DRE
5' DRE
Protein
GST-hF2
None
GST-hF2
GST-hF2
GST
Competitor/
Serum
None
100x 3'DRE
Pre-imm
Anti-F2
Anti-F2
20x-19bp
100x-19bp
20x-12bp
100x-12bp
None
100x 5'DRE
Anti-F2
1x 16bp
100x 16bp
1x 17 bp
100x 17bp
Freud-2/
Anti-F2
Freud-2

Fig. 7

Fig.III-7. Binding specificity of Freud-2/DRE complexes.

EMSA was done using purified recombinant GST-Freud-2 (GST-hF2), GST or no protein incubated with labelled 5-HT1A 3' or 5' DRE (A or B, respectively). A specific complex (lower arrowhead) was observed for GST-hF2 but not GST, which was competed with the indicated molar excess of unlabelled primers (see Table I). The 3' 19-bp and 5' 16- and 17-bp primers effectively competed at 100x, indicating that human Freud-2 specifically binds to sequences in common between these primers (Table I). To confirm the presence of Freud-2 in the complex, antiserum (2 ul) to Freud-2 C-terminal (Anti-F2) was added and a supershifted complex was observed in the presence of GST-hF2 (upper arrowhead).

III.4.4 Freud-2 repression of human 5-HT1A receptor expression

To test whether Freud-2 regulates transcription of the human 5-HT1A receptor gene, Freud-2 expression plasmid was cotransfected with human 5-HT1A promoter luciferase reporter constructs to assay transcriptional activity in either 5-HT1A receptor-expressing human SK-N-SH neuroblastoma cells or HEK cells, which lack detectable 5-HT1A receptor expression. Compared to pGL3P, the activity of the DRE containing 5-HT1A construct was low, consistent with basal repression observed at these elements (Lemonde). In both cell types, transfection of human Freud-2 significantly ($p < 0.05$) reduced the transcriptional activity of the DRE-containing 5-HT1A reporter construct (Fig. 8A, B). To examine the role of endogenous Freud-2 on transcriptional activity of the human 5-HT1A receptor gene, Freud-2 expression protein was decreased by cotransfection of CC2D1B-siRNA. Reduction of endogenous Freud-2 protein level induced a 2-fold derepression of transcriptional activity of human 5-HT1A receptor gene in HEK cells (Fig. 8C).

To determine whether reduction in Freud-2 alters endogenous 5-HT1A receptor expression, 5-HT1A-positive SK-N-SH cells were transiently transfected by either control (i.e., scrambled siRNA) or two different CC2D1B siRNAs (1-2) and the level of endogenous 5-HT1A protein was examined by Western blot assay (Fig. 9). Both siRNA-1 or the combination of siRNAs-1 and -2 reduced the expression of endogenous Freud-2 protein and increased the level of 5-HT1A protein, although siRNA1 alone was more effective in both cases. Thus, these experiments show that human Freud-2 represses 5-HT1A gene transcription in neuronal or non-neuronal cell types. Reduction of Freud-2

protein by specific CC2D1B siRNA increases 5-HT1A protein level in SK-N-SH cells that endogenously express the human 5-HT1A receptor.

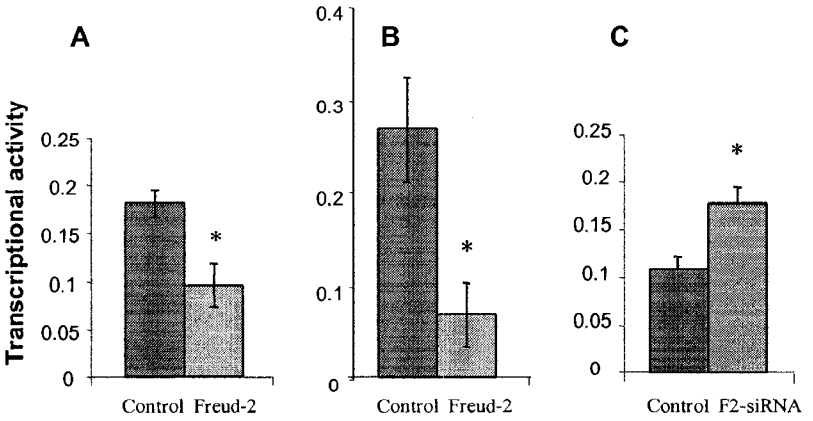
A
B
C
Transcriptional activity
0.25
0.2
0.15
0.1
0.05
0
0.4
0.3
0.2
0.1
0
*
Control Freud-2
Control Freud-2
Control F2-siRNA

Fig. 8

Fig.III-8. Repressor activity of Freud-2 at the 5-HT1A DRE.

Human embryonic kidney HEK-293 cells (A, C) or 5-HT1A-expressing human neuroblastoma SK-N-SH cells (B) were transiently co-transfected with vector (pcDNA3, Control) human Freud-2 expression plasmid (Freud-2) and either 5-HT1A-DRE-containing pGL3P luciferase reporter or control (pGL3P, lacking DRE). For siRNA experiments, cells were treated with 5 μl of scrambled control (Control) or Freud-2 siRNA (F2-siRNA). Cell extracts were collected 48-72 hr later, and luciferase activity assayed and normalized to that of β-galactosidase as relative units and transcriptional activity expressed relative to pGL3P (1). Freud-2 repressed at the 5-HT1A-DRE in both HEK-293 and SKN-SH cells, while depletion of Freud-2 partially de-repressed the 5-HT1A-DRE construct. Data represent the mean ± SEM of three independent experiments. *$p < 0.05$ compared with Control by *t*-test.

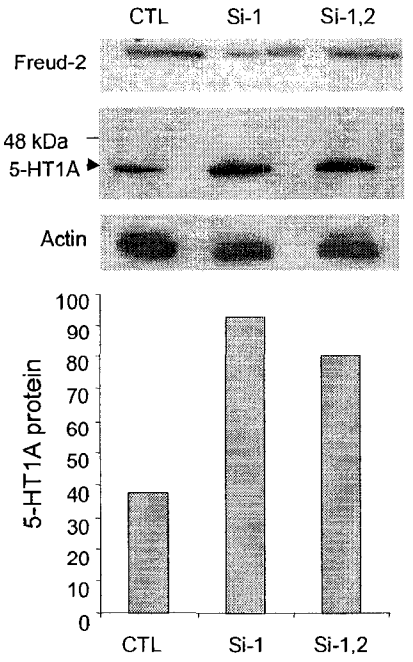
CTL
Si-1
Si-1,2
Freud-2
48 kDa
5-HT1A
Actin
5-HT1A protein
100
90
80
70
60
50
40
30
20
10
0
CTL
Si-1
Si-1,2

Fig. 9

Fig.III-9. Depletion of Freud-2 increases 5-HT1A receptor expression in SK-N-SH cells. SKN-SH cells were treated with scrambled control siRNA (CTL) or siRNAs to Freud-2 (Si-1, Si-2) and cell extracts were examined by Western blot using anti-Freud-2 (top) or anti-5-HT1A antibody (middle); the blot was probed for β-actin as loading control (bottom). Treatment with Freud-2 siRNAs reduced Freud-2 protein and increased 5-HT1A receptor expression, however the combination of siRNAs was less effective. The relative intensity of 5-HT1A protein is plotted below.

III.5 Discussion

III.5.1- Freud-2: novel repressor of post-synaptic 5-HT1A receptor expression.

In order to elucidate transcriptional mechanisms that regulate the brain serotonin system, we have focused on identification of transcription factors that regulate the 5-HT1A receptor gene. The 5-HT1A receptor is a key presynaptic regulator of serotonergic activity, and also a major post-synaptic receptor that mediates serotonin action and plays a key role in the regulation in mood (Albert and Lemonde, 2004; Gross and Hen, 2004). We previously identified the DRE as a powerful repressor region that is conserved in human and rat 5-HT1A receptor genes (Ou et al., 2000a; Lemonde et al., 2004a) and identified Freud-1/CC2D1A as a strong repressor at the DRE (Ou et al., 2003a; Rogaeva and Albert, 2007). In this study we have identified the repressor function of Freud-2/CC2D1B, a homologue of Freud-1, at the 5-HT1A receptor gene. Our results indicate that Freud-2 protein acts as a repressor of human 5-HT1A receptor gene by binding to DRE region. Recombinant purified Freud-2 protein bound specifically to both 5' and 3'-DRE, and this complex was supershifted by specific anti-Freud-2 antibody. In addition, Freud-2 repressed 5-HT1A promoter-luciferase constructs in SK-N-SH and HEK cells, suggesting a repressor role of Freud-2 in both 5-HT1A expressing neuronal and 5-HT1A negative cells. Moreover, depletion of Freud-2 protein using CC2D1B-siRNA de-repressed significantly the 5-HT1A promoter activity in HEK293 cells and increased the expression of 5-HT1A protein in SK-N-SH cells. Therefore, Freud-2 mediates neuronal and non-neuronal repression of the 5-HT1A receptor gene.

Although, like Freud-1, Freud-2 binds to the DRE and represses 5-HT1A receptor expression, Freud-2 binds at a different consensus sequence than Freud-1, which is adjacent and partly overlapping (Ou et al., 2000a). The Freud-1 FRE site defined at the rat 5-HT1A promoter (5'-CATAAAGCAAG) is similar to the 5'-TAAAAC Freud-2 minimal recognition sequence that we have identified in the human 5-HT1A DREs (Table I). Our data suggest that in cells or tissues that lack 5-HT1A receptor expression (such, both Freud-1 and Freud-2 mediate redundant repression to largely silence 5-HT1A transcription, hence the effects of over-expression or depletion of Freud-2 were modest, presumably due to the presence of Freud-1-mediated repression. Similarly, Freud-1 and Freud-2 are also expressed widely in neuronal cells and both factors appear to partially overlap in regulating the level of 5-HT1A receptor expression in neuronal cells, such as SK-N-SH cells. Consistent with this is the strong expression of Freud-2 in post-synaptic serotonergic targets regions such as prefrontal cortex and hippocampus. However, while Freud-1 plays a key role in the regulation of basal presynaptic 5-HT1A receptor expression in raphe RN46A cells, Freud-2 did not repress 5-HT1A receptor expression in these cells (data not shown). This role is supported by the very sparse expression of Freud-2 in the raphe nuclei, whereas Freud-1 is strongly expressed (Ou et al., 2003a). Our results indicate that unlike Freud-1, which regulates both pre- and post-synaptic 5-HT1A receptors, Freud-2 appears to preferentially regulate the level of expression of post-synaptic 5-HT1A receptors.

III.5-2 Implications of Freud-2 regulation of 5-HT1A expression in vivo

The strong expression of Freud-2 in forebrain regions and its role in regulation of 5-HT1A receptors suggest a role in behavioral development. In addition to mediating 5-HT actions on mood and emotion, 5-HT1A receptors on mPFC pyramidal neurons inhibit their activity (Puig et al., 2005), reducing glutamatergic input to dorsal raphe serotonergic neurons, and hence could regulate serotonergic activity (Celada et al., 2001b). Altered regulation of forebrain 5-HT1A receptors has been implicated in several forms of mental illness. In PET studies, a region- and disorder-specific reduction in the density of cortical 5-HT1A receptors is observed in depression (Drevets et al., 2000a; Sargent et al., 2000; Bhagwagar et al., 2004; Shively et al., 2006; Moses-Kolko et al., 2007) and anxiety disorders (Neumeister et al., 2004b; Lanzenberger et al., 2007), while cortical 5-HT1A receptors are increased in anorexia or bulimia nervosa (Tiihonen et al., 2004; Bailer et al., 2007). In post-mortem tissue, 5-HT1A receptor RNA and protein levels are reduced in the hippocampus of MDD and bipolar I disorder patients compared to control subjects (Lopez-Figueroa et al., 2004; Gray et al., 2006) and 5-HT1A receptor signaling is reduced in several brain regions (Hsiung et al., 2003). In 5-HT1A-/- mice, early post-natal rescue of 5-HT1A receptors in hippocampus and cortex restored anxiety phenotype to normal (Gross et al., 2002a). In mice, 5-HT1A receptors were required for SSRI-mediated hippocampal neurogenesis and anti-anxiety actions (Santarelli et al., 2003a), although this effect appears to be strain-dependent (Holick et al., 2008). The role of Freud-2 in regulation of post-synaptic 5-HT1A receptor expression suggests its role in ethiopathology of mood disorders, although the role of Freud-2 protein in reduction of 5-HT1A receptors in MDD and anxiety disorders remains to be elucidated. The expression of Freud-2 protein in glial cells of PFC is interesting since a reduction of glial cells has

been observed in cortex of depressed suicides (Rajkowska et al., 2001; Rajkowska, 2003) and is counteracted by electroconvulsive seizure (Hamidi et al., 2004; Choudary et al., 2005; Wennstrom et al., 2006). However the mechanisms involved and the role of Freud-2 in glial function remains unknown.

III.5-3 Functions of Freud-1 and Freud-2 in neurodevelopment

The homology, similar repressor function and tissue distribution of Freud-1 and Freud-2 suggest that they may have overlapping functions. Recently, a genetic deletion mutation in the Freud-1 gene has been linked to non-syndromal mental retardation (Basel-Vanagaite et al., 2006), implicating Freud-1 in cognitive development. This mutation truncates the protein, eliminating all but the three N-terminal DM14 domain, including the C2 domain that is required for Freud-1 induced transcriptional regulation (Ou et al., 2003a), suggesting that the truncated mutant would produce a non-functional or dominant-negative protein (Rogaeva et al., 2007a). Thus, Freud-1 is required for intact cognitive development, and like Freud-1, Freud-2 may participate in cognitive development.

Our data indicate that Freud-2 mediates repression of the 5-HT1A receptor gene, and that its inhibition leads to up-regulation of 5-HT1A receptor expression in neuronal cells. However, it is possible that Freud-2 may regulate additional genes. In particular, we recently characterized a DRE in the human DRD2 gene that is repressed by Freud-1 (Rogaeva et al., 2007b). The D2-DRE contains a consensus GATAAG sequence for Freud-1 binding, but also contains an adjacent TAAAAG sequence that is similar to the TAAAAC sequence we identified for Freud-2 binding to the 5-HT1A DRE. Further

studies will be required to determine whether Freud-2 also regulates dopamine-D2 receptor expression. In addition, recent studies of the single Drosophila Freud-1/2 homologue Lethal (2) giant discs indicate a new function for cytosolic Freud-1 in binding to phospholipids via its C2 domain to regulate endocytosis of Notch (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Given the extensive homology between the C2 domains of Freud-1 and Freud-2, it is likely that these proteins may also regulate endocytotic mechanisms that could play a role in neurodevelopment.

In summary, we have identified Freud-2 as a novel transcriptional repressor, which in combination with the homologue Freud-1 regulates the expression of 5-HT1A receptors in neuronal and nonneuronal cells.

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CHAPTER IV- CONCLUSION

Transcriptional regulation of the 5-HT1A receptor gene, as reviewed in the Introduction, plays a key role in the function and dysfunction of serotonin system, with implications for mental illnesses such as depression and anxiety. The 5-HT1A receptor is expressed on the soma and dendrites of 5-HT neurons as an autoreceptor, and negatively regulates the firing activity of 5-HT neurons. It also expressed as a heteroreceptor at the postsynaptic cells in several serotonin target regions, especially in the limbic system. In the limbic system, postsynaptic 5- HT1A receptors signal to inhibit excitatory input into the target cells. We have reported that two DNA-protein complexes formed when nuclear extract of L6 cells was incubated with the 31-bp-DRE probe in an EMSA. Further studies revealed that Freud-1 is one of the proteins that interacts with the first 14-bp segment within the 31-bp-DRE and mediates inhibition of 5-HT1A transcription in presynaptic raphe and other cells. I hypothesized that a second protein, which binds to the 12-bp segment adjacent to 14-bp site is important to maintain repression of the 5-HT1A receptor in either post-synaptic cells expressing this receptor or 5-HT1A non-expressing cells. Therefore, the main aim of my thesis was to identify the second transcription factor that negatively regulates expression of 5-HT1A receptor gene.

In chapter II, I identified a novel protein in mouse, which I called Freud-2, as it belongs to the same gene superfamily and shared common predicted structural features with Freud-1. Freud-2 contains several known protein domains such as the HLH domain, DM-14, putative PKA and PKC phosphorylation sites, as well as a conserved C2 domain. I first showed that mouse Freud-2 binds to 31-bp-DRE of the rat 5-HT1A receptor gene. By competition studies, I demonstrated that Freud-2 specifically interacts with 12-bp

oligonucleotide located within 31-bp DRE. By generation of a specific anti-Freud-2 antibody and supershift assay, I demonstrated the presence of the Freud-2-DRE complex in nuclear extracts. I showed that Freud-2 mRNA and protein are expressed widely in different tissues such as brain, hippocampus, as well as in peripheral tissues. Functional studies showed exciting results that indicate that Freud-2 is a specific regulator of postsynaptic 5-HT1A receptor expression. It repressed transcriptional activity of 5-HT1A promoter-reporter constructs in post-synaptic cell models such as NG-108-15 cells, but had no regulatory effects in presynaptic 5-HT1A-positive cells such as RN46A cells. This finding is consistent with previous studies that suggest regional specific regulation for 5-HT1A receptors. It has been shown that in response to antidepressant treatment only presynaptic 5-HT1A receptors were desensitized, but not postsynaptic 5-HT1A receptors. Moreover, it has been reported that NUDR negatively regulates presynaptic 5-HT1A receptor transcription, while it has opposite effects on postsynaptic 5-HT1A expression (Czesak et al., 2006), indicating that there is a regional specificity for regulation of the 5-HT1A receptor gene. To confirm my finding, knockdown of Freud-2 was performed by administering a specific SiRNA to the cells. I showed that reducing the endogenous Freud-2 by transfection of Freud-2 SiRNA derepressed the transcriptional activity of the 5-HT1A promoter. It is the first time that a repressor specific for postsynaptic 5-HT1A receptors has been identified. Taken together, these data have shown that Freud-2 is the second component that with Freud-1 mediates dual repression of the 5-HT1A receptor gene at the DRE. While Freud-1 regulates both pre- and post-synaptically expressed 5-HT1A receptors, Freud-2 is more selective to regulate post-synaptic 5-HT1A receptor expression.

The initial studies in chapter II were done using rodent Freud-2 regulation of the rat 5-HT1A promoter in rodent cells. To determine whether similar mechanisms involving Freud-2 exist in man, in chapter III I identified the human Freud-2 homologue by searching the NIH Genbank database. I provided evidence that human Freud-2 interacts with two characterized repressor elements homologue to 31-bp-DRE. I showed human Freud-2 mRNA distribution in human tissues, finding that Freud-2 is expressed in brain and peripheral tissues. Using human cells expressing or not expressing endogenous 5-HT1A receptors, I assessed the DNA binding and transcriptional function of human Freud-2. As observed at the rat 5-HT1A DRE, human Freud-2 bound to the human 5-HT1A DRE at a site adjacent to but partly overlapping the Freud-1 site. I reported that human Freud-2 is a repressor transcriptional factor in either neuronal or non-neuronal models. I also found that knockdown of human Freud-2 using SiRNA derepressed the human 5-HT1A receptor expression in 5-HT1A-postive neuronal cells. Because no human cell model of presynaptic 5-HT1A receptor expression is available, the specificity of action of human Freud-2 on postsynaptic versus presynaptic 5-HT1A regulation was not directly assessed. Further Western blot studies of human postmortem normal brain tissues revealed exciting results, which are in line with my mouse Freud-2 studies. Human Freud-2 protein highly expressed in prefrontal cortex (PFC), and hippocampus, but very low level was detectable in raphe nuclei unlike Freud-1. These results are consistent with results obtained in rodent cells, indicating that mouse Freud-2 is active on postsynaptic, but not presynaptic 5-HT1A receptor gene expression. Immunohistochemistry also confirmed that human Freud-2 is highly expressed in PFC, and hippocampus but low in raphe. Based on this evidence I suggest that human Freud-2

is a postsynaptic 5-HT1A receptor repressor. More exciting results came from immunofluorescence studies that revealed that hFreud-2 is localized in nucleus of neurons, as well in neuroglial cells. Thus, these studies reveal that Freud-2, like Freud-1, also functions as a repressor of the 5-HT1A receptor gene, suggesting that these repressors have complementary roles. In particular, while Freud-1 regulates both presynaptic and postsynaptic 5-HT1A receptor expression, Freud-2 is more selective for the postsynaptic 5-HT1A receptor. The finding that human Freud-2 regulates 5-HT1A receptor expression has very interesting implications to better understand the pathophysiology of mood disorder, and might provide a new target for antidepressant treatment.

The 5-HT1A receptor protein is first detected in the brain around embryonic stage E14. It is widely expressed all over the brain such as hippocampus, cerebellum, brainstem, cerebral cortex, and spinal cord. It is also expressed in non-neuronal cells such as astrocytes where it stimulates the release of growth factor S-100 beta, which results in regeneration and sprouting of neuronal terminals (Azmitia and Whitaker-Azmitia, 1991; Whitaker-Azmitia et al., 1993; Hillion et al., 1994; Miquel et al., 1994; Azmitia et al., 1996). Altered neuroglial interactions may be one mechanism leading to degeneration of neurons, which is one of the mechanisms that may underlie mood disorders. Abnormal regulation of neuronal or glial 5-HT1A receptors, perhaps involving Freud-1 and Freud-2, may contribute to neuroglial dysfunction and the pathophysiology of anxiety and depression.

Freud-2 is expressed in the hippocampus and might modulate 5-HT1A receptor expression in this area. Neuronal precursors are localized in sub-ventricular zone and sub-

granular zone of hippocampus in adulthood and induce neurogenesis. It has been shown that SSRI-induced neurogenesis in the hippocampus is mediated via postsynaptic 5-HT1A activation, and that this is required for behavioral improvement in mice (Santarelli et al., 2003b). Conversely, reduction of 5-HT1A neurotransmission in the hippocampus leads to impaired cognitive performance, which is seen in depression, schizophrenia, and Alzheimer's diseases (Brezun and Daszuta, 1999). Consistent with this evidence administration of fluoxetine (SSRI) increases extracellular 5-HT in the hippocampus and activates 5-HT1A receptors, which inhibit pyramidal cell excitability in the CA3 region of hippocampus (Sprouse et al., 2001). Moreover, microinjection of 8-OH-DPAT (5-HT1A agonist) increased cell proliferation in dentate gyrus of hippocampus rat (Huang and Herbert, 2005). Knockout of the 5-HT1A receptor blocked hippocampal neurogenesis and behavioral improvement induced by chronic SSRI therapy. Indeed X-irradiation of the hippocampal region in mice also inhibited the neurogenic and behavioral effects of chronic antidepressant treatment (Santarelli et al., 2003b). These results suggest that the behavioral effects of chronic antidepressant therapy are mediated through 5-HT1A receptors in the hippocampus. 5-HT1A receptor knockout mice show increased anxiety-like behaviors, and expression of 5-HT1A receptors in the forebrain (including hippocampus) but not the raphe rescued the phenotype (Gross et al., 2002b). Taken together, 5-HT1A receptor play a key role in adult neurogenesis, learning, and memory formation. I hypothesize that regulation of 5-HT1A receptor by Freud-2 may modulate hippocampus function. More studies need to be done to elucidate Freud-2 function in hippocampal neurogenesis and behavioral effects of antidepressant treatment.

In vivo studies over expressing Freud-2 in hippocampus would reveal the role of Freud-2 in hippocampal neurogenesis and behavioral function. We would expect to see less expression of 5-HT1A in hippocampus of over expressed Freud-2 animals which may result to reduce hippocampal neurogenesis and decreases the SSRI behavioral improvement. Moreover, reduction of 5-HT1A receptor expression may lead to anxiety-like behavior in transgenic model. To better understanding the function of human Freud-2 study of human Freud-2 expression in normal subjects and depressed patients will help to elucidate the role of Freud-2 in etiopathology of major depressive disorders. By examining the expression of Freud-2 in other limbic areas, the role of Freud-2 in modualting limbic system will be more prominent. In addition, looking at the regulation of Freud-2 RNA or protein by antidepressants in normal and depressed patients could implicate Freud-2 in antidepressant action and as a new target for generation of new antidepressants.

I also found that Freud-2 is expressed in prefrontal cortex, which may modulate the effect of the serotonin system on this region. It has been reported that mPFC projects to and activates DR neurons. On the other hand, DR neurons also project to mPFC and inhibit the activity of pyramidal neurons in this region through postsynaptic 5-HT1A receptor activation (Celada et al., 2001a). Electrical stimulation of DR/MnR inhibited partially the activity of PFC (Puig et al., 2005). In addition in situ studies on 5-HT1A mRNA expression revealed that this receptor is expressed in pyramidal neurons of frontal cortex and occipital cortex, as well as CA1 and in less abundant in CA3 pyramidal neurons of hippocampus. This pattern of expression suggest an involvement of 5-HT1A receptor in cognition and emotional response (Palchaudhuri and Flugge, 2005). The expression of

Freud-2 in pyramidal neurons of the PFC could implicate Freud-2 in regulation of 5-HT1A receptor expression in these cells. In addition Freud-2 might be implicated in the reduction of 5-HT1A receptors seen in regions of the PFC in patients with depression or anxiety disorders. Further studies to identify whether Freud-2 expression is altered in these patients may implicate Freud-2 dysregulation in these disorders. In addition further experiments using transgenic mice over-expressing Freud-2 in PFC region or Freud-2 conditional knockout will reveal the role of Freud-2 in emotional and cognitive responses to different aversive stimuli and conditioning paradigms. Indeed examining the response of the PFC to DRN stimulus in Freud-2 knockout will provide more evidence of post synaptic 5-HT1A regulation of Freud-2.

Freud-2 was also localized in neuroglial cells of the prefrontal cortex and it has been shown that 5-HT1A receptors are expressed in the cell body and processes of astrocytes (Whitaker-Azmitia et al., 1993; Azmitia et al., 1996). As mentioned above, astrocytes synthesize the growth factor S-100beta, which is released in response to 5-HT1A receptor stimulation. S-100beta induces neuronal terminal sprouting and neuronal regeneration. Reduction of glial number was reported in prefrontal cortex of patients with mood disorders compared to controls (Ongur et al., 1998). Moreover, this reduction also reported in deep layers of cortical regions (Cotter et al., 2001; Cotter et al., 2002). Thus, I could speculate that modulation of 5-HT1A expression in glial cells by Freud-2 might be involved in this pathophysiological aspect of mood disorders.

Depression is associated with deregulation of HPA and serotonergic system (McAllister-Williams et al., 1998; Cubala and Landowski, 2006). Hypercortisolemia in depression along with an imbalance between GR (higher GR expression) and MR receptors in limbic

system predisposes individuals to develop depression or anxiety. Transgenic mice over-expressing MR in their forebrain display reduced anxiety-like behaviors (Rozeboom et al., 2007). This imbalance led to loss of hippocampal GR and negative feedback at HPA, and increased hippocampal 5-HT1A receptor expression. The role of postsynaptic 5-HT1A receptors in regulation of HPA was reviewed in chapter I. Negative action of hypercortisolemia on expression of 5-HT1A receptor resulted in reduced postsynaptic 5-HT1A receptor expression in limbic areas such as the hippocampus (Ou et al., 2001), and may be responsible for alteration of memory formation in depressed patient. Hypercortisolemia also reduces ACTH levels by negative feedback, but 5-HT1A receptors may also impact in this phenomenon since stimulation of 5-HT1A receptors induces ACTH secretion in man. In particular, the 5-HT1A agonist ipsapirone significantly decreased serum ACTH and cortisol level in patient with unipolar depression (Lesch et al., 1990). So, alteration in 5-HT1A receptor expression and dysfunction of this receptor in depressed patient may explain the reduction in ACTH level and cortisol secretion. Thus alteration in Freud-2 protein, by altering 5-HT1A receptor expression in the limbic system may dysregulate HPA function and be involved in the pathophysiology of mood disorders.

Recent studies have also linked deregulation of postsynaptic 5-HT1A receptors to schizophrenia and bipolar disorder and effect of lithium treatment (McQuade et al., 2004; Gray et al., 2006). As a regulator of post-synaptic 5-HT1A receptor expression in the hippocampus and cortex, alterations in Freud-2 activity may contribute to a variety of mental illnesses and cognitive impairment. Future studies may address whether Freud-2 is deregulated in various mental illnesses, or whether functional genetic polymorphisms

of Freud-2 may be associated or linked with mental illness or genetic disease. In this light, the recent identification of a truncation mutant of Freud-1 that is linked to mental retardation (Rogaeva, 2007; Basel-Vanagaite 2006) suggests that other mutants or polymorphisms of Freud-1 or Freud-2 may affect cognitive development, possibly underlying deficits found in schizophrenia and mood disorders. As shown for Freud-1, Freud-2 may have gene targets in addition to the 5-HT1A receptor gene, that could mediate actions on neuronal development, however these targets remain to be identified. *In vivo* studies deleting corresponding part of Freud-2 to Freud-1 mutant will reveal the developmental role and cognitive role of Freud-2.

Taken together, Freud-2 is a novel post synaptic 5-HT1A regulator protein which negatively regulates the expression of 5-HT1A receptor gene. Expression pattern of Freud-2 in human brain studies showed that it highly expressed in the hippocampus and PFC and perhaps in other structure of limbic system which indicates that Freud-2 may have roles in modulating of limbic region in normal subjects and depressed patients and open up a new window to better understand of etiopathology of mood disorders.

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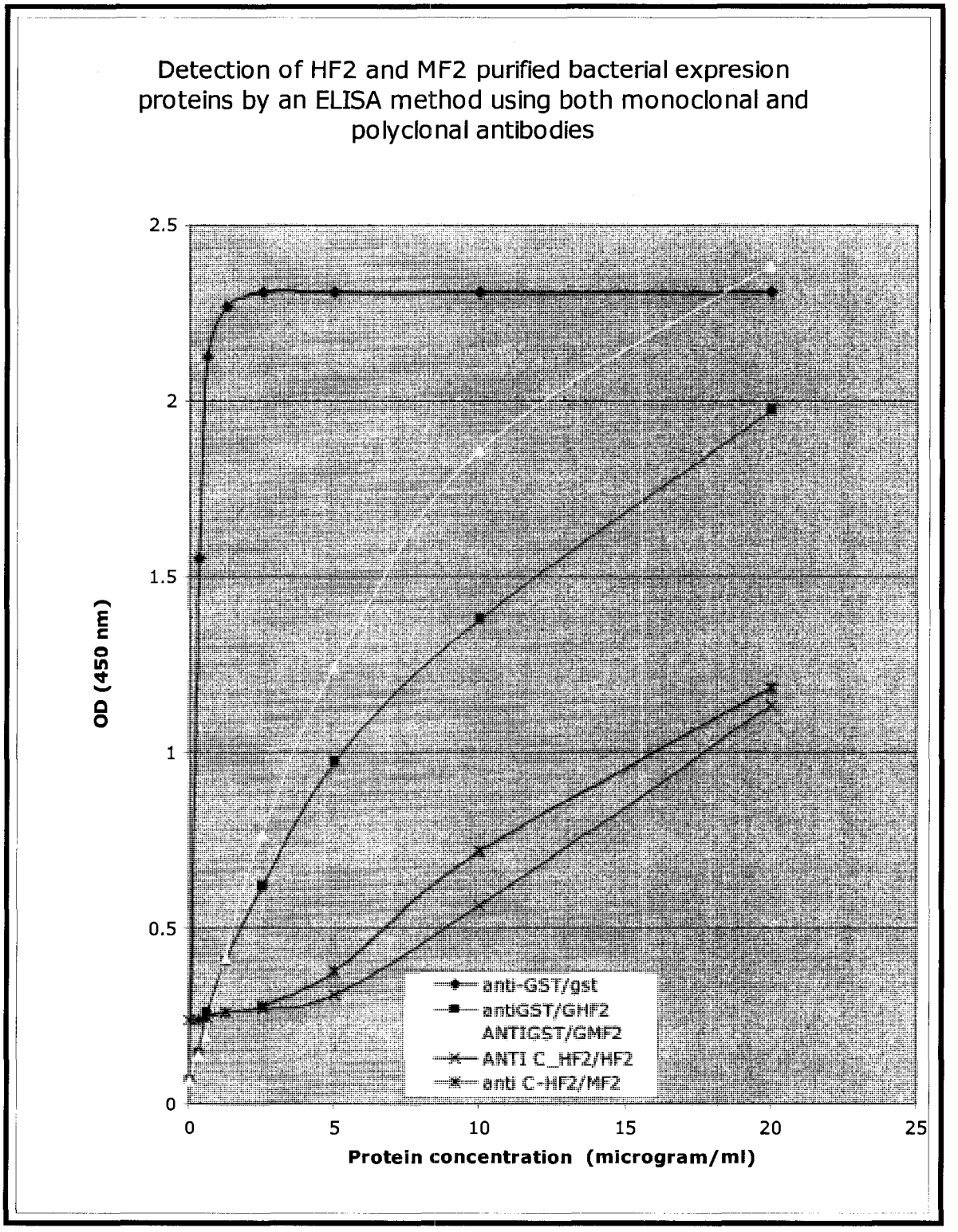
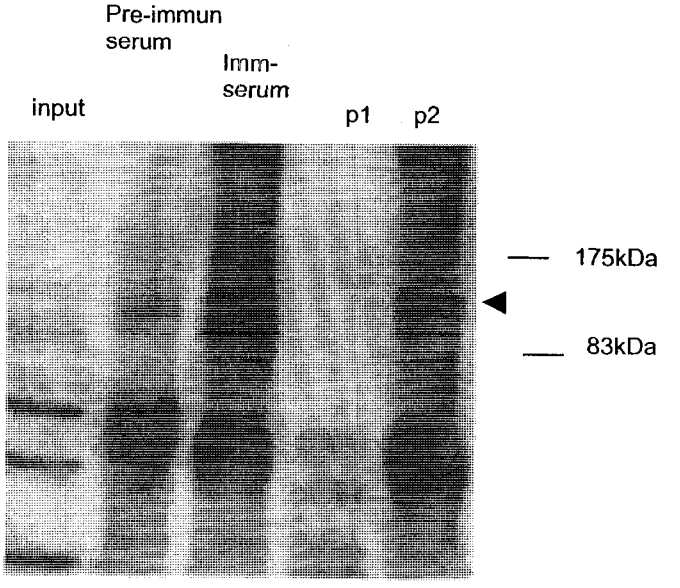
Appendices


Detection of HF2 and MF2 purified bacterial expresion proteins by an ELISA method using both monoclonal and polyclonal antibodies
OD (450 nm)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Protein concentration (microgram/ml)
anti-GST/gst
antiGST/GHF2
ANTIGST/GMF2
ANTI C_HF2/HF2
anti C-HF2/MF2

Appendix I- Quantification of antibody by ELISA

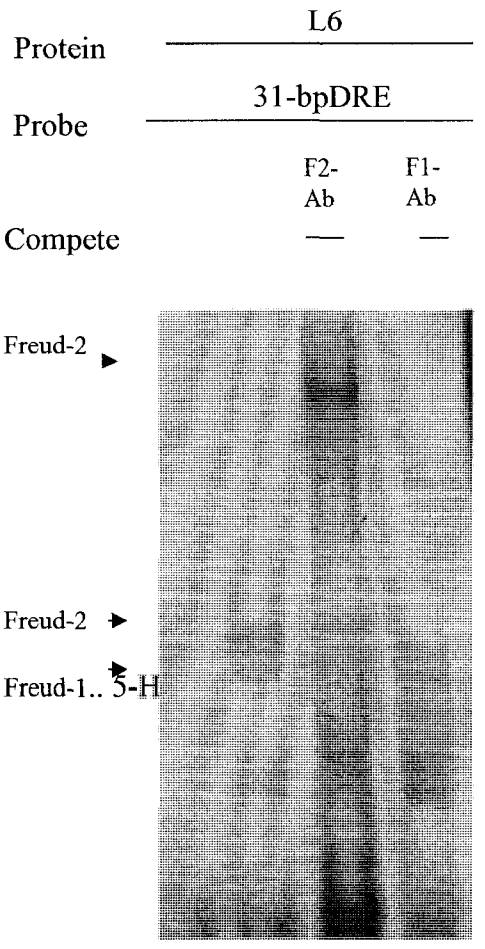
Microtiter plate was coated by nutravidin (100µl/well). After wash wells were blocked with 200 µl of 1% BSA in PBS for I hour. After three more wash with 0.1% Tween in PBS solution 100 µl of biotinylated peptide was added to each well and incubated for 10 minutes at RT. Washed plate three times with above mentined wasing solution and 1:1000 dilution of antibodies in the same dilution buffer as peptide was added to each well incubated 30 minute in 37 °C. Washed plate 4 times with above mentined washing solution then incubated with 1:5000 dilution secondary anti-rabbit antibodies and incubated for 1 hour in RT. Plate was washed 5 times more and added 100 µl/well TMB substrate (Roche). To stop the reaction 100 µl of 0.2 M H_2SO4 was added. OD was measured at 450nm.



Appendix II- immunoprecepitation of Freud-2 from L6 cell lyses.

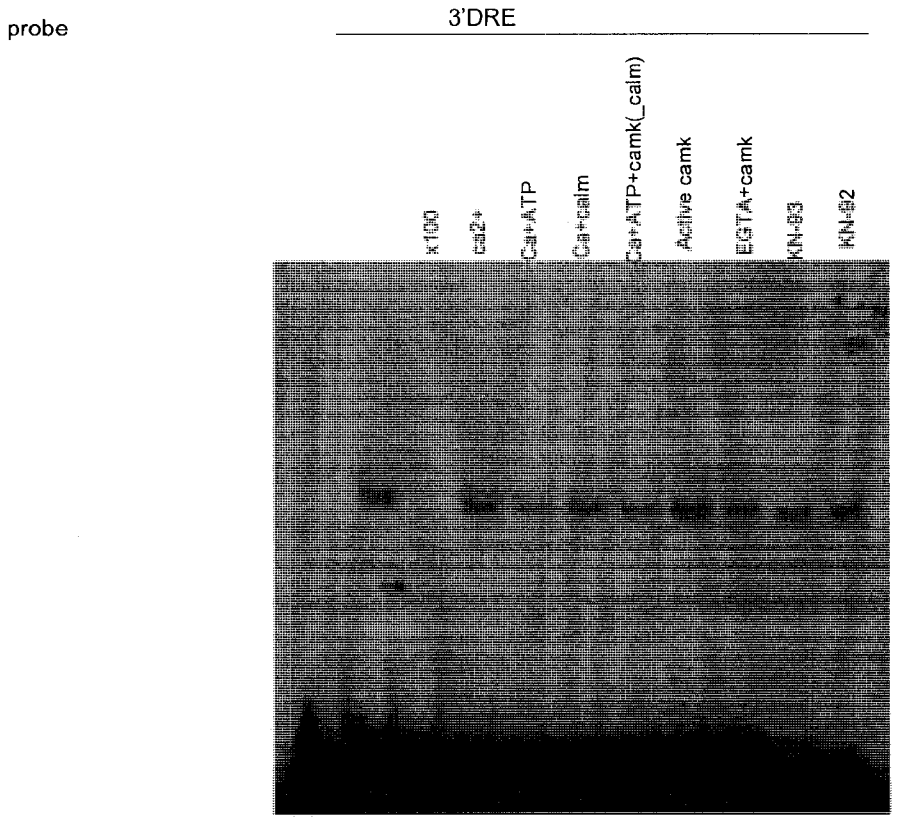
Method- Two 10 cm plates of confluent L6 cells were washed with 2xPBS. Cells were collected in REPA buffer containing protease inhibitors and incubated on ice for 30 minute. Then cells were sonicated three times at the setting of 3 for 10 second each time with 30second interval. Lysates were centifuged and supernatant diluted 10 times with ice cold PBS buffer containing protease inhibitor to the final volume of 2 ml (input). 1:10 of input was kept, remaining was incubated with pre-immune serum, immunized serum aginst the Freud-2, and purified C-F2 antibody incubated at 4° C with rotation overnight. The next day 20 µl of protein A beads was added and incubated for more 2 hours at 4 °C with rotation. Antibody/protein/beads complexes were centrifuged for 10 second at 1000xg. Proteins were eluted by 50 µl of 2x loading buffer by boiling for 5 minute in water bath. After 10 minute centrifugation, then 30 µl of supernatants were loaded in 8%

SDS-PAGE gel for Western blot assay. Specific Freud-2 band was detected using column purified C-F2 antibody generated against Freud-2 C-terminal. P1 and P2 two different samples were immunopericipitated by purified C-F2 antibody. Arrow head indicates the Freud-2 protein.



Appendix II- comparison of Freud-1 and Freud-2 antibody on DRE complexes.

Nuclear extraction of L6cells was incubated with 31-DRE demonstrated two bands,the lower band indicated Freud-1 and the upper band showed Freud-2(arrows).Freud-2 supershifted by incubation with the specific antibody against it(arrow head). Incubation of L6 nuclear extract with F1-Ab did not displace Freud-2/DRE complex.



Appendix IV- effect of calcium on Freud-2 binding to DRE

Purified Freud-2 protein was incubated with 3' DRE in a band shift assay. Freud-2/DRE interaction was enhanced by CaMKII. In the presence of calcium chelating agent, EGTA, or 10μM KN-93 (CaMK II inhibitor) the intensity of interaction of Freud-2 and DRE was decreased, while KN-92 as anegative control did not affect the interaction.