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Regulation of 5-HT1A Repressor Human Freud-1/CC2D1A by Calcium/Calmodulin Sensitive Phosphorylation

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Regulation of 5-HT1A Repressor Human Freud-1/CC2D1A

by Calcium/Calmodulin Sensitive Phosphorylation

by

Kimberly Galaraga

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ABSTRACT

Altered regulation of the serotonin system is implicated in depression and anxiety. The 5-hydroxytryptamine 1A (5-HT1A) receptor is a major regulator of the serotonin system. We have previously identified a calcium-regulated transcription factor, Freud-1, which represses 5-HT1A receptor expression in neurons. Analysis of human Freud-1 amino acid sequence revealed the presence of two putative calcium calmodulin kinase (CaMK) phosphorylation sites. We demonstrate the ability of Freud-1 to be phosphorylated by CaMKII *in vitro*. Mutations of Freud-1 phosphorylation sites reduced CaMKII mediated phosphorylation. Furthermore, CaMKII phosphorylation inhibited binding of Freud-1 to the 5-HT1A promoter elements *in vitro*. In cells, CaMKIV induced phosphorylation of Freud-1 and prevented Freud-1 induced repressor activity. Freud-1 mutants prevented the inhibitory effect of CaMKIV on Freud-1 mediated repression of 5-HT1A receptor gene. Together these results implicate phosphorylation of Freud-1 by CaMK, in calcium-dependent regulation of 5-HT1A receptor expression, could participate in antidepressant actions to restore 5-HT1A receptor levels.

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5-HIAA 5-hydroxyindoleacetic acid

5-HT 5-hydroxytryptamine (serotonin)

5-HT1A 5-hydroxytryptamine 1A receptor

5-HTP 5-hydroxytryptophan

5-HTR 5-hydroxytryptamine receptor

5-HTT 5-hydroxytryptamine transporter

5-HTTLPR serotonin transporter polymorphism

AADC aromatic amine decarboxylase

C2 protein kinase C conserved region 2

Ca calcium

CaM calmodulin

CaMK calcium calmodulin dependent protein kinase

CaMKII calcium calmodulin dependent protein kinase II

CaMKIV calcium calmodulin dependent protein kinase IV

CaMKK CaMK kinase

CBP CREB binding protein

CC2D1A coiled-coil and C2 domain containing 1A

CHIP chromatin immunoprecipitation assay

CREB cAMP response element binding protein

CRE cAMP response elements

CREM cAMP-responsive element modulator

CSF cerebrospinal fluid

DA dopamine

DBB DNA binding buffer

Deaf-1 deformed epidermal autoregulatory factor 1

DM14 Drosophila melanogaster 14

DRD2 dopamine-D2 receptor

DRE dual repressor element

DREAM DRE-antagonist modulator

DRN dorsal raphe nucleus

DSM-IV Diagnostic and Statistical Manual of Mental Disorders

EDTA ethylene glycol tetraacetic acid

EDTA ethylenediamine tetraacetic acid

EMSA electrophoretic mobility shift assay

ERK extracellular signal-regulated kinase

FRE five primed repressor element

Freud-1 five prime repressor under dual repression binding protein 1

Freud-2 five prime repressor under dual repression binding protein 2

GRK G protein coupled receptor kinase

HBS HEPES buffered saline

Hes5 Hairy/Enhancer-of-split-5

HLH helix loop helix

HVA homovanillic acid

LTP long term potentiation

MAOI monamine oxidase inhibitors

MAP microtubule associated protein

MAPK mitogen-activated protein kinase

MD major depression

MDD major depressive disorder

MRT3 mental retardation, nonsyndromic, autosomal recessive, 3

NE norepinephrine or noradrenaline

NFκB nuclear factor-kappa B

NLS nuclear localization sequence

NRSE neuron restrictive silencer element

NRSF neural restrictive silencing factor (also known as REST)

NUDR nuclear deformed epidermal autoregulatory factor

PBS phosphate buffered saline

PKA protein kinase A

PKC protein kinase C

PP2A protein phosphatase 2A

RE-1 repressor element 1

REST repressor element 1 silencer of transcription [also known as NRSF]

SH3 Src homology 3 domain

SNP small nucleotide polymorphism

SRF serum responsive factor

TCA tricyclic antidepressants

TPD tryptophan depletion

TPH2 tryptophan hydroxylase 2

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INTRODUCTION

Major Depression and Neurotransmitter Systems

Depression

Major depression (MD) has increasingly become one of the more prominent clinical issues in recent years. Major depressive disorder (MDD) has a lifetime prevalence of 15 to 20% for North American adults in the general population with women being twice as prevalent compared to men (Blier and Ward, 2003). Depressed individuals exhibit signs of abnormalities affecting mood with reduced interest or pleasure in activities, neurovegetative functions including change in appetite and disruption of sleep, cognitive thoughts such as feelings of guilt or worthlessness, and psychomotor activity such as agitation or retardation (Fava and Kendler, 2000). Although all individuals experience depression at some point in their life, major depression symptoms persist for more than two weeks and represent a change from previous functioning. All criteria for major depression, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), are important however individuals exhibit a variation of signs and symptoms (Fava and Kendler, 2000). The more prominent risk factors associated with MDD include developmental factors such as adverse childhood experiences, certain personality traits, for example heightened stress reactivity, psychosocial or life stressors, comorbid disorders, cognitive and biological factors (Riso et al., 2002). Changes in psychomotor functioning, cognitive thinking, appetite and circadian rhythm support the biological factors for major depression.

Genetics of Depression

Familial studies have reported a three fold increase in risk for major depression in first relatives of affected family members compared to the general population (Sullivan et al., 2000). The conclusions suggest a genetic predisposition to the development of depression. However, adoption studies have been more open to interpretation with three studies each showing weak, strong and no evidence. Conversely, five MDD twin studies more consistently indicated that heritability can account for MDD, although genetic factors are not solely responsible (Fava and Kendler, 2000). Complex disorders, such as MDD, presumably involve a wide array of genes that may interact with each other or be influenced by environmental factors resulting in depressive symptoms. Linkage and association studies of gene-environmental interactions have targeted 5-HTTLPR (insertion/deletion polymorphism in the promoter of a serotonin transporter) and stress and a loss of function mutation of TPH2 (a brain specific isoform of tryptophan hydroxylase – an enzyme responsible for the synthesis of serotonin) in depression however these studies have not been replicated in large scale studies (Kato, 2007). A larger sample size and new technology for comprehensive analysis and advanced methods for genotyping complex polymorphisms need to be applied for further research.

Imaging Studies

Common to all mood disorders, MDD reflects the altered functioning of certain regions in the brain. According to extensive functional neuroimaging studies, presumably major depression involves the limbic system (hippocampus, cingulate cortex), reward circuits (nucleus accumbens, amygdala, ventral tegmental area, parahippocampal gyrus, and prefrontal cortex), hypothalamus, and anterior temporal cortex (Mayberg, 1997).

Depressed patients appear to have decreased volumes of the frontal lobe, cerebellum, caudate putamen and an increase in focal white matter intensities in regions of the frontal cortex and basal ganglia (Soares and Mann, 1997). PET and SPECT studies demonstrated an overall decrease in brain metabolism but more specifically in the frontal regions of the dorsolateral and medial-prefrontal cortex, basal ganglia and cingulate cortex (Dougherty et al., 2003). Also reported was increased blood flow and metabolism in the orbital frontal cortex and amygdala based on neuroimaging analysis of depressed patients (Drevets, 1999).

Neurotransmitter Systems

Since the first catecholamine hypothesis of affective mood disorders, approximately 40 years ago, the contribution of neurotransmitter or neuroregulator systems in the pathophysiology of depression has been studied (Nemeroff, 2002; Schildkraut et al., 1965). The hypothesis describes the dysregulation of one or several neurotransmitter systems functioning independently or in concert with one another. The historical significance of the hypothesis has lead to the development of numerous antidepressant drugs with the purpose of selectively targeting monoamine systems. Substantial experimental and clinical studies have provided strong evidence for the roles of serotonin, norepinephrine, and to a lesser extent, the dopamine system in the etiology of depression (Delgado, 2000; Nemeroff, 1998; Nemeroff, 1996). All three monoamines are essential and have overlapping roles in regulating mood, emotion and cognitive function which are impaired in depressed patients (Nemeroff, 2002).

Dopamine System

Less focus has been directed to the dopamine (DA) system in relation to major depression. Dopamine neurons are organized in four major pathways: mesolimbic, mesocortical, transmitting dopamine from the midbrain to the limbic system and cortical regions respectively, nigrostriatal (from substantia nigra to striatum) and tuberoinfundibular pathway, transmitting dopamine from the hypothalamus to the pituitary gland. Consequently dopamine neurons innervate regions that are associated to behavioral and physiological functioning altered in depression. Also reported is high comorbidity with Parkinson's disease characterized by the loss of dopamine producing cells. Postmortem, neuroimaging studies have implicated the involvement of the dopamine system in the pathophysiology of depression (Delgado, 2000). *In vivo* PET studies of receptor labeling have demonstrated an increase in dopamine D2 receptor binding in the right striatum in MDD patients and another study has found higher striatal dopamine transporter density in depressed individuals (Fava and Kendler, 2000). Another study examining dopamine and its metabolite HVA (homovanillic acid) demonstrated a decrease in HVA levels in depressed patients suggesting a decrease in DA turnover is correlated with the disorder (van Praag and Korf, 1975). Also the dopaminergic system is indirectly altered by antidepressants. Some antidepressants affect dopamine metabolism and prevent its reuptake (van Praag and Korf, 1975).

Norepinephrine System

The hypothesis stating that dysregulation of the norepinephrine (NE) system is implicated in depression was first mentioned in the 1960s based on antidepressant therapeutic effects on the noradrenaline system (Blier, 2006). Noradrenergic cells in the locus coeruleus of the brainstem project to various regions including the cerebral cortex, limbic system, and the spinal cord. The norepinephrine system mediates stress reactivity and underlies the "fight or flight" response, triggering accompanying physiological reactions. Activation of noradrenergic neurons releases noradrenaline which acts on α- and β-adrenergic receptors located pre- or postsynaptically. Numerous clinical and imaging studies have provided evidence showing that dysfunction of NE neurons and changes in adrenergic receptor sensitivity contribute to the etiology of depression (Anand and Charney, 2000). An early link between the disorder and the noradrenaline system arose from a study demonstrating that the efficacy of the tricyclic antidepressant imipramine is correlated with an increase in NE levels (Schildkraut et al., 1965) by inhibiting the reuptake of noradrenaline (Blier, 2006). Treatment with reserpine, which depletes NE containing granules, is known to produce depression in normal individuals. However, serotonin and dopamine stores are also depleted (Blier, 2006). Post mortem studies showed elevated density of α2-adrenoreceptors (Ordway, 1997) that function as autoreceptors providing negative feedback regulation decreasing firing rate. A decrease in norepinephrine transporter binding levels has been reported in the locus coeruleus in depressed patients (Fava and Kendler, 2000). This may indicate that excessive stimulation of the locus coeruleus leads to depletion of endogenous NE and a corresponding increase in the levels of synthetic enzymes and upregulation of autoreceptors. There have been quite consistent reports of binding studies showing dowregulation of β-adrenergic receptors in response to antidepressants that particularly target NE levels (Anand and Charney, 2000). Decreased binding of norepinephrine transporters is observed in the locus ceruleus of depressed patients and was interpreted as downregulation due to compensatory mechanisms is response to depleted NE levels at the synapse (Klimek et al., 1997). It is well established that selective NE reuptake inhibitors (maprotiline, desipramine, reboxetine, etc.) are effective antidepressants and more so are those with both serotonin and norepinephrine reuptake properties such as duloxetine (Nemeroff, 2002). The mechanism of action of these agents provides supportive evidence that dysregulation of the NE system is involved in the etiology of depression.

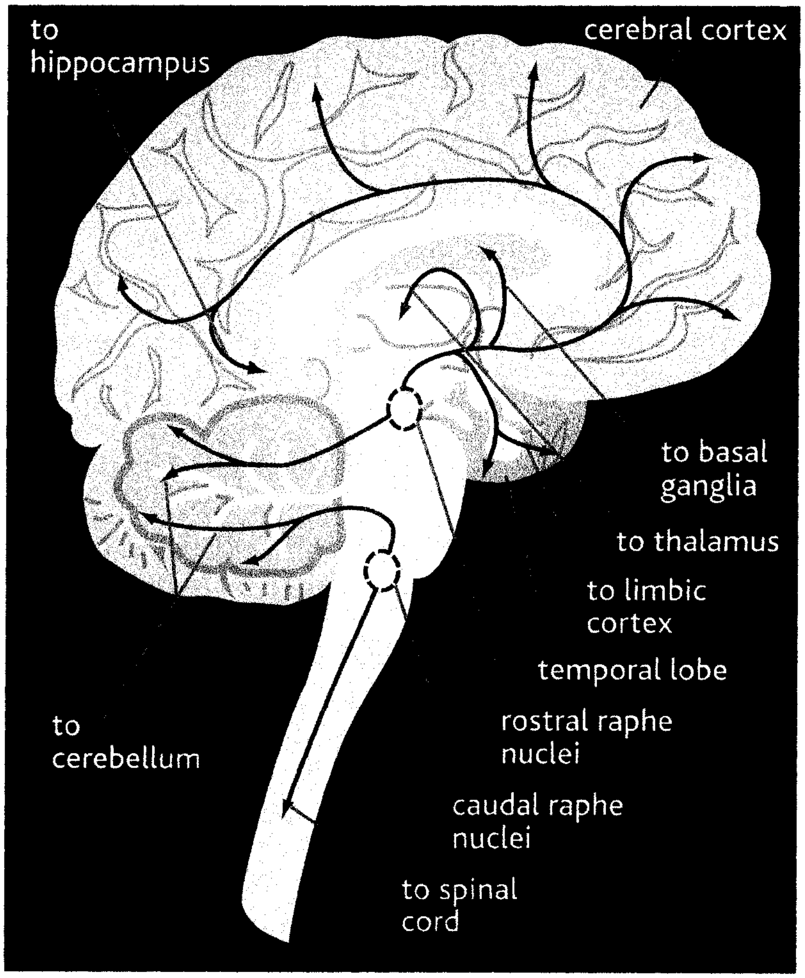
Serotonin System

Implications and Location

One of the key contributing factors of depression, and the subject of numerous studies, involves the serotonin (5-HT, 5-hydroxytryptamine) system. In 1969, the indoleamine hypothesis of depression was proposed. The hypothesis stated that depression or mania was related to decreased serotonergic activity due to less serotonin release or less available serotonin receptors that are important in serotonin signal transduction (Mann, 1999). Over the past 30 years many studies have supported the indoleamine hypothesis. The serotonin system has widespread implications in mood, sexual behavior, memory, learning, physiological functions such as sleep, appetite body temperature regulation and other disorders that include suicidal behavior, eating disorders, anxiety, aggression, obsessive compulsive disorders, alcoholism and schizophrenia (Mann, 1999; Huang et al., 2004). The serotonergic neurons are mainly clustered in the raphe nuclei located on either side of the midbrain in the brainstem (Figure 1). Each nucleus can be divided into two different regions: the rostral nucleus contains the dorsal and median raphe and projects mainly to the forebrain while

Figure 1: The serotonin pathways.

Serotonergic neurons are mainly clustered in the rostral and caudal raphe nuclei located on either side of the midbrain. Neurons projects to various regions of the brain such as the cortex, hippocampus, basal ganglia, thalamus, cerebellum and limbic regions which includes the cortical areas surrounding the brain stem. Serotonin transmission is reduced in patients with depression compared with non-depressed controls. Image obtained from http://www.cnsforum.com/imagebank/item/Neuro_path_SN_DPN/default.aspx.


to
hippocampus
cerebral cortex
to basal
ganglia
to thalamus
to limbic
cortex
temporal lobe
rostral raphe
nuclei
caudal raphe
nuclei
to spinal
cord
to
cerebellum

the caudal nucleus contains the raphe pallidus, raphe magnus, and raphe obscurus and projects to the cerebellum, brainstem and spinal cord (Lopez-Figueroa et al., 2004). The largest regions of the raphe include the dorsal and median raphe nuclei, which innervate the hypothalamus, prefrontal cortex, basal ganglia, brainstem and limbic areas such as amygdala and hippocampus that have been specifically implicated in various mood disorders (Joyce et al., 1993). Thus, the projections of the serotonin system are well located anatomically to mediate the signs and symptoms of depression.

Serotonin Synthesis

In the late 1940's, Maurice M. Rapport, Arda Green, and Irvine Page first isolated a serum tonic factor released from platelets during blood clotting as a vasoconstrictor substance and identified it as a monoamine named serotonin that was later chemically identified as 5-hydroxytryptamine (Mann, 1999; Rapport et al., 1948). Serotonin was also found in human gastrointestinal tract and platelets in blood serum, however, the concentration was the highest in the brainstem and lower in other brain regions (Mann, 1999). It became clear that aside from being involved in platelet aggregation and intestinal motility serotonin was a neurotransmitter. The synthesis serotonin is initiated by the conversion of the amino acid tryptophan into intermediary 5-HTP (5-hydroxytryptophan) by the rate-limiting enzyme tryptophan hydroxylase. Two isoforms of TPH have been shown to exist. TPH1 is found in several peripheral tissues that express serotonin (skin, gut, pineal gland) and also in the central nervous system. TPH2 is exclusively expressed in neuronal cells and the primary brain specific isoform (Walther et al., 2003). Studies have demonstrated that genetic polymorphisms in both these subtypes influence susceptibility to anxiety and depression (Zhang et al., 2005; Nash et al., 2005).

The 5-HTP is then converted to 5-HT by 5-HTP decarboxylase, ie. aromatic amine decarboxylase (AADC). The availability of tryptophan in the extracellular fluids limits serotonin synthesis. Most of the serotonin released into the synaptic space is removed by the neuron through a reuptake mechanism by serotonin transporters (5-HTT) and subsequently degraded by monoamine oxidase (MAO), which converts serotonin to its metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Serotonin Receptors

The serotonin system consists of a large family of serotonin receptors. There are 15 known mammalian receptors subtypes and 7 distinct families and that include 5-HT1A/B/D/E/F, 5-HT2A/B/C, 5-HT3A/B/C/D/E, 5-HT4, 5-HT5A/B, 5-HT6 and 5-HT7 (Barnes and Sharp, 1999; Hoyer et al., 2002; Pandey et al., 1995). Serotonin receptors are present at pre- and post-synaptic sites, and are also located on the cell body and dendrites. All serotonin receptors are G protein coupled receptors with the exception of the 5-HT3 receptor which is a ligand gated ion channel receptor. The 5-HT1 receptor class couple preferentially to $G_{i/o}$ class of G proteins to inhibit cyclic adenosine monophosphate (cAMP) formation. 5-HT1B receptors function as both terminal autoreceptors on serotonin containing neurons and heteroreceptors on nonserotonergic neurons and play an important role in regulating serotonin neurotransmission by presynaptic inhibition (Svenningsson et al., 2006). Genetic and pharmacological studies have reported a role for 5-HT1B receptors in the pathophysiology of obsessive compulsive disorder, drug addiction, depression, anxiety, aggression and sleep (Svenningsson et al., 2006; Mann, 1999). The 5-HT1D receptor is closely related to the 5-HT1B receptor and functions as autoreceptors at the nerve terminal and on the soma of serotonin neurons (Mann, 1999).

The 5-HT1D agonist, Sumatriptan, activates the 5-HT1D autoreceptors at the nerve terminals, reducing serotonin release and may explain the anti-migranous effects (Mann, 1999). The 5-HT2 class of receptors couple preferentially to $G_{q/11}$ proteins to increase the hydrolysis of inositol phosphates and elevate cytosolic calcium concentrations (Hoyer et al., 2002). Some studies have associated 5-HT2A receptors to the pathophysiology of depression. Specifically, some studies have reported higher levels of 5-HT2A receptors in the brain of suicide victims and on the platelets of depressed patients who have attempted suicide (Mann, 1999). However mechanisms of this correlation are uncertain (Mann, 1999). The 5-HT3 family of receptors are located on central and peripheral neurons and function as nonselective cation channels (Na^+, Ca^{2+} influx and K^+ efflux) leading to rapid depolariziation (Hoyer et al., 2002). 5-HT5 receptors primarily couple to $G_{i/o}$ to inhibit adenylyl cyclase activity (Barnes and Sharp, 1999). Serotonin receptor families, 5-HT4, 5-HT6, 5-HT7, all preferentially couple to G_s and function to increase cellular levels of cAMP (Hoyer et al., 2002).

Studies Associating 5-HT with Depression

There is overwhelming evidence supporting a role for reduced levels of 5-HT in the etiology of MDD. Studies measuring 5-HIAA levels in cerebrospinal fluid (CSF) demonstrated a correlation between low levels of the metabolite and the severity of depression suggesting that a decrease in 5-HIAA levels due to the reduced availability of 5-HT is associated with depressive symptoms (Risch and Nemeroff, 1992). The development of depressive symptoms results from depletion of monoamine stores by the administration of reserpine (which inhibits vesicular monoamine uptake preventing monoamine storage) or selective 5-HT depletion by a tryptophan hydroxylase inhibitor parachlorophenylalanine (Nemeroff, 2002). Interestingly, when formerly depressed patients, that have achieved remission, are subjected to acute tryptophan depletion (ATD) by a special diet most of them showed symptoms of transient relapse (Booij et al., 2005; Leyton et al., 2000) and acute melancholia was observed in normal subjects (Ellenbogen et al., 1996). This suggests that 5-HT levels must not decline in order for depressed patients to remain in remission. Evidence has also demonstrated dysregulation in the serotonin reuptake system is correlated with depression. Postmortem studies have shown a decrease in 5-HT transporter binding sites in brain tissue of depressed and suicide victims (Owens and Nemeroff, 1994). The same findings have been reported by imaging studies of drug naive or drug free depressed patients (Kasper et al., 2002). This could indicate a reduction in serotonin nerve terminals with transporter binding sites. An increase density of postsynaptic 5-HT2 receptors has been observed in the frontal cortex of depressed victims in postmortem studies (Attar-Levy et al., 1999). An increase in 5-HT2 receptor binding sites have been observed in depressed patients by imaging experiments and a significant reduction in available 5-HT2 receptors in the brain results from antidepressant treatment (Yatham et al., 1999). Blockade of 5-HT2 receptors seem to improve clinical effects of serotonin specific reuptake inhibitors (SSRIs) (Celada et al., 2004). Evidence provided by PET imaging studies showed reduced 5-HT1A receptor binding in cortical regions of depressed patients (Sargent et al., 2000; Malison et al., 1998). In previous studies it has been demonstrated that in major depressive disorder 5-HT1A mRNA levels are specifically lower in the hippocampus (Lopez et al., 1998). Also reported was an increase in 5-HT1A receptors density in the dorsal raphe nucleus of depressed suicide victims (Stockmeier et al., 1998; Mann et al., 1989). Decreased levels of postsynaptic 5-HT1A receptors could results in poor responses to 5-HT; while increased levels of presynaptic receptors would inhibit the firing of 5-HT neurons, to reduce 5-HT release. Thus, these changes in 5-HT1A receptors could contribute to reduced 5-HT neurotransmission.

5-HT1A Receptor

Receptor Structure and Characteristics

The 5-HT1A receptor is one of the most abundant subtypes that are expressed in the mammalian brain and it is thought to be a critical regulator of serotonergic activity (Lemonde et al., 2004). Aside from the correlation of 5-HT1A dysregulation with depression, 5-HT1A levels are altered in schizophrenic patients (Sumiyoshi et al., 1996). The 5-HT1A receptors are implicated in physiological functions such as blood pressure, feeding, temperature regulation, memory and disorders such as Tourette's syndrome, Huntington's disease and anxiety (Pucadyil et al., 2005). It was the first serotonin receptor to be cloned and sequenced (Albert et al., 1990; Fargin et al., 1989; Kobilka et al., 1987). The 5-HT1A receptor is encoded by an intronless gene (HTR1A) located on human chromosome 5q12.3 (Lesch and Gutknecht, 2004) and the human gene encodes a predicted protein of 422 amino acids (Pucadyil et al., 2005). The receptor belongs to the G protein coupled receptor (GPCR) superfamily involved in signal transduction pathways across the membrane. GPCRs consist of seven highly conserved alpha-helical transmembrane domains and can be activated by various ligands.

Tissue and Subcellular Localization

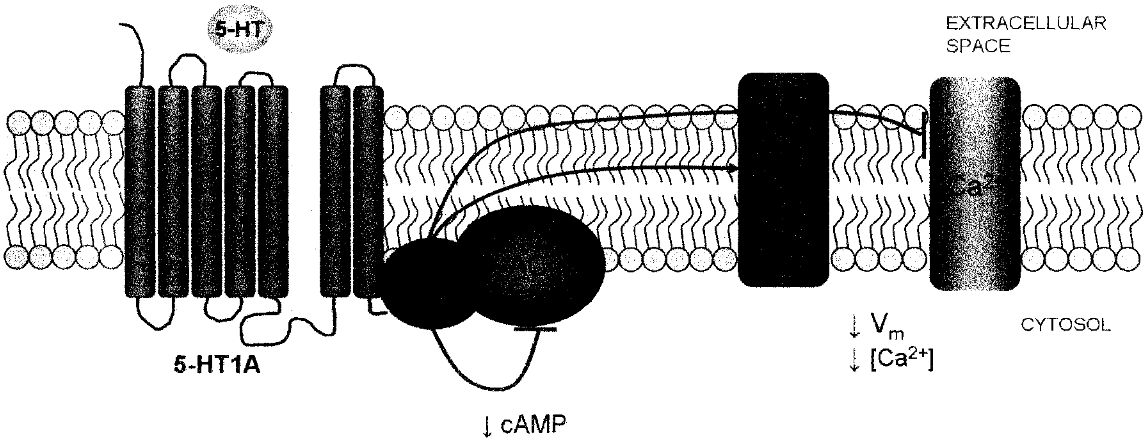
The 5-HT1A receptor is found in neuronal and non neuronal cell lines and its mRNA is primarily expressed in the brain, spleen, neonatal kidney and gut (Pucadyil et al., 2005). Experiments using radio-labeled ligands that bind specifically to 5-HT1A receptors revealed high label density in the raphe nuclei, cortex and limbic regions such as the hippocampus, raphe nuclei, amygdala, hypothalamus and lower density levels in the basal ganglia, substantia nigra and cerebellum (Palacios et al., 1990). The same 5-HT1A pattern of tissue distribution was observed using sequence specific antibodies (el Mestikawy et al., 1990). At the subcellular level the 5-HT1A receptors have been found to localize at the somatodendritic area of the presynaptic serotonin neurons in the raphe nuclei. At these regions they function as autoreceptors and regulate cell firing by feedback processes. In the hippocampus, 5-HT1A receptors are located on the postsynaptic neurons where they function as heteroreceptors that regulate neurotransmitters other than its own ligand (Ghavami et al., 1999; Palacios et al., 1990) whereas 5-HT1B receptors function as inhibitory presynaptic receptors at the nerve terminal (Morikawa et al., 2000) but both are important negative feedback loops. The diverse distribution of 5-HT1A receptors results in its involvement with brain functions and the target of various pharmacological agents.

Receptor Activation and Signal Transduction Pathways

In the nervous system, G protein signaling is initiated when neurotransmitters bind to their target transmembrane receptor proteins to initiate conformation changes that activate the associated G protein. The initial receptor bound G protein, consisting of G_α, G_β, and G_γ, is activated by exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on the α subunit. The G protein complex detaches from the receptor and the $G_{\beta\gamma}$ complex dissociates from the GTP/G_{α} subunit and can stimulate signaling cascades and various effectors to regulate cellular function. The G_{α} subunit will eventually hydrolyze the attached GTP to GDP and all the subunits reassemble allowing the cycle to begin again (Gilman, 1987). Numerous *in vivo* studies have shown that 5-HT1A mediates its physiological effects by primarily coupling to $G_{\alpha i}$/$G_{\alpha o}$ subclass of guanine nucleotide binding proteins in the hippocampus and raphe nuclei (Blier et al., 1993; Romero et al., 1994). However, the 5-HT1A receptor has been shown to also couple with the pertussis toxin-insensitive $G_{\alpha z}$ protein in the hypothalamus (Serres et al., 2000). Subsequently, 5-HT1A receptors inhibit adenylyl cyclase through pertussis toxin (PTX) sensitive G_i/G_o proteins, in effect inhibiting cAMP production and protein kinase A activation (Figure 2) (Raymond et al., 1999). The G_i protein also functions to activate potassium channels that hyperpolarize the membrane, consequently decreasing neuron firing rate. Lastly, the G protein inhibits L-type calcium channels decreasing intracellular calcium in effect decreasing neurotransmitter release (Raymond et al., 1999). The postsynaptic 5-HT1A receptors, which are particularly abundant in the hippocampus and cortex, commonly exert an inhibitory function on neuronal activity by enhancing K^+ channel activity resulting in hyperpolarization (Hamon et al., 1990; Blier and Ward, 2003). The autoreceptor related signaling is one thought to be of the main mechanisms targeted for serotonin regulation by antidepressant drugs (Blier and Ward, 2003).

Figure 2: Activation pathway of the 5-HT1A receptor.

Binding of serotonin (5-HT) activates the 5-HT1A receptor that signals via G proteins G_i/G_o to mediate inhibitory actions that include: 1) inhibition of adenylyl cyclase (AC) which inhibits cyclic adenosine monophosphate production (↓cAMP) 2) activation of potassium channels in effect hyperpolarizing the membrane ($\downarrow V_m$), decreasing neuron firing rate and 3) inhibition of calcium channels functioning consequently decreasing intracellular calcium levels ($\downarrow[Ca^{2+}]_i$) and neurotransmitter release.


5-HT
EXTRACELLULAR
SPACE
Ca^{2+}
↓ V_m
↓ $[Ca^{2+}]$
CYTOSOL
5-HT1A
↓ cAMP

Mechanism of 5-HT1A Feedback Loop

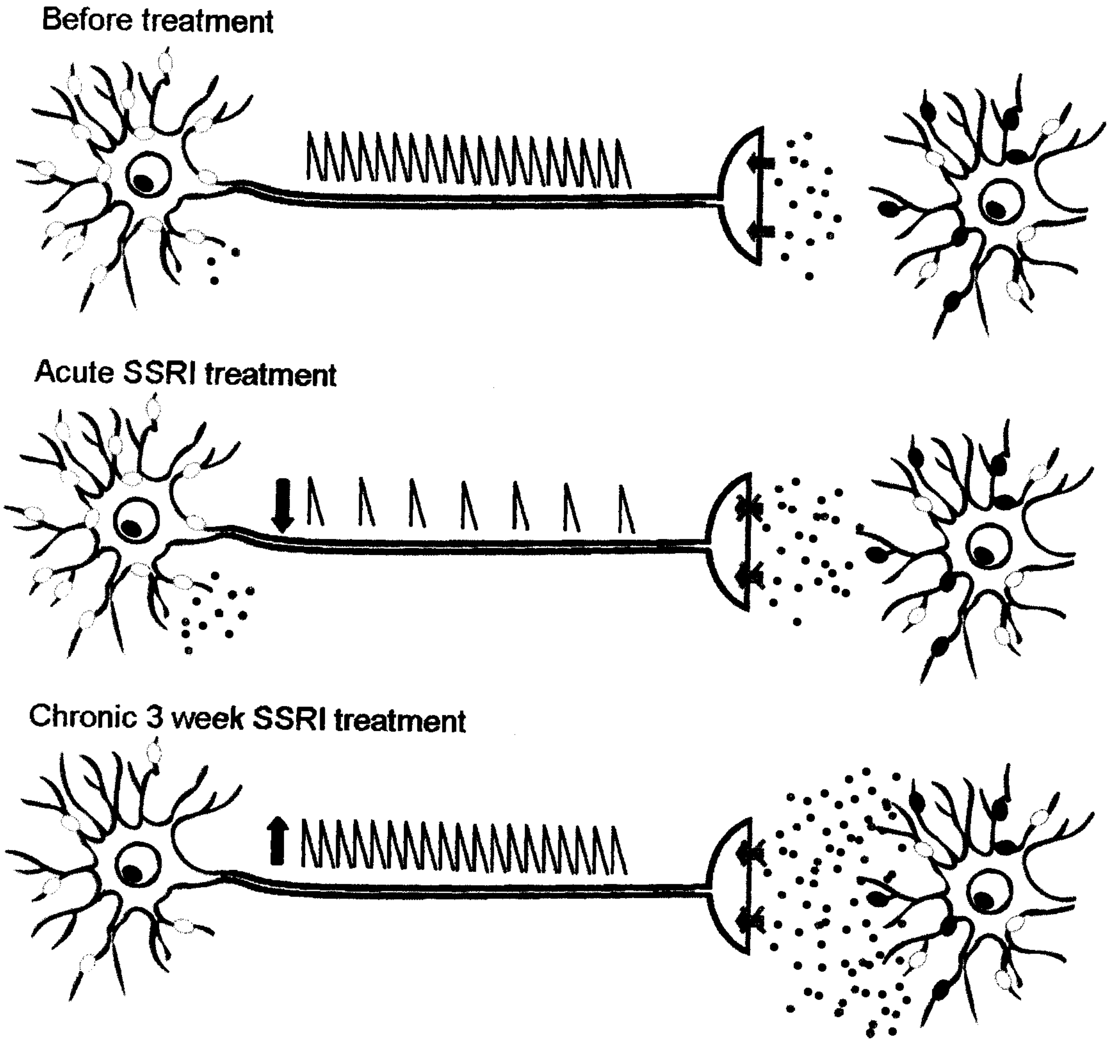
In the case of the 5-HT1A autoreceptor it mediates negative feedback inhibition of serotonergic transmission thus decreasing neurotransmitter release. As mentioned, presynaptic 5-HT1A autoreceptors are located on the soma and dendrites of serotonin neurons in the raphe nuclei. When excess amount of 5-HT is released 5-HT1A autoreceptors hyperpolarize serotonin neurons and in effect slows down neuronal firing (Blier and Ward, 2003). When 5-HT1A receptors are excessively active a decrease in serotonin release occurs in projecting neurons (Blier and Ward, 2003). Therefore the 5-HT1A receptor plays a significant role in controlling (negatively regulating) 5-HT release to various regions of the brain.

Antidepressant Action

The serotonin system is the target of various pharmacological agents to alleviate clinical depression. The earlier first choice for pharmacological treatment was tricyclic antidepressants (TCAs) (ex. Imipramine) that function by inhibiting the reuptake of the serotonin, norepinephrine and dopamine. However, their exact mechanism of action is not well understood. The mode of action of monoamine oxidase inhibitors (MAOIs) (ex. Phenelzine) is to prevent break down of monoamine neurotransmitters facilitated by monoamine oxidase and in effect maintains or increases the available stores. Serotonin specific reuptake inhibitors (SSRIs) (ex. Citalopram, Fluoxetine) prevent the reuptake of serotonin into the presynaptic cell increasing the amount of available neurotransmitter in the synapse to stimulate the postsynaptic cell. Aside from depression, SSRIs function to inhibit the serotonin transporters and has been effective in treating various mood disorders such as anxiety and obsessive compulsive disorders (Blier and de Montigny, 1999; Morilak and Frazer, 2004). Once the SSRIs rapidly penetrate the brain it is expected that serotonin transporters are immediately inhibited within minutes thus increasing 5-HT neurotransmission. However, with chronic treatment, delayed antidepressant action requires two to three weeks to ameliorate depressive behavior (Albert and Lemonde, 2004). This effect is thought to be due to the negative feedback regulation mediated by 5-HT1A autoreceptors that inhibit serotonin neurotransmission following release of serotonin at the synapse (Figure 3) (Blier and Ward, 2003). Acute antidepressant treatment inhibits the 5-HT transporter and increases serotonin levels at the synapse and at the cell body. Subsequent activation of the autoreceptors by high levels of serotonin decreases serotonergic firing and 5-HT release. After three weeks of SSRI treatment, desensitization of the presynaptic autoreceptors (due to the prolonged elevation of 5-HT levels) by internalization reduces the number of presynaptic autoreceptors (Riad et al., 2001). Postsynaptic 5-HT1A receptors that regulate firing of hippocampal pyramidal neurons do not desensitize after chronic treatment with SSRIs (Blier and de Montigny, 1987). This disinhibits the firing rates increasing serotonergic transmission and increases neurotransmitter release associated with improvement of depressed symptoms. The two to three week latency for SSRIs to relieve depressed symptoms suggests a long term adaptive change to the 5-HT system is required (Albert and Lemonde, 2004). A study demonstrates chronic treatment with an SSRI in rodents desensitizes the 5-HT1A receptors, regulating the release of 5-HT in the prefrontal cortex (Ceglia et al., 2004). The same effect on rodents is observed with TCAs and MAOIs (Gur et al., 2002). The common function of various subclasses of antidepressants is to

Figure 3: The effects of chronic antidepressant treatment on serotonin transmission.

Presynaptic 5-HT1A autoreceptors on cells bodies and dendrites of serotonin neurons and postsynaptic receptors on non serotonergic neurons (yellow dots) function through inhibitory signaling pathways. Other 5-HT receptors are located postsynaptically (blue dots). Before treatment local serotonin at the cell body activates 5-HT1A autoreceptors that regulate the serotonergic firing rate (spikes) and 5-HT release (red dots) by negative feedback inhibition. After acute SSRI treatment serotonin transporters (green arrows) are blocked (red Xs) and an increase in 5-HT levels at the cell body further activates 5-HT1A autoreceptors leading to reduced serotonergic firing (black arrow). After three weeks of chronic antidepressant treatment, the decrease in 5-HT1A autoreceptors levels, by desensitization, disinhibits the serotonergic neuron, increases firing rate and neurotransmitter release associated with improvement of depressive symptoms.


Before treatment
Acute SSRI treatment
Chronic 3 week SSRI treatment

desensitize 5-HT1A receptors therefore it must be a significant regulatory system involved in major depression.

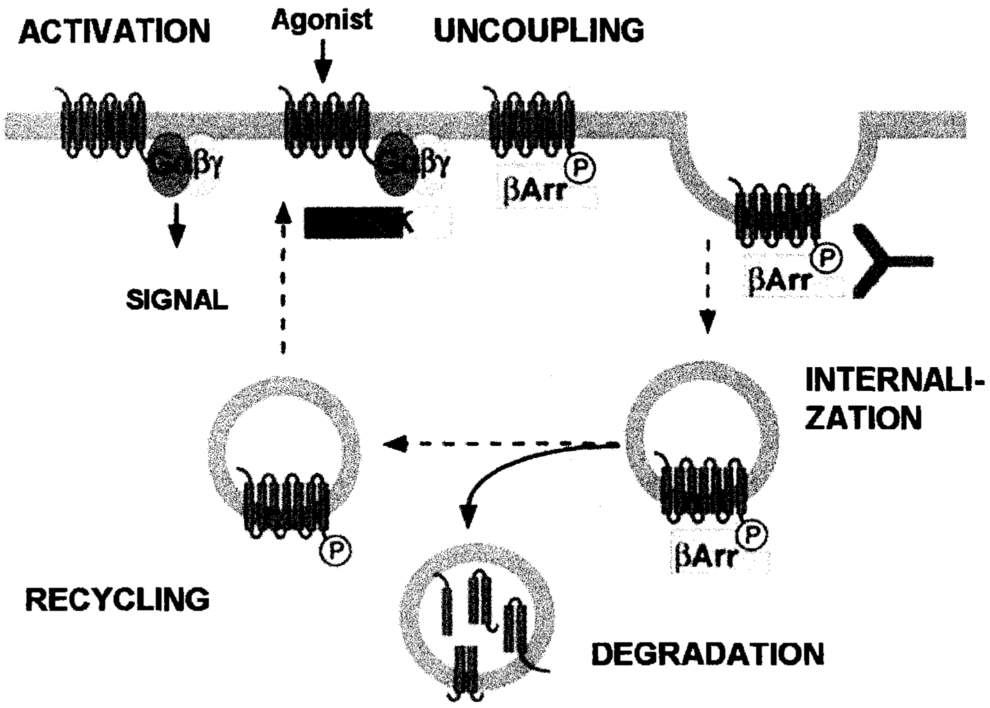
Mechanisms of 5-HT1A Receptor Desensitization

Following chronic antidepressant treatment, 5-HT1A receptor desensitization could be the fundamental basis for the adaptive changes that occur to regulate 5-HT1A autoreceptor function. The major steps of receptor desensitization are illustrated: uncoupling, internalization and degradation (Figure 4). Activation by serotonin agonist receptor uncoupling occurs within seconds. Receptor phosphorylation by G-protein coupled receptors kinases (GRK) or protein kinase A (PKA) or protein kinase C (PKC) prevents further activation by the associated G protein on the receptor (Raymond et al., 1999). This process is followed by receptor internalization, which occurs from seconds to minutes. Receptor phosphorylation and recruitment of β arrestins initiates the transportation of the receptor from the plasma membrane to the clathrin coated vesicles however the internalized receptor can activate other signaling pathways (Ferguson, 2001). The third step of desensitization is degradation or downregulation of receptor which can take from hours to days. Lysosomes then target the ubiquitinylated receptors or associated proteins and subsequently the receptors are degraded (Shenoy and Lefkowitz, 2003; Albert and Lemonde, 2004). After internalization the receptor can be directly recycled and re-used for cell signaling at the plasma membrane (Ou et al., 2003; Riad et al., 2001). Since changes in gene expression and production of new receptors occur over a longer time course we hypothesized that changes in 5-HT1A gene transcription could explain the delayed effects of antidepressant treatment

Figure 4: Schematic of receptor desensitization.

An agonist binds and activates the receptor and initiates a signal transduction (mediated by G proteins). The G-protein coupled receptor kinases (GRKs) target the agonist activated receptors and phosphorylation of the receptor makes it a target for arrestins. Arrestins bind to phosphorylated receptors causing inactivation of associated G-proteins and internalization. The arrestin-bound receptor transport into clathrin-coated pits and are internalized. Degradation or recycling of receptor follows.

Image obtained from http://www.pharmacy.rdg.ac.uk/staff/krasel/research.htm.


ACTIVATION
Agonist
UNCOUPLING
βγ
βγ
βArr
P
K
SIGNAL
βArr
P
INTERNALI-
ZATION
βArr
P
P
RECYCLING
DEGRADATION

(Albert and Lemonde, 2004). Since the two to three day time course for 5-HT1A receptor downregulation, by acute desensitization, occurs too rapidly another mechanism can explain the adaptive changes in 5-HT1A receptor numbers that occur over a few weeks after antidepressant treatment.

Dysregulation of 5-HT1A and Mental Disorders

Evidence has supported the hypothesis that altered levels of 5-HT1A autoreceptor expression has implications in depression and anxiety disorders that involve dysregulation of the 5-HT system (Lemonde et al., 2004). Studies done with 5-HT1A knockout mice exhibited enhanced serotonergic neurotransmission and results in anxiety-associated behavior (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998). This implies that 5-HT1A deficient individuals are more prone to anxiety (Toth, 2003). Conversely, post mortem studies have shown an increase in 5-HT1A autoreceptors levels in the raphe area of suicide victims with major depression compared to the control subjects of non-depressed suicide victims although there was no change in postsynaptic 5-HT1A receptor density. (Stockmeier et al., 1998; Lemonde et al., 2004). Selected antidepressants, specifically, serotonin reuptake inhibitors (SSRI), and 5-HT1A agonists desensitize the 5-HT1A autoreceptor as a mechanism of action for regulation of serotonin neurotransmission (Lesch and Gutknecht, 2004). These antidepressants function by down-regulating the 5-HT1A autoreceptors (Blier and de Montigny, 1999) which disinhibits the firing rates, increases serotonergic transmission and increases neurotransmitter release. This suggests that changes in the basal expression of the receptor may predispose individuals to depression or suicide (Blier and Ward, 2003).

Characterization of 5-HT1A Promoter

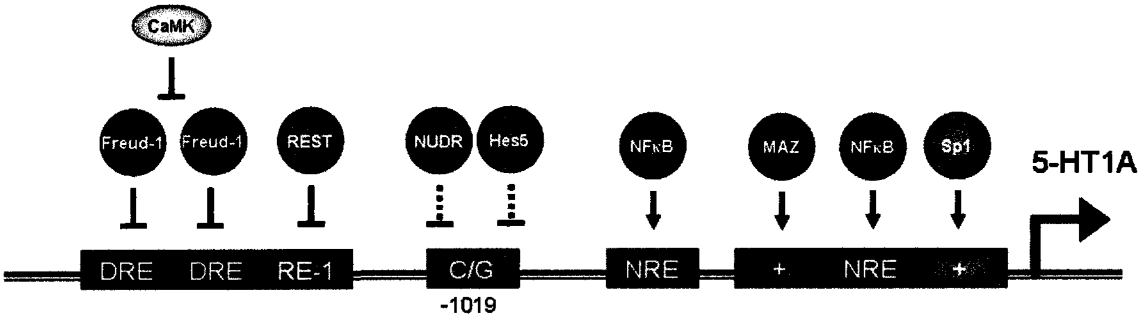
To examine transcriptional regulation of the 5-HT1A receptor, our laboratory cloned and sequenced the 5' flanking region of the rat 5-HT1A receptor gene (Storring et al., 1999). Primer extension studies and RNase protection studies verified a site of transcriptional initiation. A single brain specific transcription initiation start site was identified -967 bp upstream of the ATG translation initiation codon used in both hippocampus and rat raphe RN46A cell line (Storring et al., 1999). A consensus TATA containing element upstream of the initiation codon suggests a TATA driven promoter in the rat 5-HT1A gene. To identify novel DNA regulatory elements progressive 5' deletions of the rat 5-HT1A receptor gene fused to a luciferase reporter gene were transfected in 5-HT1A positive (RN46A raphe) and negative (L6 myoblast) rat cell lines and relative luciferase activity was quantified. RN46A cells were derived from E13 rat raphe cells and reversibly transformed by temperature sensitive SV40 large T antigen. The cells retain the basic qualities of a raphe neuron including expression of tryptophan hydroxylase, 5-HT transporters and 5-HT1A receptors (Storring et al., 1999). A specific deletion (-1590/-1519) in the 2300-luciferase construct showed a significant increase in luciferase activity compared to the -2300 construct in both neuronal and non-neuronal cell lines. This suggested that a strong repressor element is located between -1590 and -1519 that inhibits 5-HT1A transcription (Ou et al., 2000).

DNAse footprinting assay and electrophoretic mobility shift assay (EMSA) using nuclear extracts revealed the interaction between a 31bp element, located from -1524 to -1555, in the repressor region (Ou et al., 2000) and a single specific protein complex in RN46A cells and two protein complexes in L6 cells. In EMSA experiments a 14-bp element within the 31bp region was effective in competition of the protein complex observed in both neuronal and non neuronal cell lines. However the 14bp element and an adjacent 12bp element within the same 31-bp repressor region were required for competition of the protein found in both cell types and the second protein complex found in L6 cells. Therefore, the protein found in both cell types interacts with the 14-bp element and the non-neuronal specific protein mainly interacts with the 12bp element and a portion of the 14bp element. Further luciferase reporter assays using a -2300 luciferase reporter construct mutated at the 14bp region, rendering it inactive, resulted in an enhancement in luciferase activity of 36-fold compared to the -2300 control construct in RN46A cells. When both 14bp and 12bp elements were mutated, derepression on the 5-HT1A receptor gene was observed in both RN46A and L6 cells. The same trends in specific binding and repressor activities were observed in 5-HT1A receptor expressing septal SN48 and receptor negative glioma C6 cells compared to RN46A and L6 cells respectively. The model for postsynaptic regulation of the 5-HT1A receptor SN48 cell line were derived from the fusion of 21 day postnatal murin septal cells with neuroblastoma N18TG2 cells and express neurofilament protein, glutamic acid decarboxylase and 5-HT1A receptors (Lee et al., 1990; Lee et al., 1991; Charest et al., 1993). Together these results imply that the 14-bp element and the associated protein complex is crucial to suppress basal expression of 5-HT1A receptor in 5-HT1A receptor positive cells and the dual repressor region, including the other protein complex at the 12bp, element is involved in repressing 5-HT1A receptor gene expression in receptor negative cells (Ou et al., 2000). The 14-bp element plays a critical role in regulating basal 5-HT1A expression in receptor expressing neuronal cells. The dual repressor complexes are important to maintain gene inactivation in non-neuronal cells that do not express the receptor (Ou et al., 2000). The dual repressor element (DRE) in the rat 5-HT1A promoter consists of two overlapping elements the 14-bp FRE (5'-repressor element) and an adjacent 12-bp TRE (3'-repressor element). The repressor regions are highly conserved between mouse, rat and human genes. In 5-HT1A negative cells FRE and TRE bind nuclear proteins while in 5-HT1A positive cells only the FRE complex is present. The FRE is active in both neuronal cells expressing 5-HT1A and non-neuronal cells. The TRE is only active in non-neuronal 5-HT1A receptor negative cells.

Further reporter assays identified the transcriptional regulatory elements of the human 5-HT1A receptor gene. The promoter contains highly conserved DNA elements for Sp1, myc-associated gene finger protein (MAZ) and nuclear factor actor-kappa B (NF-κB) within 1-kb of the transcription start site (Parks and Shenk, 1996) (Figure 5). A TATA-less site (in human and mouse 5-HT1A genes) or a TATA containing site (in the rat 5-HT1A gene) drives transcription mediated by these enhancer regions (Albert and Lemonde, 2004). In addition, there is a PET-1 element. Pet-1, an ETS domain transcription factor, functions as an enhancer that is required for differentiation and maintenance of serotonergic neurons (Hendricks et al., 1999). Upstream of the initial enhancer region the strong repressor region, as mentioned, contains the FRE and TRE. An additional transcriptional factor, repressor element 1 silencer of transcription/neural restrictive silencing factor (REST/NRSF), sustains rest element/neuron restrictive silencer element (RE-1/NRSE) mediated repression of the 5-HT1A receptor in the absence of Freud-1/FRE functioning (Albert and Lemonde, 2004). Our lab previously identified a functional single nucleotide C(-1019)G polymorphism in the human 5-HT1A

Figure 5: Schematic representation of the human 5-HT1A transcriptional regulatory elements.

The arrows represents activation and blocked lines indicate repression; the dashed line represent C(-1019) allele conditional binding of NUDR/Hes5. The enhancer region consists of NF-κB response elements (NRE) and Sp1 and MAZ binding sites. Glucocorticoids mediate inhibitory regulation at the mineralocorticoid response element (Ou et al., 2001). The repressor region includes two tandem copies of the dual repressor element (DRE) which contains two elements, 5′-element (FRE) and 3′-element (TRE) and repressor element 1 (RE-1) which binds REST/NRSF. Transcription factor, five prime repressor under dual repression binding protein 1 (Freud-1), binds to the DRE and is regulated by calmodulin dependent protein kinase (CaMK).


CaMK
Freud-1
Freud-1
REST
NUDR
Hes5
NFκB
MAZ
NFκB
Sp1
5-HT1A
DRE
DRE
RE-1
C/G
-1019
NRE
+
NRE
+

promoter (Figure 5). In a study of depressed and control subjects the 5-HT1A homozygous G(-1019) allele was two-fold more frequent than control subjects and fourfold more prevalent in suicide victims (Lemonde et al., 2003). The polymorphic sequence was located in a 26-bp palindrome and interacting transcription factors were identified by yeast one hybrid cloning. Nuclear deformed epidermal autoregulatory factor/deformed epidermal autoregulatory factor 1 (NUDR/Deaf-1) and Hairy/Enhancer-of-split-5 (Hes5) were shown to bind to and repressed the transcriptional activity of the C(-1019) allele of the 5-HT1A promoter. NUDR mediated repression of the G(-1019) allele was abolished but Hes5-mediated repression was only partially impaired. Together all these elements play an important role in 5-HT1A regulation.

Freud-1 Transcription Factor

Discovery of mouse Freud-1

To identify DNA binding proteins specific to the 5-HT1A DRE a mouse DNA library was screened using three copies of the DRE fused to a LacZ marker by yeast one hybrid system (Ou et al., 2003). Using this approach, our laboratory identified a novel murine transcription factor, Freud-1 (FRE Under Dual repression binding protein 1) that contained two DM14 (Drosophila Melanogaster 14) and a protein kinase C (PKC) conserved region 2 (C2). The DM14 domain, first identified in the fruit fly, is a 60 aa conserved repeat sequence of unknown function. The C2 domain contains a CalB domain that mediates Ca^{2+} dependent lipid binding of PKC, phospholipases and synaptotagmin and protein-protein interactions (Perisic et al., 1998; Ou et al., 2003). Freud-1 also contains a helix-loop-helix (HLH) DNA binding domain important for its DNA binding function.

Electrophoretic mobility shift assays demonstrated specific binding of Freud-1 to the FRE (Ou et al., 2003). The C2 domain is important in Freud-1 binding activity given that a mutation of the C2 domain reduced Freud-1 DNA binding. *In vitro* binding assays using nuclear extracts also verified the presence of a second protein complex bound to the DRE in nonneuronal cells that was later assigned Freud-2. Freud-1 specific binding to the DRE was verified by supershift using Freud-1 specific antibody.

Cotransfection of Freud-1 expression plasmid and the DRE containing -2300 5-HT1A promoter luciferase reporter construct resulted in Freud-1 mediated repression of 5-HT1A transcription with a greater effect in RN46A cells compared to L6 cells (Ou et al., 2003). Repression was abolished as shown by reporter assays using a Freud-1 deletion construct causing a disruption in the CalB domain. Therefore Freud-1 repressed 5-HT1A receptor gene to a greater extent in RN46A cells compared to L6 cells and the CalB domain is required for repression (Ou et al., 2003). An intact FRE was also required for Freud-1 mediated repression. Cotransfection of antisense Freud-1 derepressed 5-HT1A reporter activity only in RN46A cells, indicative of the role of endogenous Freud-1 in repression in these neuronal cells. In nonneuronal cells the repressor region completely silenced 5-HT1A gene transcription, but also reduced basal 5-HT1A activity in neuronal cells.

Immunohistochemistry analysis demonstrated that overexpression of Freud-1 decrease the endogenous levels of 5-HT1A receptor protein in differentiated RN46A cells and an increase in 5-HT1A staining was observed in cells transfected with antisense

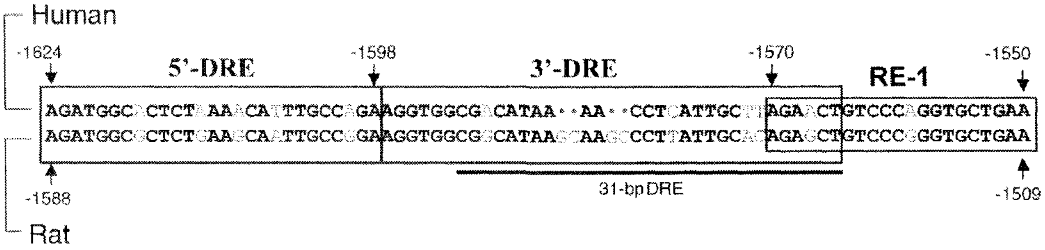
Freud-1 (Ou et al., 2003). Northern blot analysis of rat tissue confirmed Freud-1 mRNA expression in 5-HT1A-expressing rat brain areas such as the frontal cortex, mesencephalon, hypothalamus, hippocampus and midbrain as well as in peripheral tissue including the testis, pituitary, liver and kidney. In situ hybridization analysis demonstrated that high levels of Freud-1 mRNA were detected in the cell body and dendrites of hippocampal pyramidal cells and raphe nuclei (dorsal, medial and magnus). Freud-1 mRNA expression was also found in the hypothalamus, cortex and thalamus. Immunohistochemistry analysis of primary hippocampal and cortical cultures revealed Freud-1 protein was primarily found in the nucleus of 5-HT1A positive cells. In tissue Freud-1 was detected in the hypothalamus and dorsal raphe nucleus (DRN) and colocalized with 5-H1A receptors and 5-HT. These Freud-1 positive brain areas correspond to 5-HT1A expressing regions reinforcing its role as a regulator of 5-HT1A receptor expression.

Freud-1 as a 5-HT1A regulator

Additional studies of the human 5-HT1A promoter were done to characterize human Freud-1 and 5-HT1A DRE interaction (Lemonde et al., 2004). Again a series of deletions of the 6-kb human 5-HT1A promoter fused to a reporter construct were constructed in order to identify the activity of transcriptional regulatory regions. An enhancer region located -1517 to -391bp induced 5-HT1A transcriptional activity in various cell lines. However a strong repressor region located between -1790 and -1517 significantly reduced activity. This region is homologous to sequences of the rat 5-HT1A DRE (Lemonde et al., 2004). In the human 5-HT1A promoter, the repressor region lies between -1624 and -1570. The sequence alignment of rat and human repressor region contain two consecutive repeats of DREs corresponding to consensus 5'-DRE and 3'-DRE sequences that lie between -1624 and -1598 and -1598 to -1565 respectively (Figure 6). A RE-1/NRSE consensus sequence lies downstream from the DRE sites between -1570 to -1550. Deletion of sequence upstream of -1624 did not affect repressor activity indicating repressor elements were localized between -1624 and -1517. Removal of all elements resulted in significant derepression of 5-HT1A promoter activity and confirmed the importance of all of these elements. However the greatest effect on 5-HT1A promoter activity was deletion of the 3'-DRE and RE-1. Transcription factor REST is the first vertebrate silencer protein; it binds to RE-1 and represses neuronal gene transcription primarily in nonneuronal cells (Schoenherr and Anderson, 1995). RE-1 has been identified in 18 neuron specific genes and presumably functions as a negative regulator of neurogenesis (Schoenherr and Anderson, 1995). Cotransfection with a region containing the RE-1 upstream of the SV40 promoter and a REST expressing vector in RN46A cells resulted in strong repressor activity. The pattern was observed using the 5-HT1A promoter construct containing the RE-1 element confirming the importance of REST and RE-1 interaction in repressing the 5-HT1A receptor gene.

In electrophoretic mobility shift assays, *in vitro* synthesized REST formed a complex with RE-1 element. EMSA experiments using rat brain nuclear extracts formed a protein complex with the 5'-DRE and two complexes were formed with the 3'-DRE. *In vitro* transcribed and translated mouse Freud-1 was able to bind specifically to human 5'-DRE and 3'-DRE. Together these results indicate the ability of Freud-1 to bind to the human DRE elements and an additional rat brain specific protein interacts with the 3'-DRE.

Figure 6: Sequence alignment of the repressor regions of the 5-HT1A receptor gene. Shown are the alignments of the two consecutive repeats of DREs (5'-DRE and 3'-DRE), and RE-1 from human and rat 5-HT1A promoters. The repressor region between -1624 and -1550 of the human 5-HT1A gene shares 81% amino acid identity with the corresponding region -1588 to -1509 of the rat sequence. Bold nucleotides represent sequence identity. The sequence corresponding to the previously characterized 31-bp DRE in the rat 5-HT1A gene is identified. Figure and legend obtained from (Lemonde et al., 2004) with permission.


Human
-1624
5'-DRE
-1598
3'-DRE
-1570
RE-1
-1550
AGATGGCACTCTAAAACATTTGCCAGAAGGTGGCGACATAA••AA••CCTCATTGCTTAGAACTGTCCCAGGTGCTGAA
AGATGGCGCTCTGAAGCAATTGCCGGAAGGTGGCGGCATAAGCAAGCCCTTATTGCACAGAGCTGTCCCGGGTGCTGAA
-1588
31-bp DRE
-1509
Rat

Various cell lines (RN46A, SN48, L6 and HEK 293 - human embryonic kidney) transfected with SV40 promoter-luciferase constructs fused to 5-HT1A DRE or RE-1 elements were treated with histone deacetylase (HDAC) inhibitor TSA. Acetylation of histones is an important process for transcriptional activation and is implicated in REST induced gene silencing (Huang et al., 1999). FRE and TRE mediated repression was only affected by TSA in non neuronal cells where another protein complex is involved in mediating repression by the DRE. Freud-1 mediated repression was also not affected by HDAC inhibitor TSA. Repression of RE-1 element was reversed in the presence of TSA signifying the importance of HDAC activity for REST function (Lemonde et al., 2004). From further Freud-1/5-HT1A studies it has been concluded that inactivation of FRE derepresses 5-HT1A transcription in neuronal cells, however, the same effect was not observed in nonneuronal cells. All 3 elements function to repress 5-HT1A transcription in nonneuronal cells and the FRE plays a key role in repression in neuronal cells (Lemonde et al., 2004). In nonneuronal cells another protein complex, Freud-2, is more important for 5-HT1A repression (unpublished data).

From a study that has examined genes that target activators of NFκB and MAPK signaling pathways, a Freud-1 homologue has been shown to be a signal transducer in positive regulation of the I-κB kinase (IKK)/NFκB cascade study (Matsuda et al., 2003). Since NFκB upregulates 5-HT1A gene transcription (Abdouh et al., 2001; Wissink et al., 2000) Freud-1 mediated regulation of NFκB could feed back and reverse Freud-1 induced repression of the 5-HT1A receptor gene (Albert and Lemonde, 2004).

More recent studies have focused on characterization of the long isoform of human Freud-1 which is also known as CC2D1A (coiled-coil and C2 domain containing

1A), MRT3 (mental retardation, nonsyndromic, autosomal recessive 3) FLJ20241 FLJ41160 (Rogaeva and Albert, 2007). The Freud-1 family also includes another member, Freud-2/CC2D1B which share 40.8% amino acid identity with Freud-1 (Rogaeva et al., 2007a). Long Freud-1 orthologs contains conserved regions that include four DM14 (Drosophila melanogaster 14) regions of unknown function, a helix-loop-helix (HLH) domain important for DNA binding and a C2 (protein kinase C conserved region 2) calcium phospholipids binding domain important for repression activity and binding (Figure 8). The gene is located on chromosome 19p13.12-p13.2 (Basel-Vanagaite et al., 2006). A short human protein isoform has also been identified and differs by a downstream in-frame translational start site that does not include the first two Drosophila melanogaster 14 domains. The short mouse Freud-1 isoform, lacking two DM14 domains, has been previously characterized and was shown to be a transcriptional repressor of the 5-HT1A receptor gene (Ou et al., 2003) (Lemonde et al., 2004).

Northern blot analysis demonstrated that human Freud-1 RNA was expressed in most tissues with the highest expression skeletal muscle (Rogaeva and Albert, 2007). Freud-1 was ubiquitously expressed in the brain with the highest expression in the cortex, cerebellum and putamen. To examine subcellular localization and expression of Freud-1, two polyclonal antibodies were synthesized specifically to the N-terminal mouse short isoform and to the full length human Freud-1. The N-terminal antibody identified a ~67-kDa species in HEK 293 cells and also detected transiently transfected human Freud-1 long isoform (120/130kDa) that appears as a doublet, possibly due to post translational modification (Rogaeva and Albert, 2007). The full length antibody detected endogenous short and long forms of Freud-1 in HEK 293 cells. Both isoforms were also detected in cytosolic and nuclear extracts of HEK 293 and human neuroblastoma SK-N-SH cells, however, long Freud-1 was the predominant isoform. Freud-1 nuclear localization supports its role as a transcriptional regulator.

To address the functionality of human Freud-1, reporter assays were performed (Rogaeva and Albert, 2007). A7 (rat astrocytes) and NIH/3T3 (mouse embryonic fibroblast) cells were transiently transfected with Freud-1 expression plasmid and the reporter-luciferase constructs flanked with the human 5-HT1A DREs (5'-DRE, 3'-DRE, 5' and 3'-DRE) (Table 1). The DRE containing constructs alone exhibited repression due to endogenous Freud-1, (Lemonde et al., 2004) however, over expression of Freud-1 in these cell lines further repressed reporter activity. Electrophoretic mobility shift assays demonstrated that purified human Freud-1 was able to bind both rat and human 5-HT1A 5'-DRE and 3'-DRE. The interaction between endogenous Freud-1 and human 5-HT1A DREs were examined in receptor negative cells, A7 and SK-N-AS. Chromatin immunoprecipitation (CHIP) assays demonstrated that eluted fractions of Freud-1 immunoprecipitated with full length and anti-CC2D1A antibody were abundant in the 5-HT1A promoter region containing the 5' and 3'-DRE. Together these results supports the role of Freud-1 as a regulator of 5-HT1A receptor gene.

CC2D1A gene involved in nonsyndromic mental retardation

A recent study has associated the CC2D1A gene family with autosomal recessive nonsyndromic mental retardation (NMSR) that affects 1% to 3% of the population (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006). Characteristic of mental retardation (MR) include impaired intellectual functioning, limitations in cognitive, interpersonal/psychosocial and adaptive skills and the condition is present from childhood (prior to age 18) (Pratt and Greydanus, 2007). MR individuals with no physical abnormalities or neurological deficiencies are diagnosed as NMSR. A truncated protein encoded by the CC2D1A gene family has been identified in NSMR individuals by linkage analysis. The deletion mutation eliminates exons 14-16 encoding a protein lacking the fourth DM14, the helix loop helix domain and the C2 binding domain (Rogaeva et al., 2007a).

Regulation of D2R by Freud-1

The most recent findings examined the role of Freud-1 in regulating the dopamine-D2 receptor gene (Rogaeva et al., 2007b). Mouse Freud-1 was also localized in 5-HT1A negative cells in the substantia nigra which express the dopamine-D2 receptor. A Blast Search using the 5-HT1A DRE identified a conserved FRE-like sequence in the second intron of the human D2 receptor gene. EMSA using nuclear extracts from D2 receptor negative cells, HEK 293, A7 cells that express D2 receptor and D2 DRE probe resulted in formation of a protein DRE complex. A7 and HEK 293 cells transiently transfected with a D2 DRE containing plasmid exhibited reduced reporter activity indicative of D2 DRE repressor activity. By sequence analysis two uncharacterized A/G and A/C single nucleotide polymorphisms (SNP) have been identified. Reporter constructs of the D2 DRE containing either the A or G allele used in luciferase assays demonstrated that the A allele resulted in stronger repressor activity compared to the G allele in both cell lines. Addition of mouse Freud-1 specific antibody or CC2D1A antibody supershifted the protein D2 DRE complex seen by EMSA experiments and confirming the Freud-1 D2 DRE specific interaction. Additional *in vitro* binding experiments demonstrated that recombinant mouse Freud-1 protein directly interacted with the D2 DRE and had greater binding affinity for the DRE containing the A allele. Chromatin immunoprecipitation assays (CHIP) verified the endogenous Freud-1/D2 DRE interaction in HEK 293 and DRD2 expressing human neuroblastoma SK-N-AS cells. Increasing levels of DRD2 binding in were observed in A7, SK-N-AS and human retinoblastoma Y-79 cells with A7 having the lowest level of binding. Using two different antibodies, hFreud-1 and CC2D1A, decreasing protein levels were seen by western blot in the same cell lines with A7 having the highest level of protein. Also mRNA levels of DRD2 in the cells portrayed the same trend as DRD2 binding levels. This inverse relationship between Freud-1 protein expression levels and DRD2 mRNA levels supports the role of Freud-1 as a negative regulator of the dopamine D2 receptor gene. Freud-1 specific siRNA lead to a significant increase in D2DR mRNA and binding levels compared to non treated controls. The level of endogenous Freud-1 determined the impact of Freud-1 siRNA on D2DR expression levels in cells. Therefore these results support the idea that endogenous Freud-1 is crucial for the negative regulation of the human dopamine D2 receptor gene.

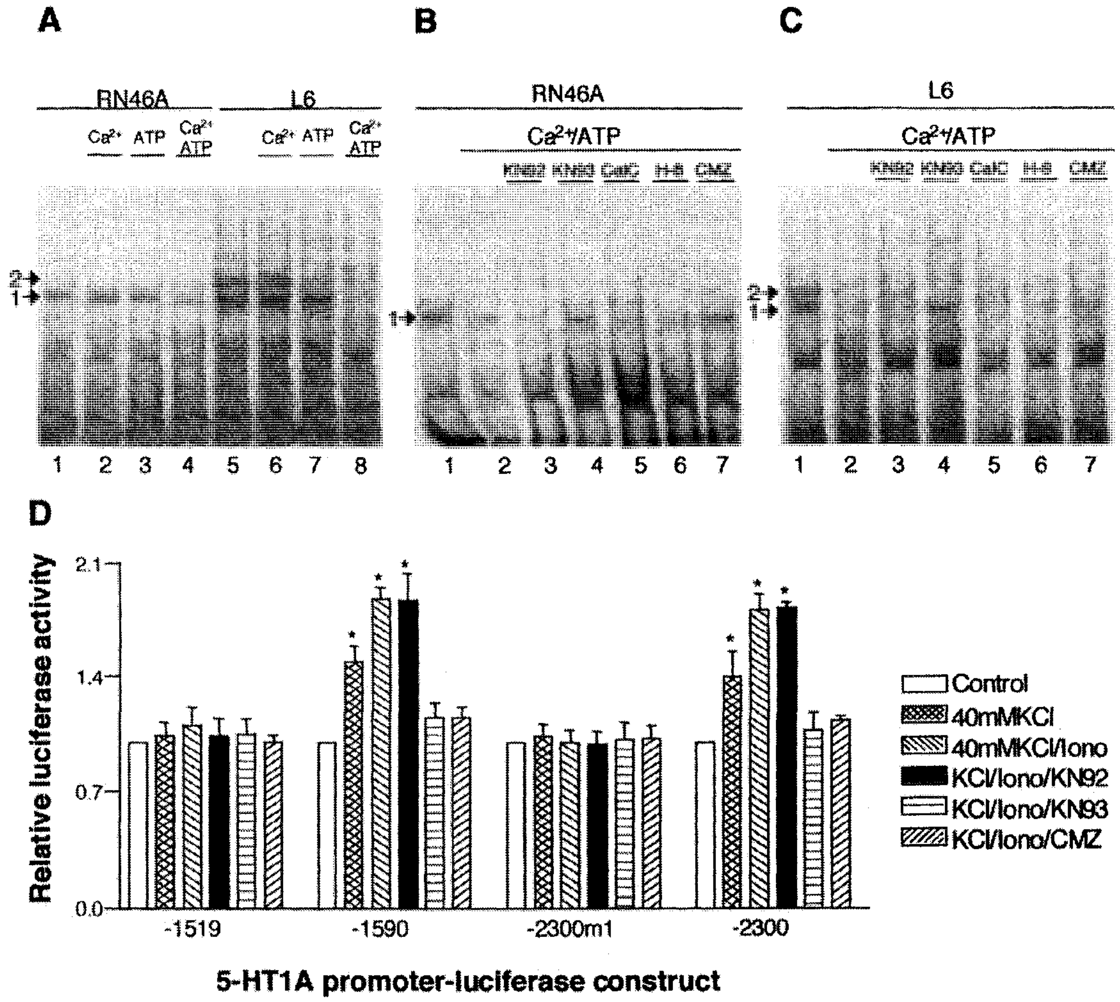
Calcium Regulation of Freud-1

Further research was carried out by our laboratory to characterize Freud-1 and specifically address regulation of 5-HT1A receptor gene by this transcription factor. The presence of the conserved C2 calcium dependent lipid binding domain in all species orthologs suggests that there is calcium dependent regulation of Freud-1 (Ou et al., 2003). Other proteins that have C2 domains are phospholipases, protein kinases C and synaptotagmin protein families and this motif has shown to be in involved in calcium dependent phospholipid binding (Basel-Vanagaite et al., 2006). Electrophoretic mobility shift assay (EMSA) was conducted to assess DNA binding ability of Freud-1. It was shown that in the presence of both calcium (Ca^{2+}) and ATP, The ability of Freud-1 to bind to its respective DNA element was inhibited compared to the binding of Freud-1 to the DRE where Ca^{2+} and ATP were absent (Figure 7). This was observed in RN46A and L6 cell lines. Freud-1, in RN46A cell line, showed no change in binding to DNA in the presence of Ca^{2+}, ATP and the non-specific CaMK inhibitor, KN93, indicating the involvement of CaMK in the inhibition of Freud-1 binding to its DNA element. Treatment with inhibitors of CaM (calmodulin) or CaMK (Ca^{2+} calmodulin dependent protein kinase) reversed calcium mediated inhibition of Freud-1. Thus, Freud-1 represents a novel calcium-regulated repressor that down regulates basal expression of 5-HT1A receptors (Ou et al., 2003).

To determine the effects of calcium mediated inhibition of Freud-1 binding to the FRE, 5-HT1A receptor gene transcription was also tested. 5-HT1A promoter luciferase constructs were treated with calcium influx inducers, ionomycin and KCl, resulting in enhancement of luciferase activity in the FRE containing construct (Figure 7) (Ou et al., 2003). This suggests that calcium signaling hinders Freud-1 repressor function. No change occurred when the constructs were treated with the inactive CaMK inhibitor. The same constructs were treated with inhibitors KN93 and CMZ. The FRE containing construct exhibited normal repressor activity indicating that the calcium mediated inhibition of Freud-1 was alleviated. This suggests that CaMK plays a role in Freud-1 FRE binding and could affect transcriptional activity of Freud-1 and 5-HT1A receptor.

Figure 7: CAM kinase attenuates Freud-1-mediated repression.

A, Ca^{2+} and ATP in combination interfere with Freud-1/DRE interaction. EMSA using the 31 bp DRE as a probe incubated with nuclear extracts from RN46A (lanes 1-4) or L6 cells (lanes 5-8) as indicated. $CaCl_2$ (lanes 2, 6), ATP (lanes 3, 7), or both (lanes 4, 8) were added into incubation buffer for 20 minutes before incubation with DRE probe. **B, C**, CAM kinase activation in nuclear extracts interferes with Freud-1-DRE binding. EMSA using DRE as a probe and nuclear extracts from RN46A cells **(B)** and L6 cells **(C)** are shown. Both $CaCl_2$ and ATP were added into the incubation before adding probe (lanes 2-7). Treatment was with 10 μM (except 50 nM CalC) KN92 (negative control for KN93; lane 3), KN93 (CaM kinase inhibitor; lane 4), CalC (PKC inhibitor; lane 5), H-8 (PKA inhibitor, lane 6), and CMZ (CaM inhibitor; lane 7), as indicated. In L6 cells **(C)** CaM or CaMK inhibitors rescued only the Freud-1-containing species (band 1). **(D)** Calcium signaling enhances 5-HT1A promoter activity in a Freud-1-FRE-dependent manner. 5-HT1A promoter-luciferase reporter constructs containing FRE (-1590 or -2300) or lacking FRE (-1519, -2300m1) were transfected into RN46A cells. Twenty-four hours after transfection, the cells were treated with 40 mM KCl or with 40 mM KCl and 1 μM ionomycin without or with 10 μM KN92, KN93, or CMZ in the medium for 16 hr. Luciferase activity is expressed as relative light units normalized to control (untreated) samples. $*p < 0.05$ in comparison with control. Note that calcium mobilizing agents had no effect in FRE-lacking reporter constructs (-1519 or -2300m1) and were blocked by CaM or CaMK inhibitors. Figure and legend were obtained from (Ou et al., 2003) with permission.


A
B
C
RN46A
L6
Ca2+
ATP
Ca2+ ATP
Ca2+/ATP
KN92
KN93
CalC
H-8
CMZ
2
1
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7
D
Relative luciferase activity
2.1
1.4
0.7
0.0
*
-1519
-1590
-2300m1
-2300
5-HT1A promoter-luciferase construct
Control
40mMKCl
40mMKCl/Iono
KCl/Iono/KN92
KCl/Iono/KN93
KCl/Iono/CMZ

The above-mentioned results suggest an interesting mechanism for activation of 5-HT1A gene transcription, which involves negative regulation of Freud-1 by calcium calmodulin kinase (CaMK). Possible candidate protein kinases include calcium calmodulin kinase II (CaMKII) or CaMKIV.

Ca2+/CaM interact with the binding region of the protein kinases that also causes a conformational change removing all inhibitory regions. As a result, the active sites of the protein kinase are exposed and permitted to bind to the substrate(Means, 2000). Specifically in CaMKII regulatory pathways, Ca2+/CaM binds to two adjacent catalytic subunits to allow one subunit phosphorylate a single amino acid.

Role of Calcium and Calmodulin

Calcium is a universal second messenger that has multifunctional roles in vesicle fusion, muscle physiology, regulating intracellular response such as glycogenolysis, mitochondrial respiration, endocytosis, neurotransmitter synthesis and gene transcription (Hudmon and Schulman, 2002). Although cells have significantly lower intracellular calcium levels compared to levels outside of the cell an increase in intracellular calcium levels can be induced by extracellular stimuli such as agonists and membrane depolarizing stimuli that allow entry of calcium via voltage gated calcium channels, ligand gated receptor channel, transporters or released from intracellular calcium stores (Hudmon and Schulman, 2002). Calmodulin, which belongs to the EF-hand family of calcium binding proteins, is used as a calcium sensor and signal transducer since various proteins are unable to bind directly to calcium. CaM can bind to up to four calcium ions with high affinity and undergoes a conformation change (Means, 2000). This interaction promotes the Ca/CaM complex to bind to specific target proteins to initiate a specific response or change in cellular function (Braun and Schulman, 1995).

Calcium/Calmodulin-Dependent Protein Kinase II

Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the many Ca/CaM activated effectors that alter the function of proteins in the cell by phosphorylation. The enzyme was first described in late 1970's (Schulman and Greengard, 1978) and has since been the topic of over 12 000 papers (Griffith, 2004). CaMKII is a serine/threonine kinase that phosphorylates numerous substrates including synapsin, myosin light chains, tryptophan hydroxylase, microtubule associated protein and casein (Hudmon and Schulman, 2002).

Isoforms and Protein Structure

CaMKII is encoded by four genes (α, β, γ, δ) with the α isoform mainly found in the forebrain and β is the major isoform in the cerebellum (Hanson et al., 1989). CaMKII isoforms are highly conserved in all mammalian species. Furthermore, human and rat α isoforms have identical protein sequences (Hudmon and Schulman, 2002). The molecular weight ranges from 54 kDa (α) to 58 – 72 kDa (δ, γ, β). Isoforms from rat brain include homologous α (54 kDa), β (60 kDa), β' (58 kDa), γ (59 kDa) subtypes (Hanson et al., 1989). Alternate splicing of CaMKII genes generates the various species. CaMKII isoforms include a kinase catalytic domain at the N terminal, a regulatory domain that includes an autoinhibitory segment and a calmodulin binding motif that both overlap and at the C terminal region is an association domain possibly responsible for subunit-subunit interaction and regulating cellular localization (Hanson et al., 1989).

Phosphorylation sites are shared between isoform and these sites exist in the autoregulatoy domain T286 (Thr 287 β, γ, δ) and Thr305/Thr306 (Thr306/Thr307 β, γ, δ). CaMKII monomers arrange into a holoenzyme composed of 8-12 subunits and the association domains are organized in two stacked hexameric rings forming a central core (Means, 2000). The catalytic and regulatory domains radiate outward from the center in a polarized arrangement forming a wheel-and-spoke like structure. The calcium/calmodulin complex binds to the outer regions of the holenzyme (Means, 2000). Presumably the units function independently for catalytic activity but collectively important for intraholoenzyme autophosphorylation, intracellular targeting and self assembly (Hudmon and Schulman, 2002).

Tissue Specificity and Subcellular Localization

CaMKII isoforms vary in their tissue, cellular and subcelluar localization. The kinase is ubiquitous expressed in most tissues but predominantly present in neurons and makes up 1-2% of total protein in some brain regions (Griffith, 2004) and 2% of hippocampal protein (Hanson et al., 1989). The α isoform primarily occupy the forebrain and the β isoform are the dominant forms in the cerebellum. (Hanson et al., 1989). The α isoform is almost exclusively found in neurons whereas β is also found in glia (Soderling et al., 2001). Alternate splice variants of the different isoforms contain a nuclear localization sequence and nuclear CaMKII has proved to be important in calcium regulated transcription of various genes including brain derived neurotrophic factor and atrial natriuretic factor mediated by phosphorylation of transcription factor such as CCAAT enhancer binding protein (C/EBP) (Soderling et al., 2001).

Mechanisms of Activation/Inactivation

Common to kinases such as CaMKII and PKC, which are regulated by second messengers, activation is attained by displacement of the autoinhibitory domain from the active site. The autoregulatory domain maintains CaMKII in the basal state by preventing binding of substrates (Hudmon and Schulman, 2002). The regulatory properties of CaMKII activation revolved around autophosphorylation. Initial autophosphorylation requires calcium and calmodulin, which bind to the autoinhibitory domain and lead to disinhibition of the kinase (Yamauchi, 2005; Waldmann et al., 1990). Autophosphorylation at residue 286, in the αCaMKII isoform, allows the kinase to remain autonomously active in a calcium independent manner, it further disrupts the autoinhibitory domain, and other autophosphorylation sites are revealed in the calcium binding domain which prevents further binding of calcium or calmodulin (Griffith, 2004). Autophosphorylation is an intramolecular reaction between adjacent subunits within the same CaMK holoenzyme (Hanson et al., 1994). Autophosphorylation also increases the affinity for the calmodulin complex, extending kinase activation time and maintains the kinase in an active state even after the calmodulin complex has dissociated from the kinase. Following initial autophosphorylation of Thr286, calcium independent/autonomous autophosphorylation at Thr305, Thr306 results in the loss of ability of CaMKII to bind to calcium/calmodulin leading to reduced kinase activity (Yamauchi, 2005).

Function

CaMKII has a broad range of substrates and phosphorylates many proteins in the brain and peripheral tissue. The major consensus phosphorylation site for CaMKII is

RXXS/T (Yamauchi, 2005). High concentration levels of CaMKII in neuronal tissue supports its many roles in neuronal function. CaMKII phosphorylated substrates are involved in many cellular processes including neurotransmitter synthesis, neurotransmitter release, regulating membrane currents, cellular transport, cytoskeletal organization, synaptic plasticity, learning and memory and gene expression (Hudmon and Schulman, 2002). It has been shown that, in the presence of calcium and ATP, CaMKII interacts with syntaxin, a key component of the exocytitic machinery important for neurotransmitter release (Yamauchi, 2005). CaMKII also phosphorylates microtubule associated proteins (MAP) which are important in maintaining cellular morphology, intracellular trafficking and establishing neurite outgrowth of neurons. CaMKII and MAP interaction inhibits actin polymerization, reduces affinity for tubulin and induces microtubule disassembly (Yamauchi, 2005). CaMII phosphorylates various substrates involved in synaptic plasticity and is also necessary for the induction of long term potentiation (LTP) specifically in the CA1 region of the hippocampus (Griffith, 2004). Activated CaMKII can phosphorylate the AMPA receptor to potentiate its current (Soderling et al., 2001). CaMKII also imposes negative effects on transcription mediated by cAMP response element-binding (CREB). Phosphorylation residue Ser133 is a known substrate for CaMKII whereas Ser 142 is simultaneously phosphorylated and induces a dominant negative effect (Soderling et al., 2001).

Calcium Calmodulin Dependent Kinase IV

Another one of six identified CaM-dependent kinases is CaMKIV that also targets variable substrates. Similar to CaMKII, CaMKIV is a serine/threonine specific kinase.

CaMKIV was initially described as being predominantly abundant in the granular cells of the cerebellum which is the reason for its original name and notation 'CaMK-kinase Gr' and it was later named CaMKIV (Ohmstede et al., 1989; Krebs, 1998).

Structure

CaMKIV is expressed in two variably spliced monomeric isoforms, M_r 65 000 (α) and of M_r 67 000 (β), with the β isoform having a 28 amino-acid N-terminal extension (Sakagami and Kondo, 1993). Common to the CaMK family, is the organizations of the domains. CaMKIV consists of a catalytic domain, an autoinhibitory binding domain and a calcium/CaM binding domain (Soderling et al., 2001). Although the sequence for the regulatory and catalytic domain is similar between CaMKII and CaMKIV, they only share 50% amino acid identity. The CaMKIV gene spans 42 kb and consists of 12 exons and 11 introns (Krebs 1998). The CaMKIV gene encodes for the similar calmodulin-binding protein calspermin, exclusively expressed in postmeiotic male germ cells for which the function is unknown (Sun et al., 1995).

Tissue Specificity and Subcellular Localization

In contrast to CaMKII or calcineurin which are ubiquitously expressed, CaMKIV expression is restricted to regions in the brain to the thymus, particularly T-lymphocytes and, to a lesser extent, the spleen and post mitotic male germ cells (Ohmstede et al., 1989; Frangakis et al., 1991; Means et al., 1991). Both CaMKIV isoforms are expressed in the brain while the β isoform is predominantly expressed in the cerebellum (Krebs, 1998). CaMKIV has been shown to be localized in the nucleus supporting its role in transcriptional regulation (Jensen et al., 1991; Matthews et al., 1994). In rodent brain, CaMKIV expression is developmentally regulated and CaMKIV expression is not seen until embryonic day 16 (E16) and reaches maximal expression at E18 (Krebs, 1998; Krebs et al., 1997).

Mechanisms of Action

Similar to CaMKII, calcium-calmodulin binds to CaMKIV to relieve autoinhibitory inhibition (Chow et al., 2005). Binding of Ca^{2+}/CaM exposes Thr200 in humans, T196 in mouse, in its activation loop to allow a low level of kinase activity (Tokumitsu and Soderling, 1996; Chatila et al., 1996). Intermolecular phosphorylation of Ser12 and Ser13 also relieves a novel form of inhibition and phosphorylation by Ca^{2+}/CaM dependent kinase CaMKK at Thr200 increases total protein kinase activity and exhibits Ca^{2+}/CaM independent activity (Chatila et al., 1996; Means, 2000). Mutation studies have demonstrated that Thr200 phosphorylation is required for activation by CaMKK, to stimulate autonomous activity which, together, induces CaMKIV transcriptional function (Chow et al., 2005). The protein phosphatase 2A (PP2A) can exist in a stable complex with CaMKIV and dephosphorylate CaMKIV on Thr200 to negatively regulate CaMKIV transcriptional function (Chow et al., 2005).

Function

CaMKIV mediated transcription is implicated in the regulation of many genes: TNF family members such as CD40L, FasL, and TNFα, BDNF orphan members of the steroid receptor subfamily such as RORα and COUP-TF (Means, 2000). Consistent with its expression in nucleus, CaMKIV regulates several transcription factors including CREB, CREM (cAMP-responsive element modulator), SRF (serum responsive factor), retinoid-related orphan receptor and chicken ovalbumin upstream promoter-transcription factor 1 (Chow et al., 2005; Chatila et al., 1996). Other transcription related components such as histone deacetylase 4 (HDAC4) and CREB binding protein (CBP)/p300 are also phosphorylated by CaMKIV (Enslen et al., 1994; Matthews et al., 1994). CaMKIV mediated phosphorylation of CREB at Ser133 is required for CREB/CBP/p300 binding resulting in transactivation for late phase of LTP in hippocampal neurons and LTD in cerebellar Purkinje cells (Anderson and Kane, 1998; Means, 2000). CaMKIV directly phosphorylates CBP at Ser 301 which is required along with CREB phosphorylation for NMDA-induced transcription in hippocampal neurons (Impey et al., 2002). Transcription factor MEF2 has also been described as a substrate for CaMKIV *in vitro* and CaMKIV has shown to activate MEF2 reporter genes (Corcoran and Means, 2001). CaMKIV can also inhibit MEF2 and HDAC4 interaction which enhances MEF2 transcription (Lu et al., 2000).

CaMK Kinase

The regulatory mechanism of specific CaMKs involved CaMK kinase (CaMKK). Ca^{2+}/CaM binding is required for CaMKK mediated phosphorylation of CaMKI/CaMKIV (Corcoran and Means, 2001). CaMKK α and β isoforms have been identified in mammals and are products of distinct genes and either isoform has been shown to phosphorylate CaMKI and CaMKIV *in vitro* (Soderling et al., 2001). CaMKKα mRNA is found in thymus and spleen, whereas CaMKKβ is present in all tissues that express CaMKIV (Corcoran and Means, 2001). CaMKK phosphorylates threonine located in the activation loop between protein kinase subdomains VII and VIII (Means, 2000).

Phosphorylation of Regulatory Transcription Factors

There are various examples of calcium and CaMK regulated transcription factors that modulate gene expression through signal transduction pathways. One example is a neuronal transcriptional repressor, DRE-antagonist modulator (DREAM), that binds to the downstream regulatory element (DRE) of the human prodynorphin gene involved in memory acquisition and pain (Carrion et al., 1999). Upon calcium stimulation DREAM binding and repressor activity is inhibited. Another study has examined the interaction between CaMKII and transcription factor NF-κB involved in regulating cytokine production and participates in inflammation, immune and stress related responses and regulation of cell survival (Meffert et al., 2003). It was found that basal synaptic input activated NF-κB in response to calcium elevation and CaMKII activation. The calcium regulated phosphatase, calcineurin, dephosphorylates nuclear factor of activated T-cells (NFAT) family of transcription factors and promotes translocation to the nucleus where it become transcripitional active (Rao et al., 1997; Willingham et al., 2005). Another study has shown that CaMKII mediated phosphorylation of Ets-1 inhibits DNA binding by reinforcing autoinhibitory mechanism (Cowley and Graves, 2000). The CaMKII and CaMKIV has been implicated in regulation of gene expression by playing a role in phosphorylation of cAMP-response element binding protein (CREB), a major transcription factor that is involved in neuroplasticity, cell survival and cognition (Tiraboschi et al., 2004). CaMKIV primarily phosphorylates CREB at Ser133 only, whereas, CaMKII phosphorylates CREB at Ser133 and Ser142 (Sun et al., 1994). CaMKIV mediated phosphorylation at Ser133 induced CREB activation. CaMKII mediated phosphorylation at Ser 142 blocks CREB activation, which seems to involve destabilization of its association with CREB binding protein (CBP) (Means, 2000). Although many examples of calcium and CaMK regulated transcription factors have been described, Freud-1 provides a new example of a CaMK regulated repressor that in inactivated by phosphorylation.

Objectives and Hypothesis

Based on the above results that Freud-1 is inactivated by a CaMK-dependent mechanism, I hypothesized that human Freud-1 is directly phosphorylated by calcium calmodulin dependent kinase CaMKII and CaMKIV at the CaMK consensus site(s). CaMK phosphorylation of Freud-1 disrupts its binding to the DRE of the 5-HT1A promoter and subsequently inhibits Freud-1 mediated repression of the 5-HT1A receptor gene. The following experiments also address the importance of CaMKII and CaMKIV in regulation of Freud-1 activity. The objectives are to determine: the ability of CaMK to phosphorylate human Freud-1, the CaMK sites involved in CaMKII mediated phosphorylation, the effects of phosphorylation of Freud-1 on the protein's ability to bind to the 5-HT1A DRE and to test whether CaMKII induced phosphorylation of Freud-1 inhibits Freud-1 mediated repression of the 5-HT1A receptor gene.

Experimental Approach

To examine whether CaMKII directly phosphorylates human Freud-1, an *in vitro* CaMKII kinase assay was performed. CaMK consensus phosphorylation sites of Freud-1 were identified using the Scansite program and the importance of each site was assessed, again by *in vitro* CaMKII kinase assay. Pull-down assays, using phosphoserine and phosphothreonine antibodies, were carried out to determine whether CaMKII or CaMKIV phosphorylates Freud-1 in cells. Electrophoretic mobility shift assays allowed us to assess the ability of phosphorylated Freud-1 to bind to the 5-HT1A DRE. Lastly reporter assays using three different luciferase-reporter constructs were used to determine if CaMK mediated phosphorylation of Freud-1 inhibits its function as a transcriptional repressor.

METHODS

Plasmid Construction and Mutagenesis

The human Freud-1 cDNA expression plasmid (Genbank accession no. AB097002) was obtained from Dr. Matsuda (ASAH1 KASEI Corporation, Shizuoka, Japan) (Matsuda et al., 2003), amplified by polymerase chain reaction (PCR) and subcloned into EcoR1 sites of pcDNA3 (Invitrogen, Burlington, ON) and pTriEx4 (Novagen, Madison, WI). Human Freud-1 mutated constructs were generated using the QuickChange® II XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The forward and reverse primers (IDT, Coralville, IA) with the point mutations underlined were as follows: (S644A): 5'-TTGAGCAAAGGACTTCGCCGTCATCAAGATCTTCCC-3', 5-GGGAAGATCTTGATGACGGCGAAGGTCCTTTGCTCAA-3'; (S644D): 5-'TTGAGCAAAGGACTTCGACGTCATCAAGATCTTCCC-3', 5'-GGGAAGATCTTGATGA CGTCG AAGGTCCTTTGCTCAA-3'; (T780A): 5'GGGAAGATCTTGATGACGTCGAAGGTCCTTTGCTCAA-3', 5'-TTGAGCAAAGGACCTTCGACGTCATCAAGATCTTCCC-3'; (T780D): 5'-ATCCTTGAGGTCCTGGATGGTCGCCGGCCCGACGGG-3', 5'-CCCGTCGGGCCGGCGACCATCCAGGACCTCAAGGAT-3'. Primers were purified by separating DNA on a 19% polyacrylamide gel and isolating concentrated primers. Oligonucleotides were eluted with TE and quantified by spectrophotometry.

The plasmids pGL3P(5'-DRE), pGL3P(3'-DRE) and pGL3P(5'+3'-DRE) were synthesized by annealing complementary oligonucleotides (Table 1) flanked by SacI and NheI sites and subcloned into SacI/NheI sites in the PGL3-Promoter (Promega,

Table 1: Sequence of oligonucleotides for 5-HT1A and D2DR receptors DNA elements

Shown are the sequences for the dual repressor elements (DREs) for the human 5-HT1A, human D2DR promters and E2F which is a non specific competitor. These oligonucleotides were used for electrophoretic mobility shift assays (EMSA) and for generating reporter constructs.

Name	Sequence
h5-HT1A 5'-DRE	5'-AGATGGCACTCTAAAACATTTGCCACA-3
h5-HT1A 3'-DRE	5'-AGGTGGCGACATAAAACCTCATTGCTTAGAACT-3'
h5-HT1A 5'+3'-DRE	5'-AGATGGCACTCTAAAACATTTGCCACAAGGTGGCGACATAAAACCTCATTGCTTAGAACT-3'
hD2DR DRE	5'-CGCGTGGGATAAGCAAGCCCTTCTTGTAAAAGTTTTAAGAACAATACA-3'
E2F	5'-TATAGTGTACTCTACTATTCTGCTC-3'

Madison, WI) (Lemonde et al., 2004). The 200-bp SV40 human 5-HT1A promoter constructs, -1517, and -1790ΔRE-1 were obtained by insertion of a 273-bp StuI/NheI (1790 to -1517) fragment into NheI site of PGL3P and the RE-1 deletion was produced using the QuickChange® II XL Site Directed Mutagenesis Kit (Stratagene) (Lemonde et al., 2004). The Gal4 human Freud-1 fusion constructs were subcloned into the EcoRI site of pBXG-1 (Bremner et al., 1995) (gift from Dr. R Bremner, University of Toronto) in frame with the Gal4 DNA-binding domain (DBD) (1-147 amino acids) and the mutations were generated with the QuickChange® II XL Site Directed Mutagenesis Kit (Stratagene) (Lemonde et al., 2004). Reporter constructs X_2G_2P and G_5P containing two LexA and two Gal4 DNA elements or five Gal4 elements, respectively, upstream of the SV40 promoter were synthesized by digesting the X_2G_2P or G_5P vector with MluI/SalI and ligating the insert into MluI/XhoI cut PGL3-Promoter vector (Lemonde et al., 2004).

CaMK constructs were kindly provided by AR Means (Duke University, Durham, NC) and originally obtained from Dr. H. Schulman (Stanford University, Stanford, CA). Constructs provided include wild type rat αCaMKII inserted into the SRα promoter-based eukaryotic expression vector (Takebe et al., 1988) and the kinase deficient mutant form K42M. Deletion of the wild type construct was carried out to synthesize the control vector. Point mutation was done to produce the constitutively active form by mutating the threonine 286 autophosphorylation site to aspartic acid. CaMKIV constructs were also obtained including human wild type CaMKIV/Gr, subcloned into the pSG5 vector (Stratagene), the catalytically dead mutant K75E and the constitutively active form Δ1-317. The pSG5 control vector was synthesized by deletion of human wild type CaMKIV/Gr. All plasmids were purified by cesium chloride density gradient centrifugation or with PureYield™ Plasmid Maxiprep System (Promega), verified by ABI/PRISM automated sequencing system and quantified spectrophometrically and ethidium bromide staining.

Cell Culture and Transient Transfection

Human embryonic kidney (HEK) 293 cells and human neuroblastoma cells (SK-N-SH; provided by Dr. Lakshmi Devi, New York University, NY) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wisent, St-Bruno, QC) supplemented with 10% Fetal Calf Serum (Wisent) and 1% penicillin/streptomycin (Wisent). Cells were grown at 37°C in 5% CO_2. HEK 293 cells were sustained in Corning polystyrene 6 well plates (Sigma-Aldrich, Oakville, ON) and Primeria 6 well plates (Falcon, Franklin Lakes, NJ) were used to transfect SK-N-SH cells. Cell lines were grown to 50-60% confluency and the medium was replaced 12 hours before transfection. Cells were transiently transfected by calcium phosphate co-precipitation using 0.5 µg of reporter construct, 0.5 µg of human Freud-1 expression plamid or vector, 0.5 µg of CaMK or vector and 0.1 µg/well of pCMVβgal (Clontech, Mountain View, CA) to correct for transfection efficiency. For protein expression 5 µg of plasmid/10cm plate was used. The DNA was diluted with sterile water (270 µl of water added to 4.5 µg of DNA), 30 µl of 2.5 M $CaCl_2$ was added and 300 µl of 2 x HBS (HEPES buffered saline; 10 g/L HEPES) was added to mixture. A total volume of 200 µl was added to each well in a 6 well plate. For pull-down assay and immunoprecipitation cells were transfected with 3 µg of DNA in a 10 cm plate. Rat raphe (RN46A) cells were maintained in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM L-glutamine (Wisent), 1%

penicillin/streptomycin (Wisent) and incubated at 33°C in 5% CO2. Primeria 6 well plates were used for transfection. RN46A cells at 75% confluency were differentiated at 39°C for 6 days Cells were maintained in differentiation DMEM F12 medium (Wisent) containing 1% FBS (Wisent), 1% of 100x N2 Supplement (Invitrogen), 375 g of pyruvic acid (Sigma-Aldrich), 27.5 mg of ovalbumin (Sigma-Aldrich), 1% penicillin/streptomycin (Wisent) and 1% of 1 x glutamine (Wisent) . RN-46A cells were transiently transfected using lipofectamine and Plus reagent (Invitrogen) using 1.5 μl of lipofectamine and 1.5 μl of Plus reagent per 1 μg of DNA. Transfection reaction was in half the volume of OPTIMEM medium for 3 hours and the remainder volume of medium was added.

Freud-1 Protein Expression and Purifications

E. coli BL21 (DE3) (Novagen) was transformed with Freud-1 expression plasmid pTriEX4- hFreud-1, grown overnight and induced at $OD_{600} = 0.6$ with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent) at 37°C for 3 hours. Cells were lysed with lysis buffer (1 mM imidazole, 50 mM NaH_2PO_4, 500 mM NaCl and 1 mg/ml of lysozyme) and sonicated (3 x 10s burst at 75 W with 10s cooling in between). Cell debris and protein were separated by ultracentrifugation and supernatant was added to Ni-NTA matrix column (Qiagen) and equilibrated with lysis buffer. The column was washed twice with wash buffer (20 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl) and the protein was eluted with elution buffer (500 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl). Elutions were collected and analyzed by SDS-PAGE on a 10% gel. Fractions containing human Freud-1 were dialyzed over night against 1x phosphate buffered saline (1 x PBS) or DNA-binding buffer (DBB) containing 20 mM HEPES pH 7.9, 0.2 mM EDTA, 0.2 M EGTA, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and 2 mM dithiothreitol and stored at -80°C. The protein was quantified using BCA Protein Assay Kit (Pierce, Rockford, IL) and analyzed by western blot.

CaMKII kinase assay

CaMKII (500U/µl) (New England Biolabs Inc. Pickering, ON) was first activated in the presence of 1x CaMKII buffer, 2 mM $CaCl_2$, 1.2 µM Calmodulin and 100 µM ATP and incubated at 30°C for 10 minutes. The CaMKII activation reactions were added to the substrate reactions that contained 1 µg of purified protein, 1x CaMKII buffer, and 1 µCi gamma-labeled ATP and incubated at 30°C for 30 minutes. The samples were resolved on a 10% SDS PAGE gel, stained with Coomassie Blue overnight and destained overnight with solution containing 10% glacial acetic acid, 10% isopropanol. The gel was dried in the heated vacuum drier for 30 minutes at 80°C and samples were visualized by autoradiography.

CaMKII Activity Assay

CaMKII activity was assayed in cell lysates using CaMKII assay kit (Upstate Biotechnology, Mississauga, ON) according to the manufacturer's protocol. Total protein (100 µg) was added to CaMKII specific substrate peptide KKALRRQETVDAL cocktail containing 500 µM Autocamtide 2 and 40 µg/ml calmodulin along with 10 µl of assay dilution buffer II (ADBII) (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$), 10 µl of PKA/PKC inhibitor cocktail and 100 μCi of $MgCl_2$ – [γ-^{32}P] ATP. The reaction was incubated for 10 minutes at room temperature with rotation. The phosphorylated substrate was separated from the residual [γ-^{32}P] ATP using P81 phosphocellulose paper. The papers were washed with 0.75% phosphoric acid and radioactivity was measured by scintillation counting. CaMKII activity was measured as counts per minute per 100 ng of protein and calculated as picomoles as per the manufacturer's instructions.

Pull- down Assay

After 48 hours cells were washed with 1xPBS and collected by centrifugation. The cells were re-suspended in lysis buffer with 1x Complete protease inhibitors (Roche, Laval, QC) and incubated on ice for 30 minutes. Cells were sonicated and the lysate was centrifuged to separate cellular debris. Ni-NTA Spin Columns (Qiagen, Mississauga, ON) were equilibrated with 600 μl of lysis buffer and centrifuged for 2 min at 2000 rpm. Cleared lysate was loaded in the column and centrifuged for 2 minutes at 2000 rpm. Ni-NTA spin columns were washed 3 times with 600 μl of wash buffer and centrifuged. Protein was eluted with 200 μl of elution buffer 3 times and centrifuged to collect purified protein. Lysate and purified protein were quantified using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) and subjected to western blot analysis.

Immunoprecipitation (CREB)

Transfected cells were collected, washed with 1xPBS and lysed with RIPA buffer for 2 hours rotating at 4°C. Cells were sonicated and centrifuged at maximum speed for 10 minutes. To pre-clear the lysate, the supernatant was added to 60 μl of Protein A 4

Fast Flow (GE HealthCare, Baie d'Urfe, QC) and incubated for 30 minutes at 4°C with rotation. One-tenth of the samples were kept as input and 1:250 of CREB antibody was added to the remaining sample and incubated overnight at 4°C with rotation. Protein A beads (20 μl of 50% bead slurry) were added and incubated for 2 hours at 4°C with rotation. Samples were centrifuged for 30 seconds, 1000 g at 4°C and pellet was washed five times with 500 μl of 1x lysis buffer. Protein was eluted with 60 μl of 2.5 SDS loading buffer and analyzed by western blot.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides (Table 1) were purified by separating on a 19% polyacrylamide gel, annealed at 100°C for 5 minutes and labeled with [α^{32}P] dCTP using 2.5 U of Klenow (New England Biolabs) and purified with Sephadex G-50 column (GE HealthCare). Purified protein was pre-incubated with or without double stranded competitor DNA oligonucleotides: E2F (Table1) in a 25 μl reaction containing DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 M EGTA, 100 mM KCl, 5mM $MgCl_2$, 5% glycerol and 2 mM dithiothreitol, pH 7.9) and 250 ng of herring sperm DNA (Roche) at room temperature for 30 minutes. [^{32}P]-labeled probe (50, 000 cpm/sample) was added and incubated for an additional 30 minutes at room temperature. The reaction was separated on a 4% polyacrylamide gel at 4°C. The gels were dried and exposed to film for 7 days at -80°C with intensifying screens. Purified protein treated with activated CaMKII, was prepared as previously mentioned for CaMKII kinase assay. Purified protein and kinase was incubated at 30°C for 10 minutes before DNA binding reaction.

Antibodies and Western blot analysis

Cells were collected 48 hours after transfection, washed with 1 x PBS and collected by centrifugation. Cells were re-suspended in RadioImmuno Precipitation Assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 0.1% w/v SDS, 0.5M EDTA, 1% w/v NP-40, 0.5% w/v deoxycholate sodium salt, 10 mM sodium fluoride, 10 mM disodium pyrophosphate) with 1x Complete protease inhibitor (Roche) and incubated on ice for 30 minutes. Cells were lysed by sonication and protein containing lysate was isolated. Samples were quantified by BCA Protein Assay Kit (Pierce). Prior to loading, 1x SDS loading buffer was added to each sample, boiled at 95°C for 5 minutes and separated by SDS-PAGE on a 12% polyacrylamide gel at 150 V, for 1 h. The protein was transferred onto a Polyvinylidene fluoride (PVDF) membrane (NEN, Waltham MA) for 1 hour and 10 minutes at 250 mA, washed overnight for 1 hour at 4°C in 2% blocking solution (Roche) in Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 8.0). The blots were incubated for 1 hour with HRP conjugated s-tag antibody (Novagen) at a dilution of 1:5000 in blocking solution and membranes were washed at room temperature in TBS-T (TBS containing 0.1% Tween 20) 3 times for 10 minutes. The blots were developed using BM Chemiluminescence Blotting Substrate (Roche) Other primary antibodies used include CREB (1:5000 overnight, Cell Signaling Technologies Inc. Danvers, MA), phospho-CREB Ser133 (1:5000 overnight, Cell Signaling Technologies), phosphoserine (1 :5000 overnight, Sigma-Aldrich), phosphothreonine (1 :5000 overnight, Sigma-Aldrich), CaMKII (1:5000 overnight, Santa Cruz Biotechnology Inc. Santa Cruz, CA), CaMKIV (1:5000 overnight, Cell Signaling Technologies Inc.), β-actin (1:5000 2 hours, Sigma-Aldrich). Prior to adding secondary antibody, membranes were washed twice with blocking solution for 10 minutes at room temperature and once with TBS-T for 10 minutes. Secondary antibodies used include horseradish peroxidase-linked anti-rabbit (1:5000 for 2 hours, Cell Signaling Technologies Inc.) and anti-mouse (1:5000 for 2 hours, Jackson Laboratories Bar Harbor, MA). Protein expression was quantified with Adobe Photoshope 6.0 using fixed size selection tool and a histogram.

Luciferase and β-galactosidase assays

Cells were harvested 48 hours after transfection and washed with 1xPBS. Triplicate samples were extracted with 100 µl/well of reporter lysis buffer (Promega) After one freeze thaw cycle the cells were centrifuged (maximum speed for 10 minutes at 4°C). Supernatant was collected and 30 µl of each sample was added to 100 µl of luciferase buffer composed of 4.5 ml of 20 mM tricine, pH 7.8, 2.67 mM $MgSO_4$, 1.07 mM solid $(MgCO_3)_4$ $Mg(OH_2)\cdot 5H_20$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM CoA, 530 µM ATP added to 0.5 ml of 10X 4.7 mM luciferin (Invitrogen) in 20 mM tricine (pH 8.0) and assayed for luciferase activity using LMax II 348 (Molecular Devices, Sunnyvale CA) (absorbance at 595λ). The β-galactosidase activity was assayed by adding 30 µl of sample to 200 µl of β-galactosidase substrate buffer (1xPBS, 0.5mM $MgSO_4$, 1.35ml of β-mercaptoethanol), 10% of 4 mg/ml chlorophenolred-ß-D-galactopyranoside (CPRG; Calbiochem, San Diego, CA). To correct for transfection efficiency luciferase values were normalized to β-galactosidase activity. We thank Dr. John Copeland for use of his luminometer and spectrophotometer.

Statistical analysis

Data are presented as mean + SEM. Student's t-test (2 tailed, unpaired equal variance) was used when two groups were compared and significance was represented by for $^*p<0.05$, $^{**}p<0.005$ and $^{***}p<0.0005$ respectively.

RESULTS

Human Freud-1 is Phosphorylated by CaMKII *In Vitro*

Based on previous evidence that Freud-1 is regulated by calcium/calmodulin dependent activity (Ou et al., 2003) our initial approach to examine the calcium dependent regulation of Freud-1 was to determine the role of CaMKII in Freud-1 phosphorylation. His-tagged wild type human Freud-1 was expressed in E. coli bacteria, purified and was used as a substrate for *in vitro* phosphorylation by CaMKII. Freud-1 was efficiently phosphorylated by rat recombinant αCaMKII as shown by [^{32}P] incorporation (Figure 8A). In the presence of CaMKII, an intense band was observed corresponding to the molecular weight of human Freud-1 (130kDa) as previously characterized (Rogaeva and Albert, 2007). In the same reaction (lane 2), several bands of lower molecular weight appeared to be phosphorylated and are likely to be Freud-1 degradation products. As a positive control for CaMKII activity, histone H1 was phosphorylated. In the absence of CaMKII, [^{32}P] incorporation of Freud-1 was not observed, confirming that CaMKII is exclusively responsible for phosphorylation. Coomassie blue stain of the protein was performed as a loading control (Figure 8B). These results provide evidence that Freud-1 is phosphorylated by CaMKII *in vitro*.

Freud-1 Site Directed Mutagenesis on CaMKII Phosphorylation Residues

Examination of the human Freud-1 protein sequence by Scansite (http://scansite.mit.edu) (Figure 9) revealed two conserved CaMKII phosphorylation consensus sites (RXXS/T) that border the C2 calcium phospholipids binding domain

Figure 8: Human Freud-1 is phosphorylated by CaMKII

As shown by *in vitro* kinase assay. A) Histone 1 (black arrow) and Freud-1 (red arrow) are phosphorylated in the presence of CaMKII. In the absence in CaMKII, Freud-1 is not able to phosphorylate. [^{32}P] incorporation was measured as an index of phosphorylation and visualized by autoradiography. B) Coomassie staining was used as a loading control. Data is representative of >3 independent experiments.

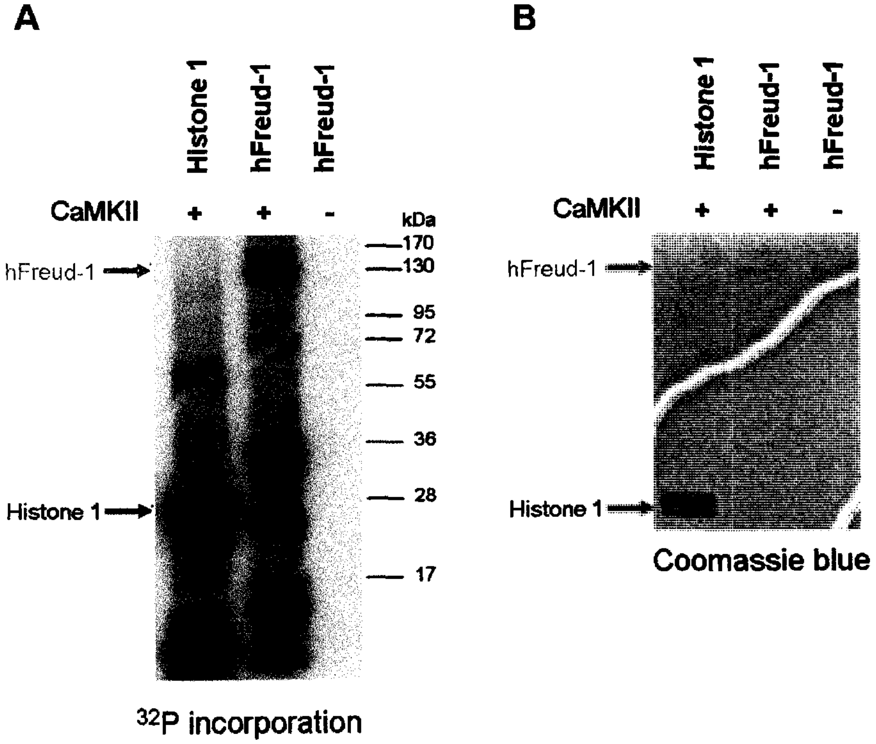
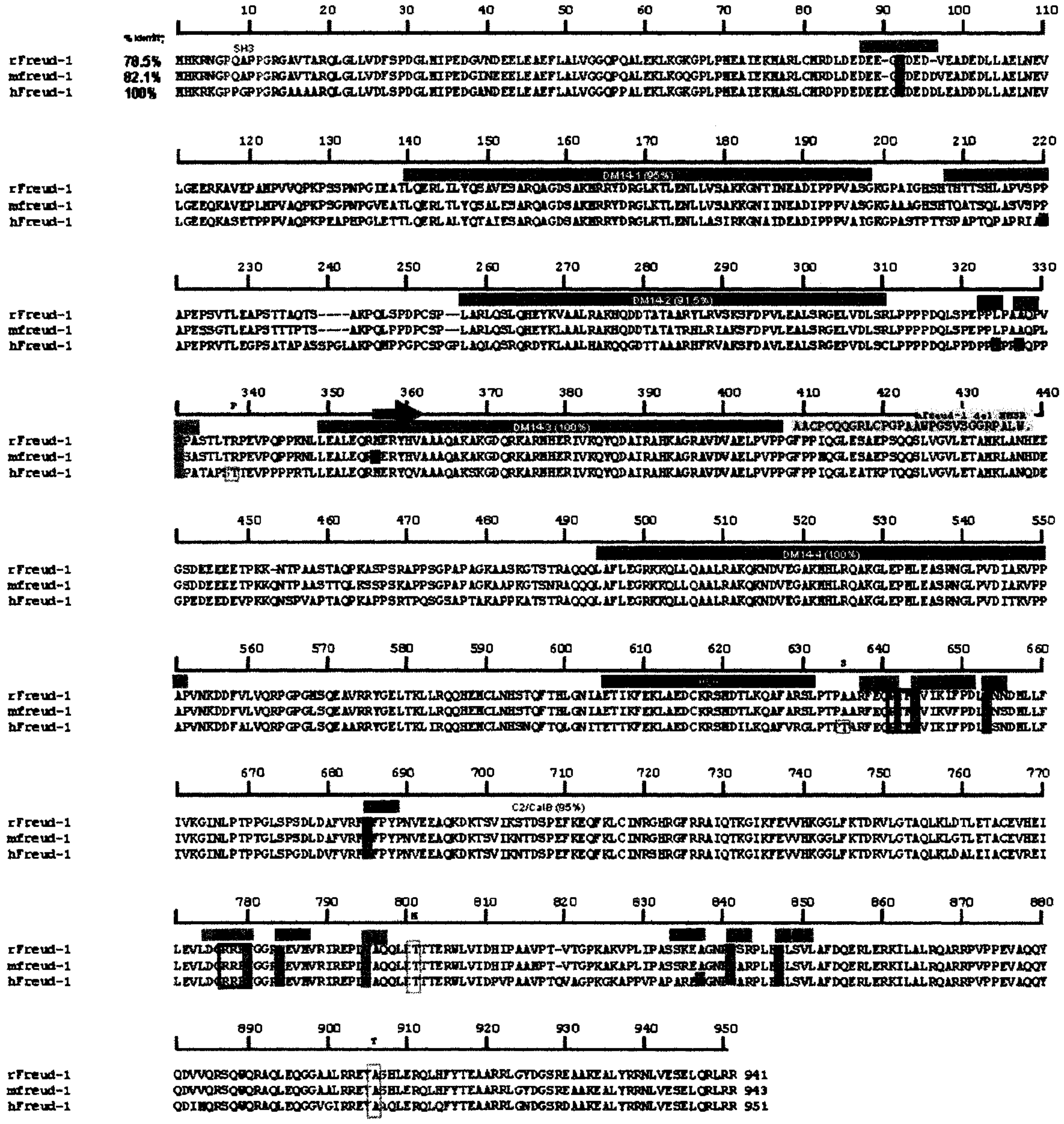

A
B
Histone 1
hFreud-1
hFreud-1
CaMKII
+
+
-
kDa
170
130
95
72
55
36
28
17
hFreud-1
Histone 1
32P incorporation
Histone 1
hFreud-1
hFreud-1
CaMKII
+
+
-
hFreud-1
Histone 1
Coomassie blue

Figure 9: Protein sequence alignment of Freud-1 orthologs

illustrating the Drosophila melanogaster (DM14) domains in red, the calcium lipid binding domain (C2/CalB) domain in yellow, the helix loop helix (HLH) domain in dark blue, kinase sites in cyan and ERK/MAPK phosphorylation sites in green. The translational start site of the mouse short isoform is represented by the purple arrow. The pink box corresponds to the frame shift amino acid sequence translated as a result of the deletion mutation in the CC2D1A gene associated with NSMR. The Src homology 3 domain (SH3) is shown in yellow. The two putative conserved CaMKII phosphorylation sites of interest are outlined in purple. A CaMK phosphorylation site found only in humans is located at position 220. Domains identified by NCBI blast (http://www.ncbi.nlm.nih.gov/BLAST/) conserved domain alignment (Marchler-Bauer et al., 2003). Sites identified by Scansite (http://scansite.mit.edu) search (Obenauer et al., 2003). The HLH site was identified by HELIXTURNHELIX (http://www.bioweb.pasteur.fr/docs/EMBOSS/helixturnhelix.html).

SNP polymorphisms represented by grey boxes were identified from http://www.ensembl.org/.


% identity
rFreud-1 78.5%
mFreud-1 82.1%
hFreud-1 100%
SH3
DM14-1 (95%)
DM14-2 (91.5%)
DM14-3 (100%)
DM14-4 (100%)
C2/CalB (95%)
hFreud-1 del mesa
AACPCQQGRLCPGPAAWPGSVSGGRPALW.
941
943
951

(Figure 10). The C2 domain is essential Ca^{2+}-dependent lipid binding of PKC, phospholipases, and synaptotagmin and protein-protein interactions (Clark et al., 1991). The first site, at position 644 (RTFS) includes the putatively phosphorylated amino acid serine (S) and the second site positioned at 780 (RRPT) includes an inducible phosphorylated threonine (T). To assess the contribution of each phosphorylation site to CaMKII mediated phosphorylation, several substitution point mutations of wild type Freud-1 were designed and synthesized (Figure 10). Putative amino acids that are able to accept a phosphate group (serine or threonine) were either mutated to neutral non phosphorylatable alanine or aspartic acid, which mimics the negative charge of a phosphorylated residue (Marechal et al., 1999; Knauf et al., 1996). The His/S-tagged human Freud-1 mutants were purified and assessed for protein expression in bacterial extracts by western blot. Freud-1 mutants S644A, T780A, S644A/T780A and S644D/T780D exhibited a significant increase in protein expression after induction with IPTG (Figure 11A).

Serine 644 is Important in CaMKII Mediated Freud-1 Phosphorylation

To determine the importance of each site for CaMKII mediated Freud-1 phosphorylation, we assessed of the ability of Freud-1 mutants to incorporate radio-labeled phosphates by *in vitro* kinase assay. The level of phosphorylation for S644A was significantly reduced by 40% compared to wild type Freud-1 indicating the significance of S644 in Freud-1 phosphorylation by CaMKII (Figure 11B, 11C). Freud-1 T780A mutant was equally phosphorylated compared to wild type. This suggests that serine 644

Figure 10: Schematic structure of the human Freud-1 protein

containing the four Drosophila Melanogaster 14 domains (DM14), a helix-loop-helix domain (HLH), and a calcium phospholipids binding domain (C2). The two conserved consensus CaMK phosphorylation sites positioned at 644 and 780 are shown with red arrows and Freud-1 mutations constructs are illustrated with the mutated amino acid in bold.

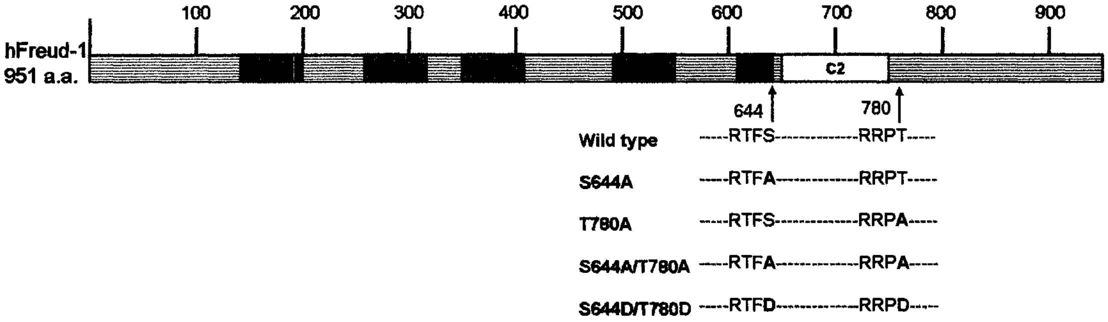
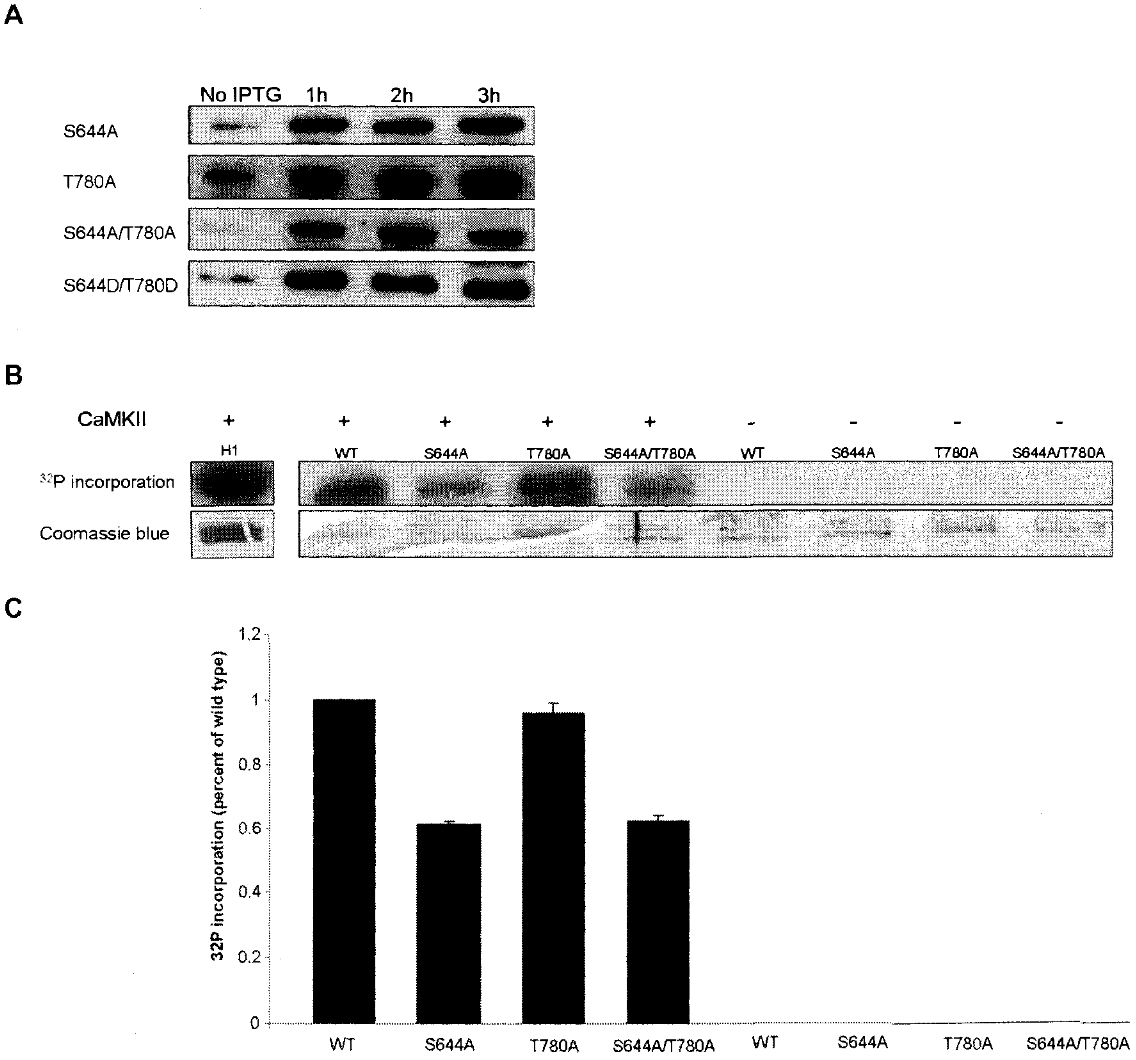

100
200
300
400
500
600
700
800
900
hFreud-1
951 a.a.
C2
644
780
Wild type -----RTFS--------------RRPT-----
S644A -----RTFA--------------RRPT-----
T780A -----RTFS--------------RRPA-----
S644A/T780A -----RTFA--------------RRPA-----
S644D/T780D -----RTFD--------------RRPD-----

Figure 11: Contribution of each phosphorylation site in CaMKII mediated phosphorylation of human Freud-1.

A) Bacterial expression of purified Freud-1 mutants. Protein expression was induced by treatment with IPTG for 3 hours. Samples were taken prior to induction (No IPTG) and every hour thereafter (4 samples total). In all four human Freud-1 mutants a significant increase in expression is shown after induction. Expression levels of purified protein were analyzed by western blot using S protein HRP conjugate antibody. B) *In vitro* kinase assay illustrating phosphorylation of Freud-1 mutants by CaMKII. Histone 1 (H1) and all mutants (S644A, T780A, S644A/T780A) were phosphorylated by CaMKII. C) Representative quantification of Freud-1 phosphorylation relative to [^{32}P] incorporation of wild type (WT) protein. Data represents Mean ± SE of 3 separate experiments.


A
No IPTG
1h
2h
3h
S644A
T780A
S644A/T780A
S644D/T780D
B
CaMKII
+
+
+
+
+
-
-
-
-
H1
WT
S644A
T780A
S644A/T780A
WT
S644A
T780A
S644A/T780A
³²P incorporation
Coomassie blue
C
32P incorporation (percent of wild type)
1.2
1
0.8
0.6
0.4
0.2
0
WT
S644A
T780A
S644A/T780A
WT
S644A
T780A
S644A/T780A

Is the target of CaMKII phosphorylation. Although site 780 did not seem to be involved in CaMKII mediated phosphorylation it may be an important target of CaMKIV for Freud-1 phosphorylation. We expected the S644A/T780A mutant would not be able to incorporate radio-labeled phosphate in the presence of CaMKII, however, reduced phosphorylation was observed. No phosphorylation was observed in the absence of CaMKII. Histone H1 protein was used as a positive control for CaMKII mediated phosphorylation, as seen by the strong band corresponding to H1 molecular weight. Equal loading of protein was detected by Coomassie blue staining. Together these findings demonstrate the importance of the putative phosphorylation site S644 in addition to the contribution of CaMKII to the phosphorylation of Freud-1.

Overexpression of CaMKII and CaMKIV in Cells

To examine CaMK and Freud-1 interaction in cells, we initially assessed CaMKII and CaMKIV protein expression in transiently transfected SK-N-SH cells. CaMKII constructs provided include wild type rat αCaMKII inserted into the SRα promoter-based eukaryotic expression vector and the catalytically inactive mutant form K42M due to its inability to bind to calmodulin (Rich and Schulman, 1998). Deletion of the wild type construct was carried out to produce the control vector. To synthesize the constitutively active form of CaMKII threonine 286 was mutated to aspartic acid to mimic autophosphorylation. Autoinhibition can be disrupted by threonine 286 autophosphorylation which allows the enzyme to function independent of Ca^{2+} (Waldmann et al., 1990). CaMKIV constructs were also obtained including human wild type CaMKIV/Gr subcloned into the mammalian expression pSG5 vector, the catalytically dead mutant K75E and the constitutively active form CaMKIV Δ1-317. Lysine 75 is involved in ATP binding and mutation to glutamic acid inactivates the enzyme (Chatila et al., 1996). Leucine 317 is located proximal to the CaM binding domain and truncation at this residue produces a Ca^{2+}/CaM independent enzyme (Chatila et al., 1996).

Levels of CaMKII in cell lysates from non transfected and CaMK transfected cells were assessed by western blot. Endogenous CaMKII was not detected in non transfected SK-N-SH cells or cells transfected with CaMKII vector (Figure 12A, 12B, left panel). However CaMKII expression was observed in the cells transfected with either WT, K42M or T286D. Possibly the antibody is not sensitive enough to detect low endogenous levels of the kinase although basal CaMKII activity was present. In HEK 293 cells endogenous CaMKII was present in the non transfected and vector control samples and slightly greater expression was seen in WT, K42M or T286D transfected cells (Figure 12A, 12B, right panel). In both cell lines, CaMKII migrated as a doublet of approximately 50 kDa. CaMKIV expression was also assessed using a CaMKIV antibody generated against the N terminus of the protein. Endogenous CaMKIV was not detected in SK-N-SH cells, although CaMKIV expression was observed in cells transfected with WT, K75E and Δ1-317. (Figure 13A, 13B) The truncated active mutant Δ1-317 was detected at 35 kDa compared to the wild type and kinase deficient mutant (K75E) that migrated at 60 kDa. Expression in HEK 293 cells was not detected due to the low transfection efficiency using both lipofectamine/Plus reagent and calcium phosphate methods of transfection. For this reason Freud-1 and CaMK interaction were further examined in SK-N-SH cells.

Figure 12: Protein expression of αCaMKII in transfected SK-N-SH and HEK 293 cells

A total of 1 μg of DNA was used per plate of cells for transient transfections A) Vector (SRα), wild type CaMKII (WT), kinase deficient mutant (K42M) and constitutively active kinase (T286D) expression levels were analyzed by western blot using anti CaMKII antibody and anti-rabbit as the secondary antibody. Expression of βactin was used as a loading control. B). Protein expression of 3 experiments for each cell line was quantified and normalized to non transfected control (Non) and is shown as Mean ± SE.

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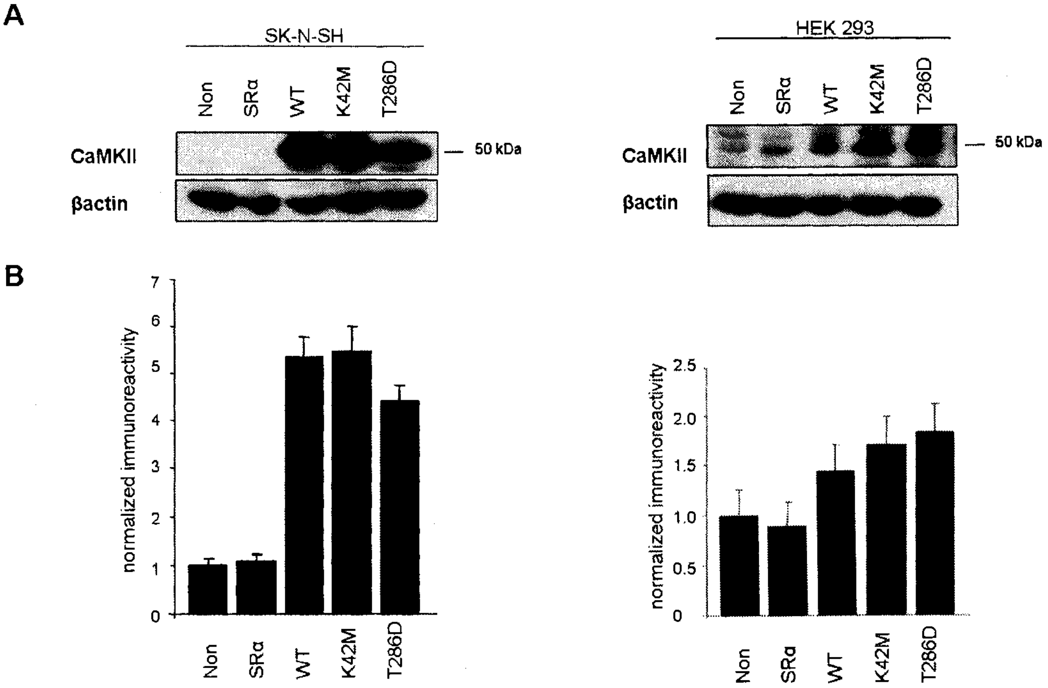
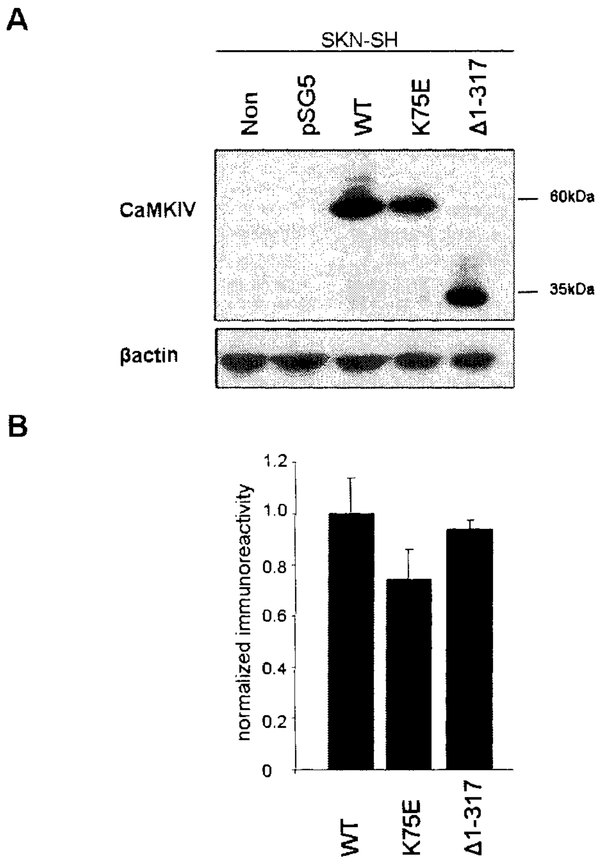

A
SK-N-SH
Non
SRα
WT
K42M
T286D
CaMKII
βactin
50 kDa
HEK 293
Non
SRα
WT
K42M
T286D
CaMKII
βactin
50 kDa
B
normalized immunoreactivity
0
1
2
3
4
5
6
7
Non
SRα
WT
K42M
T286D
normalized immunoreactivity
0
0.5
1.0
1.5
2.0
2.5
Non
SRα
WT
K42M
T286D

Figure 13: Protein expression of CaMKIV/Gr in transfected SK-N-SH cells

A total of 1 μg of DNA was used per plate of cells for transient transfections A) Vector (PSG5), wild type CaMKIV (WT), kinase deficient mutant (K75E) and constitutively active kinase (Δ1-317) expression levels were analyzed by western blot using anti CaMKIV antibody and anti-rabbit as secondary. B). Expression of βactin was used as a loading control. Protein expression of 3 experiments for each cell line was quantified and normalized to non transfected control (Non) and is shown as mean ± SE.


A
SKN-SH
Non
pSG5
WT
K75E
Δ1-317
CaMKIV
60kDa
35kDa
βactin
B
normalized immunoreactivity
1.2
1.0
0.8
0.6
0.4
0.2
0
WT
K75E
Δ1-317

CaMKII and CaMKIV Exhibit Kinase Activity in Cells

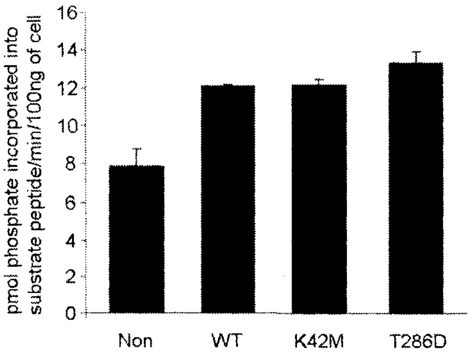
To quantify CaMKII enzyme activity in SK-N-SH cells, cells transfected with the various CaMKII constructs were assessed by CaMKII kinase activity assay. Cell lysate from non transfected cells or cells transfected with overexpressed wild type CaMKII, K42M and T286D constructs were separately examined. Incorporation of radio-labeled phosphate into a CaMKII specific substrate did not differ between WT, K42M and T286D samples. However, all samples exhibited an increase in CaMKII activity compared to the non transfected control although not significantly (Figure 14A).

To further investigate CaMKII and CaMKIV activity endogenous CREB was immunoprecipitated from cells transfected with wild type or constitutively active forms of each kinase and CREB phosphorylation was examined by western blot. CaMKII mediates CREB phosphorylation predominantly at serine 142 (Wu and McMurray, 2001; Bartsch et al., 1998) but also at serine 133 *in vitro* (Impey et al., 2002) and CaMKIV is involved in phosphorylating CREB at serine 133 (Ribar et al., 2000; Kitsos et al., 2005). Both wild type CaMKII and constitutively active T286D showed a significant increase in serine phosphorylation compared to non transfected control (Figure 14B) with the constitutively active kinase exhibiting an slight increase in serine phosphorylation compared to wild type CaMKII. Serine 133 phosphorylation was also increased (upper band) in CaMKII transfected cells compared to non transfected cells. The antibody also detects the phosphorylated form of the CREB related protein activating transcription factor 1 (ATF-1) (lower band). Both CaMKIV WT and Δ1-317 transfected samples showed a significant increase in general serine phosphorylation and serine 133 phosphorylation compared to non transfected control. The same amount of CREB was

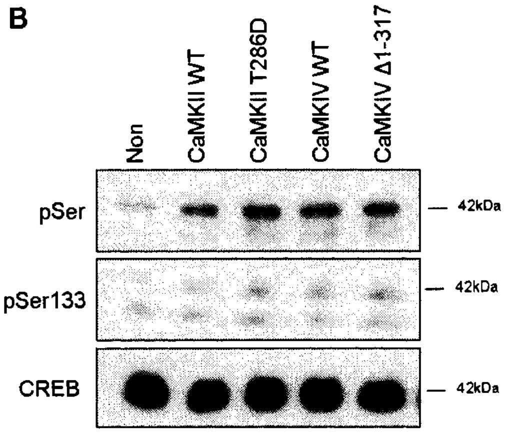
Figure 14: CaMK activity in transiently transfected SK-N-SH cells.

A) CaMKII kinase activity assay. Lysate from cells transfected with wild type CaMKII (WT), kinase deficient mutant (K42M) and constitutively active kinase (T286D) were analyzed for CaMKII mediated phosphorylation of a specific CaMKII peptide substrate. A total of 100 ng of lysate per sample was tested for [^{32}P] incorporation of substrate. Quantification represents 2 experiments. B) Western blot analyses of CaMKII and CaMKIV mediated CREB phosphorylation. CREB antibody was used to immunoprecipitate endogenous CREB from SK-N-SH cells transfected with wild type or constitutively active CaMKII or CaMKIV. Phosphorylation of serine was detected by immunoblotting with phospho-serine antibody (1:5000) and anti-mouse (1:5000) as secondary. Phospho-CREB (S133) antibody (1:5000) was used to detect specific phosphorylation at position 133. Anti-rabbit antibody (1:5000) was used as a secondary. The blots shown were quantified as a mean range + SE of 2 independent experiments.

A


pmol phosphate incorporated into substrate peptide/min/100ng of cell
0
2
4
6
8
10
12
14
16
Non
WT
K42M
T286D

B


Non
CaMKII WT
CaMKII T286D
CaMKIV WT
CaMKIV Δ1-317
pSer
pSer133
CREB
42kDa
42kDa
42kDa

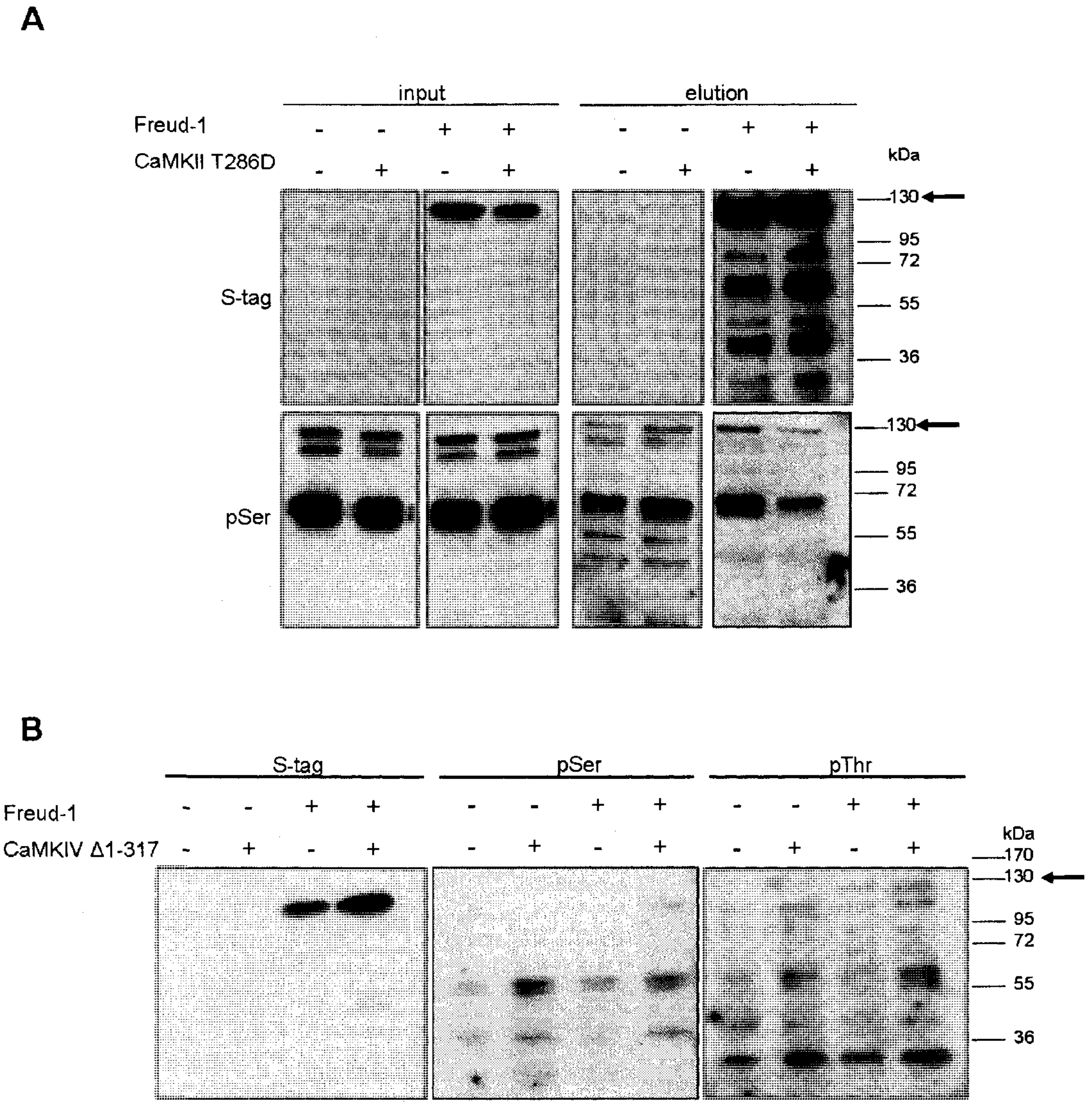
detected in all samples. Together these results indicated that the wild type and constitutively active forms of CaMKII and CaMKIV display kinase activity in cells compared to non transfected cells and it has previously been shown that the calcium independent T286D mutant is 36% constitutively active (Waldmann et al., 1990). The CaMKII kinase deficient mutant did not show reduced kinase activity and this could be due to the level of expression compared to endogenous CaMK.

Freud-1 is Phosphorylated in Cells by CaMKIV

To determine if Freud-1 is phosphorylated in neuronal cells, we performed Freud-1 pull down assays. SK-N-SH cells were co-transfected with His/S-tagged wild type Freud-1 and constitutively active CaMKII T286D. Only serine phosphorylation was assessed since it is the principal target for Freud-1 phosphorylation. Freud-1 was successfully isolated and expressed in cells in both input and elution fractions (Figure 15A). However, a general phospho-serine antibody detected various non specific bands at the same molecular weight as Freud-1. The phospho-serine antibody recognized His-tagged Freud-1 only with coexpression of activated CaMKIV Δ1-317 (Figure 15B). The phospho-threonine antibody also detected CaMKIV treated Freud-1 to a greater extent compared to Freud-1 coexpression with CaMKIV vector (Figure 15B). These results verified the role of CaMKIV in Freud-1 phosphorylation in cells. Both serine and threonine are phosphorylated by constitutively active CaMKIV.

Figure 15: CaMKII and CaMKIV mediated phosphorylation of Freud-1 in SK-N-SH cells.

Lysate from transfected cells were used in pull down assay. A) SK-N-SH cells were co-transfected with control vector (SRα) or constitutively active CaMKII (T286D) and His-tagged Freud-1 or PTriEx4 vector. Input and elution were analyzed by western blot for Freud-1 expression by s-tag antibody and serine phosphorylation (pSer antibody) B) Cells were transfected with control vector (PSG5) or constitutively active CaMKIV (Δ1-317) in addition to Freud-1 or control vector. Elution was analyzed by western blot for Freud-1 expression by s-tag antibody, serine phosphorylation and threonine phosphorylation (PThr antibody, 1:5000) Anti-mouse secondary antibody was used (1:5000). Freud-1 migration is designated by the black arrows. Data represent 2 independent experiments.


A
input
elution
Freud-1
CaMKII T286D
kDa
130
95
72
55
36
S-tag
pSer
B
S-tag
pSer
pThr
Freud-1
CaMKIV Δ1-317
kDa
170
130
95
72
55
36

Human Freud-1 Binds to the 5-HT1A DRE

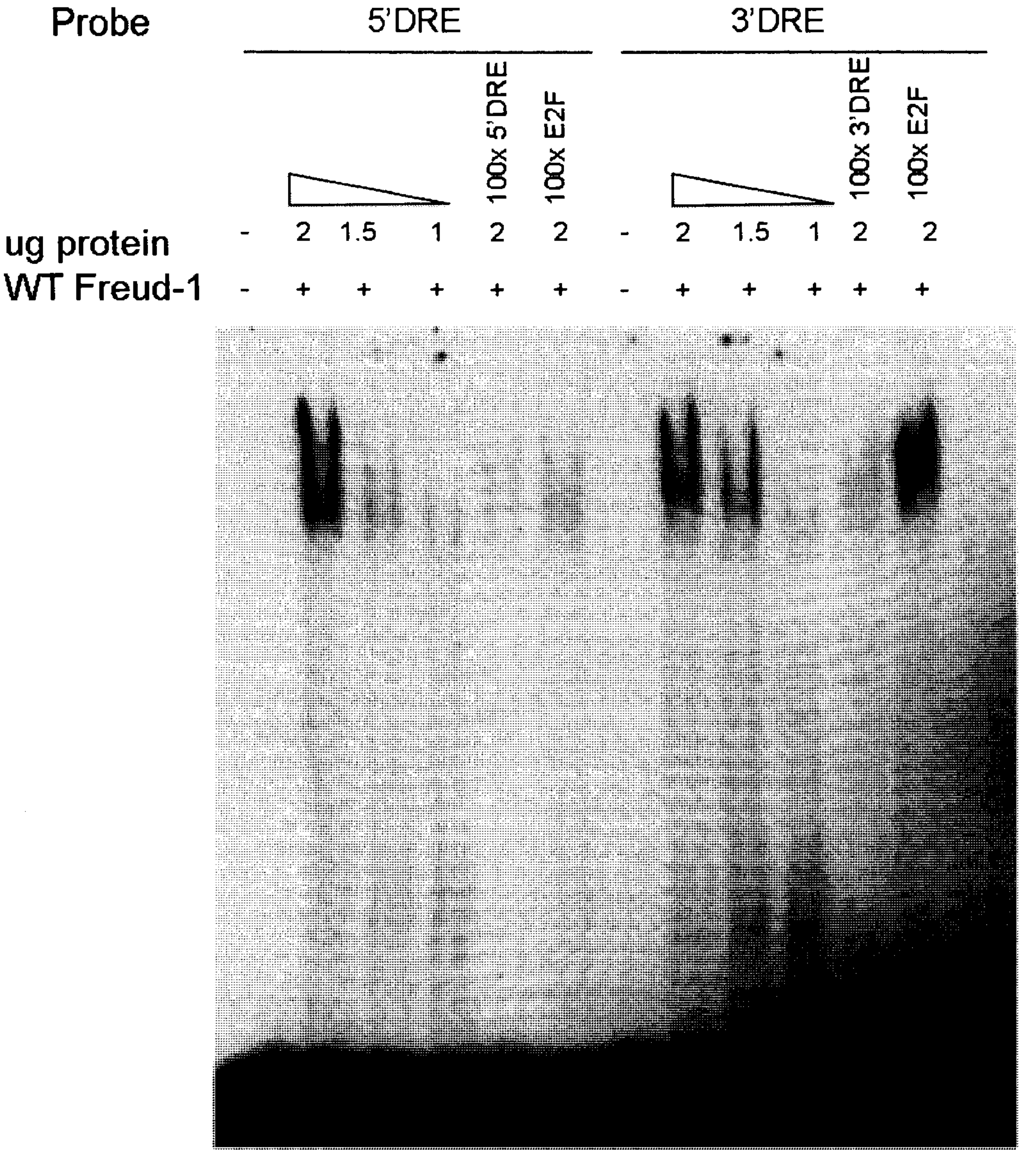
To determine the effects of CaMKII mediated Freud-1 phosphorylation on DNA binding activity we initially assessed human Freud-1 interaction with the 5-HT1A DRE *in vitro*. Purified Freud-1 was incubated with radio-labeled complementary 5'-DRE and 3'-DRE oligonucleotides and assessed by electrophoretic mobility shift assay. A 5-HT1A-DRE protein complex was observed and the interaction with both 5'-DRE and 3'-DRE is concentration dependent (Figure 16) as previously shown (Rogaeva and Albert, 2007). The protein complex was efficiently competed by 5'-DRE and 3'-DRE specific competitors. Surprisingly, the 5'-DRE complex was also competed with the non specific E2F primers but E2F did not compete with the 3'-DRE complex. Together these results confirm the interaction between Freud-1 and human 5-HT1A DRE binding *in vitro*.

CaMKII Phosphorylation of Freud-1 Decreases DNA Binding Activity *In Vitro*

Electrophoretic mobility shift assays were used to determine if CaMKII mediated phosphorylation of Freud-1 would affect protein DNA binding. We hypothesize that CaMKII mediated phosphorylation of Freud-1 will inhibit Freud-1 and 5-HT1A DRE binding *in vitro*. To test whether kinase reaction conditions are compatible with the binding reaction we assessed binding of wild type Freud-1 dialyzed in DNA binding buffer (DBB) or 1xPBS in the presence of CaMK buffer. Freud-1 protein dialyzed in DBB appeared to completely inhibit its binding to the 5'-DRE in the presence of CaMK buffer (data not shown).

Figure 16: Freud-1 binds to the 5-HT1A DRE.

Bacterially purified wild type human Freud-1 was analyzed by EMSA using the 5'-DRE and 3'-DRE of the human 5-HT1A receptor. Decreasing concentrations of protein were used. 100-fold of unlabeled specific (5'-DRE), (3'-DRE) and non-specific (E2F) competitors were added. Data are representative of 2 independent experiments.


Probe
5'DRE
3'DRE
100x 5'DRE
100x E2F
100x 3'DRE
100x E2F
ug protein - 2 1.5 1 2 2 - 2 1.5 1 2 2
WT Freud-1 - + + + + + - + + + + +

Freud-1 protein dialyzed in 1xPBS appeared to bind in the presence of CaMKII buffer. Protein dialyzed in 1xPBS dialyzed was more suitable for the kinase reactions since DBB contains calcium chelators EGTA and EDTA. Purified Freud-1 was incubated with CaMK buffer at 30°C in the presence or absence of CaMKII. Heating the kinase-protein reaction to 30°C required for kinase activation reduced Freud-1-DRE binding compared to non heated Freud-1 (figure 17, lane 2). CaMKII treated Freud-1 exhibited reduced binding compared to the untreated Freud-1 DRE complex (Figure 17). Similar results were observed for both 5'-DRE and 3'-DRE. This data indicates that CaMKII-mediated phosphorylation of Freud-1 *in vitro* reduces Freud-1 binding activity, specifically, the interaction with the 5-HT1A 5' and 3'-DRE.

Wild Type and Mutant Freud-1 Binds to the 5-HT1A DRE and D2DR DRE

Freud-1 mutations of CaMKII phosphorylation sites were carried out to examine changes in Freud-1 binding ability. Freud-1 mutants were bacterially purified and dialyzed in 1xPBS and used in 2 separate EMSA experiments. As previously shown, wild type Freud-1 binds to the 5-HT1A 5' and 3'-DRE and D2DR DRE (Figure 18A, 18B) (Rogaeva and Albert, 2007; Rogaeva et al., 2007b). The mutant S644A also binds to the 5-HT1A DRE and D2DR DRE however a lower band was observed (Figure 18A) and two DNA-protein complexes were observed (Figure 18B). The T780A mutant appears to be stuck in the well possibly due to protein-DNA aggregation (Figure 18A) and migrates as a lower DNA-protein complex (Figure 18B). The double S644A/T780A mutant also migrates as a lower DNA protein complex. These results imply that mutation of either or both CaMKII phosphorylation sites does not affect Freud-1 binding activity although we

Figure 17: CaMKII reduces Freud-1 5-HT1A DRE binding.

Purified wild type human Freud-1 dialyzed in 1xPBS was phosphorylated by CaMKII *in vitro* and analysed by EMSA using end labeled probes (5'-DRE) and (3'-DRE). Kinase reaction included 1xCaMKII buffer and was heated at 30°C. Data shown are representative of 2 independent experiments.

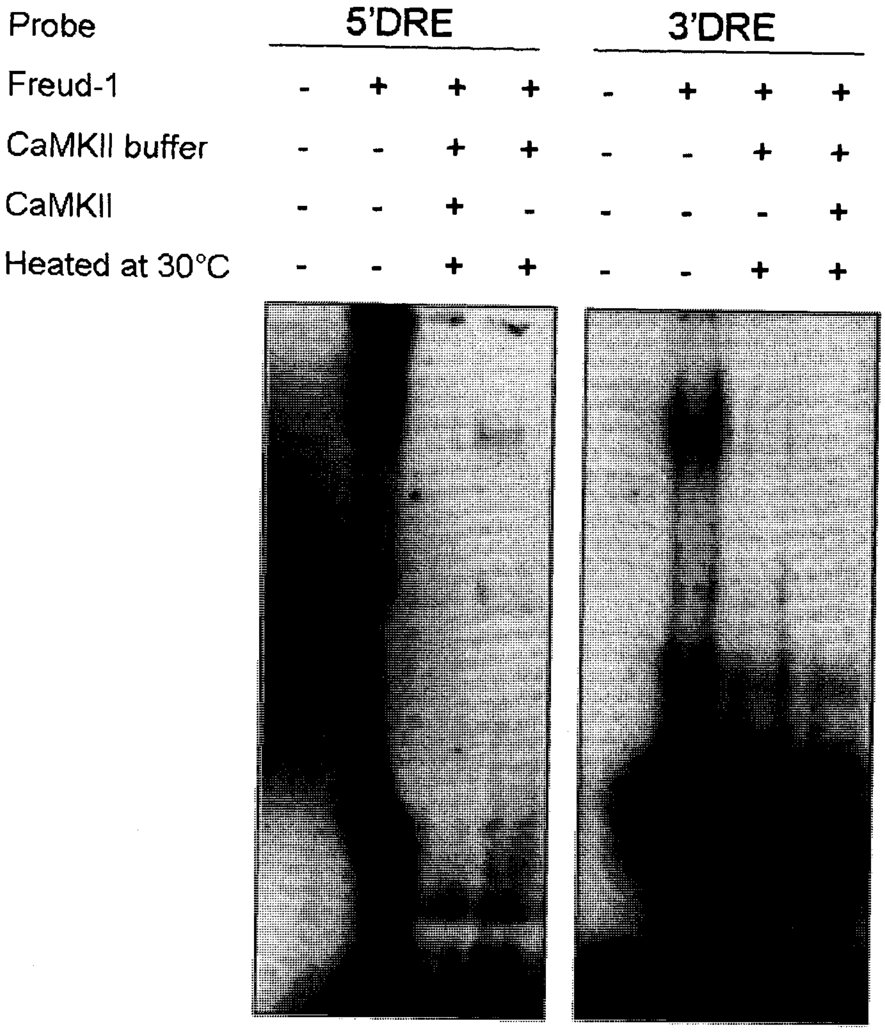
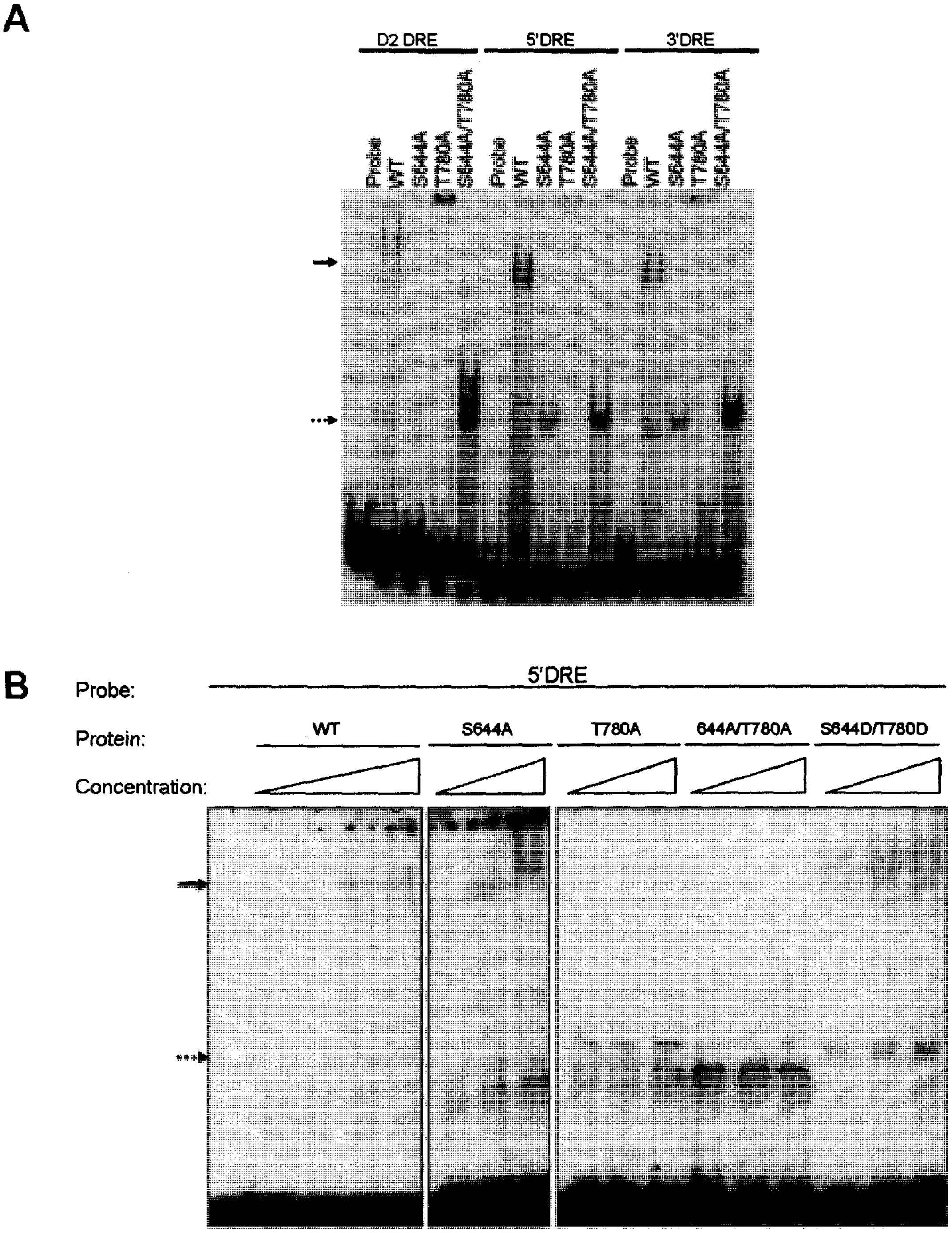

Probe
5'DRE
3'DRE
Freud-1 - + + + - + + +
CaMKII buffer - - + + - - + +
CaMKII - - + - - - - +
Heated at 30°C - - + + - - + +

Figure 18: Freud-1 mutants bind to the D2DR DRE and 5-HT1A DRE.

A) Bacterially purified wild type human Freud-1 and mutants (S644A), (T780A) and (S644A/T780A) were analyzed by EMSA using radio-labeled human dopamine D2 receptor DRE (D2 DRE), 5' human 5-HT1A DRE (5'-DRE) and human 3' 5-HT1A DRE (3'-DRE). A total of 2 µg of purified protein was used in the reaction. Data represents 2 experiments. B) EMSA of wild type and mutant Freud-1 protein dialyzed in 1xPBS. Increasing concentrations of protein were tested in binding to the labeled 5'-DRE.


A
D2 DRE
5'DRE
3'DRE
Probe
WT
S644A
T780A
S644A/T780A
Probe
WT
S644A
T780A
S644A/T780A
Probe
WT
S644A
T780A
S644A/T780A
B
Probe:
5'DRE
Protein:
WT
S644A
T780A
644A/T780A
S644D/T780D
Concentration:

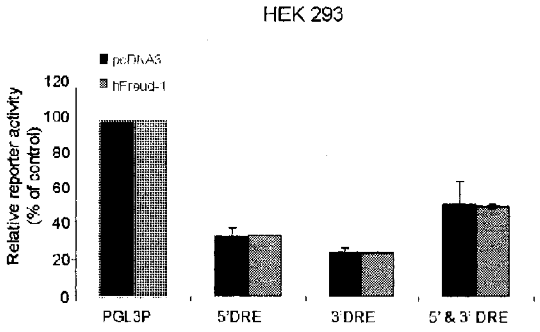
observed two protein DNA complexes. The lower migrating protein complexes could be a degraded or monomer form of the higher complex or the higher complex could be a homodimer. Surprisingly, the S644D/S780D mutant that mimics phosphorylated residues also exhibited binding activity, specifically with the 5-HT1A 5'-DRE, and appeared as both the higher and lower protein DRE complex. Possibly the negative charge on the mutated aspartic acid was not strong enough to mimic the negative charge of the phosphorylated serine or threonine or the mutation could have disrupted protein folding and in effect lost the negative charge at the phosphorylation site.

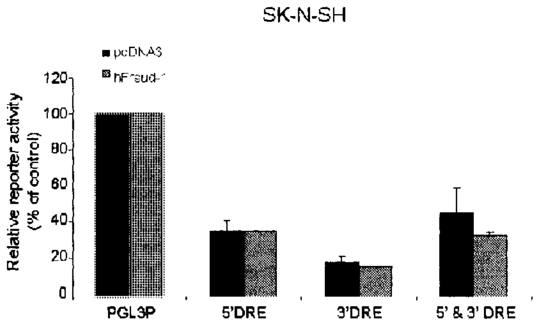
5-HT1A DRE Mediates Repression of the 5-HT1A Receptor Gene

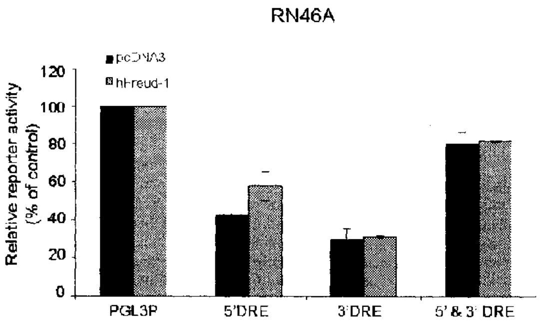
Reporter assays were performed to test the functional importance of CaMK-mediated Freud-1 phosphorylation in repression of the 5-HT1A promoter. Various reporter systems in different cell lines were examined. Cells were transfected with SV40 promoter-luciferase construct fused to 5'-DRE, 3'-DRE or both 5' and 3'-DREs of the human 5-HT1A promoter. In cell lines tested, HEK 293, SK-N-SH and RN46A cells, basal 5-HT1A activation was repressed and strongly influenced by the presence of 3'-DRE, 5'-DRE and both 5' and 3'-DREs respectively, compared to vector control (Figure 19). All reporter constructs exhibited strong repressor activity in all cells however the presence of Freud-1 did not affect repression. Extremely low reporter activity results from endogenous Freud-1 repressor function in which further repression upon transfection of exogenous Freud-1 cannot be observed. Nonetheless, the 5' and 3'-DREs confer repression upon the heterologous SV40 promoter, due to endogenous Freud-1. These experiments suggest that endogenous Freud-1 is maximal in these cell lines, hence transfected Freud-1 did not augment repression of these DRE constructs.

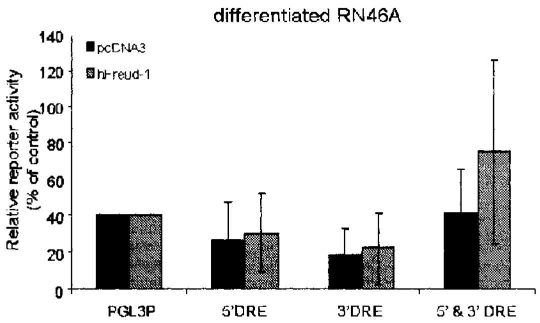
Figure 19: Basal repression of human 5-HT1A transcription is mediated by 5-HT1A 5' and 3'-DREs.

Cells (SK-N-SH, HEK293, RN46A and differentiated RN46A) were co-transfected with vector (pcDNA3) or human Freud-1 expression construct and SV40 promoter-luciferase constructs flanked with 5'-DRE or 3'-DRE or both. Samples were assessed for luciferase activity and normalized to β-galactosidase activity to correct for transfection efficiency. Data were obtained from > 2 separate experiments for each cell line in which triplicate transfections were performed. Data are presented as mean + SEM.


HEK 293
pcDNA3
hFreud-1
Relative reporter activity (% of control)
120
100
80
60
40
20
0
PGL3P
5'DRE
3'DRE
5' & 3' DRE


SK-N-SH
pcDNA3
hFreud-1
Relative reporter activity (% of control)
120
100
80
60
40
20
0
PGL3P
5'DRE
3'DRE
5' & 3' DRE


RN46A
pcDNA3
hFreud-1
Relative reporter activity (% of control)
120
100
80
60
40
20
0
PGL3P
5'DRE
3'DRE
5' & 3' DRE


differentiated RN46A
pcDNA3
hFreud-1
Relative reporter activity (% of control)
140
120
100
80
60
40
20
0
PGL3P
5'DRE
3'DRE
5' & 3' DRE

Calcium Inhibits DRE Induced Repression of the 5-HT1A Receptor Gene

Experiments were done to examine the effect of intracellular calcium on 5-HT1A activation. Our neuronal cell line was transfected with the 5'and 3'-DRE-SV40 promoter construct and treated with 40 mM of KCl which induces calcium influx and a calcium ionophore that also induces calcium influx and releases intracellular calcium stores (Ou et al., 2003). De-repression of luciferase activity was observed in 5'and 3'-DRE transfected cells treated with calcium inducers compared to vehicle treated control (Figure 20). Thus calcium signaling enhances transcription from the DRE sites in SK-N-SH cells, consistent with calcium-mediated inhibition of Freud-1 repressor activity. These experiments provide the first evidence that the DRE elements can confer calcium-dependent regulation on a heterologous (SV40) promoter.

Human Freud-1 Confers Repressor Activity in Cells

To further study the role of calcium in Freud-1 mediated repression a second reporter system was tested. Constructs consisting of the yeast Gal4 DNA binding domain (DBD, amino acids 1-147) fused to human Freud-1 (Gal4-DBD hFreud-1) were originally synthesized. Also produced were promoter-luciferase constructs with either two copies of LexA and Gal4 DNA elements (X_2G_2P) or five Gal4 DNA elements (G_5P) upstream of the SV40 promoter (Figure 21A). Gal4 DNA elements recruit the Gal4 DBD and it has been previously demonstrated that the prokaryotic repressor LexA binds to the LexA elements to induce strong repressor activity and is used as a control for repression (Lemonde et al., 2004). The Gal4 system permits the assessment of transcriptional activity while bypassing transcription factor binding ability. SK-N-SH and

Figure 20: 5-HT1A transcription is enhanced in a calcium dependent manner.

SK-N-SH cells were co-transfected with control vector (PGL3P) or SV40 promoter-luciferase constructs flanked with 5' and 3'-DRE. Cells were also treated with vehicle (DMSO) or 40 mM KCl and 1μM ionomycin (Calbiochem) for 4 hours prior to cell collection. Triplicate samples were subjected to reporter assays and corrected for transfection efficiency with co-transfection of pcMVβgal. Data represent mean + SEM values from 3 independent experiments. *$p<0.05$ (two tailed unpaired t-test) in comparison with vehicle control.

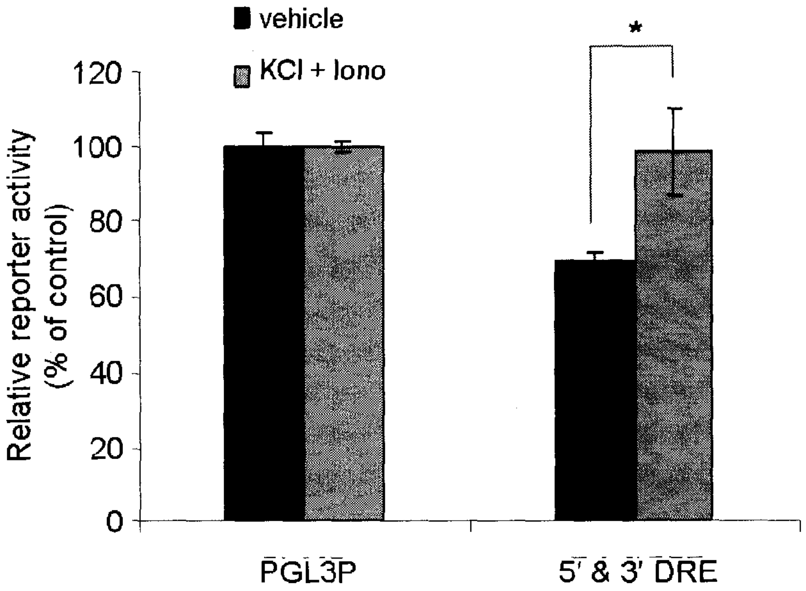
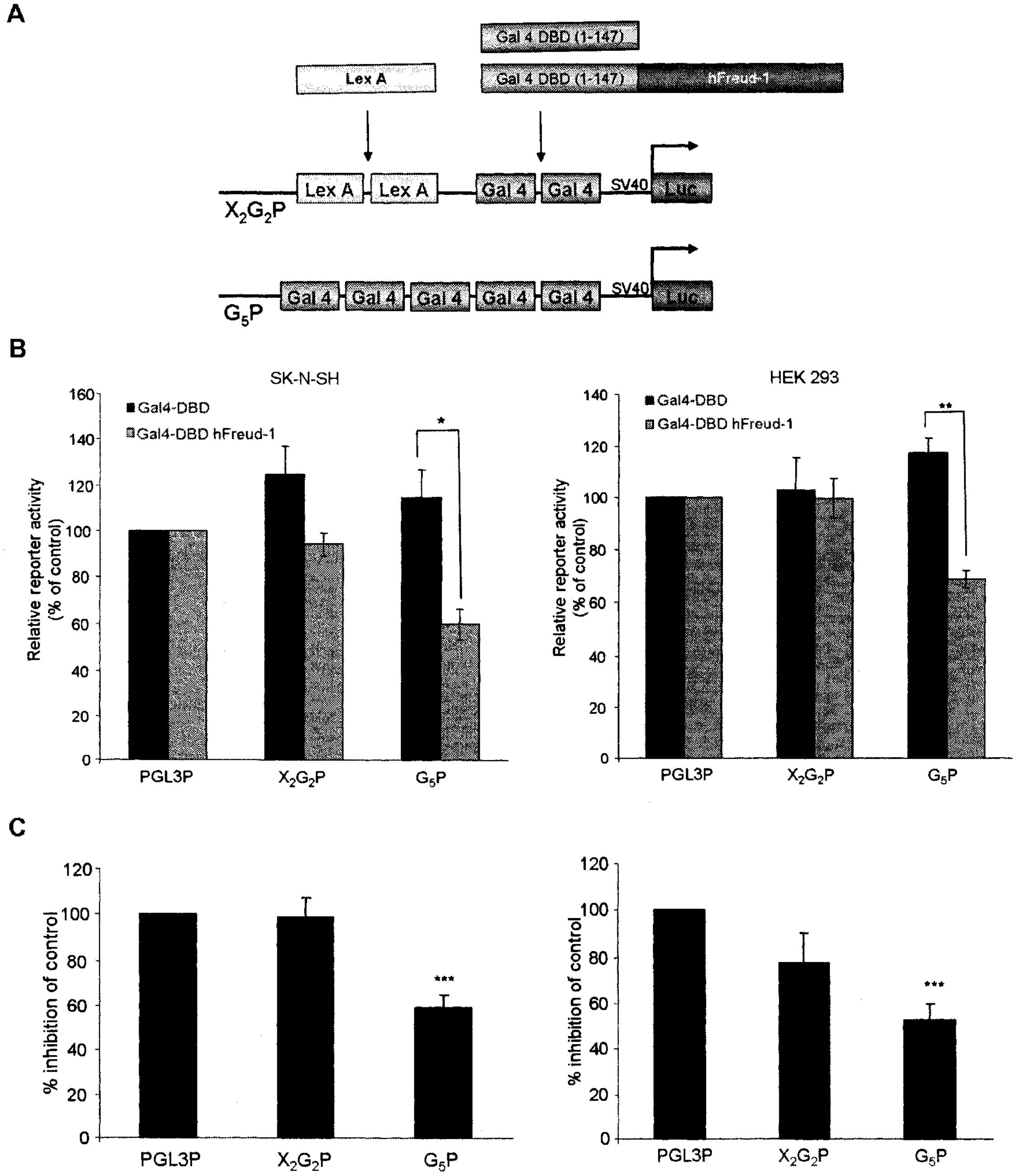

vehicle
KCl + Iono
*
Relative reporter activity
(% of control)
120
100
80
60
40
20
0
PGL3P
5' & 3' DRE

Figure 21: Human Freud-1 mediates repression in cells.

A) SK-N-SH and HEK 293 cells were co-transfected with Gal4-DBD or Gal4-DBD-Freud-1 plasmid with control vector (PGL3P), X_2G_2P containing two LexA sites and two Gal4 sites upstream of the SV40 promoter-luciferase or G_5P containing five Gal4 sites. Samples were assessed for reporter activity and normalized to β-galactosidase activity to correct for transfection efficiency. B) The same data are illustrated as % luciferase activity compared to Gal4-DBD control. Data represent triplicate transfections of each sample and is presented as mean + SEM of 3 independent experiments. *$p<0.05$, ** $p<0.005$, *** $p<0.0005$ (two-tailed unpaired t-test) compared to PGL3P vector or as indicated by bracket.


A
Gal 4 DBD (1-147)
Lex A
Gal 4 DBD (1-147)
hFreud-1
Lex A
Lex A
Gal 4
Gal 4
SV40
Luc
X2G2P
Gal 4
Gal 4
Gal 4
Gal 4
Gal 4
SV40
Luc
G5P
B
SK-N-SH
HEK 293
■ Gal4-DBD
▨ Gal4-DBD hFreud-1
Relative reporter activity (% of control)
0
20
40
60
80
100
120
140
160
*
**
PGL3P
X2G2P
G5P
C
% inhibition of control
0
20
40
60
80
100
120
***
PGL3P
X2G2P
G5P

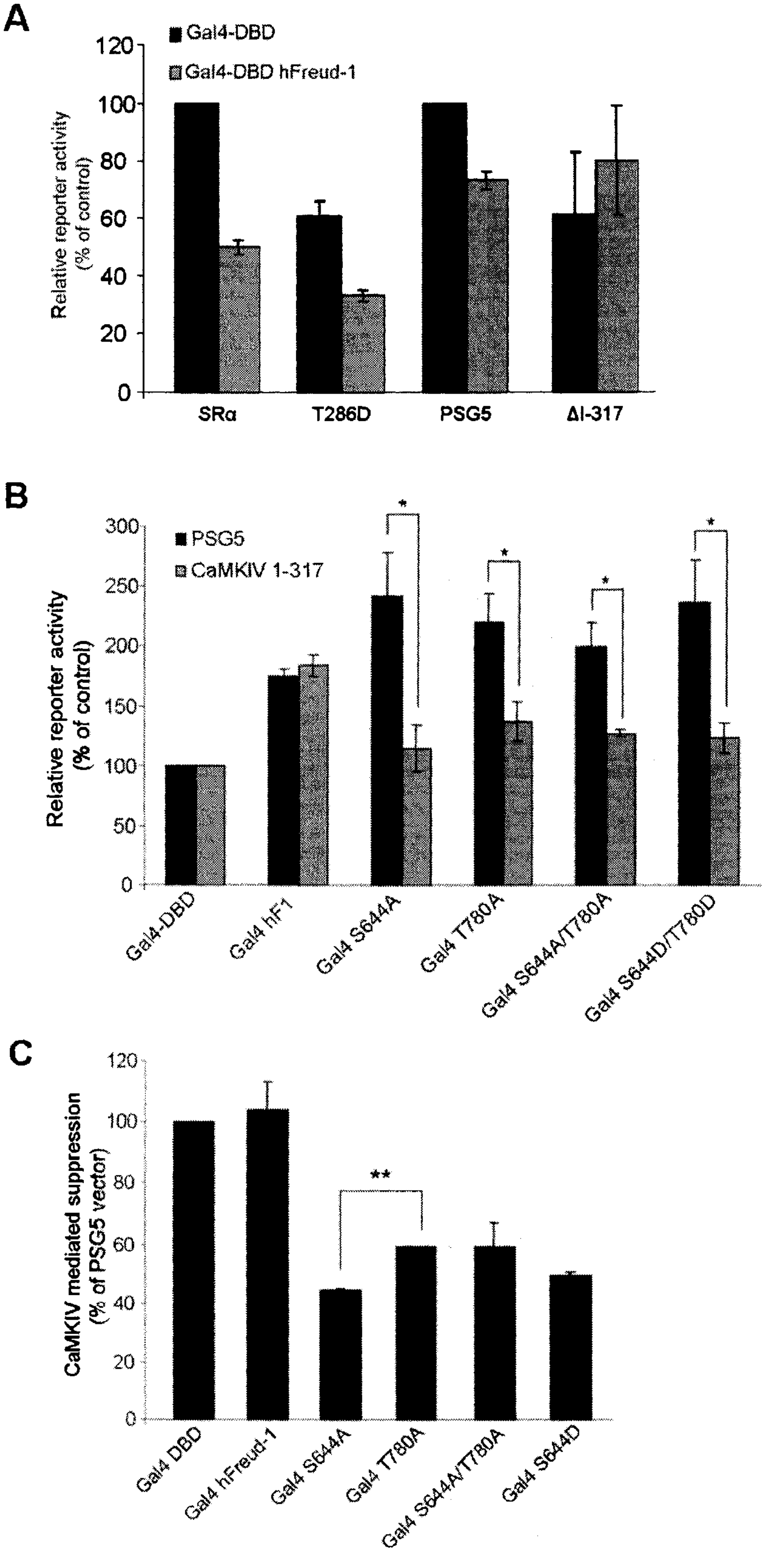
HEK293 cells were co-transfected with either Gal4-DBD vector (pBXG-1) or Gal4-DBD hFreud-1 and X_2G_2P, G_5P or PGL3P control and luciferase activity was measured. In SK-N-SH cells Gal4-DBD Freud-1 repressed transcriptional activity of the X_2G_2P construct but significant Freud-1 mediated transcriptional repression of the G_5P construct was also observed (Figure 21B, 21C left panel). In HEK 293 cells, Freud-1 only induced repression of the G_5P construct (Figure 21B, 21C, right panel). These results demonstrate strong repressor activity of human Freud-1 in both SK-N-SH and HEK 293 cell lines. This result confirms previous results observed using the short isoform of mouse Freud-1 (Lemonde et al., 2004).

Active CaMKIV Attenuates Freud-1 Induced Repression

We next examined whether active CaMKII or CaMKIV influences Freud-1 mediated repression of the G_5P construct. SK-N-SH cells were co-transfected with G_5P, Gal4-DBD or Gal4-DBD Freud-1 and CaMK constructs. Repression was observed in both CaMKII vector and constitutively active CaMKII T286D transfected Gal4-DBD Freud-1 samples compared to Gal4-DBD transfected samples (Figure 22A). Active CaMKII did not manipulate Freud-1 mediated repression in SK-N-SH cells. CaMKIV vector treated samples also demonstrated Freud-1 repression. However, in the presence of active CaMKIV Δ1-317, Gal4-DBD Freud-1 de-repressed reporter activity (Figure 22A), indicating a complete block of Freud-1 repressor activity. We next tested whether CaMKIV mediated Ser644 or T780 phosphorylation alters Freud-1 induced repression by examining whether Freud-1 mutants in these sites are resistant to CaMKIV-induced de-repression. SK-N-SH cells were transfected with G_5P, Gal4-DBD, Gal4-DBD Freud-1 or

Figure 22: Active CaMKIV attenuates Freud-1 induced repression.

A) Constructs used in transfection of SK-N-SH cells were reporter construct G_5P, Gal4-DBD or Gal4-DBD-Freud-1 plasmid and CaMKII vector (SRα), constitutively active CaMKII T286D, CaMKIV vector (PSG5) or constitutively active CaMKIV Δ1-317. Samples were assessed for reporter activity and normalized to β-galactosidase activity to correct for transfection efficiency Data represents triplicate transfections of each sample and is presented as mean + SEM of 3 independent experiments. B) Mutation of Freud-1 S644 and T780 attenuates de-repression by CaMKIV. SK-N-SH cells were transfected with G_5P, CaMKIV vector (PSG5) or CaMKIV Δ1-317 and Gal4 DBD, Gal4 DBD hFreud-1 or Gal4-DBD Freud-1 mutants. Values are expressed as % luciferase activity of control PSG5 vector. Triplicate transfections of 2 independent experiments are represented in the data as mean + SEM. $**P<0.005$.


A
Gal4-DBD
Gal4-DBD hFreud-1
Relative reporter activity (% of control)
0
20
40
60
80
100
120
SRα
T286D
PSG5
ΔI-317
B
PSG5
CaMKIV 1-317
Relative reporter activity (% of control)
0
50
100
150
200
250
300
*
*
*
*
Gal4-DBD
Gal4 hF1
Gal4 S644A
Gal4 T780A
Gal4 S644A/T780A
Gal4 S644D/T780D
C
CaMKIV mediated suppression (% of PSG5 vector)
0
20
40
60
80
100
120
**
Gal4 DBD
Gal4 hFreud-1
Gal4 S644A
Gal4 T780A
Gal4 S644A/T780A
Gal4 S644D

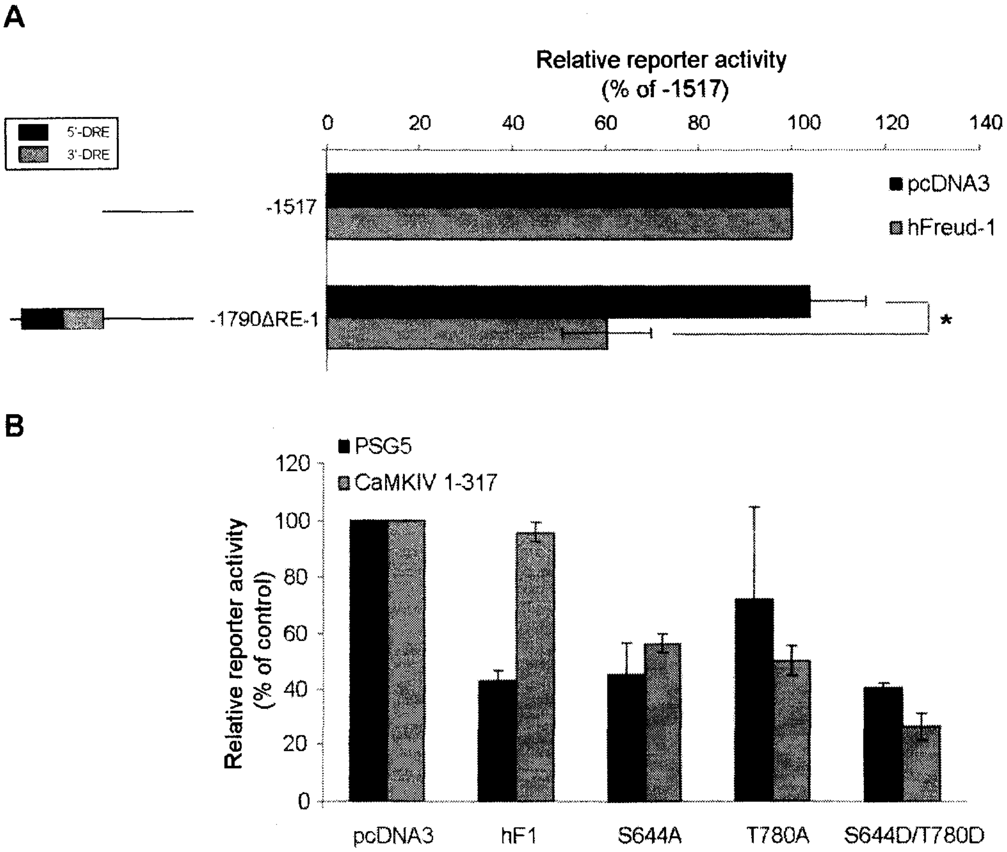
Gal4-DBD Freud-1 mutants along with CaMKIV vector, PSG5, or active CaMKIV Δ1-317. Again, no repression was observed in Gal4-DBD Freud-1 mutant samples in the presence of active CaMKIV (Figure 22B, 22C). Conversely, all of Freud-1 mutant constructs, S644A, T780A, S644A/T780A repressed transcriptional activity in the presence of constitutively active CaMKIV. Freud-1 S644A mutant induced further repression compared to the T780 mutant (Figure 22C). This suggests that both serine 644 and threonine 780 are both involved in CaMKIV induced Freud-1 phosphorylation but serine 644 may be the principle residue phosphorylated by CaMKIV. *In vitro* phosphorylation studies of the mutants support these findings, consistent with the EMSA experiments (Figure 18B), The S644D/T780D mutant repressed 5-HT1A transcriptional activity and did not mimic the effects of Freud-1 phosphorylation as expected.

Freud-1 Induced Repression of the Human 5-HT1A Promoter is DRE Dependent

A third reporter system using human 5-HT1A promoter-luciferase constructs was examined to eventually test the contribution of CaMKIV in Freud-1 mediated repression. A -1790 luciferase construct (originally obtained from Sylvie Lemonde; Lemonde et al., 2004) includes the repressor region of the human 5-HT1A gene. The -1790ΔRE-1 construct includes the 5'-DRE and 3'-DRE elements and the REST element (RE-1) was deleted (Figure 23A). The -1517 construct does not include any repressor elements and was used as a negative control. SK-N-SH cells transfected with -1790ΔRE-1 and Freud-1 expression plasmid exhibited strong repressor activity compared to pcDNA3 control vector (Figure 23A). Samples transfected with wild type Freud-1 and constitutively active CaMKIV exhibited de-repression compared to samples transfected with Freud-1 and

Figure 23: Active CaMKIV attenuates Freud-1 mediated repression of the human 5-HT1A receptor promoter.

A) SK-N-SH cells were co-transfected with human 5-HT1A promoter-luciferase constructs, -1517 control or -1790ΔRE-1 containing the 5' and 3'-DRE with the RE-1 deleted and Freud-1 expressing plasmid or control vector pcDNA3. Samples were assayed for reporter activity and corrected for transfection efficiency with co-transfection of pcMVβgal. B) SK-N-SH cells were co-transfected with -1790ΔRE-1, wild type or mutant Freud-1 and constitutively active CaMKIV or PSG5 control vector. Data represents triplicate transfections of each sample and is presented as mean + SEM of 3 independent experiments.


A
Relative reporter activity
(% of -1517)
0
20
40
60
80
100
120
140
5'-DRE
3'-DRE
-1517
-1790ΔRE-1
pcDNA3
hFreud-1
*
B
PSG5
CaMKIV 1-317
Relative reporter activity
(% of control)
0
20
40
60
80
100
120
pcDNA3
hF1
S644A
T780A
S644D/T780D

CaMKIV vector control (Figure 23B). However, Freud-1 mutants S644A and T780A showed no effect on 5-HT1A transcription when treated with active CaMKIV. The S644D/T780D double mutant also showed no effect of CaMKIV on Freud-1 induced repression of 5-HT1A activity consistent with the aspartic acid mutant unable to mimic phosphorylated residues. Together these results demonstrate that CaMKIV phosphorylates Freud-1 and blocks Freud-1 mediated repression of the 5-HT1A gene.

DISCUSSION

CaMKII and CaMKIV Regulated Freud-1, Repressor of 5-HT1A Receptor Gene

Previous studies have demonstrated the relevance of regulation of 5-HT1A receptor expression in association to mental illnesses such as anxiety and depression (Albert et al., 1996; Blier and Ward, 2003). The three week latency of antidepressant action has suggested another mode of 5-HT1A receptor regulation other than receptor desensitization. To date, our lab has examined, specifically, the transcriptional mechanisms in regulating 5-HT1A receptor expression and characterized the role of Freud-1, a novel repressor of the 5-HT1A receptor gene (Ou et al., 2003; Lemonde et al., 2004; Rogaeva and Albert, 2007). Although preceding studies, using pharmacological analysis, have implicated the role of CaMK in regulating Freud-1 it is unclear whether Freud-1 is directly phosphorylated or if CaMK phosphorylates a Freud-1 associating protein (Ou et al., 2003).

In this study we have established a mechanism of calcium dependent regulation of human Freud-1 by CaMKII and CaMKIV mediated phosphorylation. CaMKII phosphorylates Freud-1 *in vitro* at the two identified CaMK consensus sites (Ser644 and Thr780), although, CaMKII targets Ser644 for phosphorylation. Transfection of constitutively active CaMKIV induced serine and threonine phosphorylation of Freud-1 in SK-N-SH cells, providing evidence that both sites are involved in CaMKIV mediated phosphorylation. In addition, phosphorylation of Freud-1 by CaMKII inhibited binding to the 5'- and 3'-DRE of the 5-HT1A receptor. Mutation of phosphorylated residues to alanine did not affect Freud-1 binding ability to both 5-HT1A and D2DR DRE. Mutation of Ser644 and Thr780 to aspartic acid was as active as the alanine mutants, and did not appear to mimic the negative charge of phosphorylated residues as expected. As previously demonstrated, we found that the 5'- and 3'-DREs of the 5-HT1A receptor promoter conferred substantial basal repression, presumably due to activity of endogenous Freud-1 (Lemonde et al., 2004; Rogaeva and Albert, 2007). In the cell lines that were assessed, overexpression of Freud-1 did not result in further repression of luciferase activity, perhaps due to substantial levels of basal repression that may have been saturating. Inducing calcium influx and release of calcium stores, by treatment with high K^+ and a calcium ionophore, derepressed transcription of the human 5-HT1A receptor gene in SK-N-SH cells. These results support the calcium dependent regulation of Freud-1 repressor activity. Constitutively active CaMKIV counteracted Freud-1 mediated repression of 5-HT1A receptor expression at the 5-HT1A DREs. This finding was determined using the Gal4 luciferase reporter constructs as well as the human 5-HT1A promoter luciferase construct. Furthermore, Freud-1 mutants, S644A, T780A, S644A/T780A were resistant to CaMKIV phosphorylation providing evidence for roles of both sites in phosphorylation. Together, these findings support the role of CaMKII and CaMKIV mediated regulation of human Freud-1 activity.

Our data revealed that the Freud-1 mutant S644A/T780A exhibited minimal phosphorylation by CaMKII. This suggests that possibly non specific phosphorylation by CaMKII is shown. A lesser known and different CaMKII phosphorylation sequence S/TXD has been described as a target site for the kinase (Yamauchi, 2005). A CaMK phosphorylation site Ser220 in human Freud-1, (Figure 9) that is not conserved in mouse or rat Freud-1, could possibly be involved in phosphorylation by CaMKII/CaMKIV *in vitro*. Serine/threonine to alanine mutations at these sites would allow us to assess their potential contribution to CaMK phosphorylation. *In vitro* kinase experiments assessing CaMKIV mediated Freud-1 phosphorylation could have provided additional evidence for the importance of these sites, although CaMKIV mediated phosphorylation of Freud-1 was examined in cells.

Although overexpression of CaMKII and CaMKIV in both cell lines was detected by antibodies, endogenous CaMK expression was not detected in SK-N-SH. Using a substantially greater concentration of cell lysate would be one way to detect endogenous CaMK. The combined evidence that inducing calcium influx and intracellular release, by high K^+ depolarization and treatment with ionophores, results in derepression of 5-HT1A DRE mediated reporter activity implicates the involvement of calcium regulated kinase(s) in dysregulation of 5-HT1A receptor expression. So the presence of endogenous CaMKs would be essential for mediating the observed effects. Assessment of constitutively active CaMKII (T286D) kinase activity demonstrated differing results using two different experimental methods. The kinase activity assay may not have been the most sensitive method of assessment since a low concentration of non-purified kinase was tested. Although a difference in kinase activity was observed between non transfected cells and T286D overexpressed cells, the difference was not statistically significant. CREB phosphorylation did confirm T286D kinase activity however examination of specific Ser142 phosphorylation, the predominant target of CaMKII, (Wu and McMurray, 2001) would better support exclusive CaMKII mediated phosphorylation of CREB. Despite the inability to detect CaMKII-mediated phosphorylation of Freud-1 in cells, serine and threonine Freud-1 phosphorylation was induced by CaMKIV. A Ser644 and Thr780 specific antibody would have been able to differentiate phosphorylation of specific sites.

Other anti-serine antibodies were also tested but demonstrated nonspecific bands at the migration level of Freud-1. In summary, these results indicate that although CaMKII phosphorylated Freud-1 at Ser644 *in vitro*, its activity to phosphorylate Freud-1 *in vivo* was less than that of CaMKIV, which phosphorylated both serine and threonine sites of Freud-1.

To determine that effect of CaMKII phosphorylation on Freud-1/DNA interaction, EMSA studies were done and showed that CaMKII reduced Freud-1 binding. However, heating Freud-1 protein at 30°C in this assay substantially reduced binding to the 5-HT1A DRE (Figure 17) even in the absence of CaMK buffer (data not shown). However CaMKII was able to phosphorylated purified Freud-1 *in vitro* at 25°C to the same extent as suggested conditions of 30°C (closest to optimal physiological temperature) (data not shown). Performing the phosphorylation reaction at room temperature prior to the binding experiments would have shown an enhanced difference in binding activity. An interesting additional experiment would be to examine the effects of CaMKIV mediated phosphorylation of Freud-1 on its ability to bind to the 5-HT1A DRE. *In vitro* binding of the Freud-1 mutants were tested in two independent experiments using different stocks of purified protein with similar results (Figure 18). The next step would be to test binding of these mutants when phosphorylated by CaMKII or CaMKIV *in vitro*. Since Ser644 is the major site of CaMKII phosphorylation we would expect reduced Freud-1 T780A/DRE interaction, a slight reduction in S644A/DRE interaction and no effect on S644A/T780A binding activity.

Using three different luciferase construct systems provided complementary evidence supporting the primary role of CaMKIV in attenuating Freud-1 mediated repression. The more physiologically relevant system, involving the human 5-HT1A promoter-luciferase construct, assesses not only transcriptional repression by Freud-1 but also its ability to bind to the promoter. Whereas the Gal4-DBD/Gal4 reporter system only examines Freud-1 repressor activity independent of DNA binding since binding occurs due to the yeast Gal binding domains. Using both systems the ability of Freud-1 to repress transcription was inhibited by CaMKIV phosphorylation indicating that phosphorylation not only inhibits Freud-1 binding but also blocks its repressor activity. By contrast, reporter assays using constitutively active CaMKII, Freud-1 and the 5-HT1A promoter showed no effect on Freud-1 repressor activity (data not shown). Even though CaMKII phosphorylation *in vitro* reduced Freud-1 binding to the 5-HT1A DRE, the function of Freud-1 was not altered by CaMKII in cells, consistent with the lack of effect of CaMKII on Freud-1 phosphorylation *in vivo*. The CaMKII-induced CREB phosphorylation provides an important positive control, indicating that CaMKII was active. These findings indicate the role of CaMKIV in regulating Freud-1-mediated repression of 5-HT1A transcription. Consistent with its role in transcription, CaMKIV is located predominantly in the nucleus and is known to influence transcriptional activation related to long term memory (Ou et al., 2003; Impey et al., 2002; Kasahara et al., 2001; Soderling, 2000). The nuclear localization of CaMKIV versus the cytosolic localization of CaMKII could explain why CaMKIV is the likely contributor to Freud-1 phosphorylation, due to its endogenous location *in vivo*.

Biological Roles for CaMK Dependent Regulation of Freud-1

Our findings indicate that human Freud-1 represents a repressor that is inactivated by CaMKIV mediated phosphorylation. Previously, it has been shown that the short isoform of mouse Freud-1 is inhibited *in vitro* by calcium and ATP (Ou et al., 2003) and that calcium is involved in Freud-1 regulation. A more physiologically relevant system of study would be to examine human presynaptic serotonergic neurons instead of the cell model used in this study. In this system we would expect to see calcium-dependent induction of 5-HT1A receptor expression. In raphe neurons, glutamate activates NMDA receptors and increases calcium dependent serotonin release (Celada et al., 2001; Lee et al., 2003). The calcium/CaMKIV mediated inactivation of Freud-1 would provide a mechanism to increase 5-HT1A receptors as a negative feedback regulator of serotonin. On the other hand, the 5-HT1A receptor in raphe cells inhibits their firing and block N-type calcium channels (Penington et al., 1993), with the net result of reducing intracellular calcium concentrations, and possibly leading to an activation of Freud-1 repression to reduce 5-HT1A gene transcription. Thus, a CaMKIV-Freud-1 dependent mechanism could mediate the action of 5-HT increase (e.g. following treatment with SSRI) to down-regulate 5-HT1A receptor expression, thus, increasing serotonergic activity. Oppositely, inactivation of Freud-1 results in an upregulation in 5-HT1A receptor gene expression. This could also contribute to altered neuronal development and cognitive abilities associated with mental retardation in which Freud-1 is mutated (Rogaeva and Albert, 2007).

An increase in calcium influx or release of calcium stores activates CaMKII and CaMKIV. While both CaMKII and CaMKIV phosphorylate the two sites, it is only known that CaMKII inhibits Freud-1 binding to the 5-HT1A DRE. If CaMKIV regulates

Freud-1 by the same mechanism as CaMKII then we can infer that CaMKIV affects Freud-1 binding activity. However, CaMKIV inactivates Freud-1 repressor activity even when binding to the DNA is already established using the Gal4-DBD. This suggests that even if CaMKIV does not interfere with Freud-1/DRE binding, CaMKIV inhibits Freud-1 mediated repression of the 5-HT1A receptor gene. Two mechanisms of Freud-1 mediated regulation have been described: 1) Freud-1 regulates 5-HT1A receptor transcription by altering Freud-1 protein levels. High Freud-1 protein levels negatively regulate basal 5-HT1A expression (Ou et al., 2003) and 2) CaMKIV mediated phosphorylation enhances 5-HT1A transcription by inactivation of Freud-1. Either Freud-1 protein levels or calcium mediated signaling determines Freud-1 activity to regulate 5-HT1A expression along with other 5-HT1A regulators (Ou et al., 2003).

Future Studies

Additional experiments could be done to strengthen this particular study. Immunoprecipitation of endogenous Freud-1 from cells treated with KCl and ionomycin could be assessed for Ser644 and Thr780 specific phosphorylation using specific antibodies. Cells could also be treated with a CaMK inhibitor, KN-93 to demonstrate that endogenous CaMK is involved in Freud-1 phosphorylation. Immunoprecipitated endogenous Freud-1 in the presence of transiently active CaMK could also be assessed for phosphorylation detection. Kinase deficient CaMK could also be used as negative controls for pull down or immunoprecipitation assays. EMSA experiments using SK-N-SH nuclear extracts, transiently transfected with active CaMK, would determine whether endogenous Freud-1 phosphorylated by CaMKII or CaMKIV alters binding activity.

Addition of full length Freud-1 or CC2D1A antibody to the binding reaction will confirm Freud-1 specific interaction with the 5-HT1A DRE if the complex is supershifted. Although SK-N-SH cells represent a human neuronal 5-HT1A expressing cell model, a human presynaptic 5-HT1A expressing cell line would be the closest representative of physiological relevance. However such a cell line is not known. To examine CaMK mediated effects on endogenous Freud-1 and 5-HT1A DRE interaction, chromatin immunoprecipitation assays could be performed using a human cell line treated with calcium inducers or transfected with active CaMK. To assess CaMK mediated phosphorylation on endogenous Freud-1 activity QPCR analysis could be carried out. 5-HT1A expressing cells can be treated with active CaMK and quantified for endogenous 5-HT1A mRNA expression levels as an index of 5-HT1A receptor expression. Measuring 5-HT1A protein levels could be also done to confirm these changes, but may produce more variable results due to post transcriptional modifications.

To expand on the current study, the involvement of other kinases in phosphorylation of Freud-1 could be examined. The role of CaMKI, belonging to the same CaMK family as CaMKII and CaMKIV, could be assessed for Freud-1 phosphorylation since the CaMKs have broadly overlapping substrate specificities and the same phosphorylation consensus sequence (RXXS/T) (Means, 2000). Calcium and calmodulin are required for CaMKI activation, however, it is not autophosphorylated and does not have autonomous activity (Ramirez et al., 1997). CaMKI is present in all mammalians cells, however, it has been shown to be expressed in the cytosol and lacks a nuclear localization sequence (NLS) (Means, 2000). The role of CaMKK in regulating CaMKIV by phosphorylation could be addressed. Transfecting cells with additional

CaMKK expression plasmid could result in an increase in CaMKIV activity and enhanced effects of Freud-1 activity. Preliminary results focused on casein kinase II mediated phosphorylation of Freud-1 have shown that casein kinase II phosphorylates Freud-1 *in vitro* (unpublished results). The role of PKA and PKC could also be examined. However, it would be important to first demonstrate that these kinase affect Freud-1 function.

Although CaMKII does not alter Freud-1 function, the kinase along with the other CaMKs can play an indirect role in regulation of 5-HT1A receptor expression. Preliminary analysis of Freud-1 associating proteins or co-repressors have directed our focus on BAF 155 and BAF 170 associated with the mammalian SWI/SNF complex (Unpublished data; Rogaeva). Large enzymatic complexes such as SWI/SNF are involved in mechanisms that alter histone-DNA interaction to facilitate transcription factor binding (Kadam et al., 2000). Preliminary studies have shown a calcium dependent Freud-1/BAF interaction by immunoprecipitation assays (Unpublished data; Rogaeva). CaMK could phosphorylate a Freud-1 associating protein to alter Freud-1 activity. For example, CaMKIV has been shown to directly phosphorylate the CREB binding protein (CBP) to mediate CPB/CREB dependent transcriptional activation (Impey et al., 2002).

Since Freud-1 is also a regulator of the dopamine D2 receptor (Rogaeva et al., 2007b), CaMKIV mediated phosphorylation of Freud-1 can potentially modify D2DR expression. As an activator of NF-κB activity, phosphorylated Freud-1 can alter NF-κB mediated regulation of 5-HT1A gene transcription; inhibition of Freud-1 mediated NF-κB induction can mediate repression of the 5-HT1A receptor.

CONCLUSION

In summary human Freud-1 represents a novel example of a CaMK regulated transcription factor of the 5-HT1A receptor gene. CaMKIV is an important calcium mediated regulator of Freud-1 repressor. Although CaMKII phosphorylated Freud-1 *in vitro*, only CaMKIV phosphorylated human Freud-1 in cells. CaMKII phosphorylation *in vitro* reduced Freud-1 binding ability, specifically to the 5-HT1A DRE. Two CaMK phosphorylation sites were identified that contribute to Freud-1 regulation by CaMKIV. Consistent with its localization in the nucleus, CaMKIV inhibits Freud-1 repressor activity at the 5-HT1A receptor promoter. Together, these results indicate that Ca-CaMKIV induced Freud-1 phosphorylation provides an important mechanism to regulation 5-HT1A receptor transcription. Future studies to address that role of Ca-dependent mechanisms in regulation of 5-HT1A receptor expression *in vivo* will advance our knowledge of 5-HT1A gene transcription, and how this might be implicated in major depression and anxiety.

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APPENDIX

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