

**SPECIFIC MOTIFS RESPONSIBLE FOR
PROTEIN-PROTEIN INTERACTION
BETWEEN CANNABINOID CB1 AND
DOPAMINE D2 RECEPTORS**

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ABSTRACT

Studying protein-protein interactions has been vital for understanding how proteins function within the cell, how biological processes are strictly regulated by these interactions, and what molecular mechanisms underlie cellular functions and diseases. Recent biochemical and biophysical studies have provided evidence supporting that G protein-coupled receptors (GPCRs) can and do interact with one another to form dimers or larger oligomeric complexes, which may determine the structure and function of GPCRs, including receptor trafficking, scaffolding and signaling. This may help to understand the physiological roles of GPCRs and mechanisms underlying certain disease pathologies and to provide an alternative approach for drug intervention.

Cannabinoid CB1 and dopamine D2 receptors are the most common GPCRs in the brain and exert a mutual regulation in brain functions involved in learning, memory and drug addiction. There is structural and functional evidence supporting the idea that CB1 and D2 receptors physically interact with each other in hippocampal and striatal neurons to modulate their functions. Direct evidence supporting a physical interaction between the CB1 and D2 receptors was obtained from cultured HEK293 cells stably coexpressed with both receptors.

This research project was designed to critically test the hypothesis that a specific protein sequence (i.e. motif) in the D2 receptor is responsible for *in vitro* protein-protein interactions between the CB1 and D2 receptors. To reach this goal, fusion proteins

containing various domains and motifs of the CB1 and D2 receptors were prepared and then used first to determine the domains of the CB1 and D2 receptors responsible for *in vitro* protein-protein interactions between CB1 and D2 receptors, and then to identify the specific motifs in the D2 receptor responsible for *in vitro* CB1 coupling with the D2 receptors. The major method used in this study is *in vitro* pull-down assay, which uses a purified and tagged bait protein to generate a specific affinity support that is able to bind and purify a prey protein from a lysate sample. The present study provides the first evidence that CB1 intracellular C-terminal (CB1-CT) and D2 intracellular loop 3 (D2-IL3) can directly interact with each other, and that the specific motifs “D2-IL3(IV1)” and “D2-IL3(IV3)” in the D2 receptor are likely responsible for their *in vitro* coupling with the CB1 receptors.

The results of the present study are invaluable for future research exploring *in vivo* protein-protein interaction between the CB1 and D2 receptors in the rat striatum by co-immunoprecipitation. Specifically, future studies will determine whether the identified specific motifs “D2-IL3(IV1)” and “D2-IL3(IV3)” in the D2 receptor are indeed critical for their *in vivo* coupling with the CB1 receptors.

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LIST OF ABBREVIATIONS

AB	Acrylamide/Bisacrylamide
ABP	Actin-binding Protein
APS	Ammonium Persulfate
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CB1	Cannabinoid type I receptor
CD	Circular Dichroism
cDNA	complementary DNA
CNS	Central Nervous System
Co-IP	Co-immunoprecipitation
CT	C-terminal
D2	Dopamine receptor 2
DA	Dopamine
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescent Luminol
E.coli	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic Acid

EL	Extracellular Loop
Erk	Extracellular response kinases
EtBr	Ethidium Bromide
FP	Fluorescence Polarization
FRET	Fluorescence Resonance Energy Transfer
GABA	γ -aminobutyric acid
GFP	Green Fluorescent Protein
GPCRs	G-protein-coupled receptors
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase
HEK	Human Embryonic Kidney
HRP	Horseradish Peroxidase
5-HT	Serotonin
IB	Immunoblotting
IL	Intracellular Loop
IPTG	Isopropylthio- β -galactoside
ITC	Isothermal Titration Calorimetry
LB	Luria-Bertani
MAP	Mitogen-Activated Protein
MS	Mass Spectrometry
NAC	Nucleus Accumbens

NDRG4	N-myc Downstream-regulated Gene-4
Ni-NTA	Nickel-Nitrilotriacetic Acid
NMR	Nuclear Magnetic Resonance
NT	N-terminal
PAGE	PolyAcrylamide Gel Electrophoresis
PCA	Protein fragment Complementation Assays
PBI	Plant Biotechnology Institute
PBS	Phosphate Buffered Saline
PC12	Phaeochromocytoma Cell Line
PCR	Polymerase Chain Reaction
P-D	Pull-down
PVDF	Polyvinylidene Fluoride
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SPR	Surface Plasmon Resonance
SSTR	Somatostatin Receptor
TBST	Tris Buffered Saline with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
Δ 9-THC	Δ 9-tetrahydrocannabinol
VTA	Ventral Tegmental Area
X-GAL	5-bromo-4-chloro-3-indolyl- β -galactoside

1 INTRODUCTION

1.1 Protein-Protein Interaction

Protein-protein interactions, specific interactions between two or more proteins, play diverse central roles in numerous biological processes in cells. Studying protein-protein interactions has been vital for understanding how proteins function within the cell, how biological processes are strictly regulated by these interactions, and what molecular mechanisms underlie cellular functions and diseases. Protein-protein interaction is one of the main research fields in current functional proteomics. The availability of entire genome sequences of many organisms, proteomics-based protein profiling studies, and various high-throughput analysis techniques catalyze resurgence in protein-protein interaction exploration.

1.1.1 Types of Protein-Protein Interactions

The structural and functional diversity of protein-protein interactions is primarily based on the composition, affinity, and whether the association is transient or stable.

Homo- and hetero-oligomeric interactions Protein-protein interaction can occur between identical or different protein units (i.e. homo- or hetero-oligomers). Oligomers of identical or homologous protein units can be organized in an isologous or a heterologous way with structural symmetry (Goodsell and Olson, 2000). An isologous association involves the same surface on both monomers related by a 2-fold axes of symmetry and can

further oligomerize using a different interface (e.g. form a dimer of dimers with three 2-fold axes of symmetry) (Nooren and Thornton, 2003b). In contrast to an isologous association, heterologous assemblies use different interfaces that, without a closed (cyclic) symmetry, can lead to infinite aggregation (Nooren and Thornton, 2003b).

Obligate and non-obligate interactions Two different types of protein-protein interactions can be defined on the basis of whether the interaction is obligate or non-obligate. In an obligate protein-protein interaction, the involved protomers are often expressed simultaneously and co-localized upon synthesis, but they are not identified as stable structures on their own *in vivo*. It has been shown that such obligate complexes, which are generally also functionally obligate, are true for homo-oligomers but may occur in hetero-oligomeric interactions. Non-obligate interactions of protomers that exist independently are involved in many of the hetero-oligomeric complexes, such as intracellular signaling complexes and antibody-antigen, receptor-ligand and enzyme-inhibitor complexes, most of which perform a regulatory role as their biological function. However, some homo-oligomers can also assemble to form non-obligate interaction structures (Jones and Thornton, 1996; Nooren and Thornton, 2003b).

Transient and stable interactions Protein-protein interactions can be characterized as transient or stable interaction based on the lifetime of the interaction complex. Stable interaction is usually a permanent interaction that exists as multi-subunit complexes, whereas transient interaction, as the name implies, is on/off or temporary *in vivo* and typically requires a set of conditions to trigger the interaction. For example, hemoglobin

and core RNA polymerase are two stable multi-subunit complex interactions, whereas the interaction between $G\alpha$ and $G\beta\gamma$ subunits of G protein is transient. Both transient and stable interactions can be either weak or strong. Transient interactions are expected to be involved in the majority of cellular processes including protein modification, transport, folding, signaling, cell cycling, etc. Structurally or functionally obligate protein-protein interactions are generally stable, whereas non-obligate protein-protein interactions may be stable or transient (Nooren and Thornton, 2003a; 2003b).

Many protein-protein interactions do not belong to distinct types. Rather, a continuum of protein-protein interaction can exist between obligate and non-obligate interactions. Furthermore, the stability of all the protein-protein interaction complexes extremely depends on the physiological environments, so that an interaction may be mainly transient *in vivo* but becomes stable under certain cellular conditions (Jones and Thornton, 1996; Nooren and Thornton, 2003b).

1.1.2 Effects of Protein-Protein Interactions

A single protein, which generally resides in a crowded environment with many potential binding partners, can interact with diverse partners under different cellular conditions to form dynamic interaction networks, resulting in different biological outcomes. The measurable effects of protein-protein interactions can be demonstrated in several different ways.

They serve a regulatory role Either an upstream or a downstream action in intracellular signaling can be operated by protein-protein interactions (Nooren and Thornton, 2003a).

They create new binding sites Changing fragment organization related to the physiological domain swapping equilibrium or moving a substrate between or among subunits in protein-protein interactions may result ultimately in an intended end product to create some new binding sites for further interactions (Nooren and Thornton, 2003a; 2003b).

They alter kinetic properties of enzymes This may be the consequence of subtle changes of protein-protein interactions at the level of an allosteric effect or substrate binding (Nooren and Thornton, 2003a).

They activate or inactivate a protein Interactions with different binding partners can lead to a change in affinity and specificity of a protein for its substrate, which may activate or inactivate a protein and even present a new function that neither protein can exhibit alone (Nooren and Thornton, 2003a).

1.1.3 Control of Protein-Protein Interactions

Interactions between proteins can be controlled by the protomer's localization, altering local concentration and binding affinity, which may be determined by the expression, secretion, post-translational modification, translocation and stability of protein components,

ligands, and the physicochemical environment. The most important ways for the control of protein-protein interactions involve the following mechanisms.

Encounter An encounter of the interacting surfaces is necessary for the association of two or more proteins, which are required to be co-localized in time and space. Such encounters may occur between protein components, co-expressing or co-localizing within a compartment or residing in different compartments. Translocation by directed diffusion or transport is essential for the encounters of these proteins from different locations.

Local concentration Regulation mechanisms influencing the effective local concentration of proteins include gene-expression or secretion levels, temporary storage, protein degradation, diffusion or transport, and the local molecular environment. The local concentration can be increased through the localization by adjacent domains in multidomain proteins and the anchoring of proteins in a membrane (e.g. transmembrane protein oligomerization) or other structural complex (Nooren and Thornton, 2003b).

Post-translational modifications Post-translational modifications of proteins through enzymatic activity, such as phosphorylation, palmitoylation and glycosylation, can prevent or induce interactions with other proteins. For example, proteins containing the bromo domain can only bind with acetylated but not unmodified histones (Jones and Thornton, 1996).

Ligands Protein-protein interactions can be regulated by small molecules or other proteins, which are required for interactions to occur or disrupt interactions. For example, GTP/GDP exchange controls the $G\alpha$ - $G\beta\gamma$ subunit assembly of the hetero-trimeric G protein:

binding of GTP to G proteins motivates their subunits' dissociation while binding of GDP promotes their subunits' association.

Local physicochemical environment The mutual binding affinity of components of a protein-protein interaction complex can be affected by the alteration in physiological conditions, including the concentration of ions, chemicals or proteins, as well as the changes in PH and temperature.

1.1.4 Techniques for Studying Protein-Protein Interactions

Discovery and verification of biologically relevant protein-protein interactions are the primary steps for understanding how, where and under what conditions the proteins can interact with each other *in vivo* and the functional implications of these interactions. To address the vast challenge of mapping protein-protein interactions, a variety of methodologies to expedite research in this area have recently been developed in the fields of molecular biology, biochemistry, proteomics and bioinformatics.

1.1.4.1 Molecular Biology and Biochemistry Based Methods

Traditionally, protein-protein interactions have been studied by genetic, biophysical and biochemical techniques, such as yeast two-hybrid system, pull-down assay, co-immunoprecipitation (co-IP), cross-linking reagents, blot overlay or far western blot, fluorescence gel retardation assay (Park and Raines, 2004), gel-filtration chromatography (Wilton et al., 2004) and ubiquitin-based split-protein sensor (Droit et al., 2005).

Yeast two-hybrid system This genetic method takes advantage of the eukaryotic transcription process to make predictions about protein-protein interactions. This method is based on the fact that an interacting protein pair will be able to bring together the DNA binding domain and activation domain of a transcription factor *in vivo* to create a functional activator of transcription. The interaction can be identified by the expression of linked reporter genes, which may be *lacZ* gene for color selection and auxotrophic *LEU2*, *HIS3* and *ADE2* genes for growth selection (Cho et al., 2004).

Co-immunoprecipitation This immunoprecipitation experiment is designed to purify a bait protein antigen together with its interacting partners employing a specific antibody against the bait protein (Bartlett et al., 2005; Liu et al., 2000).

Pull-down assay This affinity chromatography method uses a purified and tagged or labeled bait protein to generate a specific affinity support, which will be able to bind and purify a prey protein from a lysate sample or other protein-containing mixtures (Lee et al., 2002; Zou et al., 2005).

Blot overlay or far western blot This technique includes fractionating proteins on SDS-PAGE, blotting proteins to PVDF or nitrocellulose membrane, and then detecting with a probe of interest, which is typically a bait protein properly labeled with radioisotope, biotin or chemiluminescence. When the detection is simply visualized with a specific antibody, this technique is often referred to as “Far Western blot” (Hall, 2004; Liu et al., 2000).

Cross-linking reagents Nearest neighbors, who are suspected to interact with each other *in vivo* or *in vitro*, can be trapped in their complexes by chemical cross-linking reagents for further study (Grabarek and Gergely, 1990; Sinz, 2003).

1.1.4.2 Mass Spectrometry (MS) Based Methods

This kind of technique is applied in concert with affinity-based methods, such as Co-IP and pull-down assays, to isolate interacting complexes and identify the component proteins employing standard mass spectral approaches. Briefly, MS-based methods generally involve the selective purification and enrichment of a bait protein and its binding partners from lysate samples, the digestion of isolated proteins into peptides using a protease such as trypsin, the analysis of peptide mixtures by mass spectrometry and the identification of interacting proteins by mass searching of bioinformatics databases (Figeys et al., 2001).

1.1.4.3 Protein Microarrays

The production of functional protein microarrays promises an innovative assay platform for the screening and identification of specific protein-protein interactions from complex mixtures. Most of the current protein chips are carried out through the bait proteins immobilized on a surface to capture the target proteins in the sample solution. Due to their flexibility and multiplexing capacity, these highly parallel assays are not biased towards abundant proteins and the experimental conditions can be well controlled. For

example, the stringency of the binding activities can be adjusted by introducing different cofactors or inhibitors in the binding assays (Korf and Wiemann, 2005).

1.1.4.4 Bioinformatics Methods

The development of experimental techniques for identifying protein–protein interactions has created the dilemma of how to effectively utilize the extensive amount of data gathered by these large-scale studies. Thus, as complementary ways for the high throughput experimental techniques, systematic bioinformatics methods have been developed for the study of protein-protein interactions, which include well known and novel approaches: data mining, annotation by sequence similarity, phylogenetic profiling, metabolic pathway mapping, gene neighbor and domain name fusion analyses (Droit et al., 2005). These methods integrate the information from different approaches to build the protein-protein interaction network that can predict protein function and define how macromolecules interact within complex networks.

In addition, a variety of cutting-edge technologies are utilized in the techniques described above for studying protein-protein interactions, which include nuclear magnetic resonance (NMR), fluorescence resonance energy transfer (FRET), surface plasmon resonance (SPR), circular dichroism (CD), isothermal titration calorimetry (ITC), fluorescence polarization (FP), protein fragment complementation assays (PCA), various two-hybrid systems and computational analysis (Greenfield, 2004; Northup, 2004; Obenauer and Yaffe, 2004; Park and Raines, 2004; Velazquez-Campoy et al., 2004). All the

readily reproducible methods demonstrate how to explore protein interaction partners, monitor protein-protein interactions as they occur in living cells, identify interaction interfaces, and qualitatively or quantitatively measure protein-protein interactions *in vivo* or *in vitro*.

1.1.5 Protein-Protein Interactions of GPCRs

All G protein-coupled receptors (GPCRs) share a common general structure constituted by seven transmembrane (TM) helices linked by three extracellular and three intracellular loops, an extracellular amino terminal, and an intracellular carboxyl tail (Ji et al., 1998). This topology of GPCRs allows several potential faces for their protein-protein interactions. Because the extracellular loops are short, the N-terminal, which can be very extensive, is likely to dominate the extracellular interactions of GPCRs. With respect to the intracellular regions, both the C-terminal and third intracellular loop, which can be of considerable size, are the domains on which attention has been focused for the intracellular interactions of most GPCRs. Examples include the C-terminal and third intracellular loop of β -adrenoceptors, the C-terminal of 5-HT₂ receptors and the third intracellular loop of the dopamine D2 receptor (Milligan and White, 2001).

Most GPCRs contain sequence motifs that are able to direct protein-protein interactions and, thus, have the theoretical capacity to selectively interact with a host of other proteins. In fact, recent studies have identified several interaction domains on both extracellular and intracellular faces of GPCRs, which are responsible for the

protein-protein interactions of GPCRs, and undoubtedly, more domains wait to be defined (Lee et al., 2002; Liu et al., 2000; Scarselli et al., 2001; Zou et al., 2005). GPCR-protein assemblies, including interactions between receptor and receptor, receptor and receptor-interacting proteins, or receptor and G-protein, should be viewed as dynamic complexes ranging from transient interactions to more stable interactions, all of which vary with cell background and contribute to the finely tuned processes of downstream cell signaling (Milligan and White, 2001). Recently, there has been great interest in protein-protein interactions between individual GPCRs that induce the formation of homo- or hetero-dimer/oligomers.

More recent biochemical and biophysical studies have provided several lines of evidence that GPCRs can and do interact with one another to form dimers or larger oligomeric complexes, which involve both homo-dimer/oligomers containing two or multiple copies of the same gene product and hetero-dimer/oligomers comprising more than one receptor. It is now widely accepted that GPCRs homo-dimerize/oligomerize for ligand binding and signal transduction to exert their functions. Data support that most members of the GPCR superfamily exist or can exist as homo-dimers/oligomers, which are revealed in dopamine, opioid, somatostatin and thyrotropin-releasing hormone receptors, β_2 -adrenergic receptor, muscarinic m_3 acetylcholine receptor, CB1 cannabinoid receptor, etc (Lee et al., 2003; Milligan, 2001; Wager-Miller et al., 2002). In addition, a range of hetero-dimer/oligomeric interactions have been detected, which include not only those occurring between closely related GPCR subtypes, such as γ -aminobutyric acid

(GABA)_BR1 and GABA_BR2 receptors, serotonin 5-HT_{1D} and 5-HT_{1B} receptors, somatostatin SSTR1 and SSTR5 receptors, dopamine D2 and D3 receptors, κ and δ opioid receptors, as well as μ and δ opioid receptors, but also more distantly related GPCRs, such as somatostatin SSTR5 and dopamine D2 receptors, dopamine D1 and adenosine A1 receptors, α_2 adrenergic and M₃ muscarinic receptors, as well as angiotensin AT1 and bradykinin B2 receptors (Jordan and Devi, 1999; Lee et al., 2003; Marshall, 2001; Milligan, 2001; Park et al., 2004; Scarselli et al., 2001; Vazquez-Prado et al., 2002). Two models to describe the dimerization of GPCRs have been proposed: the domain-swapped dimer model whereby transmembrane domains 1-5 of one protein couple with transmembrane domains 6 and 7 from the other protein to form the binding sites in the dimer, and the contact dimer model, which is formed by lateral packing of the individual polypeptides and may be the most likely structure for heterodimer formation (Marshall, 2001; Milligan, 2001).

Although the full functional and physiological significance of many interactions between GPCRs is not completely understood, current information indicates that these interactions might determine the structure and function of GPCRs, which have implications for receptor trafficking, scaffolding and signaling. This may help to understand the physiological roles of these receptors, and may underlie certain disease pathologies and provide an alternative approach for drug intervention.

1.2 Cannabinoid CB1 Receptor

Cannabinoids (marijuana, cannabis) have a long history of consumption for recreational and medical reasons. The effects of cannabinoids are mediated by two subtypes of G protein-coupled cannabinoid receptors: CB1 and CB2 receptors. The present study focuses on CB1 receptors, which are found primarily in the CNS, with the highest density in the hippocampus, cerebellum and basal ganglia. This distribution pattern correlates well with the effects of cannabinoids on memory, perception and the control of movement (Ameri, 1999). CB1 receptors bound by cannabinoids can modulate inhibition of adenylate cyclase activity, stimulation of inwardly rectifying potassium channels, inhibition of N- and P/Q-type voltage-dependent calcium channels, and activation of mitogen-activated protein (MAP) kinase pathway (Howlett, 1998).

1.2.1 Structure and Property of CB1 Receptor

The cDNA sequence encoding the 473-amino-acid protein product of the CB1 receptor was isolated from a rat cerebral cortex cDNA library (Matsuda et al., 1990) and a human brain stem cDNA library (Gerard et al., 1991). The gene locus for the human CB1 receptor has been genetically mapped to chromosome 6q14-q15 (Hoehe et al., 1991).

The CB1 receptor exhibits the basic structural features predicted for a GPCR (Figure 1.1) (Bramblett *et al.*, 1995). The apparent molecular weight of the CB1 receptor is 64 kDa when detected with an antipeptide antibody in immuno-blots. Reducing the apparent

weight of this receptor to 59 and 53 kDa by the treatment with endoglycosidases F and/or H indicates that it is glycosylated (Song and Howlett, 1995).

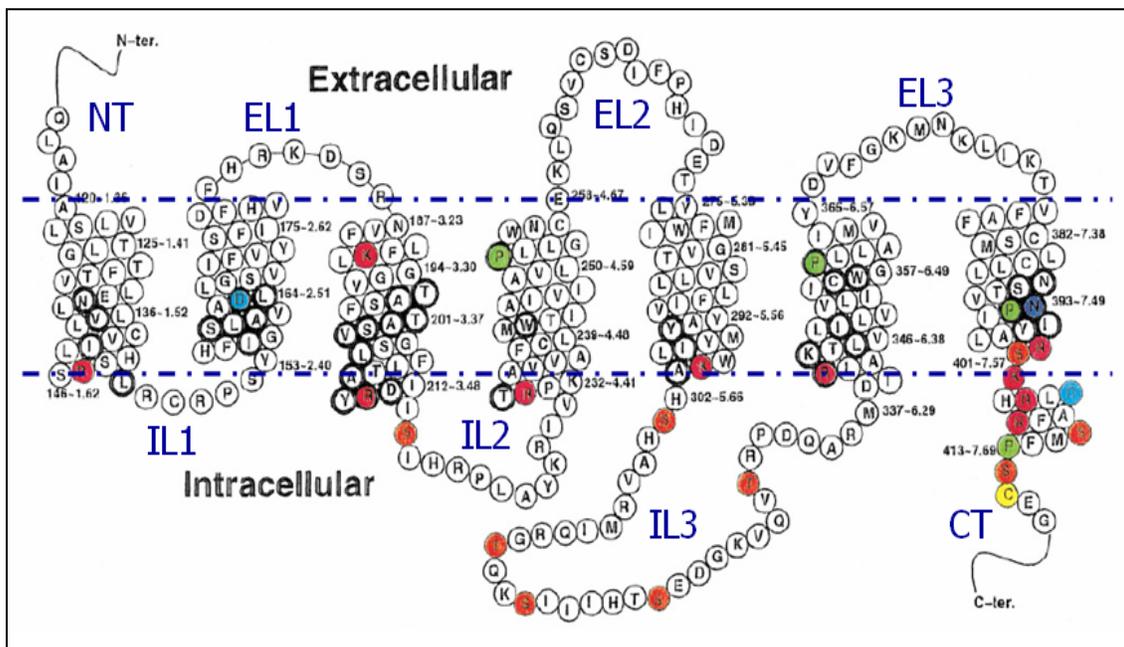


Figure 1.1: The cannabinoid CB1 receptor model of Reggio comprising seven hydrophobic transmembrane domain regions that extend through the plasma membrane, three extracellular loops (EL1/EL2/EL3), three intracellular loops (IL1/IL2/IL3), an extracellular N-terminal (NT) and an intracellular C-terminal (CT) [Modified from Howlett, 1998].

1.2.2 Signal Transduction Mediated by CB1 Receptor

Cannabinoid CB1 receptors couple to multiple signal transduction pathways that inhibit adenylate cyclase activity, modulate ion channels, and activate MAP kinase pathway (Figure 1.2).

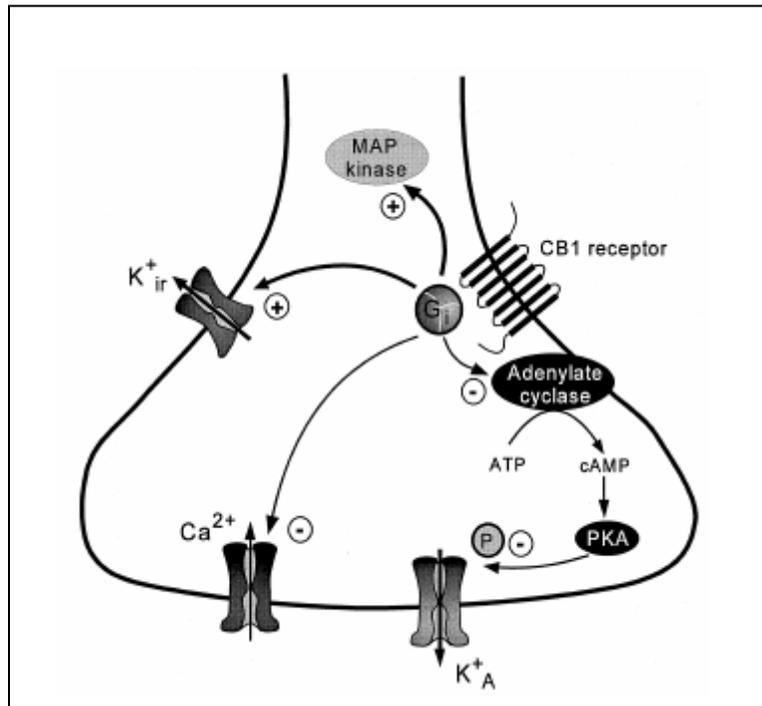


Figure 1.2: Signal transduction mechanisms stimulated by the CB1 receptor in a presynaptic nerve terminal. Activation of the CB1 receptor stimulates its coupling to G protein, which transduces the stimulation of MAP kinase, and the inhibition of adenylate cyclase, thereby attenuating the production of cAMP. Moreover, the G protein directly couples CB1 receptors to N- and Q/P-type voltage-dependent calcium channels for inhibition, and to inwardly rectifying potassium channels (K^+_{ir}) for stimulation. The G protein-dependent activation of the A-type potassium current (K^+_{A}) is modulated via the inhibition of adenylate cyclase [Taken from Ameri, 1999].

1.2.2.1 Inhibition of Adenylate Cyclase

Three lines of evidence suggest that cannabinoids act on the CB1 receptors to inhibit adenylate cyclase in a reversible, dose-dependent, and stereoselective manner, resulting in the attenuation of cAMP production (Bidaut-Russell et al., 1990; Howlett et al., 1986). Firstly, cellular selectivity of an inhibition of adenylate cyclase, which is not observed in either the soluble adenylate cyclase from rat sperm or in non-membrane-bound adenylate cyclase preparations from different cell lines, provides evidence for the existence of specific receptors for cannabimimetic compounds (Ameri, 1999). Secondly, brain regions in which cannabinoids are most effective inhibitors of adenylate cyclase are hippocampus, basal ganglia and cerebellum displaying the highest density of CB1 receptors (Bidaut-Russell et al., 1990). Finally, it has been shown that the selective CB1 receptor antagonist SR141716A can antagonize the inhibition of cAMP production induced by cannabinoids (Rinaldi-Carmona et al., 1994).

The involvement of an inhibitory G protein ($G\alpha_{i/o}$) through which CB1 receptors are negatively coupled to adenylate cyclase has been clearly demonstrated, because cannabinoid-induced inhibition of adenylate cyclase can be blocked by pertussis toxin in mammalian brain and in cultured neuronal cells (Bidaut-Russell et al., 1990; Howlett et al., 1986; Pacheco et al., 1993). Moreover, cannabinoid regulation of adenylate cyclase is sensitive to divalent cations, such as Mg^{2+} or Mn^{2+} , and guanine nucleotides in a manner characteristic for other $G\alpha_{i/o}$ protein-coupled receptors (Ameri, 1999).

The CB1 receptor is also known to share G proteins with other GPCRs, such as opioid receptor, dopamine D2 receptor and GABA_B receptor, as demonstrated by additivity experiments in cultured cerebellar granule cells (Childers et al., 1992; Pacheco et al., 1993) and striatal slices (Bidaut-Russell and Howlett, 1991) (Figure 1.3).

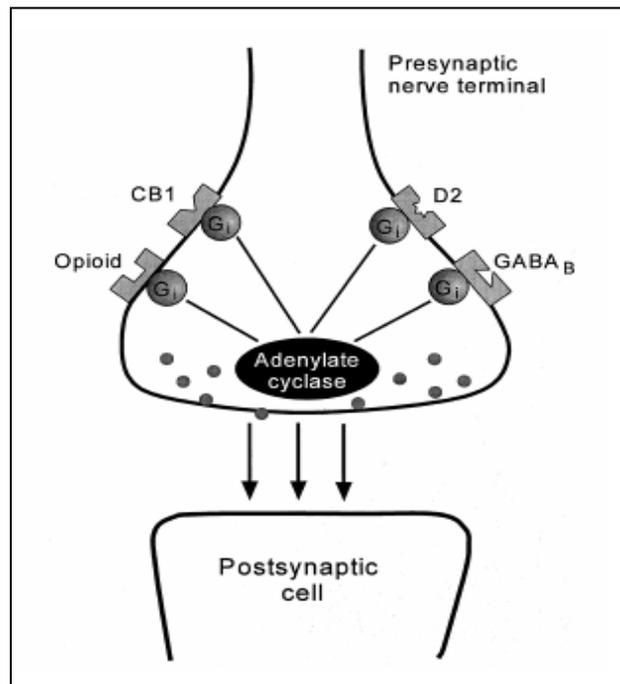


Figure 1.3: Schematic diagram illustrating the possible interaction of the cannabinoid CB1 receptor with other G_{α_{i/o}}-coupled receptors, such as opioid receptor, dopamine D2 receptor and GABA_B receptor [Taken from Ameri, 1999].

1.2.2.2 Modulation of Ion Channels

$G\alpha_{i/o}$ proteins not only mediate an attenuation of cAMP production by coupling various receptors to adenylate cyclase, but also modulate receptor activity by coupling to ion channels. The CB1 receptor has been reported to modulate the activity of N- and P/Q-type voltage-dependent calcium channels and to enhance the activation of the voltage-dependent A-type potassium channel and inwardly rectifying potassium channel (Ameri, 1999) (Figure 1.2).

It has been reported that cannabinoids exert an inhibitory effect on voltage-activated inward calcium currents executed by the N-type and P/Q-type calcium channels in primary neurons (Pan et al., 1996) and diverse neuroblastoma cell lines expressing CB1 receptors (Caulfield and Brown, 1992; Mackie and Hille, 1992). The inhibition of calcium currents by cannabinoids could be attenuated by the selective N-type calcium channel blocker ω -conotoxin GVIA and by the selective P/Q-type blocker ω -agatoxin (Caulfield and Brown, 1992; Mackie and Hille, 1992). Furthermore, electrophysiological studies have shown that inhibition of the N-type calcium current by cannabinoids is stereospecific, pertussis toxin-sensitive, and can be inhibited by the selective CB1 receptor antagonist SR141716A, indicating a CB1 receptor-mediated process (Twitchell et al., 1997). Taken together, these results demonstrate the inhibition of N- and P/Q-type voltage-dependent calcium channels mediated by CB1 receptors in neurons.

Cannabinoids have been shown to enhance the inwardly rectifying potassium current in murine tumor cells and in *Xenopus* oocytes expressing CB1 receptors (Henry and

Chavkin, 1995; Mackie et al., 1995). It has been reported that the CB1 receptor can be phosphorylated by protein kinase C, leading to a reduced ability of cannabinoids to activate inwardly rectifying potassium currents and to attenuate P/Q-type calcium currents in a cell line transfected with rat CB1 receptors (Garcia et al., 1998). Moreover, Deadwyler et al. (1993) showed that the voltage dependence of the rapidly inactivating potassium A current was significantly altered in a concentration-dependent manner by cannabinoid analogs. Recent evidence indicates that cannabinoid-induced stimulation of this potassium current is cAMP-dependent and results from CB1 receptor-mediated inhibition of adenylate cyclase and subsequent inhibition of protein phosphorylation of the A-type potassium channel protein (Figure 1.2), but not through a direct coupling of $G\alpha_i$ proteins to the A-type potassium channel (Hampson et al., 1995).

1.2.2.3 Activation of MAP Kinase

Stimulation of MAP kinase is the third well-characterized messenger system mediated by the CB1 receptor. Thus far, activation of the MAP kinase pathway induced by the CB1 receptor has only been identified in cell lines, so that it is still uncertain if this plays a significant physiological role in the CNS. It has been demonstrated that cannabinoids are highly potent activators of MAP kinase phosphorylation in stably transfected Chinese hamster ovary cells expressing human CB1 receptors. This effect can be inhibited by the selective antagonist SR141716A, implying an involvement of the CB1 receptor. Furthermore, it has been shown that the signal transduction pathway between CB1 receptor

and MAP kinase involves a pertussis toxin-sensitive G protein (G $\beta\gamma$ subunits), and is independent of the cannabinoid-induced inhibition of cAMP production (Bouaboula et al., 1995b)

1.2.3 Distribution and Function of CB1 Receptor in the CNS

The CB1 receptor is one of the most abundantly expressed neuronal receptors in the CNS and its distribution has been well characterized in rat and human brains using various techniques: receptor binding and autoradiography, reverse-transcription polymerase chain reaction (RT-PCR), northern blot, *in situ* hybridization, and immunohistochemistry (Ameri, 1999; Glass et al., 1997; Herkenham et al., 1991b; Tsou et al., 1998). These studies exhibit a widespread distribution of the CB1 receptor in those brain regions known to be tightly involved in learning and memory, brain reward, antinociception, anticonvulsion, and movement control.

Autoradiographic studies employing a radioactive cannabinoid have shown that the specific, saturable, high affinity CB1 binding sites are not distributed homogeneously in the brain. The highest density has been detected in the basal ganglia (globus pallidus, entopeduncular nucleus, substantia nigra and lateral caudate putamen) and the molecular layer of the cerebellum, which is consistent with the notable effects of cannabinoids on a decrease in spontaneous locomotor activity in rodents. High binding densities have been also demonstrated in the dentate gyrus, pyramidal cell layers of the hippocampus, and the cortex, which may provide a basis to explain an involvement of cannabinoids in the

impairment of cognition and memory, and also the anticonvulsive effects of these compounds. The intermediate levels of binding sites have been found in the nucleus accumbens, which is correlated with modulating brain reward. Small nuclei with high density of binding are also found in other areas of hypothalamus, brain stem and pituitary gland for controlling body temperature, pain and hormone function (Ameri, 1999; Herkenham et al., 1991b).

Immunohistochemical studies on distribution of the CB1 receptor in the adult rat brain have not only shown results consistent with findings of the autoradiographic studies, but also presented intriguing new data about the identification of particular neuronal cell fibers that possess CB1 receptors. Thus, CB1 receptor-like immunoreactive axons, cell bodies and dendrites have been found to be widely distributed in the forebrain and sparsely in the hindbrain and the spinal cord. The intensely stained neurons were dispersed in cortical structures including the olfactory bulb and hippocampal formation, while moderately or lightly stained neurons were detected in the amygdala and caudate-putamen. Beaded immunoreactive fibers were also found in the periaqueductal gray and dorsal horn and lamina X of the spinal cord, which are important regions in ascending pain transmission, in which the CB1 receptor is expected to be involved in the antinociception induced by cannabinoids (Ameri, 1999; Tsou et al., 1998).

1.3 Dopamine D2 Receptor

Dopamine (DA) is the most important catecholamine neurotransmitter in the mammalian brain where it is involved in a variety of functions including locomotor activity, learning and memory, emotion, reward, food intake, and endocrine regulation (Missale et al., 1998). The actions of DA are mediated via GPCRs and the cDNA sequences of five distinct G protein-coupled DA receptor subtypes (D1-D5) have been isolated and characterized. These receptors have been categorized into two groups based on sequence similarity, functional characteristics and pharmacological profiles: D1-like (D1, D5) receptors that activate adenylyl cyclase, leading to an increase in intracellular cAMP levels, and D2-like (D2, D3, D4) receptors that inhibit adenylyl cyclase and modulate calcium and potassium ion channels (Mustard et al., 2005). The D2 receptor has been found mainly in the substantia nigra pars compacta, ventral tegmental area, striatum (which includes the shell and core of the nucleus accumbens and dorsal striatum), olfactory tubercle, and pituitary gland (Bonic and Hopf, 2005; Jackson and Westlind-Danielsson, 1994).

1.3.1 Structure and Variants of dopamine D2 Receptor

The cDNA sequence encoding the D2 receptor was first isolated in 1988 (Bunzow et al., 1988) and subsequently, the existence of splice variants of this receptor was revealed in 1989 (Giros et al., 1989). It seems likely that many of the genes in the GPCR family have arisen from a single primordial gene that lost its intron by a gene-processing event (Missale et al., 1998). The gene encoding the D2 receptor is interrupted by intron, which allows the

generation of receptor variants. Indeed, the D2 receptor has two main variants, named D2(S) (415aa) and D2(L) (444aa), which are generated by alternative splicing of a 87-bp exon between introns 4 and 5 (Giros et al., 1989).

As a member of the GPCR family, the D2 receptor has seven transmembrane domains, three intra- and three extra-cellular loops, an intracellular C-terminal, and an extracellular N-terminal (O'Dowd, 1993; Missale et al., 1998). The D2 receptor has a long intracellular loop 3 (Figure 1.4) that seems to play a central role in receptor coupling (O'Dowd, 1993).

Although D2(L) differs from D2(S) by the insertion of a stretch of 29 amino acids in the intracellular loop 3, thus far very few differences have emerged between these two D2 receptor variants in terms of their distribution and function. Both of them display the same distribution pattern with the shorter form less abundantly transcribed, and reveal the same pharmacological profile, even if a marginal difference in affinities of sulpiride and raclopride for these two isoforms has been reported (Giros et al., 1989). When expressed in host cell lines, both variants inhibited adenylate cyclase but the D2(S) receptor displayed higher affinity in this effect (Missale et al., 1998). I chose to focus on the D2(L) receptor for this research project as its expression is the most abundant in the brain (Martres et al., 1992; Neve et al., 1991).

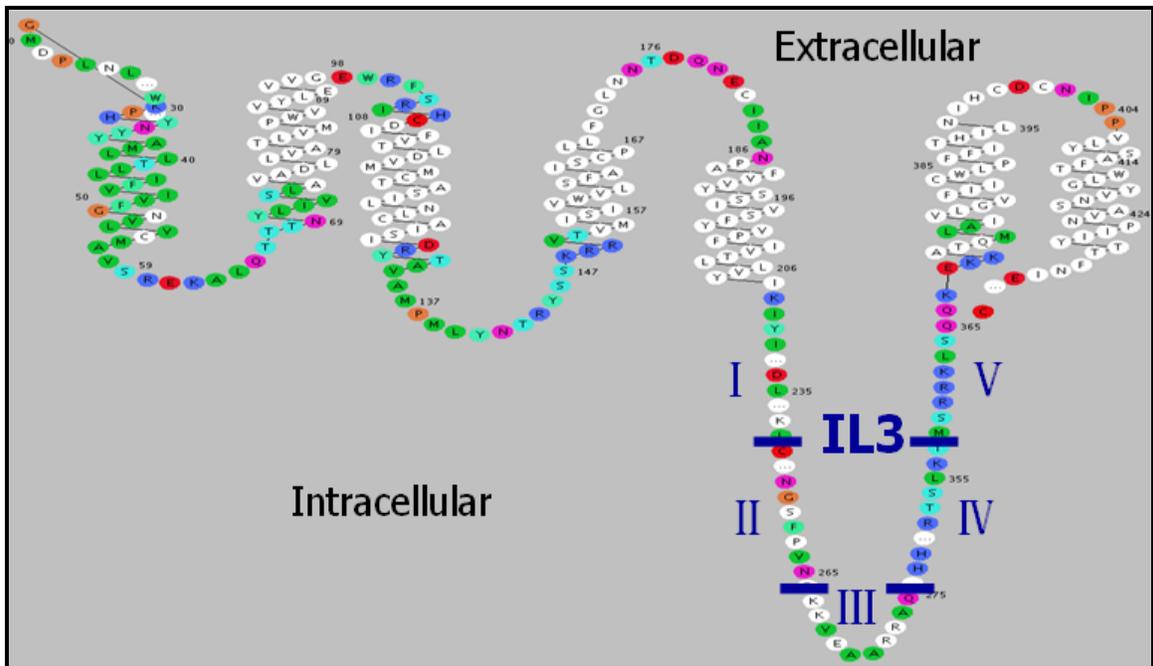


Figure 1.4: The dopamine D2 receptor model comprising seven hydrophobic transmembrane domain regions that extend through the plasma membrane, three extracellular loops, three intracellular loops (including the longest domain: intracellular loop 3, which was divided into five sections in the present study), an extracellular N-terminal and an intracellular C-terminal (Modified from <http://www.gpcr.org>).

1.3.2 Signal Transduction Mediated by D2 Receptor

Signal transduction of the D2 receptor is mediated by the heterotrimeric G proteins of $G\alpha_{i/o}$. These pertussis toxin-sensitive G proteins regulate not only adenylate cyclase via their $G\alpha$ subunits, but also ion channels, phospholipases, protein kinases, and MAP kinases, as a result of the receptor-induced liberation of $G\beta\gamma$ subunits (Bonic and Hopf, 2005). In addition to the interactions between the D2 receptor and G proteins, other protein-protein interactions (i.e. receptor oligomerization, receptor interactions with scaffolding and signal-switching proteins or other receptors) are also critical for regulation of D2 receptor signaling.

The D2 receptor inhibiting adenylate cyclase was initially shown in early 1980s in the CNS (Missale et al., 1998). Recently, several lines of evidence further confirmed that D2 receptors are $G\alpha_{i/o}$ protein linked, which can release $G\alpha$ and $G\beta\gamma$ subunits (Bonic and Hopf, 2005; Neve et al., 2004). Classically, the function of the D2 receptor has been thought to antagonize cAMP-dependent signaling, where $G\alpha_i$ subunits bind to and inhibit adenylyl cyclases, accordingly preventing production of cAMP and activation of protein kinase A (Figure 1.5 A).

D2 receptors also modulate intracellular signaling through $G\beta\gamma$ subunits, which can act on a number of intracellular targets: ion channels, phospholipases, protein kinases, and MAP kinase (Neve et al., 2004). It has been shown that direct interaction of $G\beta\gamma$ with several types of ion channels can influence the stimulation of potassium currents and inhibition of some calcium channels. $G\beta\gamma$ subunits have also been revealed to facilitate

calcium release from intracellular calcium stores (Missale et al., 1998). Moreover, G β subunits can activate MAP kinase system through several different pathways, which may involve phosphoinositide 3-kinase, Ras, and transactivation of a growth factor receptor (Bonic and Hopf, 2005) (Figure 1.5 A).

It has recently been shown that β -arrestin 2 can facilitate D2 receptor signaling in addition to its canonical role in receptor internalization. The D2 receptor may inhibit activity of the serine/threonine protein kinase (Akt) by a β -arrestin 2-dependent mechanism. This effect occurs through a newly discovered β -arrestin/ kinase/phosphatase signaling complex that is independent from the traditional cAMP-dependent pathway (Figure 1.5B). These results indicate that β -arrestin appears to be critical in signal transduction of the D2 receptor to regulate dopamine-dependent dephosphorylation of Akt and its downstream target glycogen synthase kinase (GSK) by allowing a specific association between Akt and the phosphatase PP2A (Bonic and Hopf, 2005).

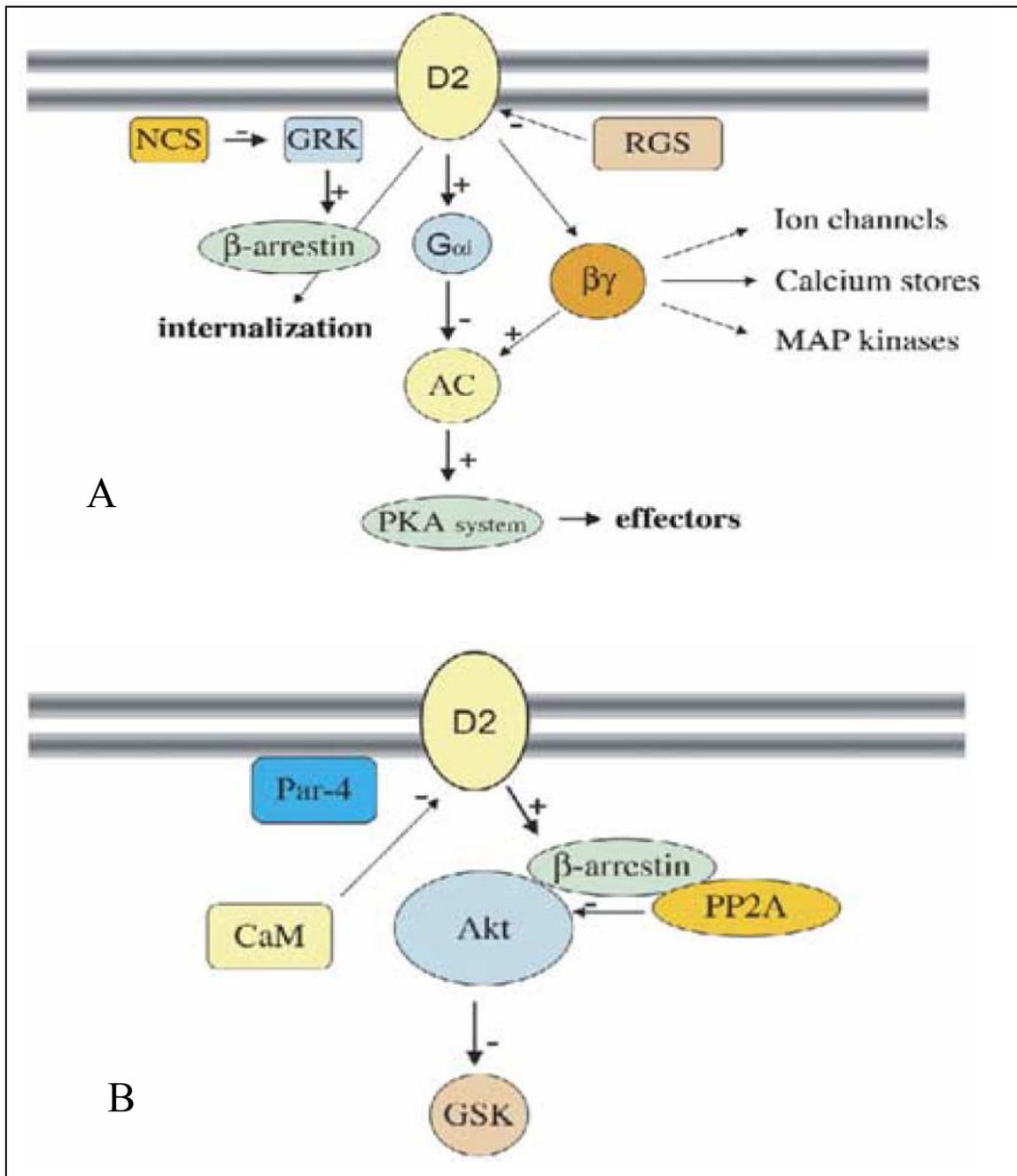


Figure 1.5: The dopamine D2 receptor intracellular signaling pathways: the inhibition of cAMP signaling through $G_{\alpha i}$ and multiple $G_{\beta\gamma}$ -dependent signaling pathways (A), as well as the inhibition of Akt signaling through the signaling complex β -arrestin 2/kinase(Akt)/phosphatase(PP2A) (B) [Taken from Bonic and Hopf, 2005].

1.3.3 Distribution and Function of D2 Receptor in the Brain

Application of *in situ* hybridization with cloned D2 receptor probes and immunohistochemical analysis with specific anti-D2 antibodies has made it possible to define the cellular and subcellular localization of D2 receptors in specific brain regions.

The D2 receptor has been preferentially located in the striatum (including the dorsal striatum and the shell and core of the nucleus accumbens) where stimulation of D2 receptors is essential for the enabling role of striatal dopamine on various behavior. The D2 receptor has also been identified in the substantia nigra pars compacta, the ventral tegmental area and the hypothalamus where dopaminergic neurons are expressed to give origins to three main pathways in the brain, i.e. nigrostriatal, mesolimbocortical and tuberoinfundibular pathways. In addition, the D2 receptor mRNAs have been found in the prefrontal, cingulate, temporal, and entorhinal cortices, septal region, amygdala, olfactory tubercle, pituitary gland, and granule cells of the hippocampus (Jackson and Westlind-Danielsson, 1994; Missale et al., 1998).

Immunohistochemical analysis has demonstrated that D2 receptors are located in medium spiny neurons of the striatum where they are more concentrated in spiny dendrites and spine heads than in the somata. D2 receptors are present in perikarya and dendrites within the substantia nigra pars compacta, and are much more concentrated in the external segment of the globus pallidus than in other striatal projections. D2 receptor immunoreactivity has also been detected in the glomerular and internal plexiform layers of the olfactory nerve, and central nucleus of the amygdala (Levey et al., 1993).

The location of D2 receptors in the above brain regions suggests their important involvement in locomotor activity, learning and memory and drug addiction. This idea is supported by recent pharmacological and behavioral studies on the relation of D2 receptors with locomotor activity (Berke and Hyman, 2000; Jackson and Westlind-Danielsson, 1994; Missale et al., 1998), learning and memory (Arnsten et al., 1995; Berke and Hyman, 2000; Missale et al., 1998; Schultz et al., 1993) and drug addiction (Missale et al., 1998; Self et al., 1996).

1.4 CB1 and D2 Receptors Are Involved in Learning and Memory

1.4.1 Cannabinoids Impair Learning and Memory via Hippocampal CB1 Receptors

The naturally occurring cannabinoids (such as Δ 9-THC) and synthetic compounds (such as HU210) are known to produce acute impairing effects on learning and memory in animals (Carlini et al., 1970; Winnicka et al., 2003) and humans (Abel, 1971). Prevention of Δ 9-THC-induced deficits in radial-maze choice accuracy and in delayed non-match-to-position tasks by SR141716A, a specific CB1 receptor antagonist, provides strong evidence that Δ 9-THC impairs working memory processes through a direct action on CB1 receptors (Beninger and Mallet, 1998; Martin and Lichtman, 1996). The important role of CB1 receptors in memory is further supported by the findings that blockade of CB1 receptors improves cognitive progresses observed in the social recognition task in rats (Terranova et al., 1996), and that recognition memory is enhanced in CB1 receptor knock-out mice (Imperato et al., 1999).

The hippocampus and its related structures appear to be pivotal for memory as its damage by surgical or chemical methods severely impairs performance in a variety of memory tasks (McLamb et al., 1988; Olton and Werz 1978). Several observations are consistent with the notion that the hippocampus regulates the disruptive effects of cannabinoids on memory (Hill et al., 2004; Martin et al., 1995). This notion is also supported by the fact that hippocampus displays a high density of CB1 receptors (Tsou et al., 1998) and high endocannabinoid levels (Di Marzo et al., 2000).

Cannabinoids have been shown to modulate hippocampal cholinergic neurotransmission, which has been suggested by ample evidence to play an important role in learning and memory: increased acetylcholine output in the hippocampus positively correlates with learning and memory (Fadda et al., 1999; Kim and Levin, 1996; Morley et al., 2000). Studies suggest that cannabinoids inhibit long-term potentiation and reduce hippocampal neurotransmitter release, in particular acetylcholine (Davies et al., 2002; Schlicker and Kathmann, 2001). Δ^9 -THC and synthetic cannabinoid CB1 receptor agonists have been reported to reduce acetylcholine release *in vivo* from the rat hippocampus (Carta et al., 1998; Gessa et al., 1998, 2000) and to inhibit electrically evoked acetylcholine release *in vitro* from hippocampal slices (Gifford and Ashby, 1996). These findings suggest that cannabinoid-induced impairment of learning and memory may be related to a reduction in acetylcholine neurotransmission in the hippocampus.

1.4.2 Hippocampal D2 Receptor System Modulates Cannabinoid-induced Learning and Memory Impairment

Memory function appears to be affected by manipulations of hippocampal dopamine D2 receptor system. Dopamine innervation of the hippocampus plays a significant role in spatial working memory and specifically, D2 receptors in the ventral hippocampus were clearly related to choice accuracy performance in the radial-arm maze (Levin and Wilkerson, 1999). Hippocampal dopamine systems may influence learning and memory function via interactions with the well-documented hippocampal acetylcholine systems.

Ingram et al. (2001) confirmed the important role of ventral hippocampal D2 receptors in complex learning, which is likely mediated by D2 receptor-induced acetylcholine release in this brain region. According to these data, it is suggestive that hippocampal D2 receptor system modulates learning and memory by regulating acetylcholine release.

Several lines of evidence suggest that hippocampal dopamine D2 receptor system is involved in the modulation of cannabinoid-induced learning and memory impairment and decreased acetylcholine release. Nomikos et al. (2003) showed that D2 receptor activation mediates high-dose cannabinoid-induced inhibitory effects on acetylcholine release. Moreover, it has been observed that cannabinoids-induced memory impairment and reduction of acetylcholine release in the hippocampus could be antagonized by the D2 dopamine receptor antagonist S(-)-sulpiride and be potentiated by the D2 dopamine receptor agonist (-)-quinpirole, indicating that hippocampal dopamine D2 receptor is critically involved in the process of cannabinoid-induced memory impairment (Gessa et al., 2000). These results imply that CB1 and D2 receptors may work together in order to regulate learning and memory, thus providing the functional evidence supporting the existence of CB1 receptors coupling with D2 receptors.

1.5 CB1 and D2 Receptors Are Involved in Drug Addiction

1.5.1 Mesolimbic Dopaminergic System in Drug Addiction

Drug addiction is a psychiatric disorder in which neuronal mechanisms underlying learning and memory are malfunctioning (Berke and Hyman, 2000; Stelt and Di Marzo, 2003). A common feature for the major drugs of abuse, such as amphetamine, cocaine and opioids including morphine and heroin, is their stimulation of the mesolimbic dopaminergic system, which has an essential role in the acquisition of natural reward and drug-seeking behavior (Spanagel and Weiss, 1999). This neurotransmitter system includes the midbrain dopaminergic projection from the ventral tegmental area (VTA) to structures closely associated with the limbic system, most prominently the nucleus accumbens shell region and the prefrontal cortex. Because of its ubiquitous involvement in the regulation of reward-related behavior, this system has been characterized as a neurochemical substrate of brain reward (Missale et al., 1998).

Pharmacological studies have shown that D2 receptors are involved in the mesolimbic dopaminergic system regulating the reward-related behavior, with its agonists or antagonists respectively stimulating or inhibiting this behavior. It has been reported that suppression of morphine-induced place preference was observed in mice that lack D2 receptors (Maldonado et al., 1997). Another study has showed that D2 receptor agonists enhanced reinstatement of cocaine-seeking behavior (Self et al., 1996). In the case of alcohol drinking, D2 receptor antagonists administered either systemically or locally into

the nucleus accumbens decrease home-cage drinking and operant responses to alcohol (Spanagel and Weiss, 1999).

1.5.2 Endocannabinoid System Modulates Mesolimbic Dopaminergic Pathway

Endocannabinoid system constituted with the cannabinoid receptors, endocannabinoids and proteins for their biosynthesis and degradation is implicated in the regulation of brain reward (Ameri, 1999). The fact that CB1 receptor is the target of cannabinoids, the psychoactive compounds of marijuana, provides support for the role of these receptors in reward related processes. Activation of mesolimbic dopamine neurons in the VTA and subsequent increased release of DA in the nucleus accumbens have been identified after systemic administration of CB1 receptor agonists, which could be blocked by selective CB1 receptor antagonists (French, 1997; Gessa et al., 1998; Wu and French, 2000). These findings suggest that endocannabinoids activating presynaptic CB1 receptors may modify dopaminergic transmission in the mesolimbic reward pathway through protein-protein interactions between cannabinoid and dopamine receptors. This working hypothesis is further supported by the findings that dopamine and endocannabinoid systems exert a mutual control on each other, by which dopaminergic D2-like receptors may regulate cannabinergic CB1 receptors in the striatum (Giuffrida et al., 1999; Stelt and Di Marzo, 2003).

1.6 Research Project

1.6.1 Background

Both CB1 and D2 receptors are highly expressed in the basal ganglia and hippocampus (Glass et al., 1997; Herkenham et al., 1991b; Tsou et al., 1998), suggesting that both receptors may interact with one another. Recent evidence further showed that CB1 and D2 receptors are co-expressed in the same neurons. In the hippocampus, coexpression of CB1 and D2 receptors was detected in the dentate gyrus, where D2 hybridization signals were detected in 88% of the low-CB1-expressing cells, and 48% of all D2-positive cells coexpress CB1 receptors (Hermann et al., 2002). In contrast, CB1 and D2 receptor mRNAs are extensively co-localized in the striatum: 73% D2-positive cells contain CB1 receptor hybridization signals (Hermann et al., 2002). These findings provide structural evidence supporting the possibility that CB1 and D2 receptors may physically interact to each other in hippocampal and striatal neurons.

This idea is further supported by various lines of evidence suggesting functional links between CB1 and D2 receptors. While stimulation of either CB1 receptors or D2 receptors leads to a decrease in cAMP accumulation, an increase in cAMP levels is promoted by switching the coupling of the CB1 receptor from $G\alpha_{i/o}$ to $G\alpha_s$ when both CB1 and D2 receptors are stimulated together (Glass and Felder, 1997; Jarrahian et al., 2004). Acute learning and memory impairment induced by cannabinoids through CB1 receptors in the hippocampus can be antagonized by the D2 receptor antagonist S(-)-sulpiride and

potentiated by the D2 receptor agonist (-)-quinpirole, suggesting that D2 receptors are involved in the modulation of cannabinoid-induced learning and memory impairment by interacting with CB1 receptors (Gessa et al., 2000). It has been shown that dopamine and the endogenous cannabinoids display complex interactions in their control of basal ganglia circuitry and in the mesolimbic reward system (Giuffrida et al., 1999; Glass and Felder, 1997; Jarrahian et al., 2004; Mailleux and Vanderhaeghen, 1993; Stelt and Di Marzo, 2003). Giuffrida et al. (1999) reported that chronic treatment with D2 receptor antagonists produced up-regulation of CB1 mRNA in the striatum.

Direct evidence supporting the existence of CB1:D2 receptor complexes comes from the recent Co-IP experiment showing a physical interaction between cannabinoid CB1 and dopamine D2 receptors in HEK293 cells stably coexpressed both receptors (Kearn et al., 2005).

1.6.2 Hypothesis

In summary, all the available lines of evidence support the notion that the CB1 and D2 receptors interact to each other both *in vitro* and *in vivo*. This research project will examine CB1 and D2 interaction in *in vitro* situation. Specifically, the central hypothesis of this project is that **specific motifs in the D2 receptor are responsible for *in vitro* protein-protein interactions between cannabinoid CB1 and dopamine D2 receptors.**

1.6.3 Objective

The purpose of this study is to identify the specific motifs in the D2 receptor that are responsible for *in vitro* coupling between CB1 and D2 receptors in HEK293 cells stably co-transfected with both receptors. Specifically, three objectives will be addressed in this project:

1. To prepare over-expressed full length CB1 and D2 receptors and purified fusion proteins containing various domains and motifs of CB1 and D2 receptors;
2. To determine the domains of the CB1 and D2 receptors responsible for *in vitro* protein-protein interactions between CB1 and D2 receptors;
3. To identify the specific motifs in the D2 receptor responsible for *in vitro* protein-protein interactions between CB1 and D2 receptors.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Vectors

In total, five original vectors were used for cloning and subcloning of the gene sequences of the CB1 and D2 receptors: two pGEX vectors (Amersham Biosciences, Piscataway, NJ) of “pGEX-2T” and “pGEX-6P-1”, “pET-28a(+)” (Novagen, Mississauga, ON), “pcDNATM3.1/*myc*-His(B)” (Invitrogen, Burlington, ON) and “pGEM-T Easy” (Promega, Madison, WI).

The pGEX vectors (Figure 2.1) are responsible for the inducible, high-level intracellular expression of genes in *E.coli* cells as fusion proteins with GST. The pET-28a(+) vector (Figure 2.2) is designed for the over-expression of the 6His fusion proteins in *E.coli* cells. The pGEM-T Easy vector (Figure 2.3) is applied to facilitate the PCR products for their subcloning. The pcDNATM3.1/*myc*-His(B) vector (Figure 2.4) is designed for the high-level expression and detection of tagged recombinant proteins in mammalian hosts.

One more vector was reconstructed from the original “pcDNATM3.1/*myc*-His(B)” with the synthetic “FLAG epitope (DYKDDDDK)” (Invitrogen, Burlington, ON) incorporated by “SacII” (5′-CCGC↓GG-3′) & “SfuI” (5′-TT↓CGAA-3′) restriction sites, named as “pcDNATM3.1/FLAG(B)” (Figure 2.5), to express the FLAG tagged fusion proteins in mammalian cells.

Except for the pET-28a(+) vector with a kanamycin-resistant gene (kan^r), all other vectors used here have an ampicillin-resistant gene (amp^r) for selective amplification of recombinant plasmids.

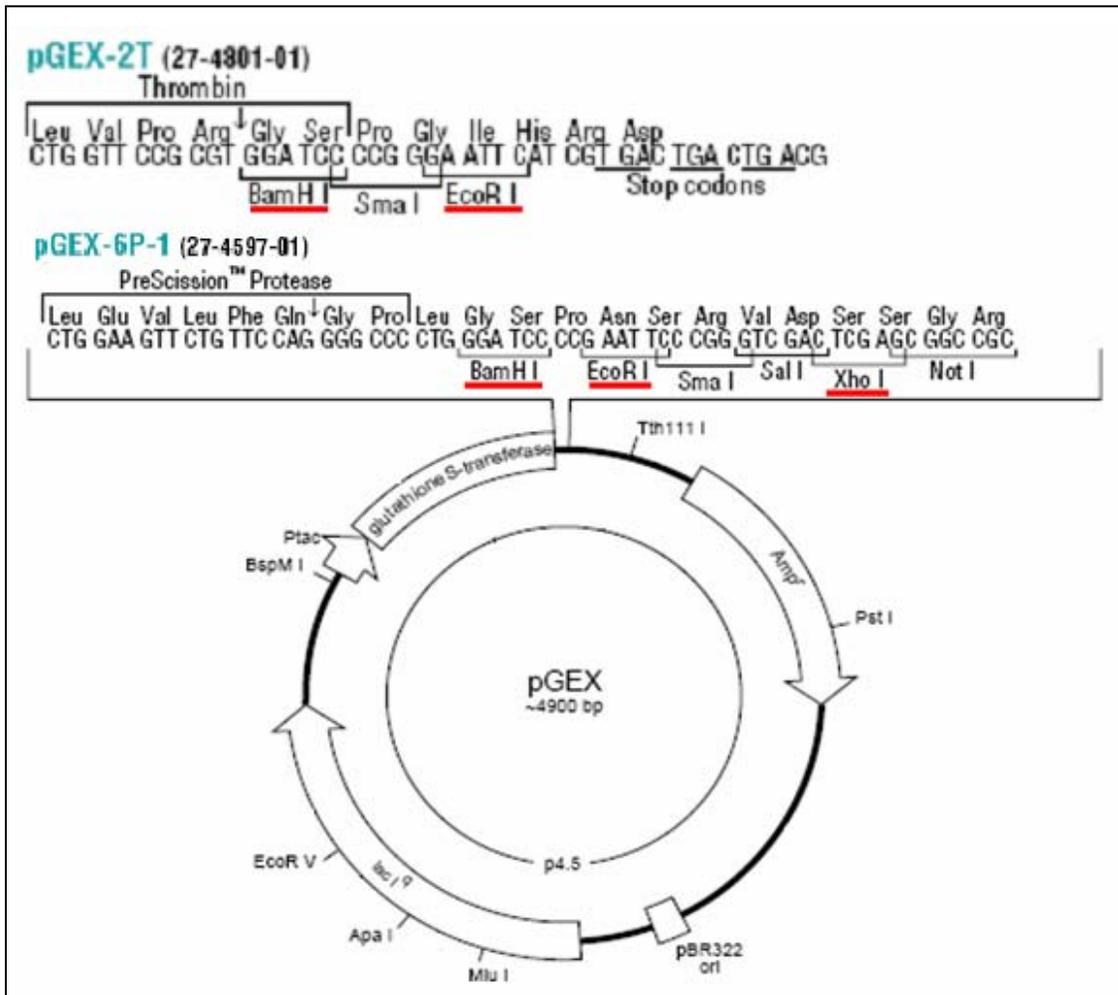


Figure 2.1: Map of pGEX vectors, “pGEX-2T” and “pGEX-6P-1”, showing the reading frames and the multiple cloning sites. The underlined restriction sites were used in this study. (Modified from Amersham Biosciences)

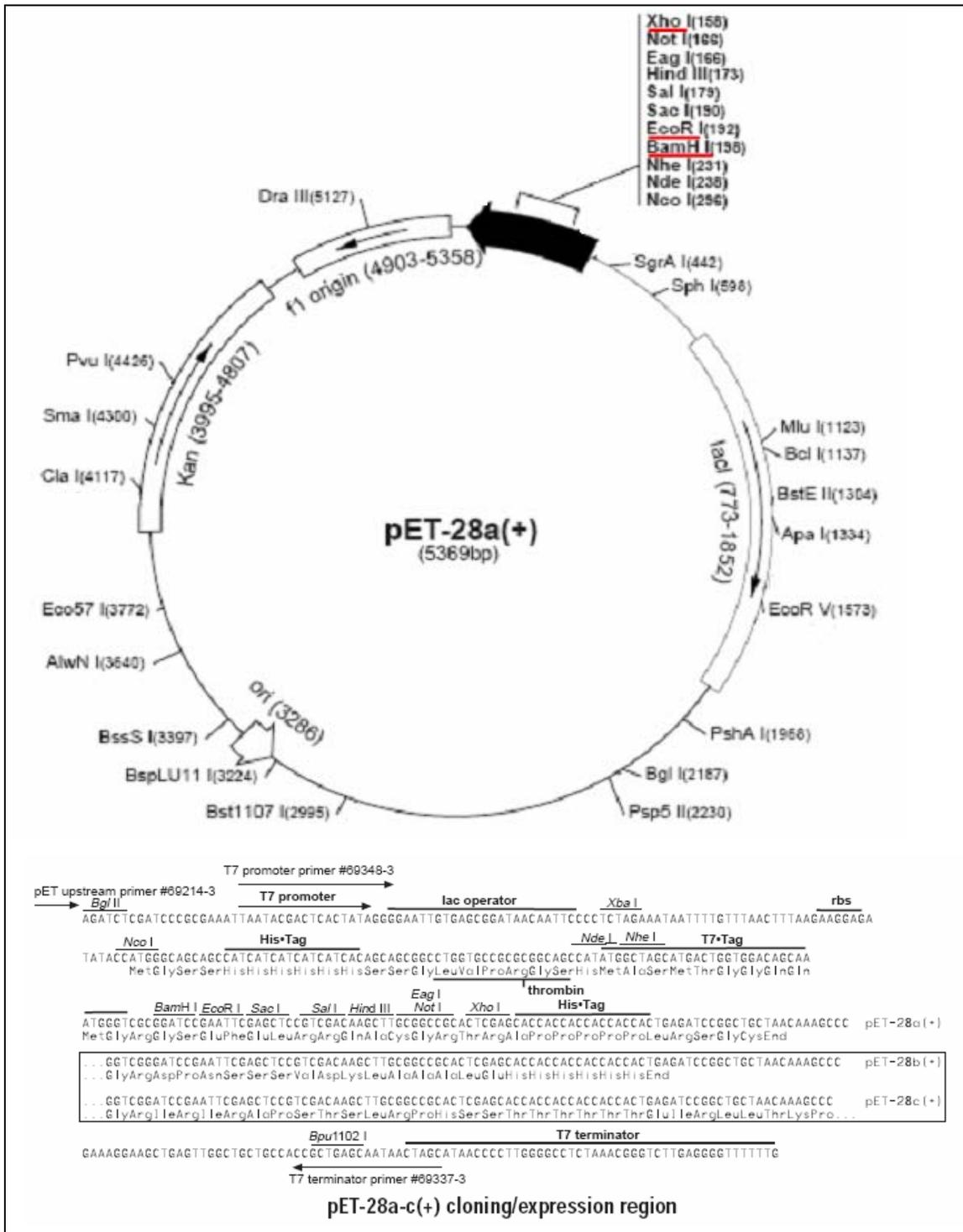


Figure 2.2: Map of pET-28a(+) vector showing the reading frame as well as the cloning and expression region. The underlined restriction sites were used in the present study. (Modified from Novagen)

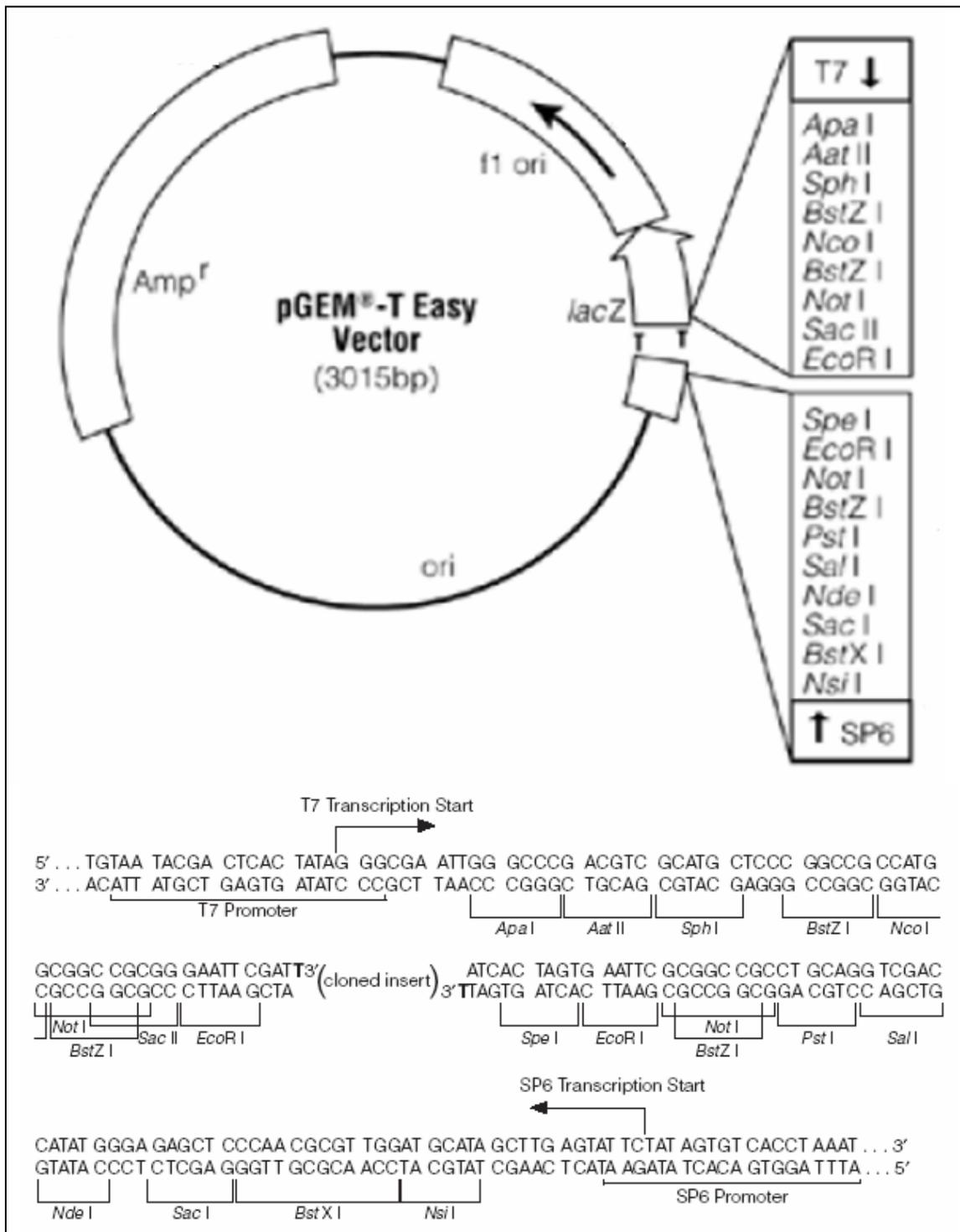


Figure 2.3: pGEM-T Easy Vector circle map illustrating the promoter and multiple cloning sequences. (Modified from Promega)

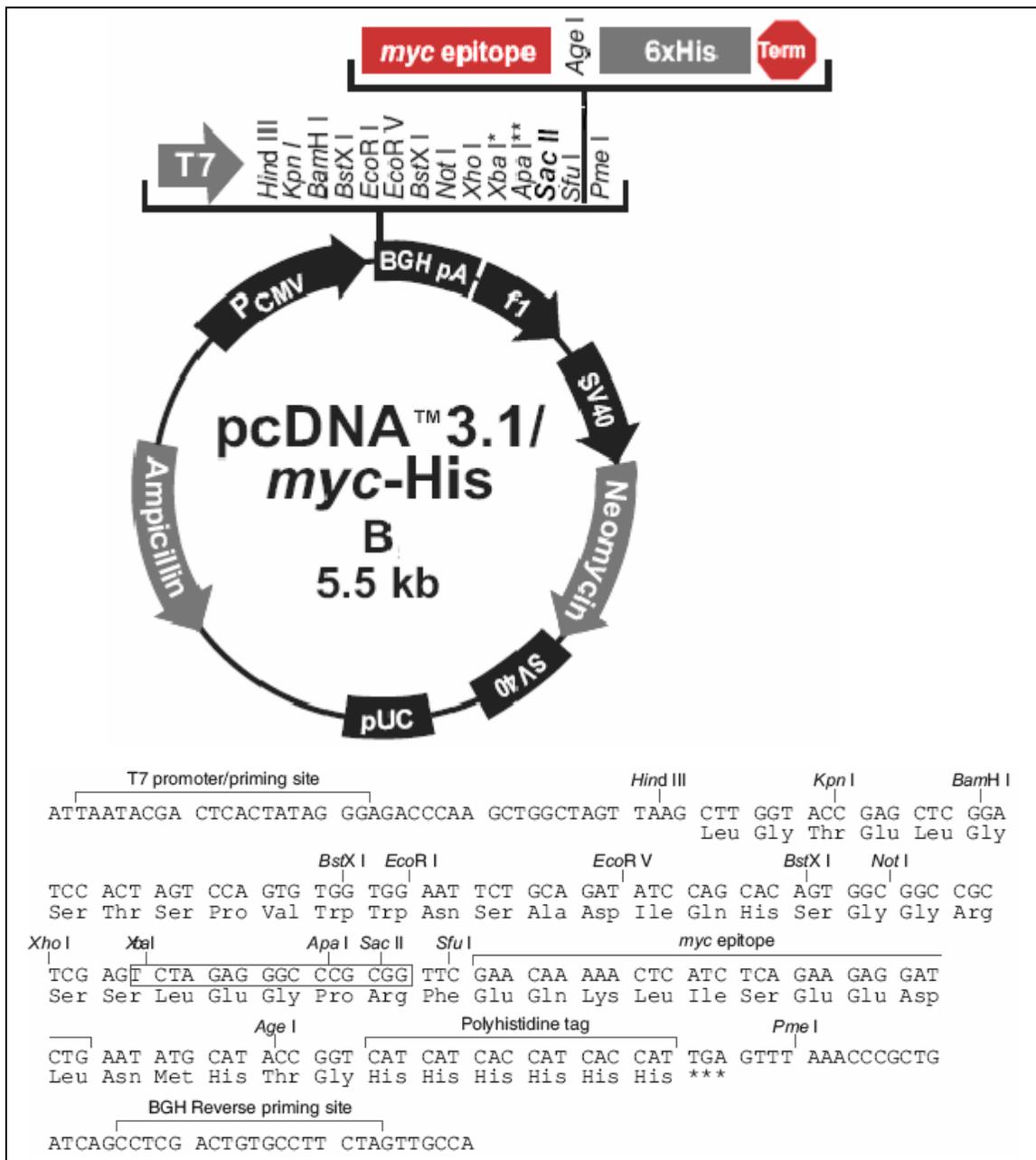


Figure 2.4: Map of pcDNA™3.1/myc-His(B) vector, illustrating the multiple cloning sites and expression regions. (Modified from Invitrogen)

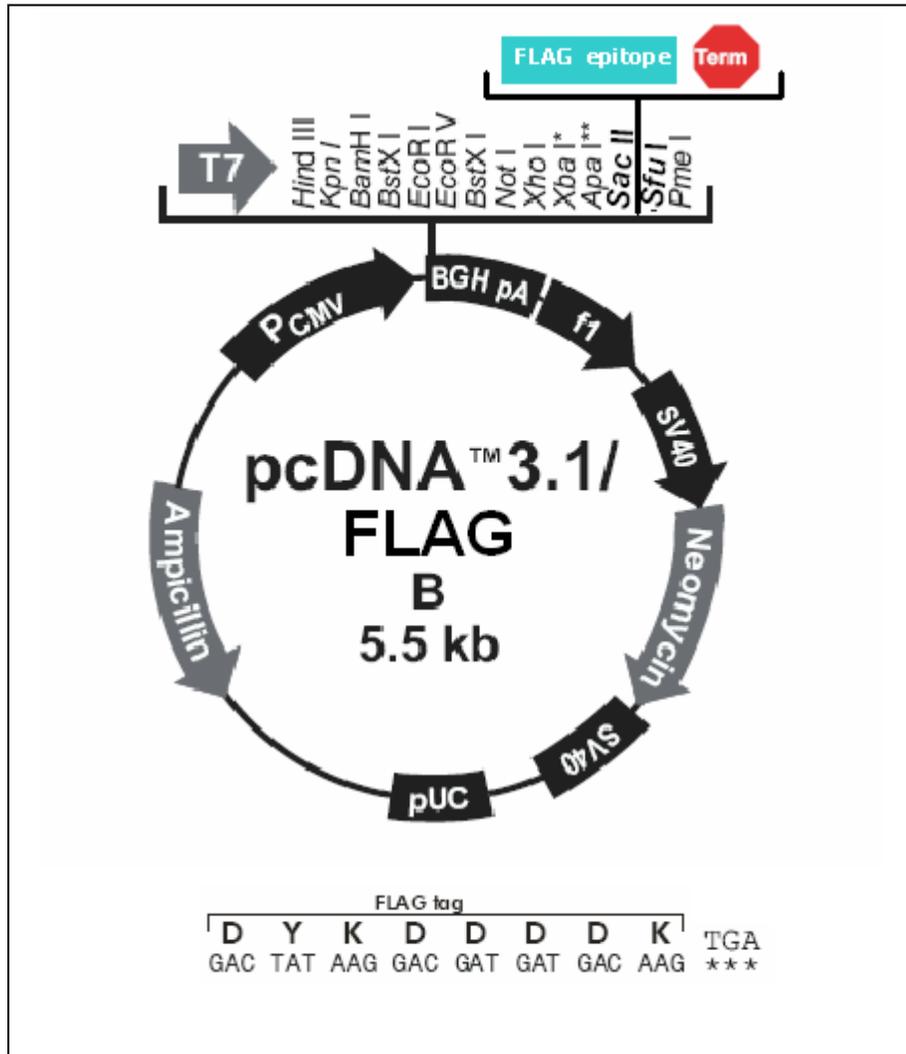


Figure 2.5: Map of pcDNA[™]3.1/FLAG(B) vector, which was reconstructed from the original “pcDNA[™]3.1/myc-His(B)” with the synthetic “FLAG epitope (DYKDDDDK)” incorporated by “SacII” & “SfuI” restriction sites. (Modified from Invitrogen)

2.1.2 Competent *E.coli* Cells

Four strains of competent *E.coli* cells were used for the transformation of plasmids: DH5 α , JM109, ER2925 and BL21. DH5 α competent cells (aliquot 50 μ l of cells for each transformation), which were directly used for transformation after purchased from Invitrogen (Burlington, ON) and had high transformation efficiency, were recommended for the transformation of routine subcloning into vectors to screen out the correct clones. The simple amplification of plasmids could be obtained from the transformation into JM109 cells (New England Biolabs, Ipswich, MA). ER2925 cells (New England Biolabs, Ipswich, MA) are useful for production of DNA to be cut with Dam, Dcm or CpG-sensitive restriction enzymes, such as “XhoI”, “SacII” and “SfuI”. BL21 and its lysogenized strain DE3 competent cells (New England Biolabs, Ipswich, MA) were respectively applied for inducing expression of GST or 6His fusion proteins after transformation.

Except for the DH5 α competent cells, the other four strains of competent *E.coli* cells were prepared by the following procedures that were performed under sterile conditions.

- a. Pick a single clone from a freshly grown LB-agar plate (2% bacto-agar, Becton Dickinson, Sparks, MD, in LB media for autoclave, 10 ml for each 10 cm plate) of *E. coli* cells incubated at 37°C overnight and disperse it in 5 ml of LB media (1% bacto-tryptone, Becton Dickinson, Sparks, MD; 0.5% bacto-yeast extract, Becton Dickinson, Sparks, MD; and 1% NaCl, EMD Chemicals, Gibbstown, NJ; adjust PH to 7.0 with 5 M NaOH and autoclave before use) in a 15 ml cultural tube.
- b. Incubate the culture at 37°C with vigorous shaking overnight.

- c. Transfer 400 μ l of this culture into 40 ml of LB media in a 250 ml flask and incubate the culture at 37°C with vigorous shaking for approximately one and half hours.
- d. Transfer the culture into a 40 ml centrifuge tube and centrifuge at 3,000 g for 3 min at 4°C. Decant the supernatant and resuspend the pellet in 20 ml of ice cold autoclaved 0.1 M CaCl₂ (EMD Chemicals, Gibbstown, NJ).
- e. Keep the resuspended solution on ice for 30 minutes and then centrifuge at 3,000g for 3 min at 4°C.
- f. Decant the supernatant and resuspend the pellet in 3 ml of ice cold 0.1 M CaCl₂. Keep the cell solution at 4°C overnight. Add 1.5 ml of 50% glycerol into the cell solution, mix well and then aliquot 200 μ l in each 1.5 ml Eppendorf tube stored at -70°C for later use.

2.1.3 Cell Culture

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, ATCC) were incubated at 37°C in a humidified atmosphere (containing 5% CO₂) and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% heat-inactivated horse serum (Gibco BRL), 25 units/ml penicillin and 25 units/ml streptomycin (Gibco BRL).

Rat pheochromocytoma (PC12) cells (ATCC) were grown in DMEM with 10% heat-inactivated horse serum, 5% fetal bovine serum (Gibco BRL), 25 units/ml penicillin and 25 units/ml streptomycin. Culture plates and flasks were coated with rat-tail collagen (BD Biosciences, Bedford, MA). Cell cultures were maintained at 37°C with 5% CO₂.

2.1.4 Animals

All procedures involving animals as well as the maintenance of the animals were executed with the approval of the University of Saskatchewan Animal Care and Use Committee (University of Saskatchewan Protocol# 20040034) and followed the guidelines from the Canadian Council on Animal Care and the National Institute of Health. The inbred adult (350~400 g body weight) male Long Evans rats were housed at a constant temperature on a 12-hr light/dark cycle, with free access to food and water.

2.2 Methods

2.2.1 Dissection of Rat Brain

In accordance with a protocol approved by the University of Saskatchewan Animal Care and Use Committee, rats were anaesthetized with sodium pentobarbital (65 mg/kg body weight) and killed by rapid decapitation. Rat brains were then quickly removed and the hippocampus and striatum were immediately dissected. Half of hippocampus was used immediately for the isolation of total RNA. The other brain samples were stored at -70°C for later use.

2.2.2 Isolation of Total RNA

The following procedures were used to extract the total RNA from the rat brain tissue or mammalian cells.

- a. Add 1.0 ml of TRIzol (Invitrogen, Burlington, ON) into a centrifuge tube containing 100 mg of rat hippocampus tissue (or cell pellet) and homogenize the tissue with a homogenizer until the sample is uniformly dissociated.
- b. Add 0.2 ml of chloroform (Sigma, St.Louis, MO) into the homogenized sample. Shake vigorously for 15 seconds and incubate for 2 minutes at room temperature. Centrifuge the sample at 12,000 g for 15 minutes at 4°C.

- c. Carefully transfer the colourless, upper aqueous phase (approximately 0.6 ml) containing the RNA into a clean microcentrifuge tube, avoiding removal of the material collected at the interface.
- d. Add 0.5 ml of isopropanol (Sigma, St.Louis, MO) into the aqueous phase and gently mix the solution well. Allow the RNA to precipitate at room temperature for 10 minutes and then centrifuge at 12,000 g for 10 minutes at 4°C.
- e. Remove the supernatant and wash the RNA pellet once with 1.0 ml of 75% ethanol (in DEPC-treated water). Mix the sample by vortexing and centrifuge at 7500 g for 5 minutes at 4°C to make sure that the pellet has sedimented before the ethanol is totally removed.
- f. Allow the RNA to dry for 10 minutes at room temperature and then dissolve the pellet with 50 µl of DEPC-treated H₂O adding 1.0 µl of the RNasin Ribonuclease Inhibitor (Promega, Madison, WI). Store the total RNA at -70°C.

2.2.3 RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) is a modification of polymerase chain reaction (PCR). The RNA strand, which is isolated from the tissue or cells, is first applied for reverse transcription into its complementary DNA (cDNA), followed by the amplification of the resulting DNA using PCR with specific primers.

2.2.3.1 Reverse Transcription of cDNA

To obtain the double-strand cDNA for PCR, reverse transcription with the above total RNA isolated from the rat hippocampus tissue was performed according to the following procedures. The total volume of reactive solution was 40 μ l.

a. Add and mix the components as below to a 0.5 ml microcentrifuge tube:

20 μ l	Total RNA
2 μ l	Oligo(dT) ₂₀ Primer (Invitrogen, 50 μ M)
4 μ l	dNTP mix (Promega, 10 mM each dATP, dTTP, dCTP, dGTP)

b. Incubate the mixture at 65°C for 5 minutes and then put on ice.

c. Collect the contents on the bottom of the tube by brief centrifugation and add:

8 μ l	5 \times cDNA Synthesis Buffer (Invitrogen)
2 μ l	0.1 M DTT (dithiothreitol)
2 μ l	RNasin Ribonuclease Inhibitor (Promega, 40 units/ μ l)
2 μ l	Cloned AMV Reverse Transcriptase (Invitrogen, 15 units/ μ l)

d. Mix the contents gently and incubate at 45°C for 60 minutes.

e. Heat the mixture at 85°C for 5 minutes to terminate the reaction and store the cDNA at -20°C.

2.2.3.2 Primer Preparation for PCR

The DNA sequences of the CB1 and D2 receptors were found from the Genbank.

CB1: accession number NM_012784, 1419bp from 153 to 1571 (Figure 2.6); D2: accession number NM_012547, 1332bp from 347 to 1678 (Figure 2.7). The genes of the following fragments were prepared by RT-PCR for cloning: full length CB1 receptor (Full CB1, 473aa), CB1 extracellular N-terminal (CB1-NT, 1~117aa), CB1 intracellular loop3

(CB1-IL3, 301~345aa) and C-terminal (CB1-CT, 401~473aa), full length long form D2 receptor (Full D2(L), 444aa), D2 intracellular loop3 (D2-IL3, 211~374aa) and five fragments of the D2 intracellular loop3 (D2-IL3 I / II / III / IV / V) (Figure 2.7).

The open reading frames of these fragments were amplified from cDNA obtained from the reverse transcription of the rat hippocampal total RNA using the PCR with specific primers. All the primers (Figure 2.8, Figure 2.9) were synthesized by Invitrogen (Burlington, ON) and flanked by special restriction enzyme sites for the cloning of the amplified DNA sequences. Except for the full length D2 receptor that includes one “BamHI” restriction sites in its DNA sequence, all the fragments were flanked by “BamHI” & “EcoRI” for subcloning. Two different PCR products of the full length D2 receptor were flanked by “EcoRI” & “XhoI” (including “TGA”) for subcloning into pGEX-6P-1 and pET-28a(+) vectors or flanked by “HindIII” & “EcoRI” (no “TGA”) for subcloning into pcDNATM3.1 vectors. There were also two different PCR products of the full length CB1 receptor. Both of them were incorporated with “BamHI” & “EcoRI”, but one included the stop code “TGA” and another did not.

All the synthesized primers were prepared with autoclaved distilled water to a final concentration of 10 μ M before use and stored at -20°C.

```

1 ttaggtcggt aggagaactt actgtgaaca ggctctttaa tttcttcaaa agatgtctcc
61 catttcaagc aaggagcacc catggctgag ggttccctcc cggcatctct ttctcagtca
121 ccttgagtct ggctaataca aagactgagg ttatgaagtc gatcctagat ggccttgca
181 acaccacctt ccgtaccatc accacagacc tcctctacgt gggctcgaat gacattcagt
241 atgaagatat caaaggagac atggcatcca aattaggata cttcccacag aaattccctc
301 taacttcctt cagggtagt cccttccaag aaaagatgac cgcaggagac aactccccgt
361 tggtcccagc aggagacaca acaaacatta cagagttcta taacaagtct ctctcgtcgt
421 tcaaggagaa tgaggagaac atccagtgtg gggagaactt tatggacatg gagtgcctta
481 tgattctgaa tcccagccag cagctggcca tcgctgtact gtccctcaca ctgggcacct
541 tcacggttct ggagaaccta ctggtgctgt gtgtcatcct gcactcccgc agtctccgat
601 gcaggccttc ctaccacttc atcggcagcc tggcagtggc cgacctcctg ggaagtgtca
661 tttttggtga cagctttggt gacttccatg tattccaccg taaagacagc cccaatgtgt
721 ttctgttcaa actgggtggg gttacagcct ccttcacagc ttctgtgggc agcctgttcc
781 tcacagccat cgacaggtac atatcattc acaggcctct ggcctataag aggatcgtca
841 ccaggcccaa ggccgttgtg gccttttgcc tgatgtggac tatcgcaata gtaatcgctg
901 tgttgctct cctgggctgg aactgcaaga agctgcaatc tgtttgctcg gacattttcc
961 cactcattga cgagacctac ctgatgttct ggattggggg gaccagtgtg ctgctgctgt
1021 tcattgtgta cgcgtacatg tacatttctt ggaaggctca cagccacgcg gtccgcatga
1081 ttcagcgtgg gaccagaag agcatcatca tccacacgtc agaagacggc aaggtgcagg
1141 tgaccgggcc tgaccaagcc cgcatggaca ttaggctggc caaaacctg gttctgatcc
1201 tggtggtggt gatcatctgc tggggccctc tgcttgcgat catggtgtat gacgtcttcg
1261 ggaagatgaa caagcttata aagacggtgt ttgccttctg cagtatgctc tgctgctga
1321 actccaccgt gaacccatc atctatgctc tgaggagcaa ggacctgaga catgctttcc
1381 gaagcatggt cccttcgtgc gaaggcaccg cacagcctct agacaacagc atgggggact
1441 cagactgcct gcacaagcac gccacaaca cagccagcat gcacagggc gcggagagct
1501 gcatcaagag caccgttaag atcgcaaggt tgaccatgct tgtgtccaca gacacgtccg
1561 ccgaggctct gtgagcctgc tgcttttgtg gc

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Figure 2.6: The DNA sequence of the CB1 receptor was found from the Genbank: accession number NM_012784, 1419bp from 153 to 1571. The shading sequences are seven transmembrane domains; the italic sequences are four extracellular domains (CB1-NT/EL1/EL2/EL3); and the underlined sequences are four intracellular domains (CB1-IL1/IL2/IL3/CT).

```

1 ctggtgcct cttgtgcgca cgctcctgc ccgccttccc gcctggtecc cgcgctgcgc
61 tcccgtctctc ccgccccgcc tegtctctgcc ccgcccgcggc cggctctactg ctccccgcgg
121 gcccagaccg gccgagcggc tgcccgccgg ggatctgaac gagtcatgcg gcggggccgg
181 aagccgaggg acccgcgag gggaccggcg gccccggacg gctgccggag gggcggccgt
241 gcgtggatgc ggcgggagct ggaagcctcg agcagccggc gccttctctg gccccgggcg
301 ccatatggct tgaagagccg tgccaccag tggccccact gcccatggatccactgaa
361 cctgtctctg tacgatgacg atctggagag gcagaactgg agccggccct tcaatgggct
421 agaagggag gcagacaggc cccactacaa ctactatgcc atgctgctca ccctctcat
481 ctttatcatc gtctttggca atgtgctggt gtgcatggct gtatccccgag agaaggcttt
541 gcagaccacc accactact tgatagtcag ccttgtctgt gctgatcttc tggtgccac
601 actggtaatg ccgtgggtt tctacctgga ggtgggtggg gagtggaaat tcagcaggat
661 tcaactgtgac atctttgtca ctctggatgt catgatgtgc acagcaagca tcctgaacct
721 gtgtgccatc agcattgaca ggtacacagc tgtggcaatg cccatgctgt ataacacagc
781 ctacagctcc aagcgcgag ttaactgtcat gattgccatt gtctgggtcc tgtccttca
841 catctctctc ccaactgctct tcggactcaa caatacagac cagaatgagt gtatcattgc
901 caacctgcc tttgtggtct actcctccat tgtctcattc tacgtgccct tcactgtcac
961 tctgtctggtc tatatcaaaa tctacatcgt cctccggaag cgccggaagc gggtcaacac
1021 caagcgcagc agtcgagctt tcagagccaa cctgaagaca ccactcaagg gcaactgtac
1081 ccaccctgag gacatgaaac tctgcaccgt tatcatgaag tctaatggga gtttcccagt
1141 gaacaggcgg agaatggatg ctgcccgcgg agctcaggag ctggaaatgg agatgctgtc
1201 aagcaccagt ccccagaga ggaccgggta tagecccate cctcccagtc accaccagct
1261 cactctcct gatccatccc accacggcct acatagcaac cctgacagtc ctgccaacc
1321 agagaagaat gggcacgcca agattgtcaa tcccaggatt gccaagttct ttgagatcca
1381 gaccatgccc aatggcaaaa cccggacctc ccttaagacg atgagccgca gaaagctctc
1441 ccagcagaag gagaagaaag ccactcagat gcttgccatt gttctcgggtg tgttcatcat
1501 ctgctggctg cccttcttca tcacgcacat cctgaatata cactgtgatt gcaacatccc
1561 accagtctctc tacagcctc tcacatggct gggctatgtc aacagtgccg tcaacccat
1621 catctacacc accttcaaca tcgagttccg caaggccttc atgaagatct tgcactgctg
1681 agtctgcccc ttgctgcaac agcagctgct tcccacctcc ctgctatgc aggccagacc

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Figure 2.7: The DNA sequence of the D2 receptor was found from the Genbank: accession number NM_012547, 1332bp from 347 to 1678. The boxed sequence is the “BamHI” restriction site. The underlined sequences are four intracellular domains (D2-IL1/IL2/IL3/CT). The light shading sequences with underline are the three fragments (I: 11~241aa, III: 271~305aa, V: 341~374aa) and the dark shading sequences with underline are the other two fragments (II: 242~270aa, IV: 306~340aa) of the D2 intracellular loop3.

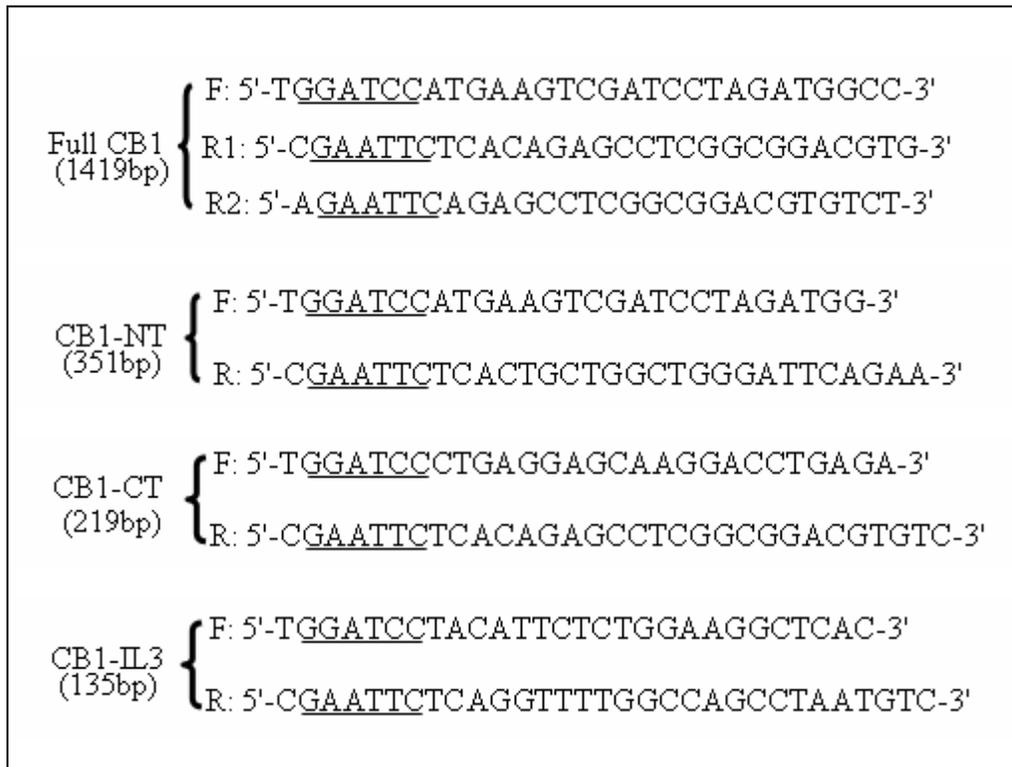


Figure 2.8: Primers for CB1 receptor fragments prepared by RT-PCR and flanked by “BamHI” & “EcoRI” restriction sites (marked by underline) for cloning.

F(forward): sense primer

R(reverse): anti-sense primer

BamHI (5' -G↓GATCC-3')

EcoRI (5' -G↓AATTC-3')

Full D2(L) (1332bp)	$\left\{ \begin{array}{l} \text{F1: 5'-TGAATTCATGGATCCACTGAACCTGTCCT-3'} \\ \text{R1: 5'-GCTCGAGTCAGCAGTGCAAGATCTTCATG-3'} \\ \text{F2: 5'-AAGCTTATGGATCCACTGAACCTGTCCT-3'} \\ \text{R2: 5'-GAATTCAGCAGTGCAAGATCTTCATGAAG-3'} \end{array} \right.$
D2-IL3 (492bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCAAAATCTACATCGTCCTCCGGA-3'} \\ \text{R: 5'-CGAATTCTCACTGAGTGGCTTTCTTCTCCTTC-3'} \end{array} \right.$
D2-IL3 (I) (93bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCAAAATCTACATCGTCCTCCGGA-3'} \\ \text{R: 5'-CGAATTCTCACTTGAAGTGGTGTCTTCAGGT-3'} \end{array} \right.$
D2-IL3 (II) (87bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCGGCAACTGTACCCACCCTGA-3'} \\ \text{R: 5'-CGAATTCTCACATTCTCCGCCTGTTCACTG-3'} \end{array} \right.$
D2-IL3 (III) (105bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCGATGCTGCCCCGCCGAGCTCA-3'} \\ \text{R: 5'-CGAATTCTCAGAGCTGGTGGTGAAGTGGGAG-3'} \end{array} \right.$
D2-IL3 (IV) (105bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCACTCTCCCTGATCCATCCCA-3'} \\ \text{R: 5'-CGAATTCTCACTTGGCAATCCTGGGATTGA-3'} \end{array} \right.$
D2-IL3 (V) (102bp)	$\left\{ \begin{array}{l} \text{F: 5'-TGGATCCTTCTTTGAGATCCAGACCAT-3'} \\ \text{R: 5'-CGAATTCTCACTGAGTGGCTTTCTTCTCCTTC-3'} \end{array} \right.$

Figure 2.9: Primers for D2 receptor fragments produced by RT-PCR and flanked by “BamHI” & “EcoRI”, “EcoRI” & “XhoI” or “HindIII” & “EcoRI” restriction sites (marked by underline) for cloning.

F(forward): sense primer

BamHI (5' -G↓GATCC-3')

XhoI (5' -C↓TCGAG-3')

R(reverse): anti-sense primer

EcoRI (5' -G↓AATTC-3')

HindIII (5' -A↓AGCTT-3')

2.2.3.3 PCR Experiments

The following “hot-start” PCR protocol (with total volume 50 μ l as an example) served as a general guideline for our PCR experiments. Optimal reaction conditions (i.e. total volumes of reactions, primers, different kinds or concentrations of DNA polymerase, incubation temperatures and times, template DNA as well as $MgCl_2$) varied and were optimized for each DNA fragment.

a. Add and mix the following components to a sterile 0.2 ml microcentrifuge tube:

5 μ l	10 \times PCR buffer (include $MgCl_2$)
4 μ l	dNTP mixture (Promega, 2.5 mM each dATP, dTTP, dCTP, dGTP)
5 μ l	10 μ M Sense primer
5 μ l	10 μ M Anti-sense primer
1 μ l	Template cDNA
20 μ l	Autoclaved distilled water

b. Cap the tube and centrifuge briefly to collect the contents on the tube bottom. Put the sample in the PCR equipment and incubate it at 94°C for 2 minutes to completely denature the template.

c. After denaturation at 94°C, add 2~5 units of *Taq* (New England Biolabs, Ipswich, MA), *Vent* (New England Biolabs, Ipswich, MA) or *Pfu* (Fermentas, Burlington, ON) DNA polymerase in 10 μ l autoclaved distilled water.

d. Perform 28~35 cycles of PCR amplification as follows:

Denature	94°C for 40 seconds
Anneal	55°C~60°C for 30 or 40 seconds
Extend	72°C for 1 minute or 1 minute 30 seconds

e. Incubate the sample at 72°C for an additional 10 minutes and maintain the reaction at 4°C. The sample could be stored at -20°C until use.

- f. Visualize the amplification products by agarose (Invitrogen, Burlington, ON) gel electrophoresis with ethidium bromide (EtBr) and DNA molecular weight standards.
- g. Purification of the amplified DNA products from the agarose gel was performed by using Gel and PCR Clean-Up Kit (Promega, Madison, WI) according to manufacturer's protocol.

2.2.4 Synthesis Fragments

The appropriate complementary single-stranded DNA oligos of the mini-genes of the CB1 and D2 receptor short fragments less than 30 amino acids were directly synthesized by "Invitrogen". Each sequence was designed to give sticky ends, using "BamHI" & "EcoRI" restriction sites, to allow insertion into the pGEX-2T vector.

The following procedures were employed to generate the double-stranded DNA fragments by annealing the synthesized complementary single-stranded DNA oligos.

- a. Resuspend the lyophilized single-stranded oligos in distilled water to a final concentration of 200 μ M before use. Prepare 10 \times Oligo Annealing Buffer (100 mM Tris-HCl, J.T.Baker, Phillipsburg, NJ, PH 8.0; 10 mM EDTA, Sigma, St.Louis, MO, PH 8.0; and 1 M NaCl) for use.
- b. Add the components as follows in a 0.5 ml sterile microcentrifuge tube to set up the annealing reaction at room temperature (total volume 20 μ l).

5 μ l	200 μ M "Sense strand" DNA oligo
5 μ l	200 μ M "Anti-sense strand" DNA oligo
2 μ l	10 \times Oligo Annealing Buffer
8 μ l	DNase/RNase-free Water

- c. Incubate the sample at 95°C for 4 minutes. Remove the tube containing the annealing reaction from water bath and allow the reaction mixture to cool to room temperature for 5~10 minutes, during which single-stranded oligos anneal.
- d. Centrifuge the annealing mixture for 3~5 seconds and mix gently. The concentration of the double-stranded DNA oligo is 50 μM.
- e. Dilute 1 μl of the 50 μM double-stranded DNA oligo mixture into 99 μl of DNase/RNase-free water to obtain a final concentration of 500 nM and vortex to mix thoroughly.
- f. Dilute 1 μl of the 500 nM double-stranded DNA oligo mixture into 99 μl of 1× Oligo Annealing Buffer as follows to obtain a final concentration of 5 nM and vortex to mix thoroughly.
- | | |
|-------|----------------------------------|
| 1 μl | 500 nM double-stranded DNA oligo |
| 10 μl | 10× Oligo Annealing Buffer |
| 89 μl | DNase/RNase-free Water |
- g. Aliquot the 5 nM double-stranded DNA oligo stock for subcloning. Store the remainder of the 50 μM, 500 nM and 5 nM double-stranded DNA oligos at -20°C.

The DNA sequences of these synthetic fragments, which include CB1 intracellular loop1, 2 and extracellular loop1, 2, 3 (CB1-IL1/IL2/EL1/EL2/EL3), D2 intracellular loop1, 2 and C-terminal (D2-IL1/IL2/CT) and three motifs of the fourth fragment of the D2 intracellular loop3 (D2-IL3 IV1/2/3), were listed as follows (all sequences with 5' -3' direction, F: sense, R: anti-sense).

CB1-IL1 (144~155aa/36bp)

F: GATCCCACTCCCAGTCTCCGATGCAGGCCTTCCTACCACTGAG

R: AATTCTCAGTGGTAGGAAGGCCTGCATCGGAGACTGCGGGAGTGG

CB1-IL2 (214~233aa/60bp)

F: GATCCGACAGGTACATATCCATTCACAGGCCTCTGGCCTATAAGAGGATC

GTCACCAGGCCCAAGTGAG

R: AATTCTCACTTGGGCCTGGTGACGATCCTCTTATAGGCCAGAGGCCTGTG

AATGGATATGTACCTGTGCG

CB1-EL1 (177~188aa/36bp)

F: GATCCGACTTCCATGTATTCCACCGTAAAGACAGCCCCAATTGAG

R: AATTCTCAATTGGGGCTGTCTTTACGGTGAATACATGGAAGTCG

CB1-EL2 (257~274aa/54bp)

F: GATCCAAGTCAAGAAGCTGCAATCTGTTTGCTCGGACATTTTCCCCTC

ATTGACGAGTGAG

R: AATTCTCACTCGTCAATGAGTGGGAAAATGTCCGAGCAAACAGATTGCA

GCTTCTTGCAGTTG

CB1-EL3 (367~378aa/36bp)

F: GATCCGACGTCTTCGGGAAGATGAACAAGCTTATCAAGACGTGAG

R: AATTCTCACGTCTTGATAAGCTTGTTTCATCTTCCCGAAGACGTGCG

D2-IL1 (60~70aa/33bp)

F: GATCCTCCCGAGAGAAGGCTTTGCAGACCACCACCAACTGAG

R: AATTCTCAGTTGGTGGTGGTCTGCAAAGCCTTCTCTCGGGAG

D2-IL2 (131~151aa/63bp)

F: GATCCGACAGGTACACAGCTGTGGCAATGCCCATGCTGTATAACACACGC

TACAGCTCCAAGCGCCGATGAG

R: AATTCTCATCGGCGCTTGGAGCTGTAGCGTGTGTTATACAGCATGGGCATT

GCCACAGCTGTGTACCTGTTCG

D2-CT (431~444aa/42bp)

F:GATCCAACATCGAGTTCCGCAAGGCCTTCATGAAGATCTTGCACTGCTGAG

R:AATTCTCAGCAGTGCAAGATCTTCATGAAGGCCTTGCGGAACTCGATGTTG

D2-IL3 (IV1) (306~317aa /36bp)

F: GATCCACTCTCCCTGATCCATCCCACCACGGCCTACATAGCTGAG

R: AATTCTCAGCTATGTAGGCCGTGGTGGGATGGATCAGGGAGAGTG

D2-IL3 (IV2) (318~329aa/36bp)

F: GATCCAACCCTGACAGTCCTGCCAAACCAGAGAAGAATGGGTGAG

R: AATTCTCACCCATTCTTCTCTGGTTTGGCAGGACTGTCAGGGTTG

D2-IL3 (IV3) (330~340aa/33bp)

F: GATCCCACGCCAAGATTGTCAATCCCAGGATTGCCAAGTGAG

R: AATTCTCACTTGGCAATCCTGGGATTGACAATCCTGGCGTGG

2.2.5 Cloning and Subcloning

DNA fragments from RT-PCR or direct synthesis were cloned and subcloned into specific vectors to get recombinant plasmids by ligation after the digestion of DNA fragments and vectors with appropriate restriction enzymes (all enzymes from Fermentas, Burlington, ON). Ligation is a process by which two DNA fragments, a small DNA insert containing target DNA and a plasmid vector, are combined together by using T4 DNA ligase (Fermentas, Burlington, ON).

All the DNA fragments described previously, except for the full length D2 receptor, were cloned into pGEX-2T vector to get the GST fusion proteins in *E.coli* cells. The pGEX-2T vectors and the fragments produced by RT-PCR were digested with “BamHI” & “EcoRI” restriction enzymes to obtain sticky ends for ligation. The synthesized and then annealed double-stranded DNA fragments with the blunt ends could be directly inserted into the digested pGEX-2T vectors. The full length D2 receptor was cleaved with “EcoRI” & “XhoI” restriction enzymes and then ligated into pGEX-6P-1 and pET-28a(+) vectors for expressing its fusion proteins with GST or 6His. The full length CB1 receptor was also subcloned into pGEX-6P-1 and pET-28a(+) vectors with “BamHI” & “EcoRI” restriction sites. To over-express the full length CB1 and D2 receptors and their “-myc” or “-FLAG” tagged recombinant proteins in mammalian cells, their gene coding sequences were inserted into pcDNATM3.1 vectors. For this subcloning, the DNA fragments of the full length D2 and CB1 receptors were respectively digested with “HindIII” & “EcoRI” and “BamHI” & “EcoRI” restriction enzymes. As to the digestion, the reaction was allowed to

incubate at 37°C for 2~3 hours whereas the reaction of the ligation was incubated at 22°C for 2 hours or at 4°C overnight.

After cloning and subcloning, the ligation reactions were transformed into DH5 α competent *E.coli* cells to select the correct insert-containing clones of each recombinant plasmid for sequencing.

2.2.6 Transformation

Bacterial transformation is a process to introduce a foreign plasmid into bacterial cells and to use the bacteria for amplification of this plasmid. The procedure of transformation includes the following steps.

- a. Remove competent *E.coli* cells from -70°C freezer and place directly on ice to thaw out for 5~10 minutes.
- b. Add 20 μ l of the ligation reaction of one recombinant plasmid or 1 μ l of selected plasmid into the competent cells and allow sitting on ice for 30 minutes.
- c. Place the mixture in a 42°C water bath for exactly 90 seconds and immediately return to ice for 5 minutes.
- d. Incubate the mixture with 600 μ l of LB media at 37°C for 45 minutes and then centrifuge at 5000 g for 5 minutes. Decant most of supernatant leaving the cells to be resuspended in approximately 100 μ l of media.
- e. Spread the cells on a LB-agar plate containing the appropriate selection components and allow the transformed cells to grow overnight in a 37°C incubator. The use of

special blue-white screening with IPTG (isopropylthio- β -galactoside, Promega, Madison, WI) and X-GAL (5-bromo-4-chloro-3-indolyl- β -galactoside, Promega, Madison, WI) was applied for the recombinant plasmids fused with DNA insert and the pGEM-T Easy vector.

2.2.7 Plasmid Purification and Identification

A single clone from a freshly grown LB-agar plate of *E. coli* cells transformed with the recombinant plasmid was cultured in 5 ml of LB media with appropriate antibiotics in a 15ml cultural tube for incubation at 37°C with vigorous shaking. The mini-prep procedure of plasmid DNA purification was then performed with the overnight cultures to isolate plasmid DNA using the manufacturer's protocol (Promega, Madison, WI).

Two methods were applied for the identification of the recombinant plasmids: restriction digestion and inducing expression. In total, 13 PCR products were cloned and subcloned into different vectors and then identified with restriction digestion. Eleven synthetic fragments were ligated into pGEX-2T vector and then identified by inducing expression.

Samples of purified plasmid DNA were digested using appropriate restriction enzymes incubated at 37°C for 2~3 hours to see if the insert was present within the vector. The digestion reaction was run on a 1% agarose gel with EtBr to visualize the cleaved DNA, which was compared to a DNA ladder of known band sizes. Once the appropriate DNA insert was visualized, the sample was prepared for sequencing. (Examples as Figure 2.10)

For inducing expression, the overnight culture described above was 1:100 sub-cultured into 5 ml of LB media adding ampicillin (100 µg/ml, Sigma, St.Louis, MO) at 37°C with vigorous shaking for 1.5 hours. The culture, cooled down to room temperature, was then treated with IPTG at a final concentration of 1.0 mM to induce protein expression and allowed to grow an additional 3~4 hours at 26°C in an orbital shaker. A pellet, obtained by centrifuging 500 µl of the culture at 5000 g for 5 minutes, was boiled for 10 minutes with an equivalent volume of 2× SDS sample buffer (50 mM Tris-HCl, PH 6.8; 4% SDS, Sigma; 50 mM DTT, Sigma; 20% Glycerol, Sigma; and 0.2% Bromophenol Blue, Sigma, St.Louis, MO). After centrifuging the boiled sample at 12,000 g for 10 minutes, 5~10 µl supernatant of the sample was loaded into SDS-PAGE gel for protein staining and destaining (see Section 2.2.9) to make sure if the fusion protein was expressed. One clone showing the inducing expressed fusion protein would be sent for sequencing. (Examples as Figure 2.11)

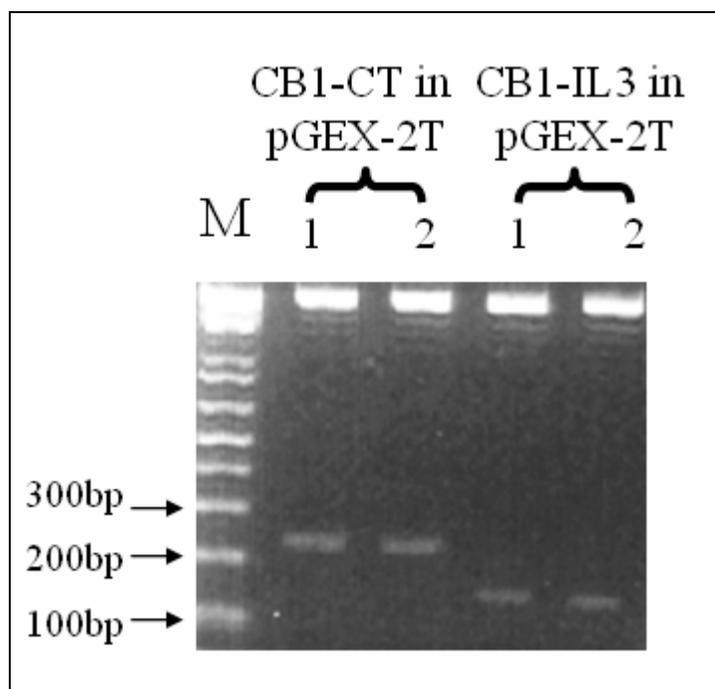


Figure 2.10: Two clones of each recombinant plasmid of “CB1-IL3 in pGEX-2T” and “CB1-CT in pGEX-2T” were identified by restriction digestion with “BamHI” & “EcoRI” restriction enzymes. “CB1-IL3” (135bp) and “CB1-CT” (219bp) were found to be successfully inserted into pGEX-2T vector in their samples. (M: DNA ladder)

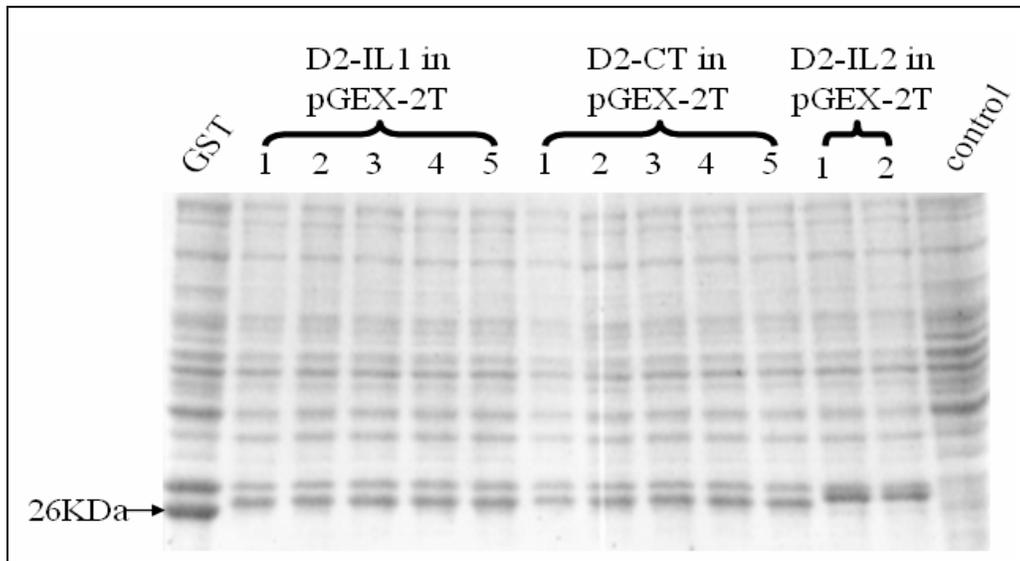


Figure 2.11: Five clones of each recombinant plasmid of “D2-IL1 in pGEX-2T” and “D2-CT in pGEX-2T” and two clones of another recombinant plasmid “D2-IL2 in pGEX-2T” were identified by IPTG inducing expression. Expressed GST protein (GST: 26KDa) and non-inducing expression sample (control) were applied as controls. Results showed that all the clones had been induced to express their own fusion proteins (GST-D2-IL1: 27KDa, GST-D2-CT: 27.5KDa, GST-D2-IL2: 28KDa), suggesting that these three DNA fragments were successfully ligated into pGEX-2T vector.

2.2.8 Sequencing

To confirm correct splice fusion without spurious PCR-generated errors, one appropriate clone for each recombinant plasmid was screened out through “restriction digestion” or “inducing expression” and sent to PBI (Plant Biotechnology Institute) for sequencing. Different primers were applied for forward and reverse sequencing of DNA fragments in diverse vectors as follows.

pGEX-2T Vector:	5' pGEX Sequencing Primer
	3' pGEX Sequencing Primer
pET-28a(+) Vector::	T7 Promoter Sequencing Primer
	T7 Terminator Sequencing Primer
pcDNA TM 3.1 Vectors:	T7 Promoter Sequencing Primer
	BGH Reverse Sequencing Primer
pGEM-T Easy Vector:	pUC/M13 Forward Sequencing Primer
	pUC/M13 Reverse Sequencing Primer

2.2.9 Protein Staining and Destaining

After loaded into SDS-PAGE gel, proteins were visualized by Coomassie Blue staining and destaining and then compared to protein standards. Staining was accomplished by soaking the gel in the stain solution (45% methanol, EMD Chemicals, Gibbstown, NJ; 10% acetic acid, BDH Chemicals, Toronto, ON; 0.25% Coomassie Blue R-250, EM Science, Gibbstown, NJ; and 45% deionized water) on a shaker for 30 minutes or overnight at room temperature. A quick staining could be performed by heating the stain/gel in a

microwave oven for ~10 seconds until the solution was sufficiently warm but did not boil, then shaking for ~10 minutes.

To destain gel, the gel was removed from the stain solution and soaked in the destain solution (30% methanol, 10% acetic acid and 60% deionized water) with gentle shaking overnight at room temperature. Also, a quick destaining was carried out by microwaving the destain/gel for 10 seconds (heating could be repeated) until sufficiently warm (did not boil), and letting stand with gentle shaking. Periodically replace with the fresh destain solution until desired destaining was achieved.

2.2.10 Over-Expression of Fusion Proteins in *E.coli* Cells

Each recombinant plasmid was sequenced to confirm the correct DNA insert sequence and then transformed into an appropriate strain of competent *E.coli* cells to over-express fusion proteins by inducing with IPTG. As mentioned previously, transformation of BL21 competent cells was applied for the over-expression of GST fusion proteins whereas transformation of DE3 competent cells was employed to over-express 6His fusion proteins.

The procedures of IPTG inducing over-expression of fusion proteins in *E.coli* cells are listed below.

- a. Pick a single clone from a freshly cultured LB-agar plate of *E. coli* cells transformed with the sequenced recombinant plasmid and disperse it in 3~5 ml of LB media with appropriate antibiotics (ampicillin, Sigma, St.Louis, MO; or kanamycin, EMD

Chemicals, Gibbstown, NJ) in a 15ml cultural tube for incubation at 37°C overnight with vigorous shaking.

- b. Sub-culture 400 µl of the overnight culture into 40 ml of LB media adding appropriate antibiotics in a 250 ml flask and incubate at 37°C with vigorous shaking for 1.5 hours.
- c. Cool down the above culture to room temperature and then induce with IPTG to a final concentration of 1.0 mM. Allow the cells to grow an additional 3~4 hours at 26°C in an orbital shaker.
- d. Take 500 µl of the culture for protein staining and destaining (see Section 2.2.7 for “inducing expression”) to confirm that the fusion protein was expressed in the *E.coli* cells.
- e. Transfer the culture into a 40 ml centrifuge tube and harvest the cells by centrifuging at 3000 g for 3 minutes at 4°C.
- f. Resuspend and wash the cell pellet with 1 ml of ice cold 1× PBS buffer (150 mM NaCl, 13 mM KCl, 10 mM Na₂HPO₄ and 1.7 mM NaH₂PO₄, PH 7.4, all chemicals from EMD Chemicals, Gibbstown, NJ). Transfer the resuspended cells to a 1.5 ml microcentrifuge tube and pellet the cells by centrifuging at 5000 g for 3 minutes.
- g. Pour off the supernatant and store the cell pellet at -20°C for later use.

2.2.11 Purification of Fusion Proteins

Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) and Ni-NTA His•Bind Resins (Novagen, Mississauga, ON) were applied for purification of GST and 6His fusion proteins, respectively. Glutathione Sepharose 4B (Glutathione immobilized on sepharose beads) is designed for the rapid and convenient single step purification of GST fusion proteins, recombinant derivatives of glutathione S-transferase. Ni-NTA His•Bind Resins contain metal(Ni^{2+})-chelating reactive nitrilotriacetic acid (NTA) groups covalently attached to a solid support and the immobilized Ni^{2+} has the remarkable affinity for a tag of six consecutive histidine residues (6His tag). Thereby, they are chosen to allow one-step purification of 6His-tagged proteins under native or denaturing condition.

Purification of GST or 6His fusion proteins was performed by the following procedures.

- a. Bacterial Cell Lysis (all steps should be done on ice)
 - a) Add 500 μl of ice cold “Bacterial cell lysis buffer” (50 mM Tris-HCl pH 8.0; 0.25% Sodium Deoxycholate, Sigma, St.Louis, MO; 0.15 M NaCl; 1% Nonidet P40, EM Science, Gibbstown, NJ; and 1% Tritoon X-100, Sigma, St.Louis, MO) containing 1 μl (1:500) of protease inhibitors (for bacterial cells, Sigma, St.Louis, MO), 1.0 mM DTT and 0.5 mg/ml lysozyme (Sigma, St.Louis, MO) to the fresh or frozen cell pellet from 1ml of bacterial culture (as described in Section 2.2.10) in a 1.5 ml microcentrifuge tube.

- b) Incubate the cell pellet on ice for approximately 30 minutes and resuspend by pipetting or gentle mixing every few minutes until the solution becomes clear;
- c) Further lyse cells with ultrasonic disruption (0.5-MHz ultrasound for 10 seconds × 20 times) and centrifuge at 12,000 g for 10 minutes at 4°C to obtain the supernatant of the cell lysate.

b. Preparation of Matrix

- a) Gently shake the bottle to resuspend the matrix of Glutathione Sepharose 4B or Ni-NTA His•Bind Resins and transfer sufficient slurry (20~30 µl) to a 1.5 ml microcentrifuge tube.
- b) Sediment the matrix by centrifuging at 5000 g for 30 seconds and then carefully remove the supernatant.
- c) Wash the matrix with 500 µl of ice cold “Bacterial cell lysis buffer” by inverting the tube several times to mix.
- d) Collect the matrix by centrifugation at 5000 g for 30 seconds and then carefully decant the supernatant.
- e) Repeat matrix equilibration steps c) and d) twice for a total of 3 washes and gently resuspend the matrix in 50 µl of ice cold “Bacterial cell lysis buffer” after the final wash.

c. Binding of Fusion Proteins

- a) Add the supernatant of the cell lysate, prepared as described above, to the equilibrated matrix and mix gently by pipetting or inverting.

- b) Incubate the sample with gentle mixing on a rotating platform for 1 hour at 4°C for binding of fusion proteins.
- d. Washing of Nonspecific Proteins (samples keep on ice)
- a) Pellet the matrix by centrifuging at 5000 g for 30 seconds and then carefully remove the supernatant.
 - b) Add 800 µl of ice cold “Bacterial cell lysis buffer” to the matrix and mix gently by inverting the tube several times.
 - c) Collect the matrix by centrifugation at 5000 g for 30 seconds and then carefully decant the supernatant.
 - d) Repeat Washing Steps twice for a total of 3 washes and gently resuspend the matrix in 50 µl of ice cold “Bacterial cell lysis buffer” after the final wash.
- e. Elution and Identification of Fusion Proteins
- a) Boil 10 µl of the above resuspended slurry for 10 minutes with 10 µl of 2× SDS sample buffer to elute the bound fusion proteins from the matrix.
 - b) Load 5~10 µl supernatant of the boiled sample, centrifuged at 12,000 g for 10 minutes, into SDS-PAGE gel for protein staining and destaining to visualize the purified fusion proteins in comparison with protein standards.
 - c) Store the remainder of the resuspended slurry at 4°C for later use.

2.2.12 Expression of Proteins in Mammalian Cells

High-level stable and non-replicative transient gene expression can be carried out in most mammalian cells through the transfection of the recombinant plasmids fused with gene DNA fragments and pcDNATM3.1 vectors. Here, two mammalian cell lines, HEK293 cells and PC12 cells, were transfected with five recombinant plasmids, “CB1 in pcDNATM3.1/*myc*-His(B)”, “CB1 in pcDNATM3.1/FLAG(B)”, “CB1(TGA) in pcDNATM3.1/*myc*-His(B)”, “D2 in pcDNATM3.1/*myc*-His(B)”,” and “D2 in pcDNATM3.1/FLAG(B)”, to transiently express the full length CB1 and D2 receptors and their “-myc” or “-FLAG” tagged fusion proteins.

To obtain sufficient plasmids for transfection, the sequenced correct recombinant plasmids were transformed into JM109 competent cells for amplification. After purification and quantitation, 50 µl of aliquot plasmid DNA were stored at -20°C for transfection. The following procedures were employed for transient transfection with LipofectamineTM 2000 reagent (Invitrogen, Carlsbak, CA).

- a. Trypsinize with Trypsin-EDTA (Gibco, BRL) and plate the cells in 6-well plates (collagen-coated plates for PC12 cells) at 1×10^6 cells per well with 2~3 ml of their normal growth medium without antibiotics on the day before transfection. The cells would be 90~95% confluence on the day of transfection.
- b. Dilute 2~4 µg of DNA into 125~250 µl of DMEM and 5~10 µl of LipofectamineTM 2000 into 125~250 µl of DMEM for each well of cells to be transfected and incubate the diluted LipofectamineTM 2000 at room temperature for 5 minutes.

- c. Mix the diluted DNA with the diluted LipofectamineTM2000 (from the last step) and then incubate at room temperature for 20 minutes to form DNA-LipofectamineTM2000 complexes.
- d. Add the DNA-LipofectamineTM2000 complexes (250~500 μ l) directly to each well and gently rock the plate back and forth to mix well.
- e. Incubate the transfected cells in a 37°C incubator with 5% CO₂ for 24~72 hours and then harvest the cells for analysis of transgene expression. For the well of cells to grow over 24 hours, replace the growth medium 6 hours after transfection.

To identify transfection efficiency, one well of cells would be transfected with the plasmids containing the green fluorescent protein (GFP) as a control. GFP, which emits green light when excited by blue light, is a spontaneously fluorescent protein isolated from coelenterates. The intrinsic fluorescence of GFP can be visualized in living cells by fluorescence correlation microscopy 24 hours after transfection. Furthermore, RT-PCR (see Section 2.2.3) could be employed for the identification of efficient transfection. The total RNAs, extracted from non-transfected cells (as a negative control) and transfected cells with empty vectors (as another negative control) or recombinant plasmids, were applied for reverse transcription into their cDNAs, followed by the amplification of DNA fragments of the full length CB1 and D2 receptors using PCR with appropriate primers.

The transfected cells were harvested from each well and centrifuged at 1000 g for 5 minutes to remove the media. The cell pellet was then washed with ice cold 1× PBS buffer and lysed by appropriate volume of ice cold “Modified RIPA lysis buffer” (50 mM

Tris-HCl pH 7.4, 0.25% Sodium Deoxycholate, 0.15 M NaCl, 1% Nonidet P40 and 1.0 mM EDTA) containing protease inhibitor (1:100 using for mammalian cells, Sigma, St.Louis, MO). The cell lysate was boiled for 10 minutes with an equivalent volume of 2× SDS sample buffer and centrifuged at 12,000g for 10 minutes. The supernatant would be loaded into SDS-PAGE gel for western blot to analyze if the transgenes were expressed in the transfected cells.

2.2.13 Western Blot (Immunoblotting, IB)

Western blot (Immunoblotting, IB), a technique for the analysis and identification of protein antigens, was performed by the following detailed procedures. Quantitation of total protein concentration was accomplished by the BCA Protein Assay Kit (Pierce, Rockford, IL) with protein standard—BSA (Bovine Serum Albumin) if necessary.

- a. Prepare SDS-PAGE gel consisting of 4% Stacking gel and 10% Resolving gel

(volumes listed below for one piece gel of 0.75 mm thickness).

10% Resolving gel (4 ml)		4% Stacking gel (1.5 ml)	
ddH ₂ O	1.6 ml	ddH ₂ O	0.86 ml
Buffer A	1 ml	Buffer C	0.375 ml
30%AB	1.34 ml	30%AB	0.25 ml
10%APS	20 µl	10%APS	15 µl
10%SDS	40 µl	10%SDS	15 µl
TEMED	4 µl	TEMED	3 µl

Buffer A: 1.5 M Tris-HCL, PH 8.8, 0.5% SDS

Buffer C: 0.5 M Tris-HCL, PH 6.8, 0.5% SDS

(Except for ddH₂O and 10% SDS, other solutions store at 4°C; 30% AB from Bio-Rad, Hercules, CA; APS, SDS, and TEMED from Sigma, St.Louis, MO)

- b. Load up to 10~20 μ l of prepared protein samples per well for gels of 0.75mm thickness and run gel in 1 \times Running buffer (25 mM Tris-HCl, 0.1% SDS and 250 mM Glycine, ICN Biomedicals, Aurora, OH) at 120 V until bromophenol blue dye reaches bottom of gel.
- c. Transfer the proteins from the gel to an Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) in 1 \times Transfer buffer (25 mM Tris-HCl, 250 mM Glycine, 0.00375% SDS and 20% Methanol) at 0.2 A for 2~3 hours on ice.
- d. Briefly wash the membrane for 30 seconds in 1 \times TBST buffer (25 mM Tris-HCl, PH 7.4, 150 mM NaCl and 0.1% Tween-20, Sigma, St.Louis, MO) and soak the membrane in 10~15ml “blocking solution” (5% instant skim milk in 1 \times TBST buffer) to cover the membrane for 30~60 minutes with gentle shaking at room temperature.
- e. Incubate the membrane in a primary antibody diluted with “blocking solution” for 2 hours at room temperature or overnight at 4°C with gentle shaking and then rinse the membrane for 3 \times 10 minutes in 1 \times TBST buffer at room temperature on a shaker.
- f. Incubate the membrane with the HRP-conjugated secondary antibody (1:2000~5000, diluted with “blocking solution”); gently shake at room temperature for 2~3 hours and then rinse the membrane with 1 \times TBST buffer for 5 \times 5 minutes at room temperature on a shaker.
- g. Incubate the membrane for 1 minute in ECLTM Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ) and expose to X-Ray film (KODAK, New Haven, Conn.) for variable exposure times. Develop the film to visualize proteins.

h. Re-expose the membrane, if required. Strip the membrane with 1× stripping buffer (65 mM Tris-HCl, PH 6.8, 2% SDS and 100 mM β-mercaptoethanol, Sigma, St.Louis, MO) for 1 hour at 60°C. Wash the stripped membrane for 3× 10 minutes in 1× TBST buffer at room temperature on a shaker prior to blocking in 5% milk solution.

2.2.14 Pull-Down Assay

The pull-down assay is an *in vitro* affinity chromatography method used to confirm the existence of protein-protein interaction predicted by other research techniques and to identify previously unknown protein-protein interaction. In a pull-down assay, a tagged or labeled bait protein is used to create a specific affinity matrix that will be able to bind and purify prey proteins from a lysate sample, which interact with the bait protein.

Two types of lysate samples were used in pull-down assays in this study. One type of lysate was prepared from the bacterial cells over-expressed GST or 6His fusion proteins (from Section 2.2.10 and preparation as Section 2.2.11.a). To avoid the false positive results produced by the contamination of the pull-down complexes through excessive concentrations of bacterial cell lysate, which dilution of bacterial cell lysate should be first decided for each pull-down assay. Another one was obtained by homogenizing rat striatum tissues (from Section 2.2.1) with homogenizer in ice cold “Modified RIPA lysis buffer” (3 ml of buffer per gram of tissue kept on ice) until the sample was uniformly dissociated. The homogenized tissue lysate was frozen at -70°C until use. To obtain the supernatant, the

homogenized tissue lysate was centrifuged at 14,000g for 15 minutes at 4°C. The total protein concentration of lysate was quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL) with protein standard (i.e. BSA).

The lysate was incubated with purified GST or 6His fusion proteins immobilized on the matrix (from Section 2.2.11) at 4°C nutating overnight. The beads were washed five times with 800 µl of ice cold lysis buffer and then centrifuged at 5000 g for 30 seconds. Eventually, the bound (prey) proteins were eluted by boiling for 10 minutes with an equivalent volume of 2× SDS sample buffer and analyzed by western blot (see Section 2.2.13). The GST protein was always employed as a negative control for all the pull-down assays in present study.

3 RESULTS

3.1 RT-PCR Reactions for Generation of CB1 Receptor and D2 Receptor Fragments

3.1.1 PCR-based Amplification of CB1 Receptor Fragments

The DNA sequences of five longer fragments (over 90 bp) of the CB1 receptor, two full length CB1 (CB1 and CB1-TGA) and CB1-NT/CT/IL3, were amplified by RT-PCR with the cDNA from the rat hippocampal tissue and their specific sense and anti-sense primers.

The full length CB1 fragments (1419 bp, Figure 3.1) were obtained by the performance of 35 cycles of PCR amplification with a denaturation temperature of 94°C for 40 seconds, an annealing temperature of 55°C for 40 seconds and an extension temperature of 72°C for 90 seconds, using *Vent* DNA polymerase, a high-fidelity thermophilic DNA polymerase. After the full length CB1 was successfully amplified, the same DNA template was used to amplify the other three CB1 fragments, i.e. CB1-NT (351 bp, Figure 3.1), CB1-CT (219 bp, Figure 3.2) and CB1-IL3 (135 bp, Figure 3.2). These PCR amplifications were performed for 28 cycles with an annealing temperature of 60°C for 40 seconds and an extension time of 60 seconds, using *pfu* DNA polymerase, which was applied for high-fidelity PCR.

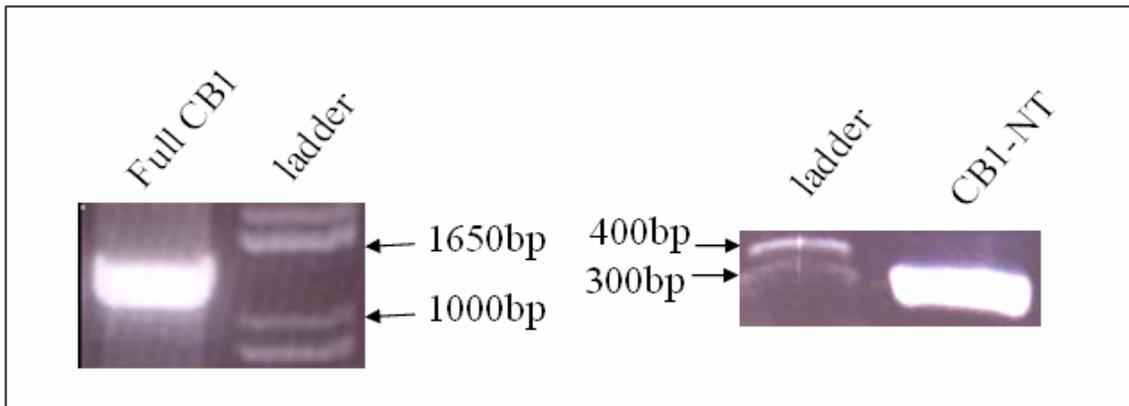


Figure 3.1: PCR products of the full length CB1 receptor (1419 bp) and CB1-NT (351 bp), electrophoresed on a 1% agarose gel and visualized with ethidium bromide (EtBr).

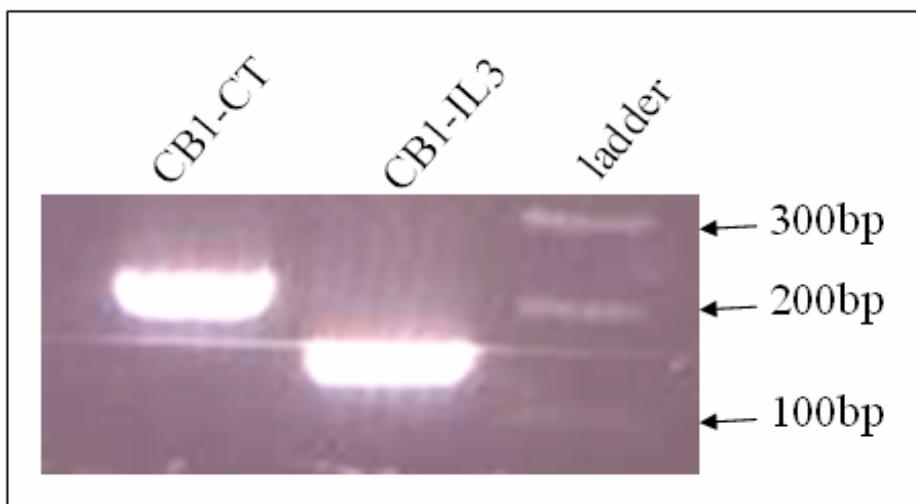


Figure 3.2: PCR products of CB1-CT (219 bp) and CB1-IL3 (135 bp), electrophoresed on a 1% agarose gel and visualized with EtBr.

3.1.2 PCR-based Amplification of D2 Receptor Fragments

In the same way, RT-PCR was employed to amplify DNA sequences of the full length D2 receptor and its longest domain, intracellular loop3 (D2-IL3), from the cDNA reversely transcribed from the rat hippocampal tissue. These samples (preparation as Section 2.2.3.3) were exposed to 30 cycles of PCR amplification with a denaturation temperature of 94°C for 40 seconds, an annealing temperature of 56°C for 40 seconds and an extension temperature of 72°C for 90 seconds, using *Vent* DNA polymerase.

As mentioned previously, there were two different PCR products of the full length D2 receptor. One PCR product flanked by “HindIII” & “EcoRI” (no “TGA”) was first cloned into pGEM-T Easy vector after the base “A” (adenosine) was added to the ends (3′) of this DNA fragment by holding the purified PCR product with 1 mM dATP, 1× PCR buffer and *Taq* DNA polymerase at 72°C for 2 hours. This fragment was then subcloned into pcDNATM3.1 vectors after digested with “HindIII” & “EcoRI” from the pGEM-T Easy vector.

The five fragments of D2-IL3 (D2-IL3 I / II / III / IV / V) were obtained by PCR from the recombinant plasmid of D2-IL3 as the template DNA, using *pfu* DNA polymerase. The annealing temperature for 25 cycles of these PCR amplifications was 60°C holding for 30 seconds, followed by a 40 seconds extension. The second fragment of D2-IL3 (D2-IL3 II) is the additional 29 amino acids in the intracellular loop3 of the long form D2 receptor.

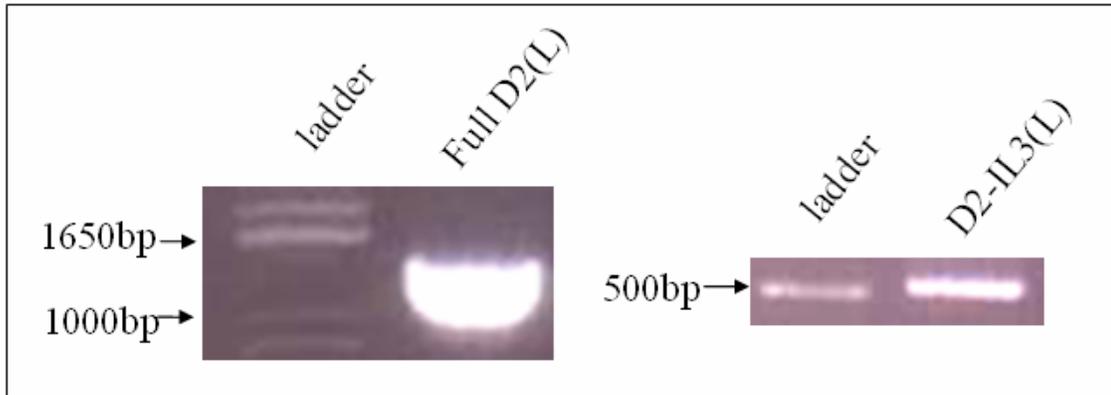


Figure 3.3: PCR products of the full length long form D2 receptor (1332 bp) and its intracellular loop3 (D2-IL3, 492 bp), electrophoresed on a 1% agarose gel and visualized with EtBr.

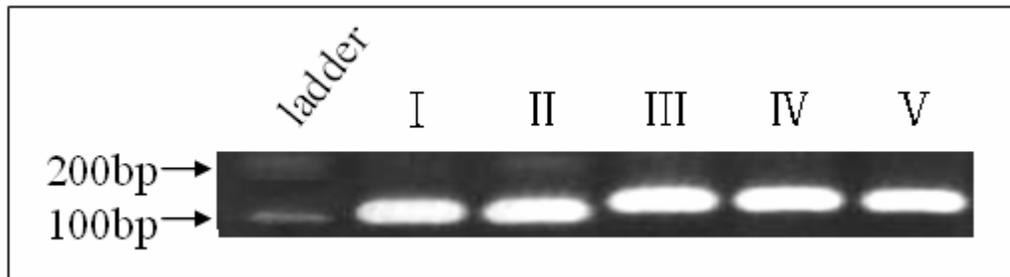


Figure 3.4: PCR products of D2-IL3(I) (93 bp), D2-IL3(II) (87 bp), D2-IL3(III) (105 bp), D2-IL3(IV) (105 bp) and D2-IL3(V) (102 bp), electrophoresed on a 1% agarose gel and visualized with EtBr.

3.2 Chromatograms Showing the Sequences of CB1 Receptor and D2

Receptor Fragments

Each DNA fragment applied in this study was sequenced to confirm the correct sequence after it was cloned into an appropriate vector.

3.2.1 Chromatograms Showing the Sequences of CB1 Receptor Fragments

In total, ten DNA fragments of the CB1 receptor were sequenced in pGEX-2T or pcDNATM3.1/myc-His(B) vector after their cloning.

One fragment of the full length CB1 receptor (no “TGA”) was directly cloned into the pcDNATM3.1/myc-His(B) vector and then forwardly sequenced with “T7 Promoter Sequencing Primer” (Figure 3.5) and reversely sequenced with “BGH Reverse Sequencing Primer” (Figure 3.6). Two bases were different with the DNA sequence found in Genbank, one at “902 bp (A instead of G)” and another at “1067 bp (T instead of C)”. These “substitutions” did not change the translated proteins. Another fragment of the full length CB1 receptor (including “TGA”) was sequenced in the pGEX-2T vector with forward “5’ pGEX Sequencing Primer” and reverse “3’ pGEX Sequencing Primer” to ensure its correct DNA sequence (data not shown).

The other eight fragments of four extracellular and four intracellular domains of the CB1 receptor were cloned into the pGEX-2T vector and then forwardly sequenced with “5’ pGEX Sequencing Primer”. Their sequencing data were illustrated below: CB1-NT (Figure 3.7), CB1-EL1 (Figure 3.8), CB1-EL2 (Figure 3.9), CB1-EL3 (Figure 3.10), CB1-IL1 (Figure 3.11), CB1-IL2 (Figure 3.12), CB1-IL3 (Figure 3.13), and CB1-CT (Figure 3.14).

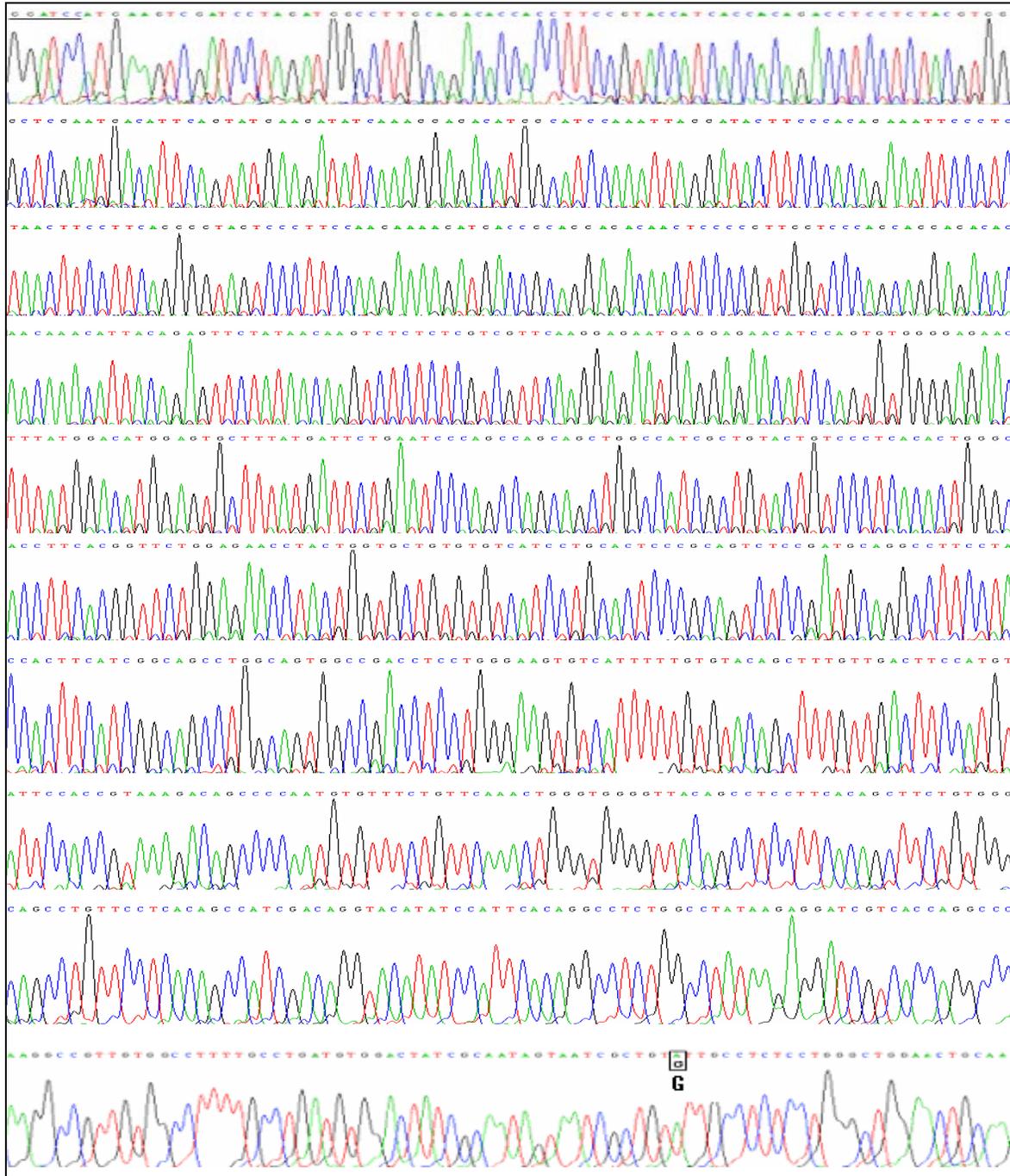


Figure 3.5: Chromatogram of coding sequence (153~928 bp) of the CB1 receptor with the restriction site BamHI (underlined) in pcDNATM3.1/myc-His(B) vector, sequenced with the “T7 Promoter Sequencing Primer”. One base was different from the sequence found in Genbank at “902 bp (A instead of G)” marked by box, but it did not change the translated protein.

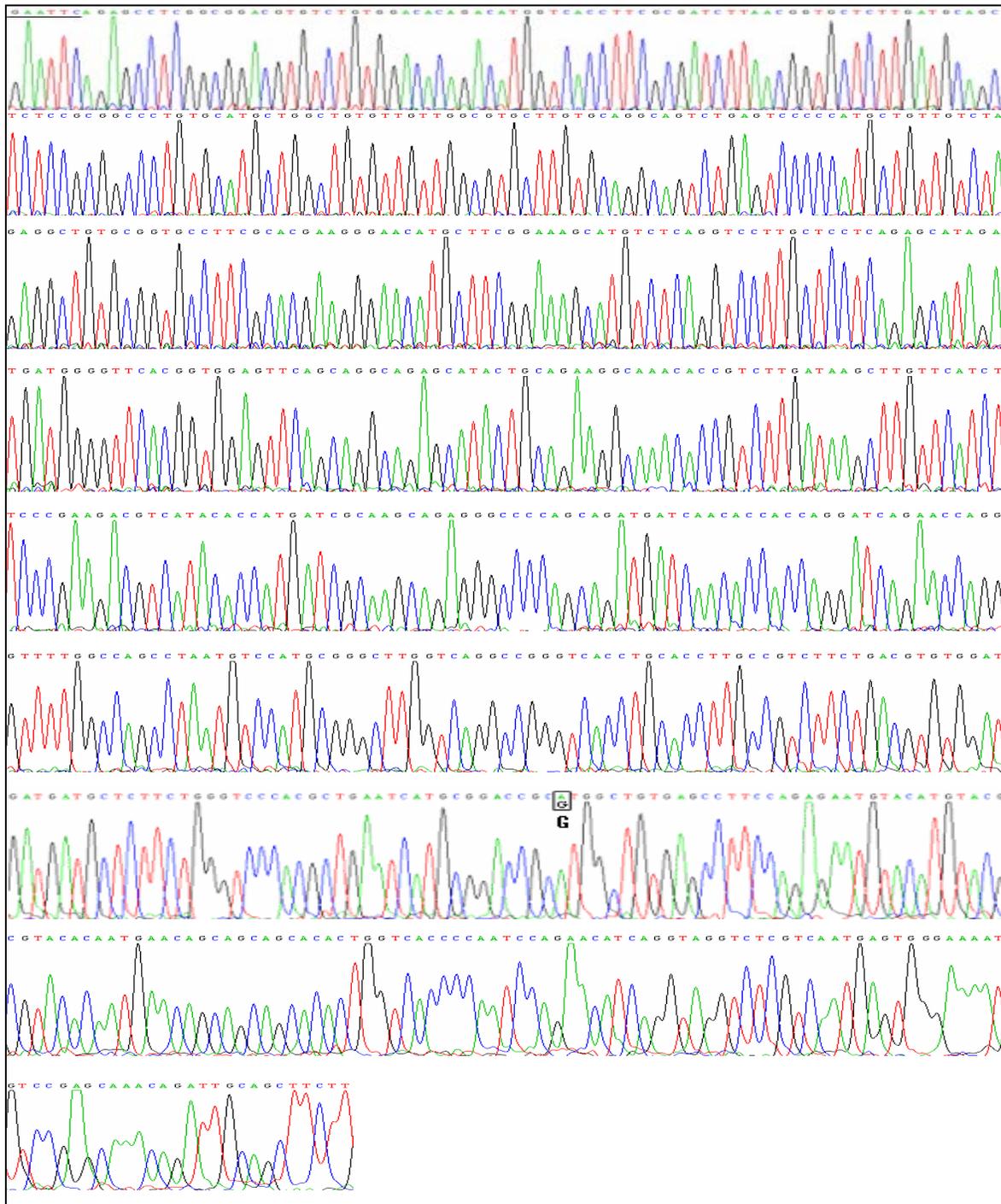


Figure 3.6: Chromatogram of coding sequence (1571~928 bp) of the CB1 receptor with the restriction site *EcoRI* (underlined) in pcDNATM3.1/myc-His(B) vector, sequenced with the “BGH Reverse Sequencing Primer”. One base was different from the sequence found in Genbank at “1067 bp (T instead of C)” marked by box, but it did not change the translated protein.

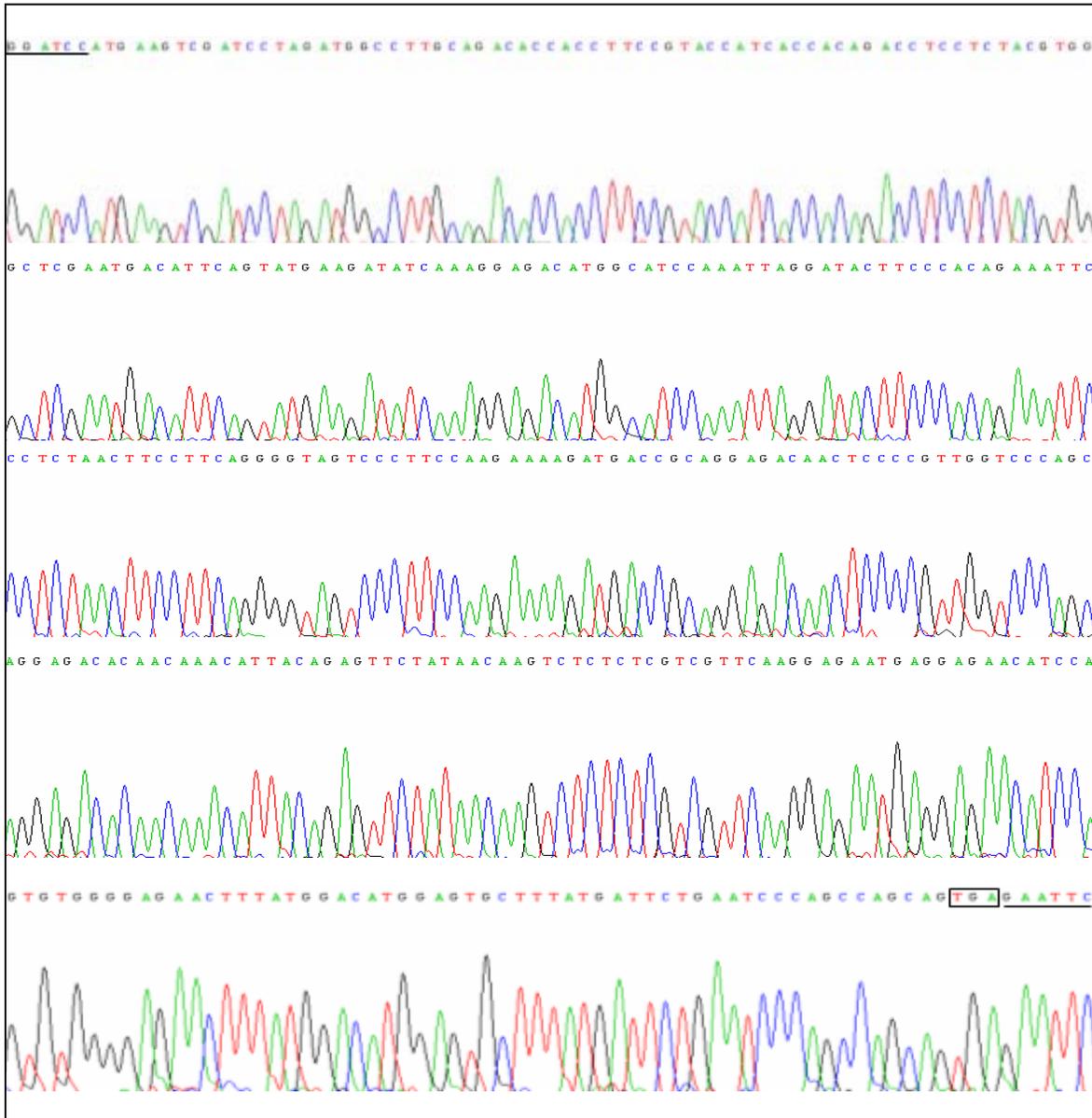


Figure 3.7: Chromatogram of the coding sequence (153~503 bp) of “CB1-NT” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

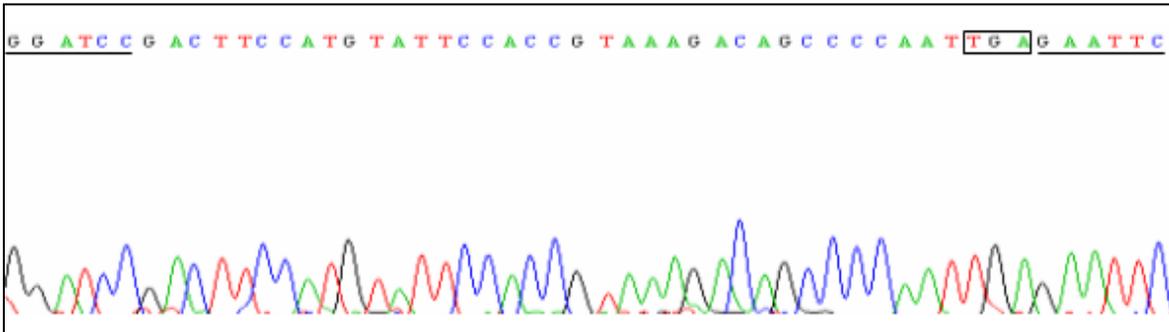


Figure 3.8: Chromatogram of the coding sequence (681~716 bp) of “CB1-EL1” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

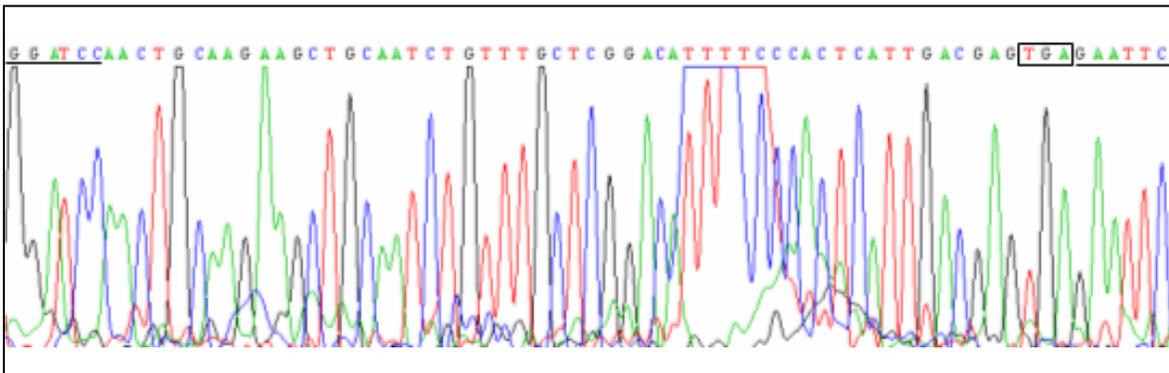


Figure 3.9: Chromatogram of the coding sequence (921~974 bp) of “CB1-EL2” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

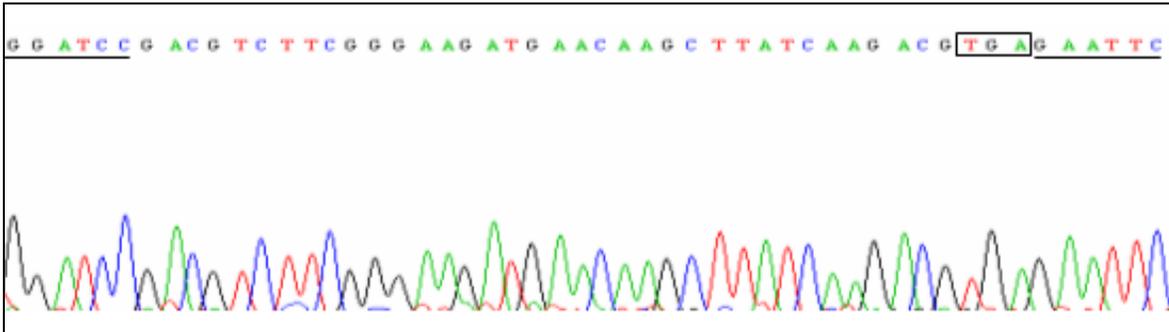


Figure 3.10: Chromatogram of the coding sequence (1251~1286 bp) of “CB1-EL3” in pGEX-2T, sequenced with the “5’ pGEX Sequencing Primer”.

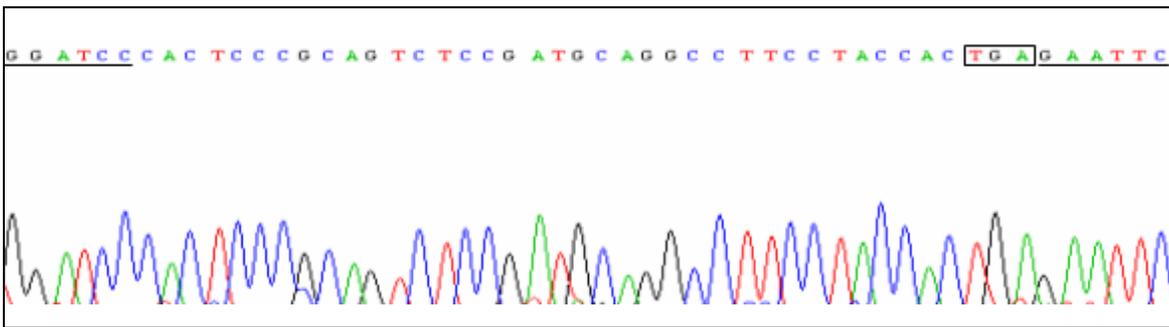


Figure 3.11: Chromatogram of the coding sequence (582~617 bp) of “CB1-IL1” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

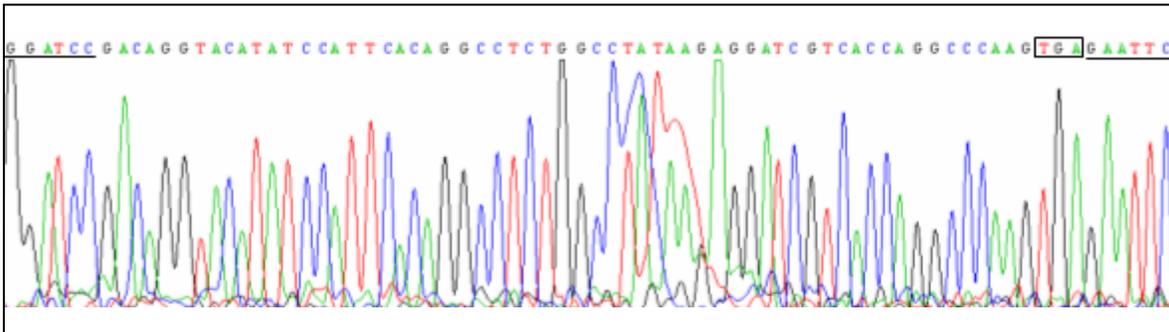


Figure 3.12: Chromatogram of the coding sequence (792~851 bp) of “CB1-IL2” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

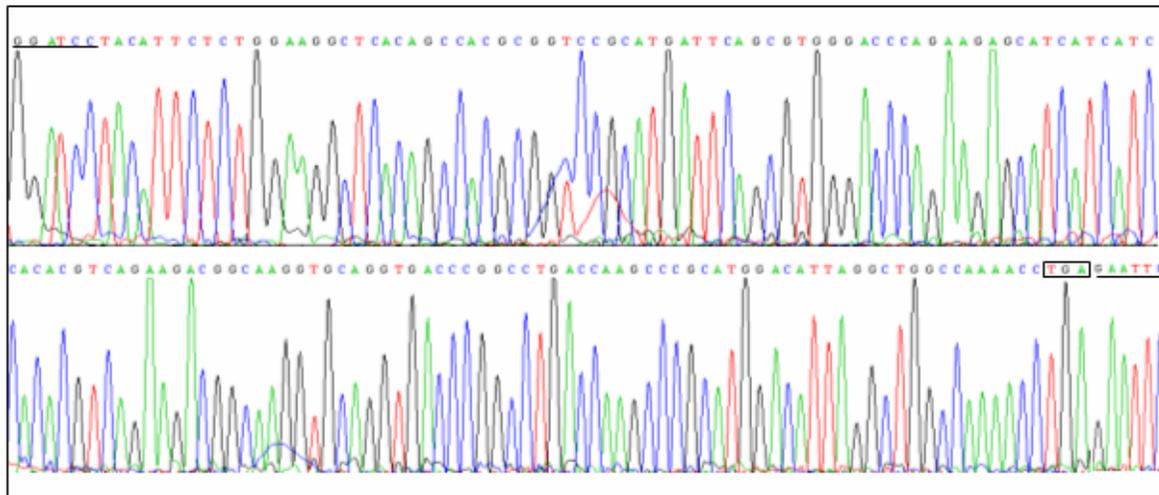


Figure 3.13: Chromatogram of the coding sequence (1053~1187 bp) of “CB1-IL3” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

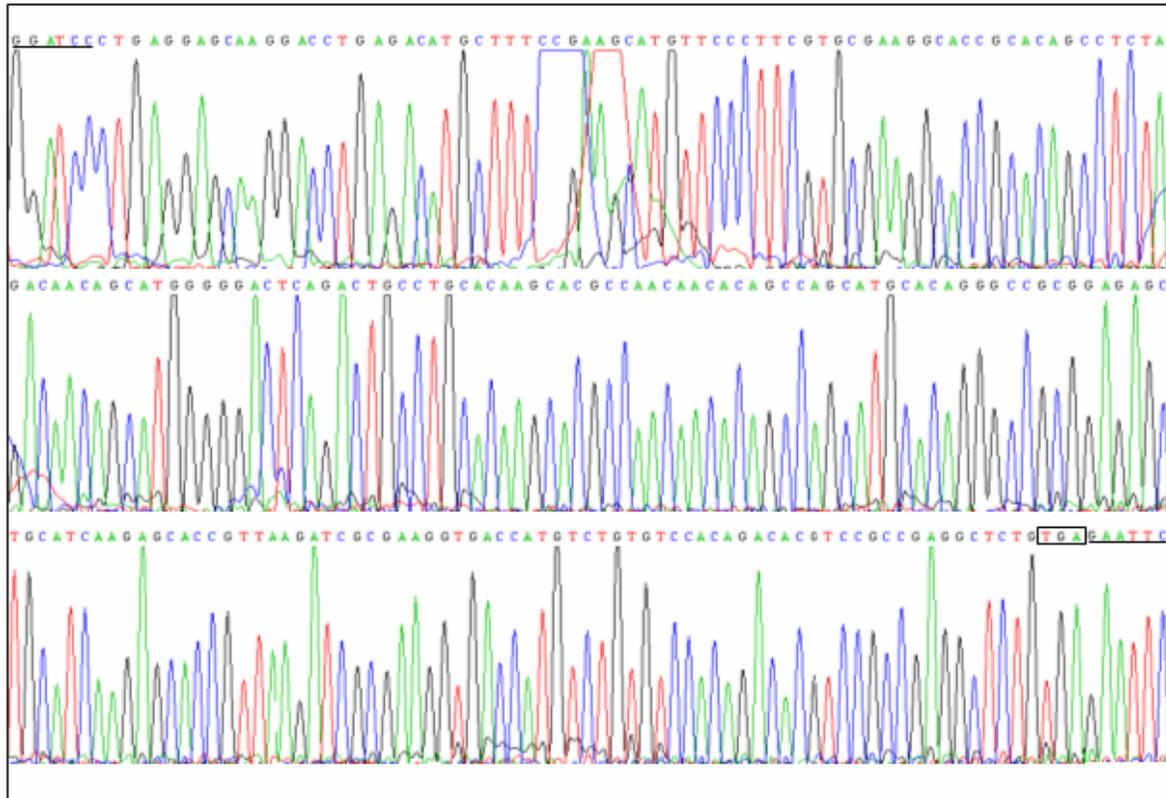


Figure 3.14: Chromatogram of the coding sequences (1353~1571 bp) of “CB1-CT” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

3.2.2 Chromatograms Showing the Sequences of D2 Receptor Fragments

In total, fourteen DNA fragments of the D2 receptor were cloned into pGEM-T Easy, pET-28a(+) or pGEX-2T vectors and then sequenced.

One fragment of the full length D2 receptor, flanked by “HindIII” & “EcoRI” (no “TGA”) for subcloning into pcDNATM3.1 vectors, was cloned into pGEM-T Easy vector, forwardly sequenced with “pUC/M13 Forward Sequencing Primer” (Figure 3.15) and reversely sequenced with “pUC/M13 Reverse Sequencing Primer” (Figure 3.16). One base was different from the sequence found in Genbank at “1210 bp (C instead of T)”, but this “substitution” did not change the translated protein. Another fragment of the full length D2 receptor, incorporated with “EcoRI” & “XhoI” (including “TGA”), was directly cloned into the pET-28a(+) vector and then forwardly and reversely sequenced with “T7 Promoter Sequencing Primer” and “T7 Terminator Sequencing Primer”, respectively. The sequencing result was the same as above: one base was different from the sequence found in Genbank at “1210 bp (C instead of T)” (data not shown).

The three short intracellular domains of the D2 receptor, i.e. D2-IL1 (Figure 3.17), D2-IL2 (Figure 3.18) and D2-CT (Figure 3.19), were sequenced in the pGEX-2T vector with forward “5’ pGEX Sequencing Primer”. The PCR product of the intracellular loop3 (D2-IL3), the longest domain of the D2 receptor, was forwardly sequenced with “T7 Promoter Sequencing Primer” after it was cloned into the pET-28a(+) vector (Figure 3.20). Similar to the sequencing result of the full length D2 receptor, one base different from the sequence found in Genbank was also found at “1210 bp (C instead of T)”. The five

fragments of D2-IL3 (D2-IL3 I / II / III / IV / V, Figure 3.21~25), produced by PCR using “D2-IL3 in pET-28a(+)” as template DNA, were cloned into the pGEX-2T vector and then sequenced with forward “5′ pGEX Sequencing Primer”. The base different from the sequence found in Genbank at “1210 bp (C instead of T)” was identified in the third fragment (D2-IL3 III). In addition, three motifs of the fourth fragment (D2-IL3 IV1/2/3), obtained by annealing with the synthetic DNA oligos, were also cloned into the pGEX-2T vector for forward sequencing (Figure 2.26~28).

In all the illustrated sequencing data, the stop codon “TGA” and the bases different from the sequences found in Genbank were boxed, while the restriction sites incorporated at two ends of each DNA fragment were underlined.

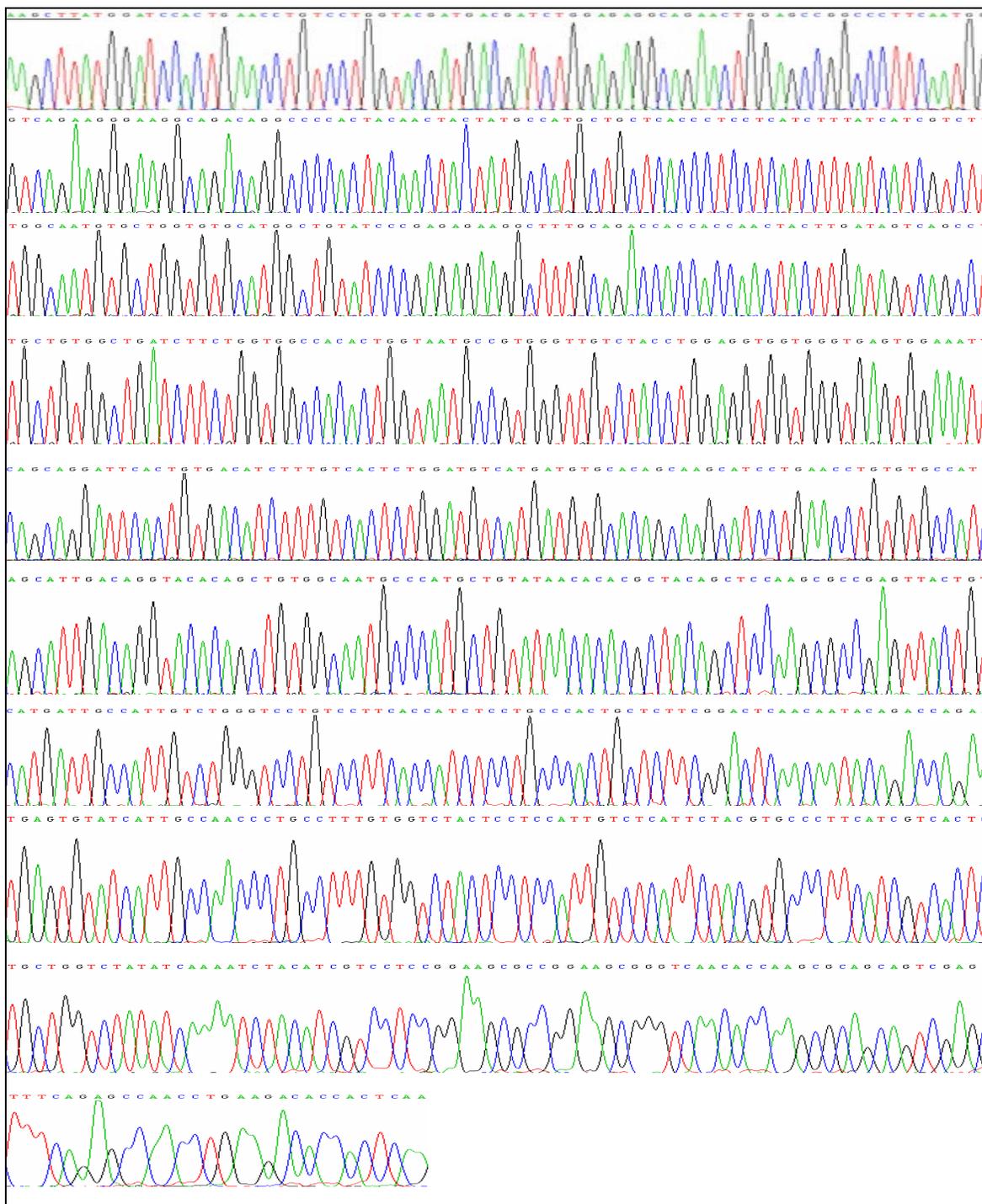


Figure 3.15: Chromatogram of the coding sequence (347~1078) of the long form of the D2 receptor with the restriction site HindIII (underlined) in pGEM-T Easy vector, sequenced with the “pUC/M13 Sequencing Primer”.

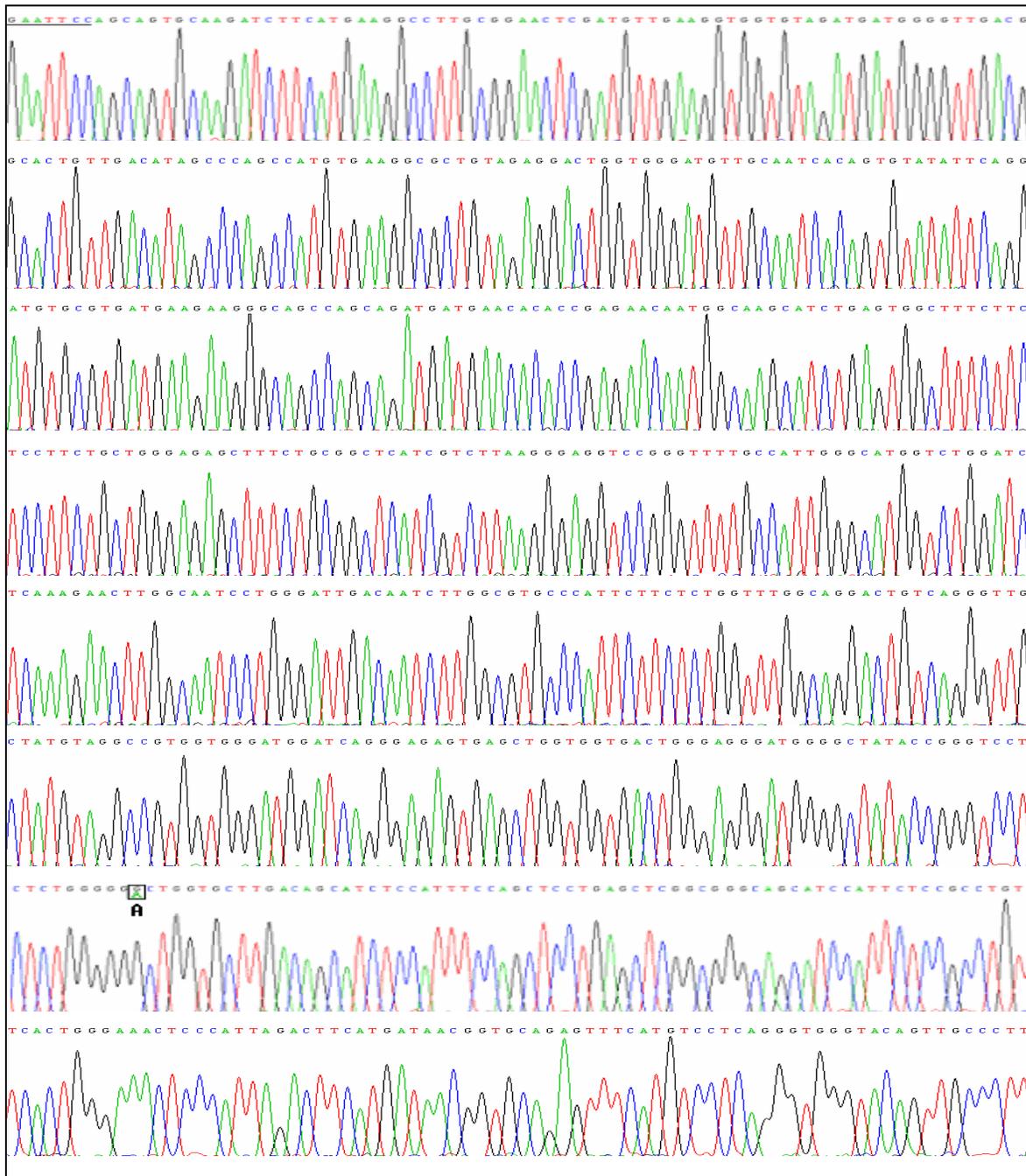


Figure 3.16: Chromatogram of the coding sequence (1678~1078) of the long form D2 receptor with the restriction site EcoRI (underlined) in pGEM-T Easy vector, sequenced with “pUC/M13 Reverse Sequencing Primer”. One base was different from the sequence found in Genbank at “1210 bp (C instead of T)”, but it did not change the translated protein.

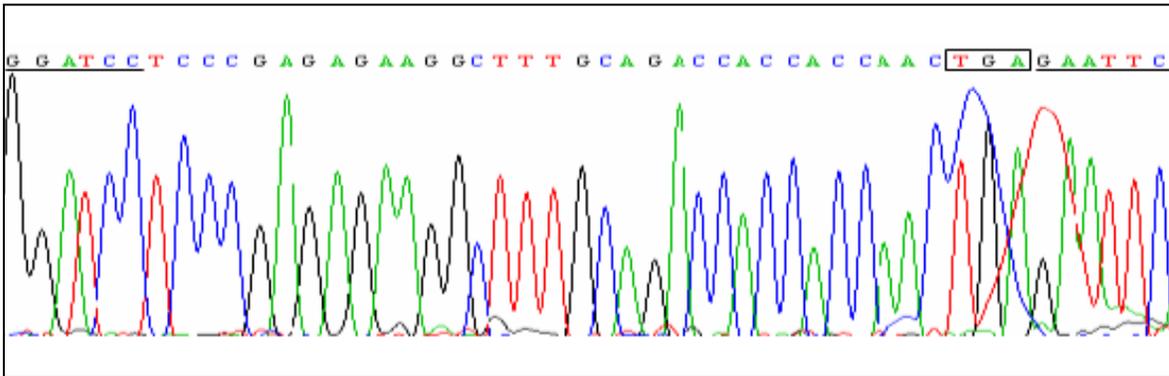


Figure 3.17: Chromatogram of the coding sequence (524~556 bp) of “D2-IL1” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

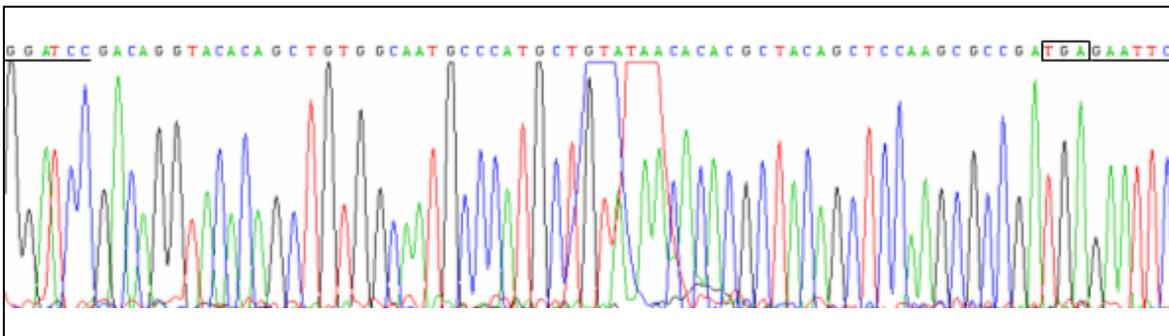


Figure 3.18: Chromatogram of the coding sequence (737~799 bp) of “D2-IL2” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

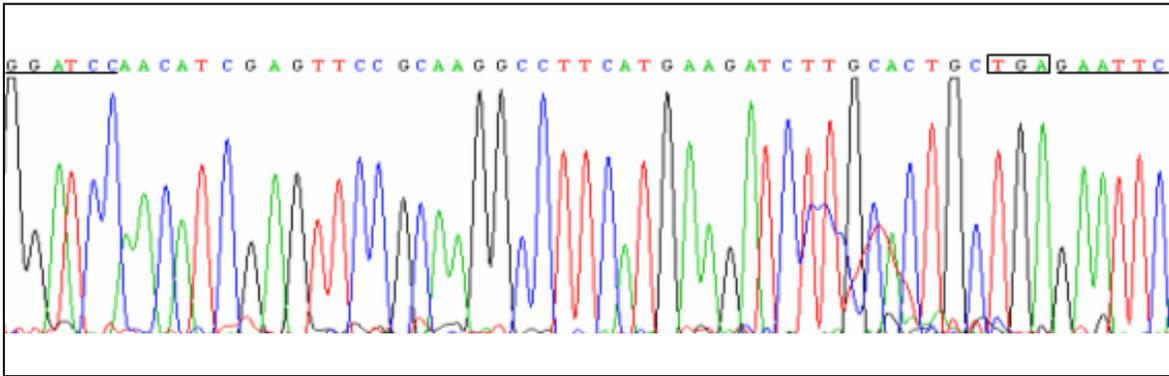


Figure 3.19: Chromatogram of the coding sequence (1637~1678 bp) of “D2-CT” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

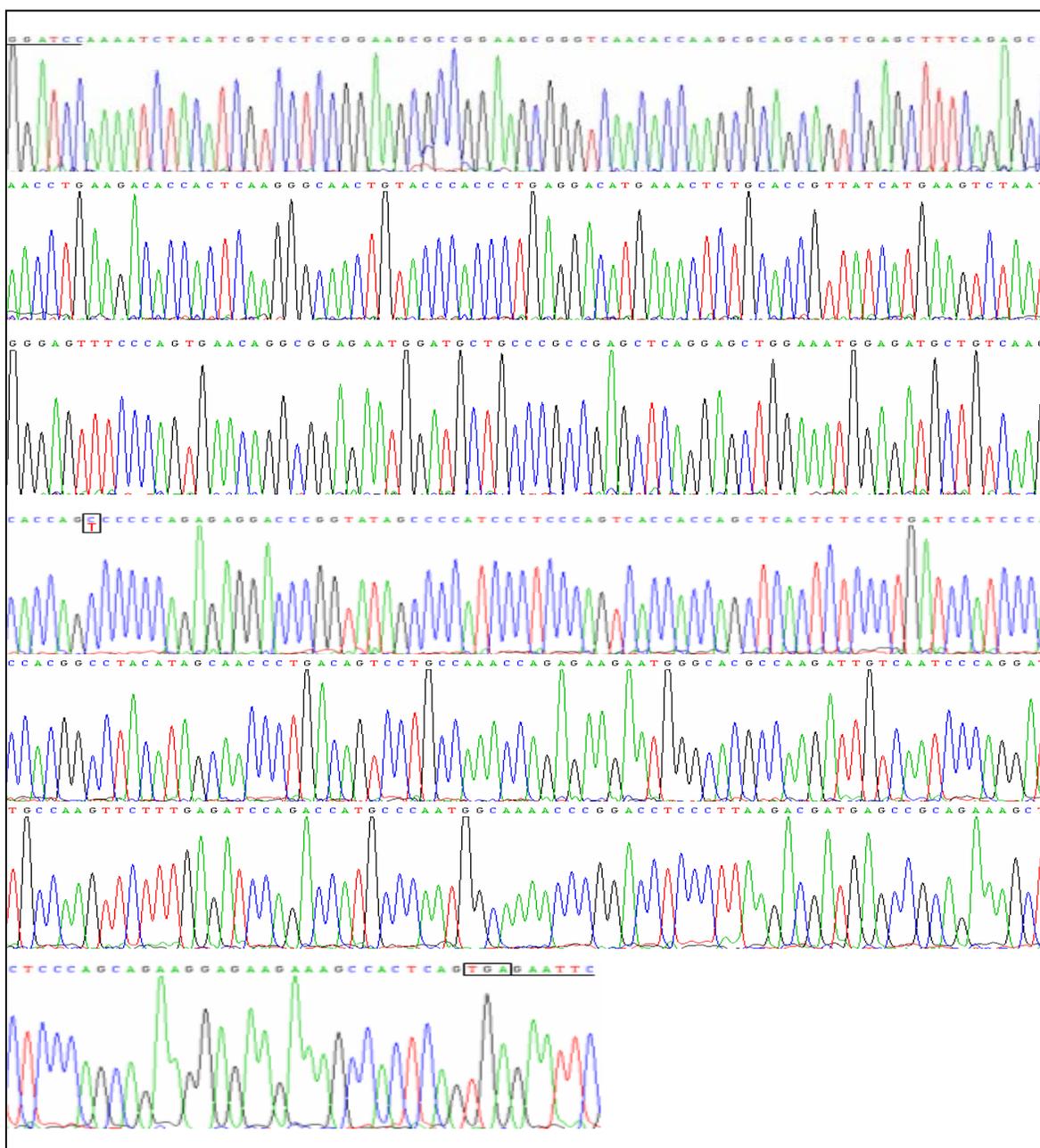


Figure 3.20: Chromatogram of the coding sequence (977~1468 bp) of “D2-IL3 (L)” in pET-28a(+) vector, sequenced with the “T7 Promoter Sequencing Primer”. One base was different from the sequence found in Genbank at “1210 bp (C instead of T)”, but it did not change the translated protein.

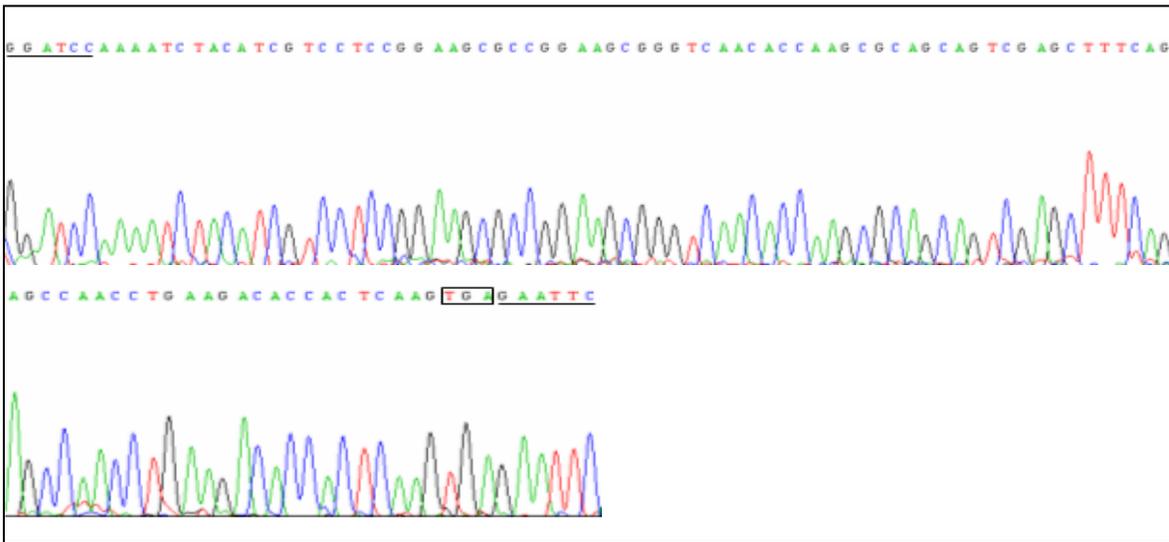


Figure 3.21: Chromatogram of the coding sequence (977~1069 bp) of “D2-IL3 (I)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

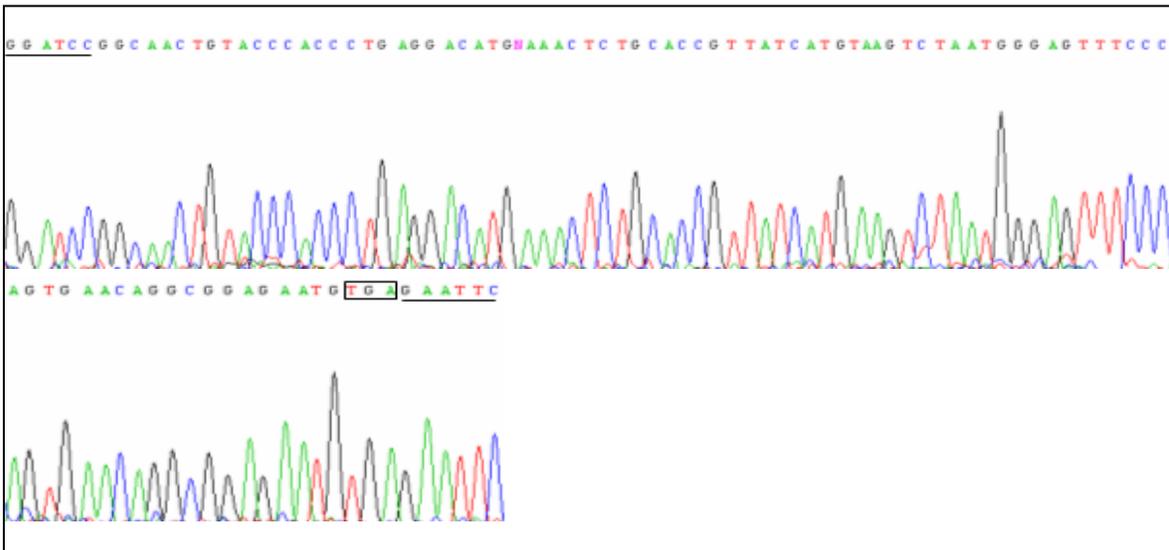


Figure 3.22: Chromatogram of the coding sequence (1070~1156 bp) of “D2-IL3 (II)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

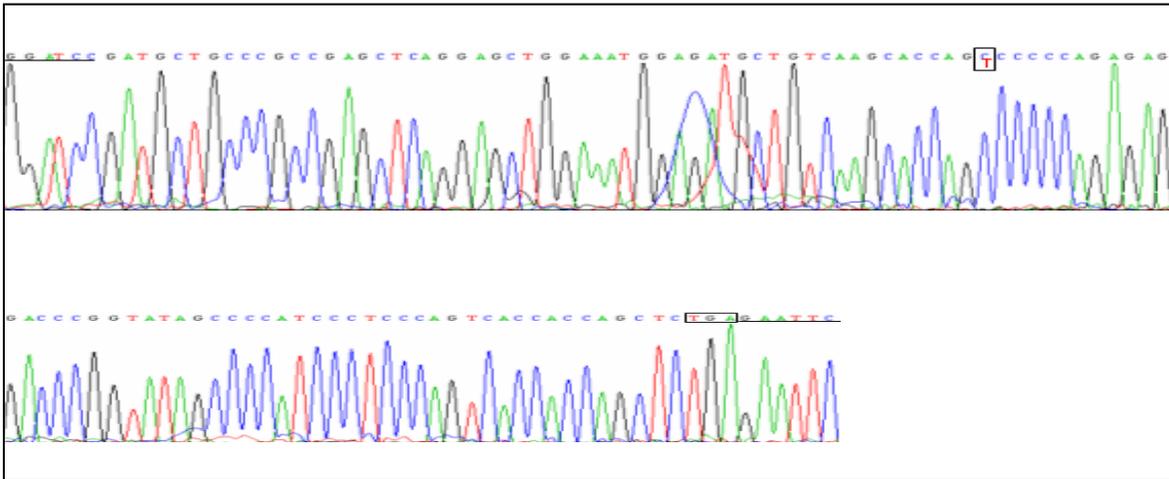


Figure 3.23: Chromatogram of the coding sequence (1157~1261 bp) of “D2-IL3 (III)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”. One base was different from the sequence found in Genbank at “1210 bp (C instead of T)”, but it did not change the translated protein.

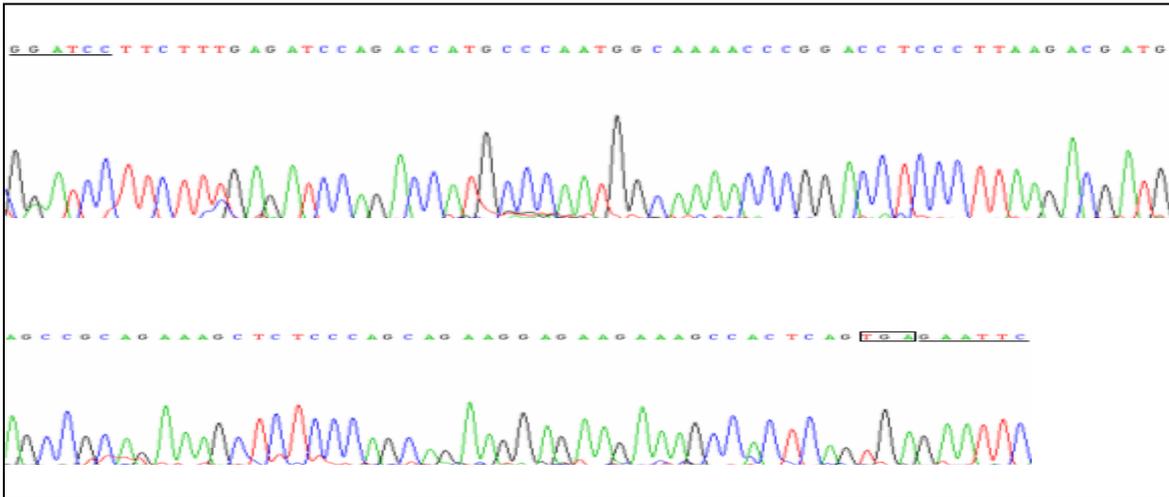


Figure 3.24: Chromatogram of the coding sequence (1367~1468 bp) of “D2-IL3 (V)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

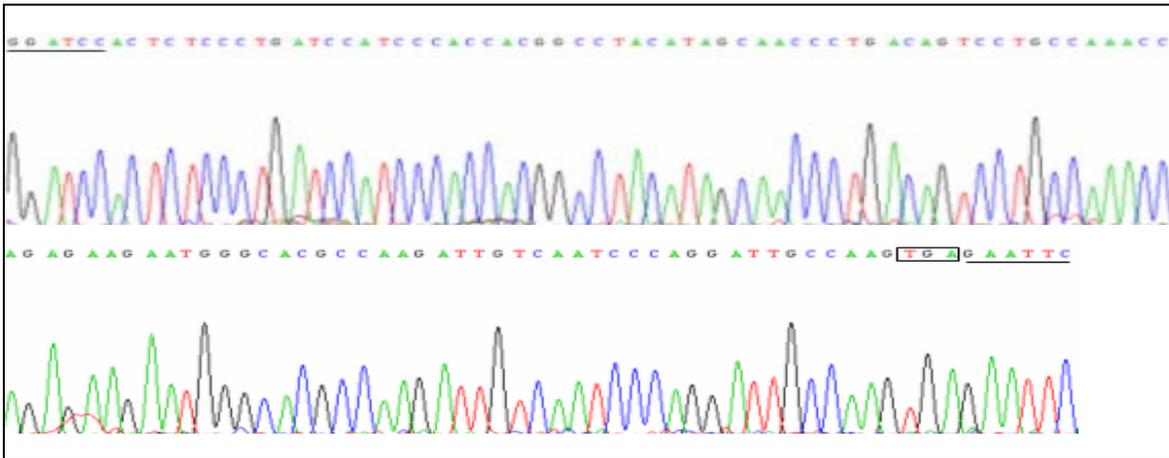


Figure 3.25: Chromatogram of the coding sequence (1262~1366 bp) of “D2-IL3 (IV)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

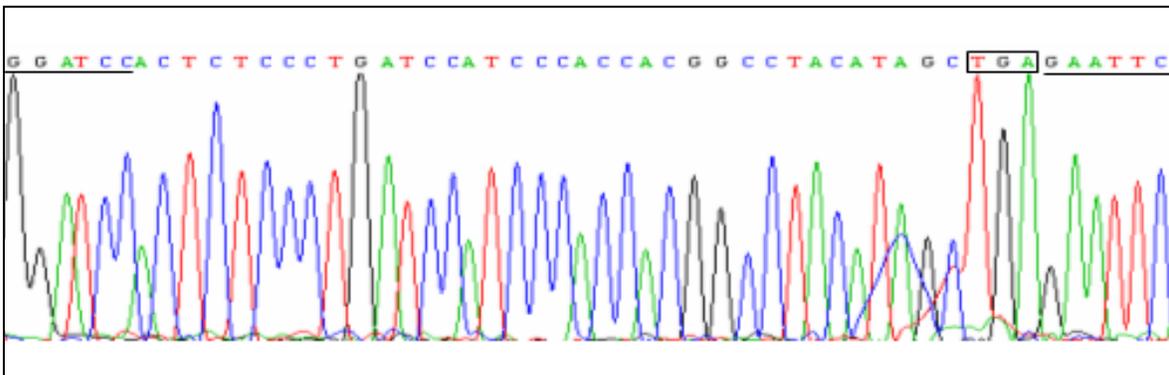


Figure 3.26: Chromatogram of the coding sequence (1262~1297 bp) of “D2-IL3 (IV1)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

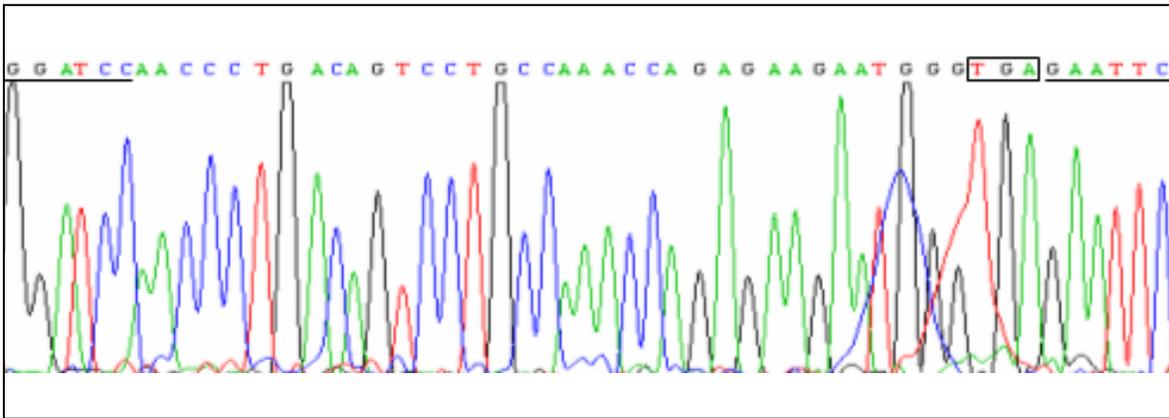


Figure 3.27: Chromatogram of the coding sequence (1298~1333 bp) of “D2-IL3 (IV2)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

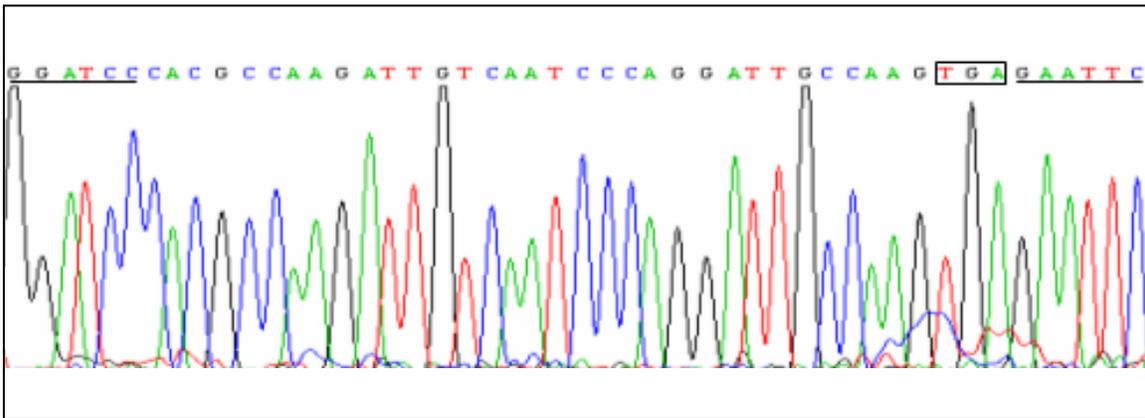


Figure 3.28: Chromatogram of the coding sequence (1334~1366 bp) of “D2-IL3 (IV3)” in pGEX-2T vector, sequenced with the “5’ pGEX Sequencing Primer”.

3.3 Over-Expression and Purification of Fusion Proteins in *E. coli* Cells

To over-express fusion proteins in *E.coli* cells, the recombinant plasmids were transformed into appropriate competent cells and then induced with 1.0 mM IPTG, followed by visualization in SDS-PAGE gel using Coomassie Blue stain. To over-express GST fusion proteins, the DNA fragments were ligated into pGEX-2T or pGEX-6P-1 vector and transformed into BL21 competent cells. However, to over-express 6His fusion proteins, the pET-28a(+) vector was used to clone or subclone the DNA fragments and DE3 competent cells were employed for the transformation of the recombinant plasmids.

The full length CB1 receptor was first cloned into pGEX-2T vector, and then subcloned into pGEX-6P-1 vector (since it could not be expressed in pGEX-2T vector) and into pET-28a(+) vector for obtaining 6His fusion protein of the CB1 receptor. The full length D2 receptor was respectively cloned into pET-28a(+) and pGEX-6P-1 vectors to get its 6His and GST fusion proteins. However, the GST or 6His fusion proteins of the full length CB1 and D2 receptors could not be induced to over-express in *E.coli* cells.

After over-expression, GST and 6His fusion proteins were purified from the *E.coli* cells, respectively, using Glutathione Sepharose 4B beads and Ni-NTA His•Bind resins (see Section 2.2.11).

3.3.1 Fusion Proteins of CB1 Receptor Fragments

The GST fusion proteins of four intracellular domains (GST-CB1-CT/IL1/IL2/IL3) and four extracellular domains (GST-CB1-NT/EL1/EL2/EL3) of the CB1 receptor were successfully over-expressed in BL21 competent cells and purified from these cells (Figure 3.29). The expected molecular weights of these GST fusion proteins are listed below: 27KDa for GST-CB1-IL1/EL1/EL3, 28KDa for GST-CB1-EL2/IL2, 31KDa for GST-CB1-IL3, 34KDa for GST-CB1-CT and 39KDa for GST-CB1-NT.

In another experiment, the DNA fragment of CB1-CT was subcloned into the pET-28a(+) vector to over-express its 6His fusion protein in DE3 competent cells, followed by purification using Ni-NTA His•Bind resins. The molecular weight of this 6His fusion protein of CB1-CT (6His-CB1-CT) was 15KDa (Figure 3.30), which could be visualized in the 14% SDS-PAGE gel through protein staining.

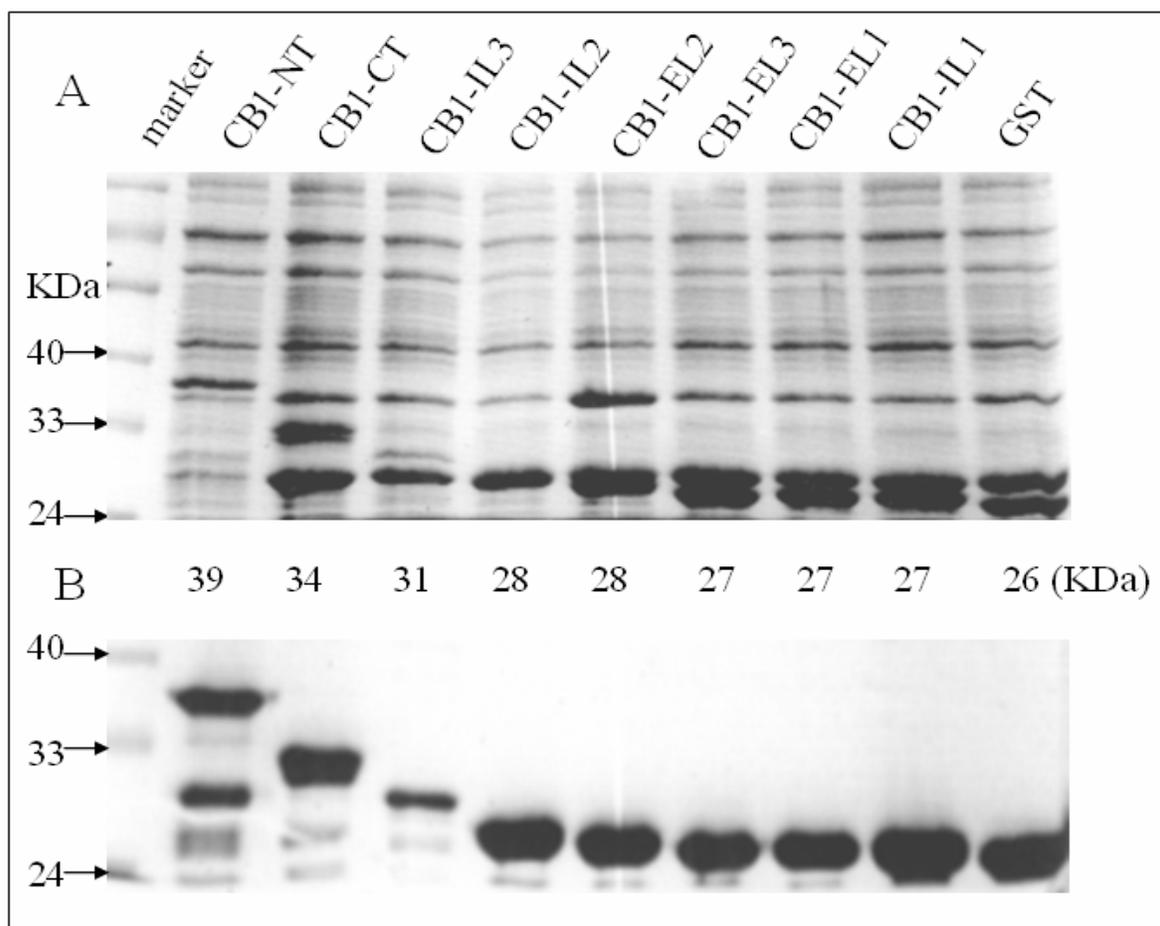


Figure 3.29: Over-expressed (A) and purified (B) GST fusion proteins of four intracellular domains (GST-CB1-CT/IL1/IL2/IL3) and four extracellular domains (GST-CB1-NT/EL1/EL2/EL3) of the CB1 receptor with their expected molecular weights listed between A and B. GST protein as a control.

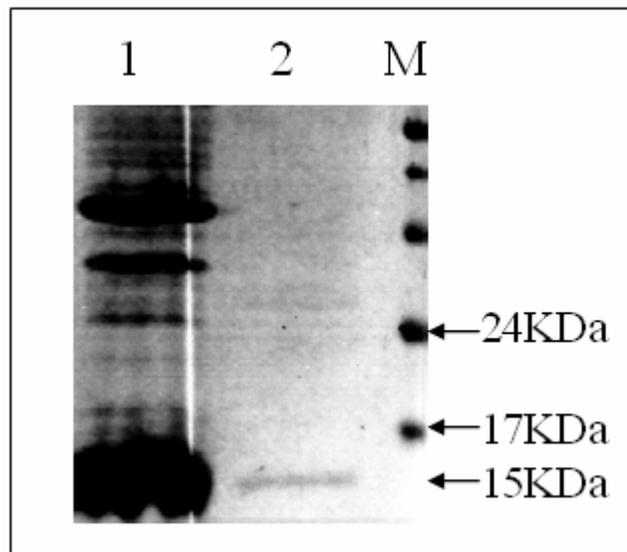


Figure 3.30: Over-expressed and purified 6His fusion protein of CB1-CT.
(6His-CB1-CT: 15KDa)

M: protein marker

1: over-expressed 6His-CB1-CT

2: purified 6His-CB1-CT

3.3.2 Fusion Proteins of D2 Receptor Fragments

The 6His fusion proteins of D2-L3 (6His-D2-IL3: 26KDa) were successfully over-expressed by inducing with IPTG in DE3 competent cells, and then purified by Ni-NTA His•Bind resins (Figure 3.31). In addition, this DNA fragment of D2-IL3 was also subcloned into pGEX-2T for over-expression and purification of the GST fusion protein of D2-IL3 (GST-D2-IL3: 44KDa) in BL21 cells (Figure 3.32). Three other intracellular domains of the D2 receptor were also inserted into pGEX-2T to over-express their GST fusion proteins (GST-D2-IL1: 27KDa, GST-D2-IL2: 28.5KDa and GST-D2-CT: 27.5KDa), which were then purified with Glutathione Sepharose 4B-beads (Figure 3.33).

GST fusion proteins of five fragments of D2-IL3 (GST-D2-IL3 I/II: 29KDa and GST-D2-IL3III/IV/V: 30KDa, Figure 3.34) and three motifs of D2-IL3(IV) (GST-D2-IL3 IV1/2/3: 27KDa, Figure 3.35) were over-expressed after cloning into pGEX-2T vector and transforming into BL21 competent cells.

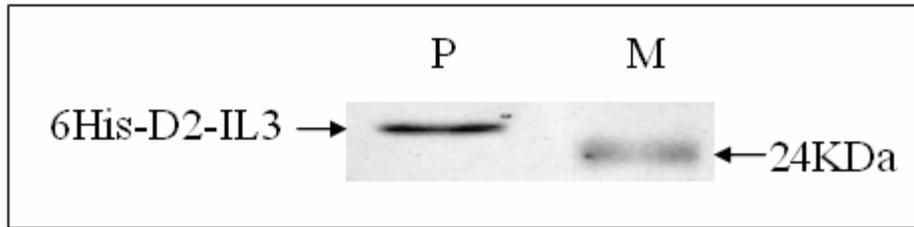


Figure 3.31: Purified 6His fusion protein of D2-IL3. (6His-D2-IL3: 26KDa)
M: protein marker
P: purified 6His-D2-IL3

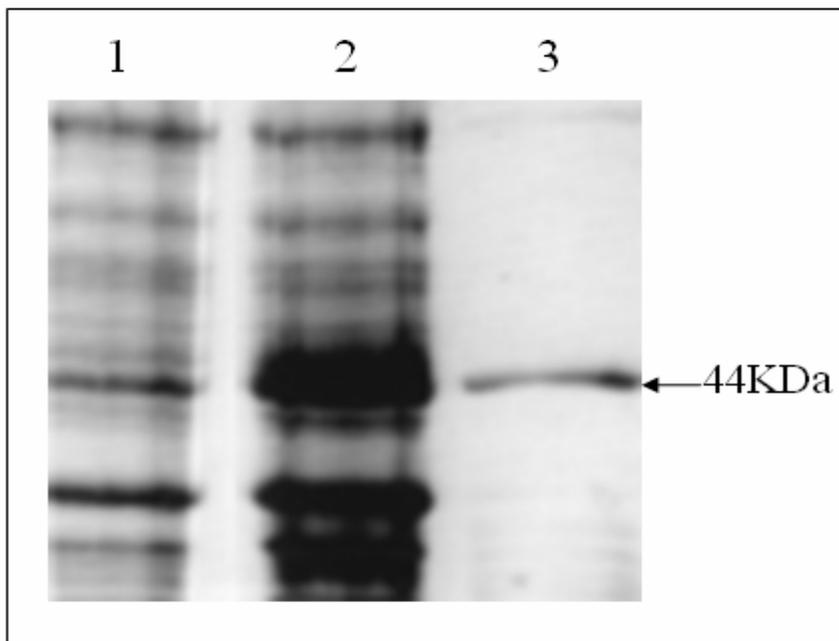


Figure 3.32: Over-expressed and purified GST fusion protein of D2-IL3.
(GST-D2-IL3: 44KDa)
1: control (non-induced sample)
2: over-expressed GST-D2-IL3
3: purified GST-D2-IL3

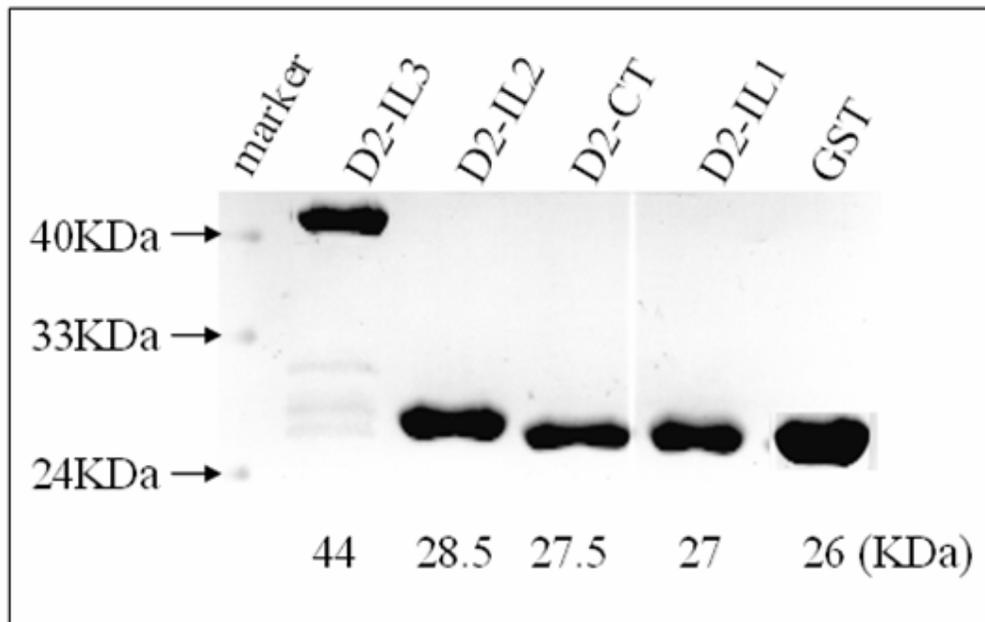


Figure 3.33: Purified GST fusion proteins of four intracellular domains of the D2 receptor (GST-D2-IL1/IL2/IL3/CT) with their expected molecular weights listed at the bottom.

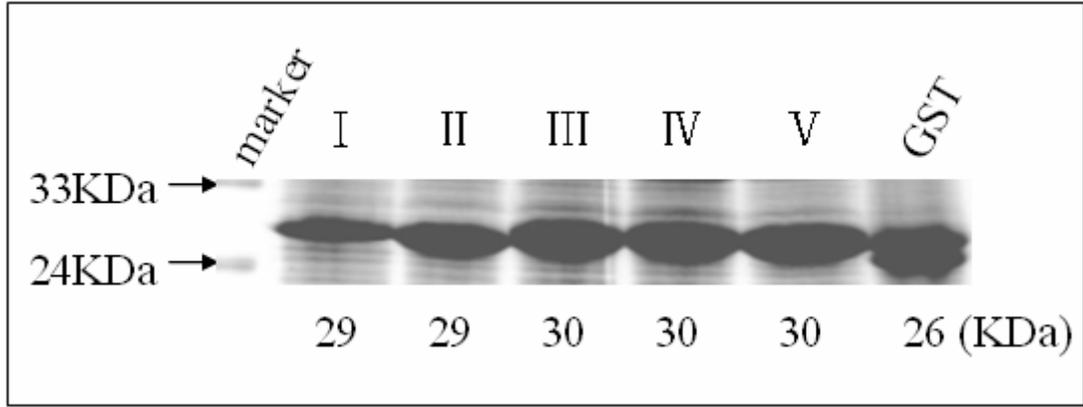


Figure 3.34: Over-expressed GST fusion proteins of the five fragments of D2-IL3 (GST-D2-IL3 I /II/III/IV/V) with their molecular weights at the bottom.

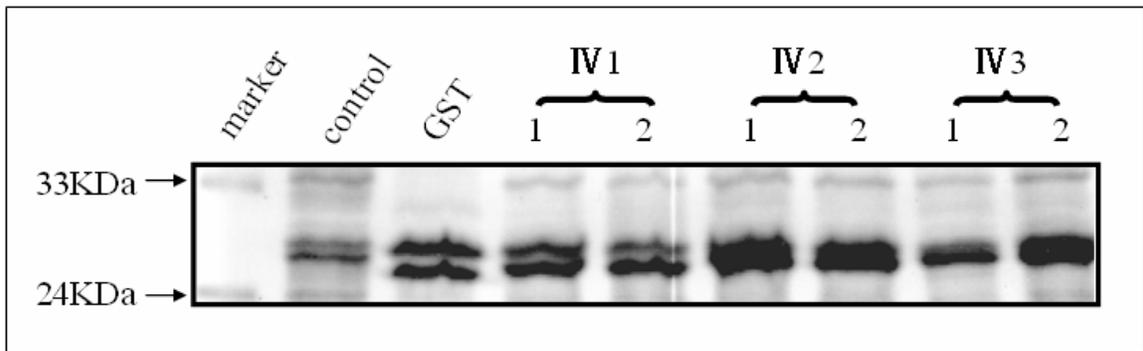


Figure 3.35: Over-expressed GST fusion proteins of the three motifs of D2-IL3(IV), GST-D2-IL3(IV1/2/3): 27KDa.

3.4 Expression of Tagged Proteins Containing the full length CB1 and D2 Receptors in Mammalian Cells

Since the full length CB1 and D2 receptors could not be over-expressed in *E.coli* cells, an attempt was made to express them and their recombinant proteins with “-myc” or “-FLAG” in mammalian cells using the recombinant plasmids, i.e. “CB1 in pcDNATM3.1/*myc*-His(B)”, “CB1 in pcDNATM3.1/FLAG(B)”, “CB1(TGA) in pcDNATM3.1/*myc*-His(B)”, “D2 in pcDNATM3.1/*myc*-His(B)”, and “D2 in pcDNATM3.1/FLAG(B)”.

3.4.1 Transfection Efficiency in HEK293 and PC12 Cells

For expression of proteins in mammalian cells, the first important step is to perform the efficient transient transfection of the recombinant plasmids.

Two mammalian cell lines (i.e. HEK293 and PC12 cells) were tried for transient transfection with LipofectamineTM 2000 reagent. To identify the transfection efficiency, the vector containing GFP was used. Figure 3.36, taken under fluorescence correlation microscopy 24 hours after transfection, showed that HEK293 cells had a much higher transfection efficiency than PC12 cells. Therefore, HEK293 cells were chosen for transfection to express proteins of the full length CB1 and D2 receptors as well as their tagged recombinant proteins in this study.

To further prove efficient transfection, RT-PCR was applied to amplify the DNA sequences of the full length CB1 (Figure 3.37, Lane 1', 2' and 3') or D2 (Figure 3.37, Lane 1, 2 and 3) receptors, using the cDNAs reversely transcribed from the non-transfected HEK293 cells (Figure 3.37, Lane 1 and 1'), HEK293 cells transfected with pcDNATM3.1/*myc*-His(B) empty vectors (Figure 3.37, Lane 2 and 2') or recombinant vectors of "CB1 in pcDNATM3.1/*myc*-His(B)" (Figure 3.37, Lane 3') and "D2 in pcDNATM3.1/*myc*-His(B)" (Figure 3.37, Lane 3). The DNA fragments of the full length CB1 or D2 receptor could be only amplified by RT-PCR using the cDNA from the HEK293 cells transfected with their recombinant vectors (Figure 3.37, Lane 3' and 3). These results clearly demonstrate an efficient transfection with LipofectamineTM 2000 reagent.

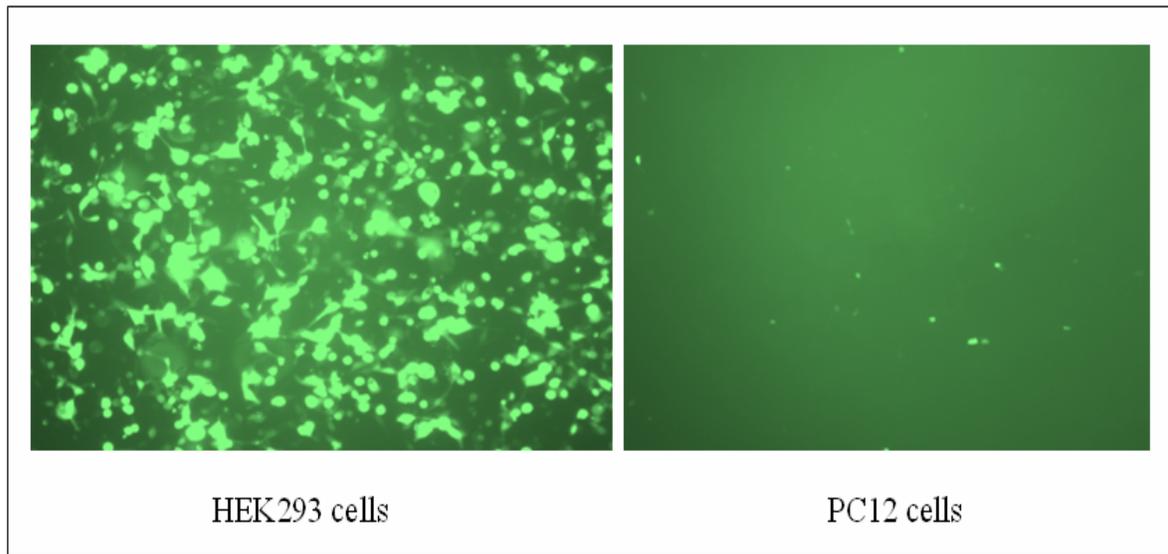


Figure 3.36: GFP identification of efficient transfection. The vector containing GFP was transfected into HEK293 and PC12 cells.

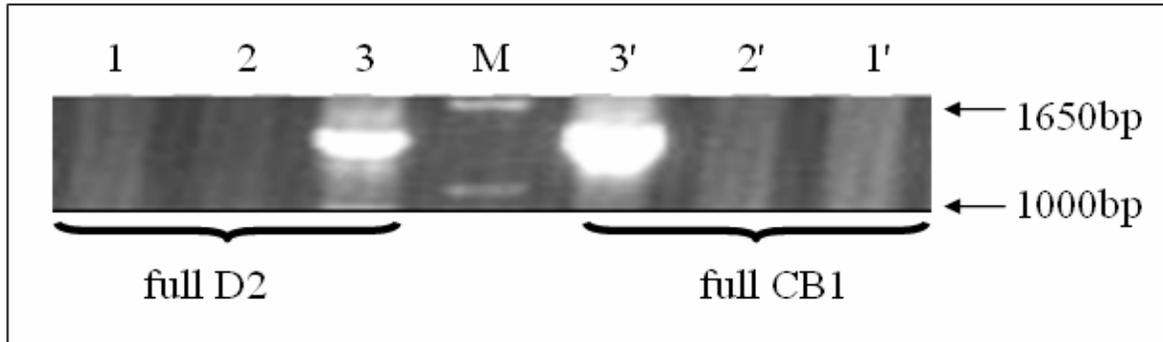


Figure 3.37: RT-PCR identification of efficient transfection. RT-PCR was applied to amplify the DNA sequences of the full length CB1 or D2 receptor, using the cDNAs reversely transcribed from the non-transfected HEK293 cells, the HEK293 cells transfected with “pcDNATM3.1/*myc*-His(B)” empty vectors or recombinant vectors of “CB1 in pcDNATM3.1/*myc*-His(B)” and “D2 in pcDNATM3.1/*myc*-His(B)”.

M: 1Kb DNA ladder

1 & 1' : cDNA from non-transfected HEK293 cells

2 & 2' : cDNA from HEK293 cells transfected with “pcDNATM3.1/*myc*-His(B)”

3' : cDNA from HEK293 cells transfected with “CB1 in pcDNATM3.1/*myc*-His(B)”

3: cDNA from HEK293 cells transfected with “D2 in pcDNATM3.1/*myc*-His(B)”

3.4.2 Expressed Proteins Were Difficult to Detect by Western Blot

Western blot was used to determine whether the proteins of the full length CB1 and D2 receptors were expressed after the transfection of relevant expression plasmids into HEK293 cells in this study (negative or similar results not shown).

As described above, five recombinant plasmids of the full length CB1 and D2 receptors were transfected into HEK293 cells for expression. For each recombinant plasmid, a dose of either 2 µg or 4 µg of transfected DNA for each well of a 6-well plate and different growing times of 24/36/48/72 hours after transfection were carried out for their expression. Then, five primary antibodies, i.e. anti-CB1 (44-310, Biosource, Camarillo, CA), anti-D2 (AB1558, Chemicon, Temecula, CA), anti-His (27E8, Cell Signaling, Danvers, MA), anti-myc (c2905, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-FLAG (#2368, Cell Signaling, Danvers, MA) antibodies, were employed to detect expressed proteins.

For the HEK293 cells transfected with “CB1(TGA) in pcDNATM3.1/myc-His(B)” or “CB1/D2 in pcDNATM3.1/FLAG(B)”, no specific bands could be identified in the western blot experiments using “anti-CB1” (1:500) or “anti-FLAG” (1:200) primary antibodies (data not shown). The western blot experiments of the “D2 in pcDNATM3.1/myc-His(B)” transfected HEK293 cells with different growing time after transfection, detected with “anti-D2” (1:500), “anti-His” (1:1000) or “anti-myc” (1:500) primary antibodies, showed some bands (Figure 3.38). However, the expected specific bands (i.e. around 53KDa) of the expressed proteins detected with these three primary antibodies could not be found. The over-expressed fusion protein of “CB1-myc” (around 56KDa) could occasionally be

detected with “anti-CB1” (1:500) primary antibody in western blot after the recombinant plasmids of “CB1 in pcDNATM3.1/*myc*-His(B)” were transfected into HEK293 cells (see Figure 3.39, Lane “CB1-*myc*”).

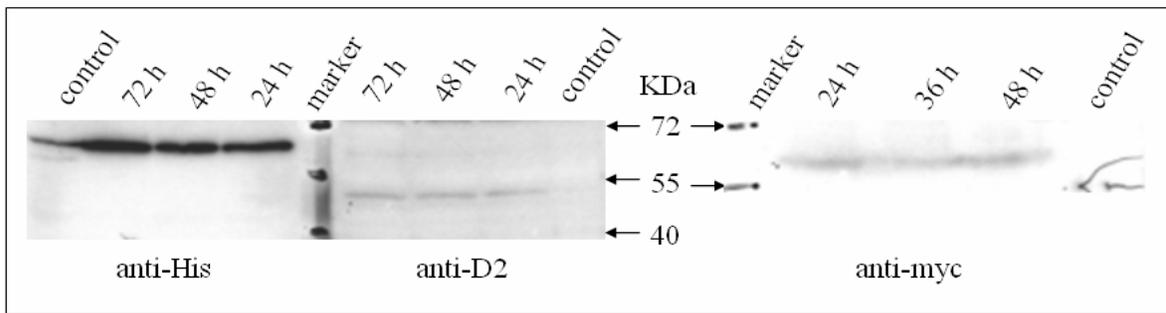


Figure 3.38: Western blot experiments of HEK293 lysates transfected with “D2 in pcDNATM3.1/*myc*-His(B)” and allowed to grow for 24, 36, 48 or 72 hours after transfection, were detected with “anti-D2”, “anti-His” or “anti-*myc*” primary antibodies. The expected 53KDa protein band could not be detected. Non-transfected HEK293 cells were used as “control”.

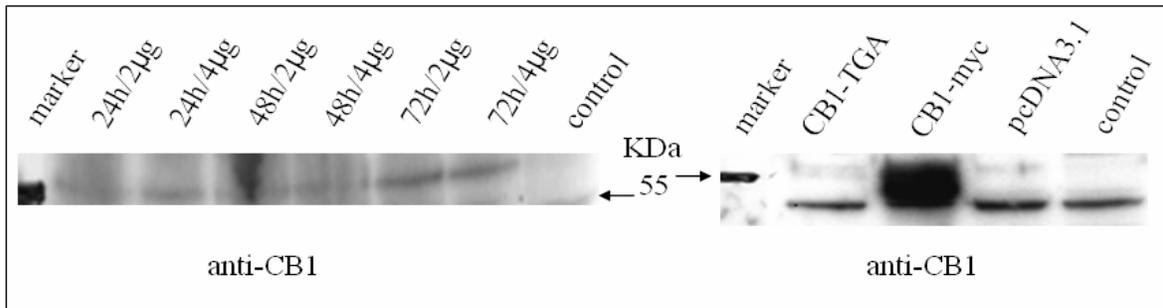


Figure 3.39: Western blot experiments of HEK293 lysates transfected, with either 2 µg or 4 µg of “CB1 in pcDNA™3.1/*myc*-His(B)” and allowed to grow for 24, 48 or 72 hours after transfection, were probed with “anti-CB1” primary antibody. The over-expressed fusion protein of “CB1-*myc*” (around 56KDa) could occasionally be identified (Lane “CB1-*myc*”). The HEK293 cells non-transfected or transfected with empty vectors of “pcDNA™3.1/*myc*-His” were used as controls.

3.4.3 Troubleshooting for the Low Expression Level of Tagged Proteins Containing Full Length CB1 and D2 Receptors

Since transient expression of the tagged fusion proteins of the full length CB1 and D2 receptors was not successful in transfected HEK293 cells, troubleshooting has been performed to try to solve the problems.

To make sure if excessive proteins were loaded into SDS-PAGE gel, different amounts of proteins were chosen for analysis. Another possibility is that the expressed proteins only existed in the floating transfected cells, and therefore the floating cells were collected alone after transfection and then analyzed by western blot. In addition, nine nucleotides (incorporating a Kozak consensus sequence to enhance gene expression) were added before the CB1 and D2 receptor open reading frames after these two DNA fragments were amplified by RT-PCR, cloned and subcloned into pcDNATM3.1/*myc*-His(B) vectors and transfected with their sequenced recombinant vectors into HEK293 cells.

However, these 3 modified experiments still did not show positive results, namely, both CB1 and D2 receptors were not found to be over-expressed (Figure 3.40). Because strong signal exists in the stacking gel shown in Figure 3.40 (Lane “20 μ l”), it is possible that the expressed fusion protein aggregated with other proteins and was unable to enter the resolving gel.

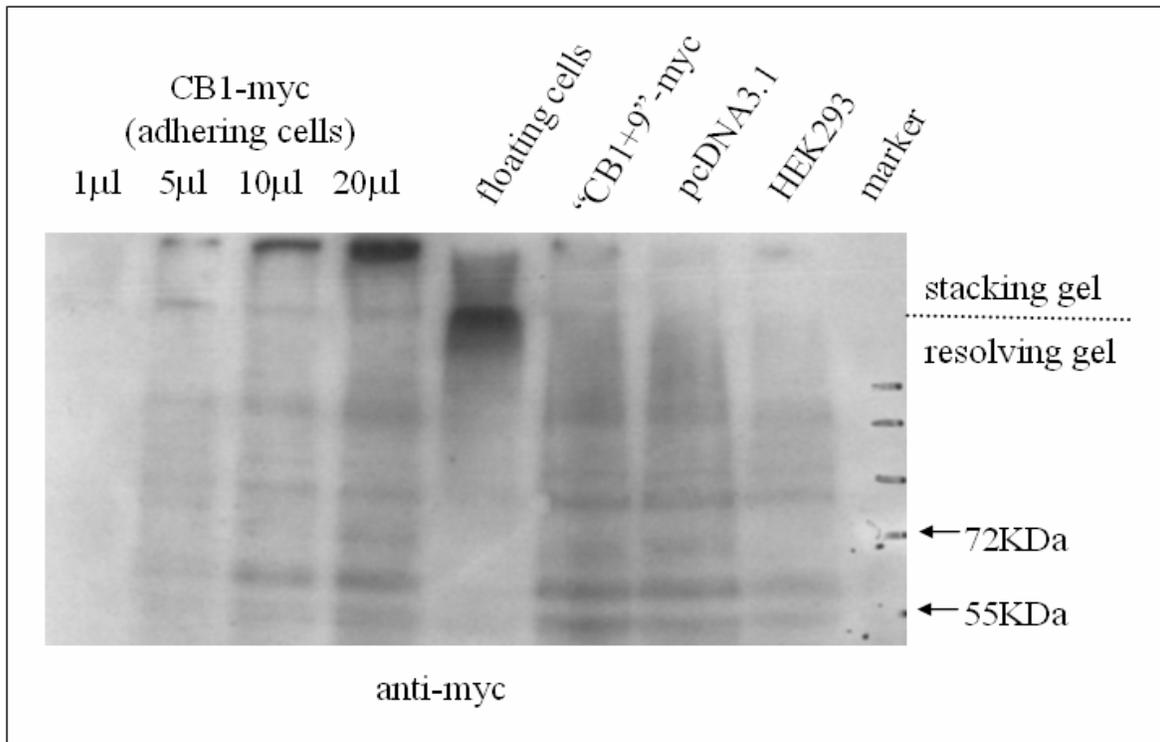


Figure 3.40: Western Blot for troubleshooting of transient expression of tagged recombinant protein (“CB1-myc”) in HEK293 cells using “anti-myc” primary antibody.

CB1-myc (adhering cells): different amounts (1/5/10/20 μl) of protein from adhering HEK293 cells transfected with “CB1 in pcDNATM3.1/*myc*-His(B)” were loaded for analysis.

floating cells: proteins from floating HEK293 cells transfected with “CB1 in pcDNATM3.1/*myc*-His(B)” were prepared for analysis.

“CB1+9”-myc: nine bases were added before the CB1 receptor gene coding sequence.

pcDNA3.1: HEK 293 cells transfected with “pcDNATM3.1/*myc*-His”.

HEK293: non-transfected HEK293 cells.

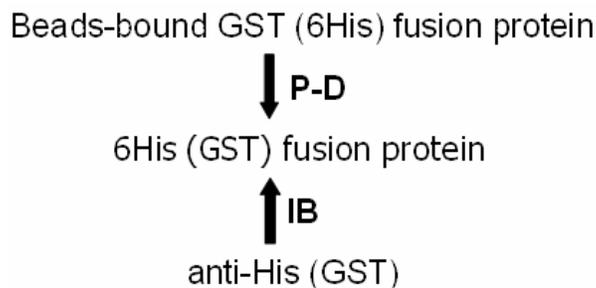
marker: protein ladder.

3.5 Pull-Down Assays

In this study, pull-down assay was employed to explore the possible protein-protein interaction between the CB1 and D2 receptors. The “GST” or “6His” tagged fusion proteins were used as bait protein in all the pull-down assays, which were classified as “*in vitro* pull-down assay” and “semi-*in vivo* pull-down assay”.

3.5.1 *In Vitro* Pull-Down Assays

For “*in vitro* pull-down assay”, the bacterial cell lysate containing the over-expressed 6His or GST fusion proteins was applied as the lysate sample. “*In vitro* GST pull-down assay” consists of two steps. First, the bead-bound GST tagged fusion proteins would act as the bait protein to pull-down prey proteins from the bacteria cell lysate containing the over-expressed 6His recombinant proteins. Then, the “anti-His” primary antibody was used in western blot to detect the prey proteins in pull-down complexes. For “*in vitro* 6His pull-down assay”, the resin-bound 6His tagged fusion proteins were employed as the bait protein to pull-down prey proteins from the bacteria cell lysate containing the over-expressed GST recombinant proteins, and then the prey proteins in pull-down complexes were detected by the “anti-GST” (26H1, Cell Signaling, Danvers, MA) primary antibody in western blot.



3.5.1.1 Pull-Down Between “CB1-CT” and “D2-IL3”

It has been shown that long intracellular fragments of GPCRs are possibly more likely to form their protein-protein interactions than short fragments (Lee et al., 2002; Liu et al., 2000; Milligan and White, 2001; Zou et al., 2005). Therefore, *in vitro* GST pull-down assay was applied to study the interaction between CB1 C-terminal (CB1-CT) or CB1 intracellular loop3 (CB1-IL3) and D2 intracellular loop3 (D2-IL3), which are the longer intracellular domains of the CB1 and D2 receptors.

The beads-bound GST tagged fusion proteins of CB1-CT (GST-CB1-CT) or CB1-IL3 (GST-CB1-IL3) were used as the bait proteins to pull-down the 6His recombinant protein of D2-IL3 (6His-D2-IL3) from the bacterial cell lysate containing the over-expressed 6His-D2-IL3 (Figure 3.41 A). The beads-bound GST protein was used as a negative control to pull-down 6His-D2-IL3 (Figure 3.41 A, Lane “GST”). Another negative control was the fusion protein of GST-CB1-CT without incubation with the bacterial cell lysate (Figure 3.41 A, Lane “control”). The result of western blot, detected with “anti-His” (1:1000) primary antibody, suggests that GST-CB1-CT could pull-down 6His-D2-IL3 from the bacterial cell lysate.

Next, the beads-bound GST tagged fusion proteins of D2-IL3 (GST-D2-IL3) were applied as the bait protein to pull-down the 6His recombinant protein of CB1-CT (6His-CB1-CT) from the bacterial cell lysate containing the over-expressed 6His-CB1-CT (Figure 3.41 B). The fusion protein of GST-D2-IL3 without incubation with the bacterial cell lysate and the beads-bound GST protein to pull-down 6His-CB1-CT were employed as

negative controls (Figure 3.41 B, Lane “control” and “GST”). The detection with “anti-His” (1:1000) primary antibody in western blot indicates that GST-D2-IL3 could pull-down 6His-CB1-CT from the bacterial cell lysate.

All the above results from *in vitro* GST pull-down assays suggest that D2 intracellular loop3 (D2-IL3) and CB1 C-terminal (CB1-CT) could directly couple to each other.

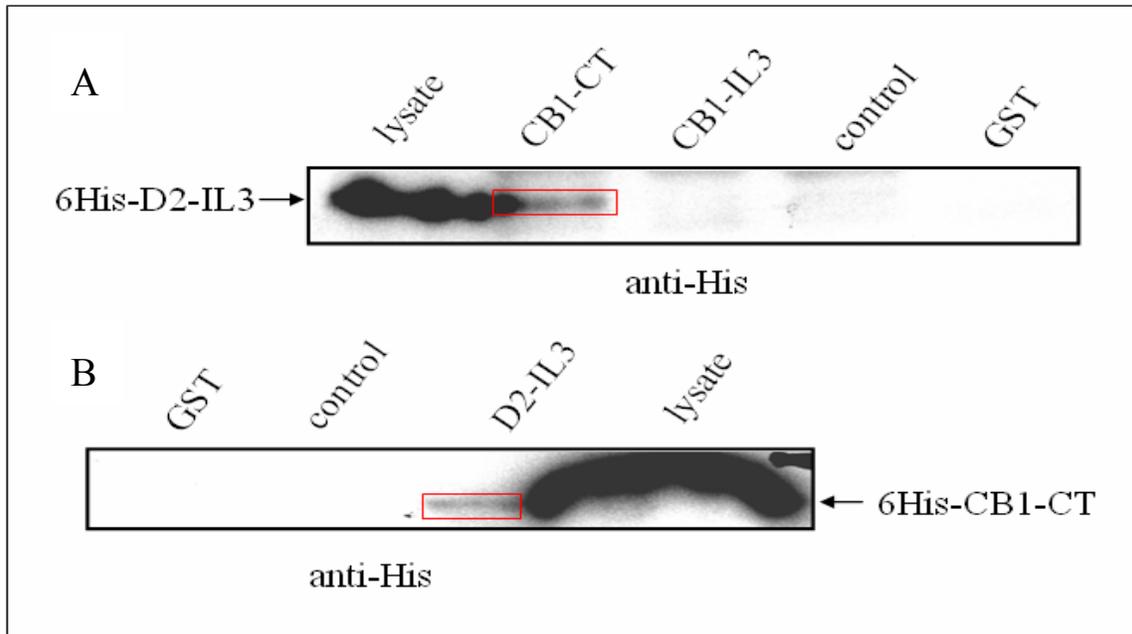


Figure 3.41: “D2-IL3” and “CB1-CT” could directly couple to each other.

A: *In vitro* GST pull-down assays were performed using “GST-CB1-CT” and “GST-CB1-IL3”. Resolved pull-down complexes were examined for the presence of “6His-D2-IL3”.

lysate: the bacterial cell lysate containing over-expressed “6His-D2-IL3”.

CB1-CT: “GST-CB1-CT” was used as bait for pull-down.

CB1-IL3: “GST-CB1-IL3” was used as bait for pull-down.

control: beads-bound “GST-CB1-CT” without incubation with the lysate.

GST: beads-bound GST protein was used as bait for pull-down.

B: *In vitro* GST pull-down assay was performed using “GST-D2-IL3”. Resolved pull-down complex was examined for the presence of “6His-CB1-CT”.

lysate: the bacterial cell lysate containing over-expressed “6His-CB1-CT”.

D2-IL3: “GST-D2-IL3” was used as bait for pull-down.

control: beads-bound “GST-D2-IL3” without incubation with the lysate.

GST: beads-bound GST protein was used as bait for pull-down.

3.5.1.2 Identification of Specific Motifs “D2-IL3 (IV1/3)”

To determine if C-terminal (CB1-CT) is the only intracellular domain of the CB1 receptor that can interact with D2-IL3, *in vitro* 6His pull-down assays were carried out. In this experiment (Figure 3.42), the resins-bound 6His tagged fusion protein of D2-IL3 (6His-D2-IL3) was used as bait protein to pull-down GST recombinant proteins of all intracellular domains of the CB1 receptor (GST-CB1-IL1/IL2/IL3/CT) from the bacterial cell lysates containing these over-expressed GST recombinant proteins; the over-expressed GST protein was employed as a negative control. The “last wash” of each pull-down complex was prepared for western blot to exclude the possibility of contamination of the pull-down complexes by excessive concentrations of bacterial cell lysates. The result from this experiment indicates that D2-IL3 could only pull-down CB1-CT, but not other CB1 intracellular domains.

The same method was employed to prove that intracellular loop3 (D2-IL3) is the only intracellular domain of the D2 receptor that can couple with CB1-CT. The resins-bound 6His tagged fusion protein of CB1-CT (6His-CB1-CT) was applied as bait protein to pull-down GST recombinant proteins of all intracellular domains of the D2 receptor (GST-D2-IL1/IL2/IL3/CT) from the bacterial cell lysates containing these over-expressed GST recombinant proteins; the over-expressed GST protein was employed as a negative control (Figure 3.43). The “last wash” of each pull-down complex was also prepared to exclude the possibility of contamination of pull-down complexes by excessive concentrations of bacterial cell lysates. The results of western blot, detected with “anti-GST” (1:1000) primary antibody, suggest that CB1-CT could only pull-down D2-IL3, but not other D2 intracellular domains.

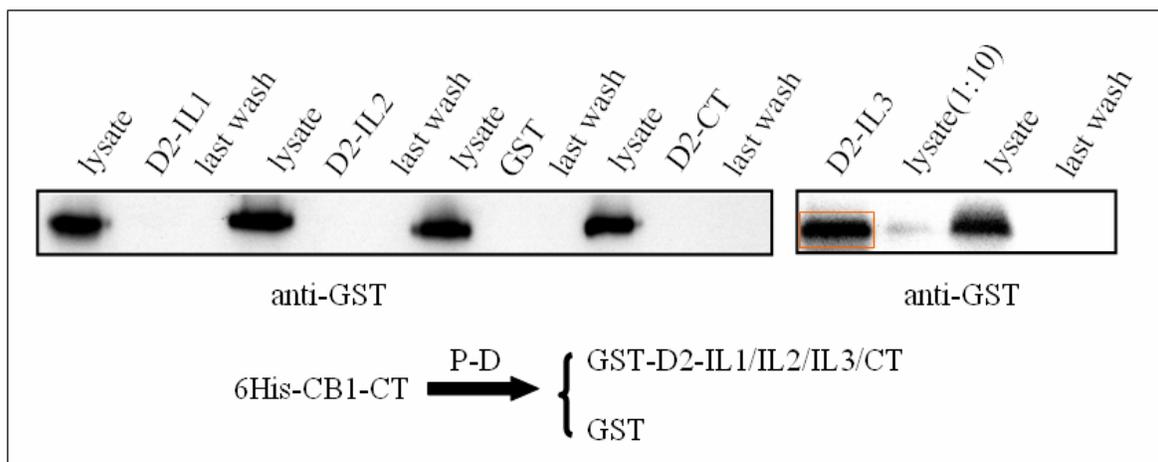


Figure 3.43: “CB1-CT” could only pull-down “D2-IL3”, but not other D2 intracellular domains. *In vitro* 6His pull-down assays were performed using “6His-CB-CT”. Resolved pull-down complexes were respectively examined for the presence of “GST-D2-IL1/IL2/IL3/CT”.

lysate: bacterial cell lysates containing over-expressed proteins of “GST” or “GST-D2-IL1/IL2/IL3/CT”.

last wash: wash buffer of the last wash of each pull-down complex.

D2-IL1: Bacterial cell lysate containing over-expressed “GST-D2-IL1” was used as a lysate sample for pull-down.

D2-IL2: Bacterial cell lysate containing over-expressed “GST-D2-IL2” was used as a lysate sample for pull-down.

GST: Bacterial cell lysate containing over-expressed “GST” was used as a lysate sample for pull-down.

D2-CT: Bacterial cell lysate containing over-expressed “GST-D2-CT” was used as a lysate sample for pull-down.

D2-IL3: Bacterial cell lysate containing over-expressed “GST-D2-IL3” was used as a lysate sample for pull-down.

Next, the intracellular loop3 of the D2 receptor, the longest domain of the D2 receptor, was divided into five fragments (D2-IL3 I / II / III / IV / V). To identify which fragment is responsible for the coupling between “CB1-CT” and “D2-IL3”, the 6His tagged fusion protein of “CB1-CT” (6His-CB1-CT) was used to pull down GST recombinant proteins of these five fragments (GST-D2-IL3 I / II / III / IV / V) from their bacterial cell lysates (Figure 3.44). The results suggest that the fourth fragment of “D2-IL3” (D2-IL3 IV) is the main fragment responsible for *in vitro* coupling between the CB1 and D2 receptors.

To further narrow down the motif responsible for the coupling between “CB1-CT” and “D2-IL3”, the fourth fragment of “D2-IL3” (D2-IL3 IV), which includes 35 amino acids, was partitioned into three motifs: D2-IL3(IV1) (12aa), D2-IL3(IV2) (12aa) and D2-IL3(IV3) (11aa). The 6His tagged fusion protein of “CB1-CT” (6His-CB1-CT) was used again to pull down the GST recombinant proteins of these three motifs (GST-D2-IL3 IV1/2/3) from their bacterial cell lysates (Figure 3.45). Results indicate that the first and third motifs in the fourth fragment of D2 intracellular loop3 (D2-IL3 IV1 and IV3) are likely the specific motifs responsible for the *in vitro* coupling between the CB1 and D2 receptors.

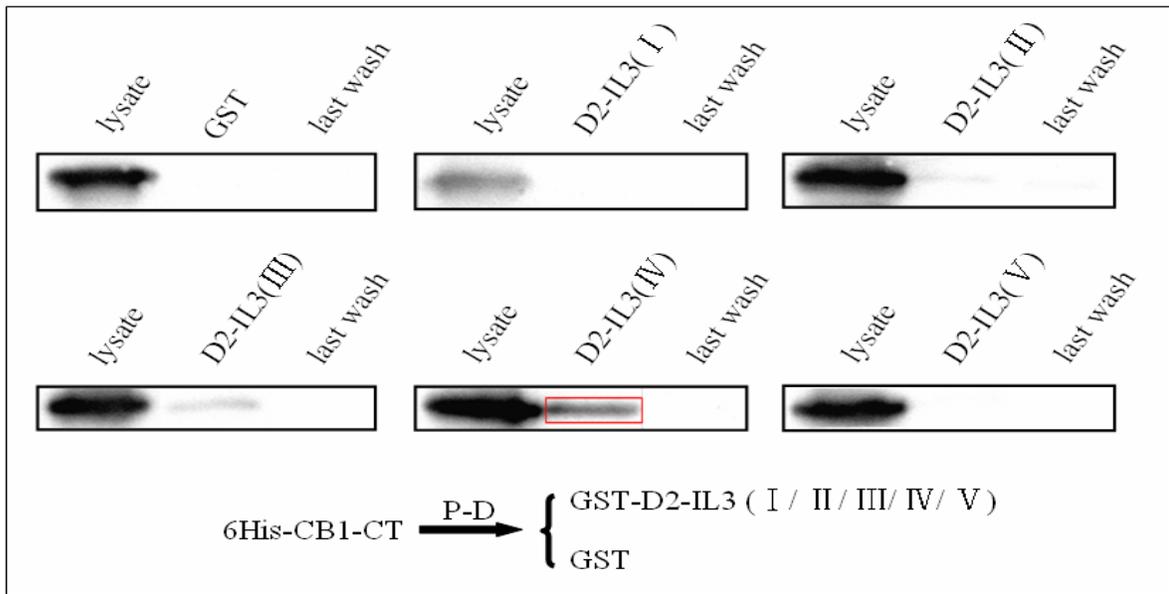


Figure 3.44: The fourth fragment of “D2-IL3” is likely the major fragment responsible for the coupling of “CB1-CT” with “D2-IL3”. *In vitro* 6His pull-down assays were performed using “6His-CB-CT”. Resolved complexes were respectively examined for the presence of “GST-D2-IL3(I / II / III / IV / V)”.

lysate: bacterial cell lysates containing over-expressed proteins of “GST” or “GST-D2-IL3(I / II / III / IV / V)”.

last wash: wash buffer of the last wash of each pull-down complex.

GST: Bacterial cell lysate containing over-expressed “GST” was used as a lysate sample for pull-down.

D2-IL3(I): Bacterial cell lysate containing over-expressed “GST- D2-IL3(I)” was used as a lysate sample for pull-down.

D2-IL3(II): Bacterial cell lysate containing over-expressed “GST- D2-IL3(II)” was used as a lysate sample for pull-down.

D2-IL3(III): Bacterial cell lysate containing over-expressed “GST- D2-IL3(III)” was used as a lysate sample for pull-down.

D2-IL3(IV): Bacterial cell lysate containing over-expressed “GST- D2-IL3(IV)” was used as a lysate sample for pull-down.

D2-IL3(V): Bacterial cell lysate containing over-expressed “GST- D2-IL3(V)” was used as a lysate sample for pull-down.

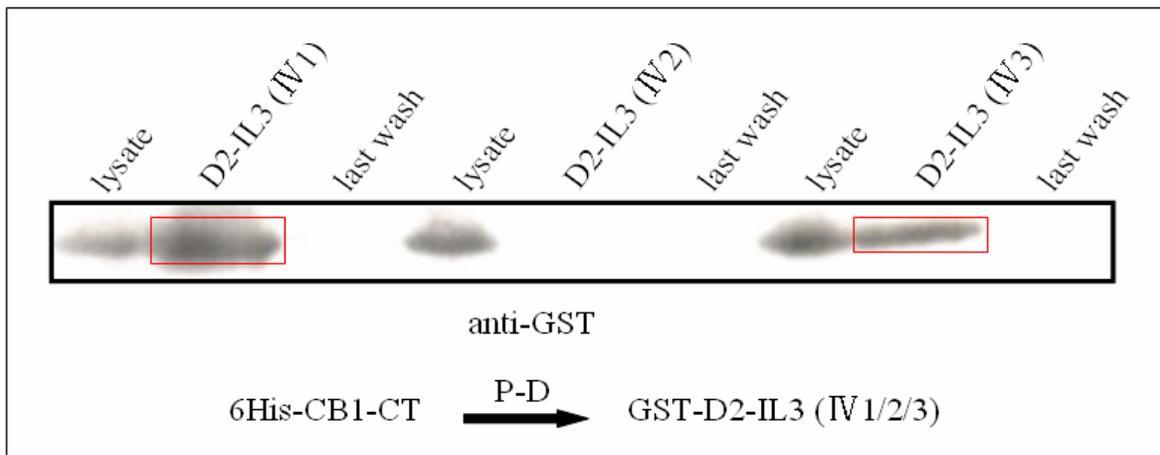


Figure 3.45: The first and third motifs in the fourth fragment of D2-IL3 (D2-IL3 IV1/3) are likely the specific motifs responsible for the coupling of “CB1-CT” with “D2-IL3”. *In vitro* 6His pull-down assays were performed using “6His-CB-CT”. Resolved complexes were respectively examined for the presence of “GST-D2-IL3(IV1/2/3)”.

lysate: bacterial cell lysates containing over-expressed proteins of “GST-D2-IL3(1/2/3)”.

last wash: wash buffer of the last wash of each pull-down complex.

D2-IL3(IV1): Bacterial cell lysate containing over-expressed “GST- D2-IL3(IV1)” was used as a lysate sample for pull-down.

D2-IL3(IV2): Bacterial cell lysate containing over-expressed “GST- D2-IL3(IV2)” was used as a lysate sample for pull-down

D2-IL3(IV3): Bacterial cell lysate containing over-expressed “GST- D2-IL3(IV3)” was used as a lysate sample for pull-down.

3.5.2 Semi-*In Vivo* Pull-Down Assays

For “semi-*in vivo* pull-down assay”, the rat striatum lysate was incubated with the purified GST tagged fusion proteins. To validate this experimental method, the GST tagged fusion protein of NDRG4 (GST-NDRG4) was used to successfully pull-down Erk proteins from the rat striatum lysate (Figure 3.46).

GST tagged fusion proteins of all the extracellular and intracellular domains of the CB1 receptor (GST-CB1-IL1/IL2/IL3/CT/NT/EL1/EL2/EL3) were purified by Glutathione Sepharose beads and then used to pull-down D2 receptors from the normal rat striatum lysate (Figure 3.47). The fusion GST fusion proteins without incubation with the lysate (Figure 3.47, Lane “control”) and the beads-bound GST protein alone (Figure 3.47, Lane “GST”) were used as controls. The western blot experiments of these pull-down assays, which were detected with “anti-D2” (1:500) primary antibody, showed that no obvious specific band of the D2 receptor (50KDa) could be detected in most pull-down complexes (Figure 3.47 A and B), except that there was a weak band close to the lysate D2 band (Lane “CB1-IL3” in Figure 3.47B). To confirm whether this represents specific band, the pull-down assay was repeated with the beads-bound GST tagged fusion protein of CB1 intracellular loop3 (GST-CB1-IL3) without incubation with the rat striatum lysate as a control. It was found that the weak band close to the lysate D2 band was a non-specific band (Figure 3.47 C). These results indicate that beads-bound GST tagged fusion proteins of CB1 fragments could not pull-down D2 receptors in *in vivo* situation, or that no protein-protein interaction occurs between CB1 and D2 receptors in *in vivo* condition.

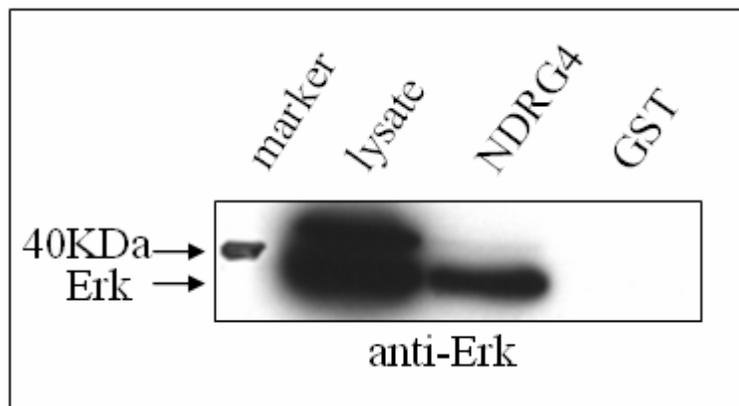


Figure 3.46: A validation of experimental method of “semi-*in vivo* pull-down assay”. GST fusion proteins of NDRG4 could pull-down Erk proteins from the rat striatum lysate.

marker: protein ladder.

lysate: normal rat striatum lysate.

NDRG4: “GST-NDRG4” was used as bait for pull-down.

GST: “GST” was used as bait for pull-down.

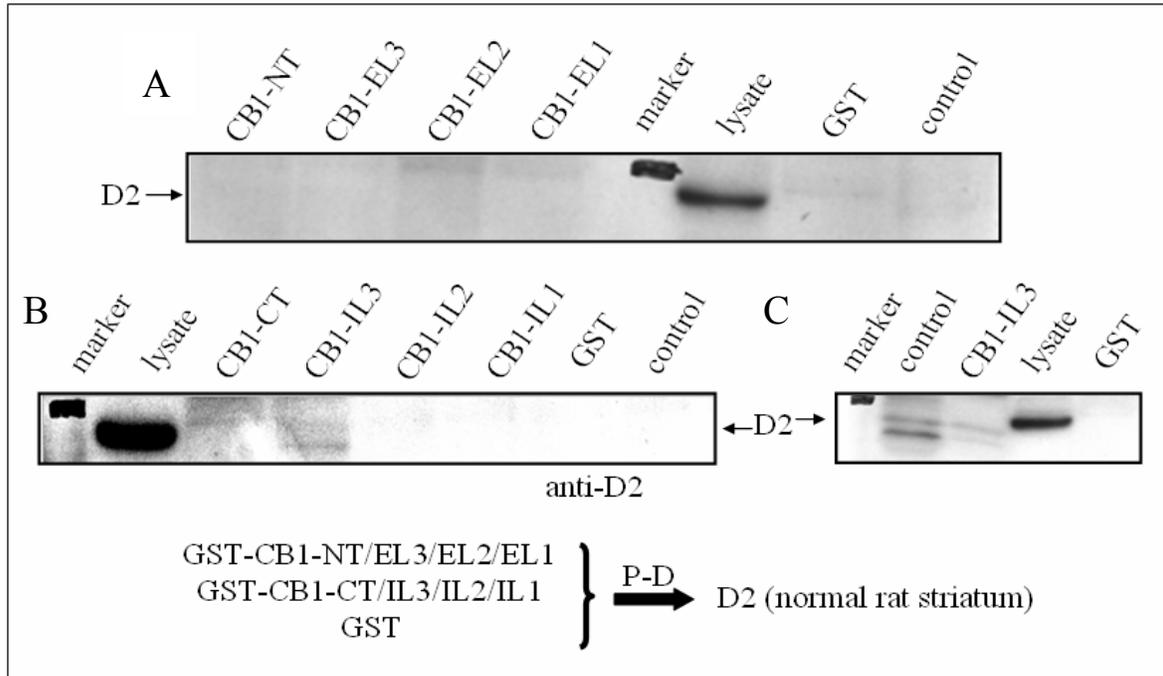


Figure 3.47: Semi-*in vivo* pull-down assays were performed using GST fusion proteins of all the extracellular and intracellular domains of the CB1 receptor (GST-CB1-IL1/IL2/IL3/CT/NT/EL1/EL2/EL3). Resolved complexes were examined for the presence of the D2 receptor. Western blot did not detect the D2 receptor in all the pull-down complexes.

marker: protein ladder.

lysate: normal rat striatum lysate (D2: 50KDa).

GST: "GST" was used as bait for pull-down.

control: beads-bound GST fusion proteins without incubation with lysate.

A: "GST-CB1- NT/EL1/EL2/EL3" proteins were used as bait for pull-down.

B: "GST-CB1-IL1/IL2/IL3/CT" were used as bait proteins for pull-down.

C: "GST-CB1-IL3" was used as bait for pull-down (repeat).

4 DISCUSSION

4.1 Over-Expression of Full Length CB1 and D2 Receptors

To determine the biological function of a protein, it is important to isolate and characterize the protein *in vitro*. Therefore, the protein of interest has to be over-expressed and then purified from a biological sample by “expression systems”, which are designed to control transcription of the cloned gene and translational efficiency and stability of the protein. These systems allow the production of large amounts of the protein relative to all other proteins in the cell. In present study, the tagged fusion proteins containing the full length CB1 and D2 receptors were tried for over-expression in *E.coli* bacteria and mammalian cell lines.

However, no obvious expression of GST or 6His fusion proteins containing the full length CB1 and D2 receptors could be identified by protein staining in three *E.coli* strains, i.e. DH5 α , BL21 and DE3. It is possible that the protein may be toxic to the cell when expressed at high levels. Alternatively, the over-expressed proteins may not be soluble when present at very high concentrations.

Unstable transient expression of the tagged fusion proteins containing the full length CB1 and D2 receptors has been detected in HEK293 and PC12 cells efficiently transfected with both receptors, but no specific bands of the recombinant proteins of “D2-FLAG” and “CB1-FLAG” could be found in the western blot experiments. Specifically, the expected specific bands (i.e. around 53KDa) of the expressed proteins “D2-myc/His” could not be

consistently detected with “anti-D2”, “anti-His” or “anti-myc” primary antibodies. The over-expressed fusion protein “CB1-myc” (around 56KDa) could occasionally be identified with “anti-CB1” primary antibody in western blot. Further troubleshooting experiments were performed: (i) excessive proteins were loaded onto the SDS-PAGE gel; (ii) lysates from adherent cells as well as from “floating” cells were examined; (iii) an additional nine nucleotides were added before the coding sequences of CB1 and D2 receptors for their expression (this additional sequence might act as a Kozak sequence for enhancing gene expression). It is found that (i) a strong signal exists in the stacking gel; (ii) the proteins were not detected in “floating” cells; and (iii) the additional nine nucleotides had no effect. These results suggest that the expressed fusion protein may aggregate with other proteins to form a huge complex that it does not resolve on the SDS-PAGE. Another possibility for the failure of detection in Western blot is a potentially inefficient antibody.

4.2 Protein-Protein Interaction between CB1 and D2 Receptors

GPCRs, which constitute by far the largest family of cell surface proteins involved in signaling across biological membranes, modulate a wide range of physiological processes and are implicated in numerous diseases. It has been shown that cells co-transfected with two types of GPCRs and treated with agonist specific for one or the other receptor displayed ligand-binding profiles much different from cells expressing either receptor individually. These results indicate that the physical protein-protein interaction between two types of receptors, i.e. heterodimerization, is an important mechanism for the modulation of GPCR function. Indeed, the heterodimerization of GPCRs has gained much support over the past several years, for instance, the dimerization between dopamine D2 and D3 receptors, κ and δ opioid receptors, somatostatin SSTR5 and dopamine D2(L) receptors, dopamine D1 and adenosine A1 receptors, α_2 adrenergic and m_3 muscarinic receptors (Jordan and Devi, 1999; Park et al., 2004; Rocheville et al., 2000b; Scarselli et al., 2001; Vazquez-Prado et al., 2002).

Both cannabinoid CB1 and dopamine D2 receptors are the most common GPCRs in the brain and are known to couple to the G protein $G\alpha_{i/o}$. Various lines of evidence support the idea that direct protein-protein interaction occurs between CB1 and D2 receptors that regulates their functions. In order for different types of receptors to physically interact, they must exist within the same cells. It has been demonstrated that the CB1 and D2 receptors are co-expressed within the same neurons in the striatum, olfactory tubercle, hippocampus and some cortical areas of adult mouse forebrain (Hermann et al., 2002).

Functional studies also indicate the existence of protein-protein interaction between CB1 and D2 receptors in animals: when animals were treated with a CB1 agonist combined with either dopamine agonist or antagonist, the dopamine ligands dramatically affected cannabinoid-induced learning and memory impairment in rats, as well as motor function in mice (Gessa et al., 2000; Wager-Miller et al., 2002). Cell culture studies further support the existence of a CB1:D2 complex that can modulate the function of both receptors: the concurrent activation of D2 receptors and CB1 receptors promotes the coupling of CB1 receptors to $G\alpha_s$, instead of $G\alpha_{i/o}$ resulting in elevation in intracellular cAMP accumulation in primary striatal neuronal culture and HEK293 cells cotransfected with D2 and CB1 receptors (Glass and Felder, 1997; Jarrahian et al., 2004). The direct evidence supporting the existence of direct protein-protein coupling between CB1 and D2 receptors comes from the observation of Kern and his colleagues that co-IP experiments showed physical interaction of CB1 receptor with D2 receptors in HEK293 cells stably expressing both receptors (Kern et al., 2005). The important question is which domain(s) and protein sequence(s) in the D2 and CB1 receptors are responsible for *in vitro* protein-protein interactions between both receptors.

Particular importance has been ascribed to the C-terminal (CT) and the third intracellular loop (IL3) of GPCRs in the formation of their di/oligomers (Milligan and White, 2001). It has been found that the D2 receptor can interact with actin-binding protein (ABP-280) via its third intracellular loop; this was originally identified in a yeast two-hybrid screen and confirmed by protein binding assays (Li et al., 2000). Agonist

stimulation of D2 receptors promotes the formation of the direct protein-protein interaction between the third intracellular loop of the D2 receptor and the ATPase N-ethylmaleimide-sensitive factor regulating AMPA glutamate receptor-mediated excitotoxicity (Zou et al., 2005). Wager-Miller et al (2002) provided biochemical and immunocytochemical evidence for CB1 dimerization using a CB1 carboxy terminus-specific antibody, which appears to have a high affinity for an oligomeric form of the CB1 receptor. They also suggested that the proximal and distal regions of the CB1 C-terminal might be important contributors to the assembly of the CB1 receptor oligomer (Wager-Miller et al, 2002).

Using *in vitro* GST and 6His pull-down assays, the present study showed that CB1 C-terminal (CB1-CT) could pull-down D2 intracellular loop3 (D2-IL3) (Figure 3.41 A), but not other D2 intracellular fragments (Figure 3.43), and consistent with this, “D2-IL3” could pull-down “CB1-CT” (Figure 3.41 B), but not other CB1 intracellular domains (Figure 3.42). These results indicate that *in vitro* direct protein-protein interaction of the CB1 receptor with the D2 receptor occurs between CB1 intracellular C-terminal (CB1-CT) and D2 intracellular loop 3 (D2-IL3).

To identify the specific fragment in the D2 receptor responsible for the protein-protein interaction between “CB1-CT” and “D2-IL3”, the intracellular loop3 of the D2 receptor was divided into five fragments (D2-IL3 I / II / III / IV / V). *In vitro* 6His pull-down assays in present study indicate that the fourth fragment of “D2-IL3” (D2-IL3 IV) is the main fragment responsible for *in vitro* coupling between the CB1 and D2 receptors (Figure 3.44).

A weaker interaction was noted for the D2-IL3(III) fragment. We chose to focus, however, on the D2-IL3(IV) fragment. To further narrow down the specific motifs for further studies, the fourth fragment of “D2-IL3” (D2-IL3 IV) was partitioned into three motifs D2-IL3(IV1/2/3) for *in vitro* 6His pull-down assays. Results suggest that the first and third motifs in the fourth fragment of D2 intracellular loop3 (D2-IL3 IV1 and IV3) are likely the specific motifs responsible for the *in vitro* coupling between the CB1 and D2 receptors (Figure 3.45).

All the evidence obtained so far supports the *in vitro* direct protein-protein interaction between CB1 and D2 receptors, but it still remains unknown whether such protein-protein interaction between CB1 and D2 receptors exists *in vivo*. To answer this question, I have performed pilot experiments, in which no specific bands of the D2 receptor could be detected when the purified GST tagged fusion proteins of CB1 fragments were employed to pull-down D2 receptors from the rat striatum lysate. In the paper reporting the physical interaction between CB1 and D2 receptors in transfected HEK293 cells, the amount of immunoprecipitated CB1 receptors markedly increased after treating the transfected cells with the D2 receptor agonist quinpirole and increasing concentrations of the CB1 receptor agonist CP 55,940 (Kearn et al., 2005). Therefore, it is possible that the coupling between CB1 and D2 receptors in the striatum may be too minute to be detected in basal *in vivo* condition, although both CB1 and D2 receptor proteins were found to exist in the same striatal neurons (Hermann et al., 2002). This working hypothesis is also supported by other evidence. An intense signal for CB1 receptor mRNA but low signal for CB1 receptor

protein was found in the striatum, whereas no hybridization signal but intense immunoreactivity could be detected in the globus pallidus and substantia nigra by *in situ* hybridization and immunohistochemistry methods (Julian et al., 2003). These results suggest that CB1 receptors are synthesized in the striatum and most of them transported to their target areas, i.e. globus pallidus and substantia nigra (Herkenham et al., 1991a; Julian et al., 2003). It is thus plausible to hypothesize that there may not be sufficient amount of CB1 receptor protein available in the striatum in basal *in vivo* situation. Alternatively, there might be no physical protein-protein interaction between CB1 and D2 receptors in the rat striatum if both CB1 and D2 receptors co-expressed in the same striatal neurons are not close enough to allow for a physical interaction or if post-translational modification regulates the interaction. Even without the *in vivo* direct protein-protein interaction between CB1 and D2 receptors, convergence in the cellular signaling pathway between both receptor systems can occur at the level of G-protein/adenylate cyclase signal transduction in the striatum for the mutual regulation of their functions (Ameri, 1999; Julian et al., 2003; Meschler and Howlett, 2001; Stelt and Di Marzo, 2003).

4.3 Future Direction

Further research employing co-IP and semi-*in vivo* pull-down assay is required to determine *in vivo* protein-protein interaction between the CB1 and D2 receptors in the rat striatum. Co-IP is a critical technique for revealing protein-protein interaction *in vivo*. In order to identify protein-protein interaction between the CB1 and D2 receptors in the rat striatum, an anti-D2 antibody will be used to immunoprecipitate an immune complex that may include physically interacted D2 and CB1 receptors, followed by application of an anti-CB1 antibody to detect CB1 receptors from this immune complex by western blot. In a parallel experiment, an anti-CB1 antibody will be used first, followed by an anti-D2 antibody. If positive results are not to be obtained from the co-IP experiments using striatum samples in basal *in vivo* condition, further experiment will be performed to investigate whether the protein-protein interaction between CB1 and D2 receptors in the rat striatum may occur or be strengthened after activation of CB1 and/or D2 receptors. In order to do so, rats will be treated with CB1 receptor agonist, D2 receptor agonist or both, followed by dissection of the striatal sample and subsequent performance of co-IP assay. In this way, agonist treatment may increase the amount of the CB1 and/or D2 receptor proteins so as to facilitate formation of CB1:D2 complexes that could be detected with co-IP assay.

After verification of protein-protein interaction between CB1 and D2 receptors in the rat striatum with co-IP assay, it is important to determine whether the identified specific motifs “D2-IL3(IV1)” and “D2-IL3(IV3)” in the D2 receptor are indeed critical for D2

receptors coupling with CB1 receptors. The ability of the purified peptides of “D2-IL3(IV1)” and “D2-IL3(IV3)” to disrupt protein-protein interaction between CB1 and D2 receptors in co-IP experiments will be examined, in much the same manner as we have used relevant peptides to block the interaction between PTEN and the 5-HT_{2C} receptor (Ji et al., 2006). If positive results are obtained, the purified peptides of “D2-IL3(IV1)” and “D2-IL3(IV3)” will be made membrane permeable (Ji et al., 2006) and then utilized to assess their suppressing effects on cannabinoid-induced memory impairment and the rewarding effects of major drugs of abuse with appropriate behavioral tests. Therefore, a better understanding of the protein-protein interaction between CB1 and D2 receptors may impact our understanding of the mechanisms that underlie the cannabinoid-induced memory impairment and the rewarding effects of major drugs of abuse. Furthermore, the specific motifs “D2-IL3(IV1)” and “D2-IL3(IV3)” may represent an effective target for use as novel therapeutic agents in dopamine-sensitive drug addictions.

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APPENDIX - VITA: YUN ZHANG

Education

- 09/91 – 07/96 Bachelor of Medicine (Excellent Undergraduate)
Nanjing Railway Medical University, Nanjing, China
- 01/05 – 10/06 Master of Science (Neuropsychiatry)
University of Saskatchewan, Saskatoon, Canada

Experience

- 07/96 – 11/01 Doctor, Gynecology/Obstetrics
- 12/01 – 05/03 Doctor-in-charge, Gynecology/Obstetrics
Changsha Railway Hospital, Changsha, China
- 04/04 – 12/04 Technician in Neuropsychiatry Research Unit
University of Saskatchewan, Saskatoon, Canada

Awards

1. *Doctoral Research Award* (2007.1~2009.12, \$66,000.00), August 2006, awarded by the Heart and Stroke Foundation of Canada
2. *College of Medicine Graduate Scholarship* (2005.9~2006.9, \$7500.00), June 2005, awarded by the College of Medicine in the University of Saskatchewan
3. *ALFRED E. MOLSTAD TRUST Discretionary Use Research Fund* (\$1500.00), July 2005, awarded by the Department of Psychiatry in the University of Saskatchewan
4. *Excellent Undergraduate*, June 1996, awarded by Nanjing Railway Medical University (it is now Medical College of Southeast University)

Publications

Papers:

1. Jiang W, Van Cleemput J, Sheerin AH, Ji S, **Zhang Y**, Saucier DM, Corcoran ME, Zhang X. Involvement of Extracellular Regulated Kinase and p38 Kinase in Hippocampal Seizure Tolerance. *J. Neurosci. Res.* 2005, 81(4):581-8.
2. Jiang W, **Zhang Y**, Xiao L, Van Cleemput J, Ji S, Bai G, Zhang X. Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *J. Clin. Invest.* 2005, 115:3104-16.
3. Ji S, **Zhang Y**, Van Cleemput J, Jiang W, Wan Q, Backstrom JR, Zhang X. Disruption of PTEN coupling with 5-HT_{2C} receptors suppresses behavioral responses induced by drugs of abuse. *Nat Med.* 2006, 12(3):324-9.

Abstracts:

4. Ji S, **Zhang Y**, Van Cleemput J, Jiang W, Wan Q, Backstrom JR, Zhang X. Cannabinoid promotes the proliferation of neural stem/progenitor cells in the rat hippocampus both in vitro and in vivo. *35th Annual Meeting Society For Neuroscience*, Nov. 2005, Washington D.C., USA.
5. Ji S, **Zhang Y**, Van Cleemput J, Jiang W, Wan Q, Backstrom JR, Zhang X. Disruption of protein-protein coupling between PTEN and serotonin 5-HT_{2C} receptor suppresses marijuana rewarding effects. *13th Annual Life & Health Sciences Student Research Conference*, Mar. 2006, Saskatoon, SK, Canada.