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CHARACTERIZATION OF THE D3 DOPAMINE RECEPTOR AND A SPLICE VARIANT, D3nf, IN SF9 CELLS

By

Jennifer Lynne Elmhurst

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Pharmacology
University of Toronto

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CHARACTERIZATION OF THE D3 DOPAMINE RECEPTOR
AND A SPLICE VARIANT, D3nf, IN SF9 CELLS.

Jennifer Elmhurst, M.Sc., 1998
Graduate Department of Pharmacology
University of Toronto

Abstract

The best characterized of the many human D3DR mRNA splice variants encodes a truncated, non-functional protein called D3nf. The D3DR and D3nf were epitope-tagged and expressed in Spodoptera frugiperda (Sf9) insect cells by recombinant baculovirus infection. The D3DR displayed a pharmacology consistent with reports in other cell lines. When co-expressed with D3nf, the density of D3DR expression was significantly lowered compared to when D3DR was expressed alone. Immunoblot analysis of membranes from Sf9 cells expressing the D3DR or D3nf alone revealed species consistent in size with protein monomers and oligomers. Photoaffinity labeling of these membranes with \[^{125}\text{I}]\text{azidonemonapride} identified the same species for the D3DR as identified by immunoblotting. No labelling of D3nf was observed, consistent with its inability to bind ligands. When the D3DR and D3nf were co-expressed, the same species were observed as when the proteins were expressed alone. Immunofluorescent studies showed that D3DR and D3nf were well expressed on the surface of Sf9 cells. These data suggest that the D3DR and D3nf form oligomers and that D3nf may decrease the capacity of D3DR at the cell surface to bind ligand.
Acknowledgements

I owe thanks to many people who helped and guided me throughout the course of this thesis. First and foremost, I thank my supervisors Dr. Susan George and Dr. Brian O’Dowd who provided helpful advice and were always very supportive of my work. I was also very lucky to work in a lab with excellent and knowledgeable technical support. I thank Tuan Nguyen for his unfailing ability to help me whenever I encountered problems with DNA manipulation. I thank Zhidong Xie for teaching me all I needed to know about recombinant baculovirus and SF9 cells. Thank you to George Varghese who was very patient in teaching me about cell culture, binding assays and cyclase assays. Judy Trogadis at the Toronto Western Hospital was very kind to teach me how to prepare cells for confocal microscopy. Thanks to Sam Lee who always more than happy to offer advice and be supportive of the many problems I encountered. His emotional support was most valuable and important to me. I also thank my friends and family, especially Matt Nowak, for bearing with my tempermentalness and complaints about my work and, most importantly, sharing in my times of happiness.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2AR</td>
<td>α₂-adrenoceptor</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>β2AR</td>
<td>β₂-adrenoceptor</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCR5</td>
<td>chemokine receptor 5</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>D1DR</td>
<td>D₁ dopamine receptor</td>
</tr>
<tr>
<td>D2DR</td>
<td>D₂ dopamine receptor</td>
</tr>
<tr>
<td>D3DR</td>
<td>D₃ dopamine receptor</td>
</tr>
<tr>
<td>D4DR</td>
<td>D₄ dopamine receptor</td>
</tr>
<tr>
<td>D5DR</td>
<td>D₅ dopamine receptor</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water, autoclaved</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleoside triphosphates (including deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>force of gravity</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin hormone releasing hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>5'-guanylylimidodiphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>ICL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>Kᵦ</td>
<td>dissociation constant of radioligand binding</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>inhibition constant of ligand binding</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>m3</td>
<td>muscarinic receptor, subtype 3</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>nAchR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>OPA</td>
<td>One-Phor-All buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em></td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane spanning domain</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween tris-buffered saline</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Dopamine Receptors

Dopamine is one of the major neurotransmitters found in the mammalian brain and is believed to be involved with locomotor control, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. It also functions in the periphery by influencing cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function and gastrointestinal motility (Missale et al., 1998). The name "dopamine" is an acronym for its chemical name dihydroxyphenylethylamine. In addition to its function as a neurotransmitter, dopamine is also a precursor in the synthesis of adrenaline and noradrenaline (Figure 1.1).

There are three major dopaminergic pathways in mammalian brain: 1) the nigrostriatal pathway, 2) the mesolimbic pathway and 3) the tuberoinfundibular system. The nigrostriatal pathway is predominant and has cell bodies originating in the substantia nigra and terminating in the caudate nucleus of the corpus striatum. The cell bodies of the mesolimbic pathway lie mainly in the A10 cell group of the midbrain and project to the limbic system, including the nucleus accumbens, globus pallidus, olfactory tubercle, septum, amygdala and cortex. The tuberoinfundibular system consists of short neurons running from the arcuate nucleus of the hypothalamus to the median eminence and pituitary gland (Lindvall and Bjorklund, 1983).

To date, five receptors have been identified which mediate dopamine's action in the brain and periphery. Initially, the dopamine receptor family was classified into two groups based on differential actions of dopamine on the enzyme adenylyl cyclase (AC); the dopamine D1 receptor (D1DR) is stimulatory to AC and the dopamine D2 receptor (D2DR) inhibitory (Kebabian and Calne, 1979). This classification held for over a decade until the advent of molecular techniques which permitted the cloning of three more novel dopamine receptor subtypes.

Dopamine receptors belong to the superfamily of G protein-coupled receptors (GPCRs). GPCRs are the largest family of receptors with over 240 members cloned to date, excluding the sensory receptors
Figure 1.1: Biosynthesis of catecholamines. The precursor is L-tyrosine, an aromatic amino acid, which is converted by tyrosine hydroxylase to dihydroxyphenylalanine (DOPA), the rate-limiting step in catecholamine synthesis. DOPA is converted to dopamine by the enzyme DOPA decarboxylase. Only adrenergic neurons contain the enzyme dopamine β-hydroxylase which catalyzes the formation of noradrenaline. The formation of adrenaline by phenylethanolamine N-methyl transferase occurs mainly in the adrenal medulla.
Members of this superfamily include receptors for other monoamines, acetylcholine, opioids, histamine, 5-hydroxytryptamine, vasopressin, chemokines and prostaglandins. The characteristic feature of GPCRs is the receptor topology which predicts a protein with seven hydrophobic transmembrane spanning domains. The sequence conservation among related GPCR subfamilies has greatly facilitated the isolation of genes for other family members through gene cloning techniques. Not surprisingly, receptors which bind the same endogenous ligand show the highest degree of sequence similarity, especially in the transmembrane spanning regions.

The first member of the dopamine receptor family was cloned in 1988 (Bunzow et al., 1988). The rat D2DR was cloned using the hamster β2-adrenoceptor (β2AR) gene as a probe. The full-length clone, unlike most of the other GPCRs cloned at that time, contained introns which interrupted the coding region of the gene. The presence of introns is now considered to be a characteristic feature of D2-like receptors. In 1990, the human D1DR gene was cloned (Sunahara et al., 1990) using the sequence of the D2DR gene and was found to be intronless. The rat dopamine D3 receptor (D3DR) gene was cloned in the same year using a probe derived from the D2DR sequence (Sokoloff et al., 1990). The D3DR was found to be similar to the D2DR in terms of its pharmacology and gene structure and was, therefore, described as "D2-like". The final known D2-like receptor, the human dopamine D4 receptor (D4DR), was cloned in 1991 (VanTol et al., 1991), also using the D2DR as a probe. Later that year, another D1-like receptor was cloned: the dopamine D5 (D5DR) or D1B receptor (Sunahara et al., 1991).

D1-like receptors, like most other GPCRs, are intronless. The preponderance of intronless GPCRs is consistent with the theory that the many genes encoding these receptors arose through gene duplication events from a common progenitor gene (reviewed in O'Dowd, 1993). The smaller size of an intronless gene would increase the likelihood that upon duplication both copies of the gene would be functional. D2-like receptors, on the other hand, contain introns. The location of the introns in D2-like genes appears to be similar to others within the subfamily and also to the opsin gene. Since opsin is the suspected progenitor to GPCRs, these observations again support the idea that GPCR genes share a common phylogenetic origin.
suspected progenitor to GPCRs, these observations again support the idea that GPCR genes share a common phylogenetic origin.

1.2 Dopamine D3 Receptors

The D3DR was first cloned from rat (Sokoloff et al., 1990), followed by the cloning of the human (Giros et al., 1990) and murine D3DR (Fishburn et al., 1993). The D3DR is considered to be D2-like due to its similarity to the D2DR with respect to its pharmacology, coupling to AC inhibition, amino acid sequence and intron-containing gene structure. The human D3DR shares 46% overall identity with the D2DR and 78% homology within the transmembrane spanning regions alone. Figure 1.2 shows the predicted structure of the D3DR. Of note is the relatively long third intracellular loop and short carboxy terminus characteristic of these receptors. This structure is also shared by the α2-adrenoceptor (α2AR), the receptor to which the D2DR has closest homology, and is, therefore, suspected to be important for coupling to inhibitory G proteins (O'Dowd, 1993). The carboxyl tail of D2-like receptors terminates with a cysteine residue. This residue has been shown to be palmitoylated in the D2DR (Ng et al., 1994b), and a conserved residue is also palmitoylated in the β2AR (O'Dowd et al., 1989). The D3DR has three potential sites which may be N-glycosylated, Asn12 and Asn19 in the amino terminus and Asn97 in the second extracellular domain. The human D3DR gene contains 5 introns and spans over 53,000 bp (Figure 1.3) (Griffon et al., 1996).

The presence of introns in the coding region of the gene allows for the possibility of alternative splicing of gene products. Indeed, several splice variants of the D3DR have been reported in the rat, mouse and human (Table 1.1). In comparison, only one splice variant is known for D2DR and arises from a splicing event which deletes 29 amino acids in the third intracellular loop (Giros et al., 1989). The resulting variants, D2L and D2S, encode functional receptors which differ in many ways from each other. For example, D2L is the predominant form in vivo, however, D2S has a 2-3 fold higher affinity for some substituted benzamide antagonists (Malmberg et al., 1993). Further, the D2S isoform is processed to the mature glycosylated species faster than D2L and some D2L remains in the ER in an unprocessed...
Figure 1.2: A model showing the predicted topographical structure of the D3DR. As with other D2-like receptors, the amino terminus is extracellular, there are seven transmembrane spanning domains and the carboxyl tail is short. Amino acids which are conserved among all D2-like receptors (D2DR, D3DR and D4DR) are shown with solid circles.
Figure 1.3: Organization of the human D3DR gene, heterogeneous nuclear RNA transcript, messenger RNA (mRNA) and receptor. Regions corresponding to exons are numbered and shaded areas indicate regions encoding transmembrane domains. Boxes represent transmembrane domains in the translated receptor.
form (Fishburn et al., 1995). There are also differences in the ability of each isoform to couple to G proteins (Liu et al., 1996; Senogles, 1994). Interestingly, a similar D3DR variant has been reported in the mouse, however, the splicing occurs via an internal acceptor site instead of a separate cassette exon as in the case of the D2DR (Fishburn et al., 1993). Comparable D3S and D3L variants in the human and rat are unlikely to exist since an analogous intron in this region appears to be missing in these species.

Many other splice variants of the D3DR have been found in rat and human which are predicted to encode truncated receptors (Table 1.1). One variant, the D3nf, is the focus of this investigation and is described below (section 1.9). Another variant, detected in both rat and human, results from the deletion of 113 base pairs (bp) in TM3 (Giros et al., 1991; Griffon et al., 1996; Snyder et al., 1991). The deletion causes a shift in the reading frame and the generation of a premature stop codon thereby resulting in a truncated protein with only two transmembrane domains. Another truncated variant has been reported in the human with a 143 bp deletion in the second intracellular loop with a premature stop codon (Griffon et al., 1996; Nagai et al., 1993). Yet another variant results from a 256 bp deletion in transmembrane domain three and has a premature stop codon (Griffon et al., 1996). An insertion event in a mouse variant adds 84 bp and an in frame stop codon after TM1 (Pagliusi et al., 1993). Finally, one variant in the rat has a 54 bp deletion in the second extracellular loop and encodes a protein with seven transmembrane spanning domains, however, it does not bind dopaminergic ligands (Giros et al., 1991). In fact, none of these splice variants, except the murine D3S and D3L, have been shown to be functional receptors in transiently transfected cells.
Table 1.1: Splice variants of the dopamine D3 receptor.

<table>
<thead>
<tr>
<th>Species</th>
<th>Insertion (+)/Deletion (-)</th>
<th>Region</th>
<th>Frame-shift?</th>
<th>Predicted Protein Size</th>
<th># TMs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>-113 bp</td>
<td>TM3</td>
<td>Yes</td>
<td>100 or 109 aa</td>
<td>2 TMs</td>
<td>(Giros et al., 1991; Snyder et al., 1991)</td>
</tr>
<tr>
<td>Human</td>
<td>-113 bp</td>
<td>TM3</td>
<td>Yes</td>
<td>109 aa</td>
<td>2 TMs</td>
<td>(Griffon et al., 1996; Snyder et al., 1991)</td>
</tr>
<tr>
<td>Rat</td>
<td>-54 bp</td>
<td>ECL2</td>
<td>No</td>
<td>428 aa</td>
<td>7 TMs</td>
<td>(Giros et al., 1991)</td>
</tr>
<tr>
<td>Mouse</td>
<td>-63 bp</td>
<td>ICL3</td>
<td>No</td>
<td>446 vs. 425 aa</td>
<td>7 TMs</td>
<td>(Fishburn et al., 1993)</td>
</tr>
<tr>
<td>Human</td>
<td>-143 bp</td>
<td>ICL2</td>
<td>Yes</td>
<td>138 aa</td>
<td>3 TMs</td>
<td>(Griffon et al., 1996; Nagai et al., 1993)</td>
</tr>
<tr>
<td>Mouse</td>
<td>+84 bp</td>
<td>ECL1</td>
<td>No</td>
<td>na</td>
<td>1 TM</td>
<td>(Pagliusi et al., 1993)</td>
</tr>
<tr>
<td>Human</td>
<td>-98 bp</td>
<td>ICL3</td>
<td>Yes</td>
<td>342 aa</td>
<td>6 TMs</td>
<td>(Schmauss et al., 1993)</td>
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<tr>
<td>Human</td>
<td>-256 bp</td>
<td>TM3</td>
<td>Yes</td>
<td>na</td>
<td>3 TMs</td>
<td>(Griffon et al., 1996)</td>
</tr>
</tbody>
</table>

na: not available
1.3 Dopamine D3 receptor distribution and localization in brain

Most of the studies which have investigated the distribution of D3DR in brain have relied on in situ hybridization to localize mRNA. Other methods have included visualization by receptor autoradiography or by immunocytochemistry. Experimentally, in situ hybridization is reliable because it is specific to the mRNA of interest, however, the results must be interpreted with caution as the distribution of mRNA may not reflect the distribution or density of translated protein. Nor does this approach show the cellular localization of proteins to axons or synapses since RNA is mostly located in the somata. The use of antibodies to detect protein product is limited because it cannot distinguish between functional and non-functional receptor populations and relies on antibody specificity to distinguish closely related proteins. The best means of detecting functional receptors is via binding analysis however, this technique is hindered by the availability of ligands to discriminate among different receptor subtypes - a limitation especially problematic for the dopamine receptors. Further, the ability of ligands to selectively label one receptor population over another appears to be dependent upon the in vitro assay conditions (Burris et al., 1995; Gonzalez and Sibley, 1995; Levant et al., 1995).

Overall, the distribution of D3DR protein and mRNA is more discrete and less abundant compared to that for the D2DR. Notably, the distribution of the D2DR at the cellular level does not appear to overlap that of the D3DR, suggesting that direct functional interactions between the two are not likely to occur (Bouthenet et al., 1991; Diaz et al., 1995). D3DR mRNA is expressed preferentially in limbic brain regions with the highest density in the granule cells of the islands of Calleja and the medial shell of the nucleus accumbens (Bouthenet et al., 1991; Diaz et al., 1995). These areas mirror the most prominent areas of D3DR binding which also include the olfactory tubercle and lobules 9 and 10 of the cerebellum (archicerebellum) (Diaz et al., 1995; Levesque et al., 1992). Since the mRNA and binding sites overlap, it is believed that receptor localization corresponds to dendrites or perikarya rather than axons of distant neurons (Diaz et al., 1995; Levesque et al., 1992). Moderately dense expression of D3DR mRNA is also observed in the mammillary nucleus, the lateral part of the substantia nigra pars compacta, cerebral cortex and amygdala (Bouthenet et al., 1991; Diaz et al., 1995). Low densities of
D3DR mRNA are found in the cerebral cortex, caudate/putamen, ventral pallidum, ventral tegmental area and cerebellar cortex (Bouthenet et al., 1991; Sokoloff et al., 1990).

1.4 Dopamine D3 receptor coupling in heterologous systems

Earlier reports of D3DR expression in different cell systems were unable to observe functional coupling or agonist detected high and low affinity binding, implying that the receptor was unable to couple to G proteins. Further investigations into the coupling of the D3DR have been fraught with inconsistencies as various researchers, even when using the same cell system, have come to different conclusions. Sokoloff's report of the cloning of the rat D3DR, for example, showed no shift in agonist binding curves in the presence of GTP or coupling to AC when expressed in Chinese hamster ovary (CHO) cells (Sokoloff et al., 1990). In 1992, the same group expressed the human D3DR in the same cell line and observed a GTP-induced shift in the agonist binding curve but made no report of functional coupling (Sokoloff et al., 1992a). In 1994, another group reported a Gpp(NH)p- induced shift of the agonist binding curve and no coupling to AC for the human D3DR (MacKenzie et al., 1994). These discrepancies could reflect differences between the rat and human D3DR which may alter coupling to endogenous G proteins. However, in 1994, yet another group reported that the rat D3DR showed both a GTP induced change in agonist affinity in CHO cells and coupling to AC (Chio et al., 1994). A clear explanation for these varied reports is not obvious.

Compared to the D2DR, the high affinity state of the receptor appears to be more stable and resistant to guanine nucleotides and Na+ (Burris et al., 1995; MacKenzie et al., 1994). This observation may explain the higher affinity of the D3DR for agonists compared to the D2DR. In the same way, the magnitude of guanylnucleotide-induced shifts in agonist binding curves may be smaller for D3DR than D2DR because the low-affinity state of the receptor has a much higher affinity for agonists (Chio et al., 1994; Pilon et al., 1994).

Table 1.2 summarizes what has been examined to date in terms of D3DR coupling in different systems. Some of the studies are contradictory and vary not only in terms of the presence of coupling to
effector systems but also in the efficacy of coupling. In general, the D3DR appears to be more restricted and less efficacious in coupling to effector mechanisms compared to the D2DR. Also, the fact that effector coupling in some cases can be abolished by pretreatment with pertussis toxin (PTX) suggests that the receptor may couple to Go or Gi (Chio et al., 1994; Liu et al., 1996; Pilon et al., 1994; Potenza et al., 1994; Seabrook et al., 1994; Werner et al., 1996).

The contradictory results observed for the D3DR emphasize that caution must be used when drawing conclusions from heterologous systems concerning the physiological function of receptors in vivo. The availability and stoichiometry of G proteins, effector enzymes and overall cellular environment in cell lines may differ from what exists for the D3DR in vivo. Cell systems are, however, invaluable for discovering candidate coupling systems for a receptor and for studying second messenger cascades because native tissues expressing D3DR are difficult to study and may express more than one subtype of dopamine receptor.
Table 1.2: D3 receptor coupling in heterologous systems.

<table>
<thead>
<tr>
<th>Heterologous Cell Line</th>
<th>D3DR Species</th>
<th>Signaling Pathway</th>
<th>PTX Sensitive</th>
<th>GTP-shift</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6 glioma</td>
<td>Rat</td>
<td>↑extracellular acidification (via Na/H antiporter)</td>
<td>No</td>
<td>ND</td>
<td>(Cox et al., 1995)</td>
</tr>
<tr>
<td>CCL1.3</td>
<td>Human</td>
<td>↔cAMP</td>
<td>Yes</td>
<td>ND</td>
<td>(MacKenzie et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↔inositol phosphate production</td>
<td>No</td>
<td></td>
<td>(Tang et al., 1994)</td>
</tr>
<tr>
<td>CHO</td>
<td>Rat</td>
<td>↔cAMP</td>
<td>No</td>
<td></td>
<td>(Sokoloff et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>↔cAMP</td>
<td>Yes</td>
<td></td>
<td>(MacKenzie et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>↔arachidonic acid release</td>
<td>No</td>
<td></td>
<td>(Freedman et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>↔cAMP</td>
<td>No</td>
<td></td>
<td>(Chio et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>↑mitogenesis</td>
<td>ND</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑extracellular acidification</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COS-7</td>
<td>Rat</td>
<td>↔cAMP</td>
<td>No</td>
<td></td>
<td>(Sokoloff et al., 1990)</td>
</tr>
<tr>
<td>GH4Cl</td>
<td>Human</td>
<td>↔AA metabolism</td>
<td>Yes</td>
<td></td>
<td>(Seabrook et al., 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↔cAMP</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>↔inositol phosphate turnover</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↔intracellular calcium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↔potassium current</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK-293</td>
<td>Rat</td>
<td>↔cAMP</td>
<td>Yes</td>
<td></td>
<td>(Burris et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>↓cAMP</td>
<td></td>
<td></td>
<td>(Robinson and Caron, 1996)</td>
</tr>
<tr>
<td>MN9D</td>
<td>Human</td>
<td>↔cAMP</td>
<td>No</td>
<td></td>
<td>(Tang et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↔inositol phosphate production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑branching and length of neurites</td>
<td></td>
<td></td>
<td>(Swarzenski et al., 1994)</td>
</tr>
<tr>
<td>Heterologous Cell Line</td>
<td>D3DR Species</td>
<td>Signaling Pathway</td>
<td>PTX Sensitive</td>
<td>GTP-shift</td>
<td>Reference</td>
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<tr>
<td>------------------------</td>
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</tr>
<tr>
<td>NG108-15</td>
<td>Human</td>
<td>↑ c-fos gene activation ↑[^3H]thymidine incorporation ↔cAMP</td>
<td>Yes</td>
<td>Yes</td>
<td>(Pilon et al., 1994)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td>↓calcium current</td>
<td>Yes</td>
<td>ND</td>
<td>(Seabrook et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓cAMP</td>
<td>Yes</td>
<td>ND</td>
<td>(Griffon et al., 1997)</td>
</tr>
<tr>
<td>Sk-N-MC</td>
<td>Human</td>
<td>↔cAMP</td>
<td>Yes</td>
<td>ND</td>
<td>(MacKenzie et al., 1994)</td>
</tr>
<tr>
<td>Xenopus melanophores</td>
<td>Human</td>
<td>↑melanososome aggregation ↓cAMP</td>
<td>Yes</td>
<td>ND</td>
<td>(Potenza et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓whole cell K⁺ current</td>
<td>Yes</td>
<td>ND</td>
<td>(Liu et al., 1996)</td>
</tr>
<tr>
<td>Xenopus oocytes</td>
<td>Human</td>
<td>Couple to inwardly rectifying K⁺ channel (GIrK)</td>
<td>Yes</td>
<td>ND</td>
<td>(Werner et al., 1996)</td>
</tr>
</tbody>
</table>

ND: not determined
1.5 Dopamine D3 receptor physiological function

Determining the function of the D3DR, or any other dopaminergic receptor, in organisms has been, and continues to be, a challenge. Most experimental evidence comes from studies which use so-called "selective" D3DR ligands in behavioural models, however, few dopaminergic ligands can truly discriminate among dopamine receptors, especially at higher concentrations. For example, 7-OH-DPAT has been considered to be a selective D3DR ligand for many years based on the higher affinity of 7-OH-DPAT for heterologously expressed D3DR over D2DR (Freedman et al., 1994; MacKenzie et al., 1994; Tang et al., 1994). Some studies have pointed out that the selective affinity of 7-OH-DPAT and other ligands are sensitive to in vitro assay conditions (Burris et al., 1995; Gonzalez and Sibley, 1995; Levant et al., 1995), hence the reported higher affinity for D3DR may not persist in vivo. Other observations of D3DR function in organisms come from the use of knock-out animals in which the D3DR is effectively deleted from the genome (Accili et al., 1996; Xu et al., 1997). Such animals may prove invaluable for elucidating which dopaminergic functions are mediated via D3DR versus other D2-like receptors. However, interpretations must be made with caution as it is possible that behavioural changes in knock-out animals may be caused from developmental anomalies arising from D3DR loss or from compensatory changes in other neurotransmitter systems which adapt to the loss of D3DR.

Many studies, using a variety of experimental techniques, have implicated the D3DR as a modulator of locomotor activity. Stimulation of the D3DR appears to inhibit locomotor activity in an opposite manner to the D2DR which is believed to increase locomotion. Accordingly, many studies have observed a bi-phasic effect of 7-OH-DPAT (Daly and Waddington, 1993; Svensson et al., 1994) or PD 128907 (Pugsley et al., 1995) on locomotion in rats with lower doses inhibiting and higher doses stimulating locomotion. Consistent with this idea, one strain of D3DR knock-out mice demonstrates increased locomotor activity, rearing and hyperactivity (Accili et al., 1996). Another knock-out strain shows increased activity in a novel environment (Xu et al., 1997).

The D3DR may also play a role mediating effects of reinforcement and reward suggesting that it may be a potential target for pharmacotherapies for drug abuse and dependence. For instance, 7-OH-
DPAT has been shown to decrease self-administration of cocaine (Caine and Koob, 1993; Caine et al., 1997) and also enhance the reinforcing properties of cocaine (Parsons et al., 1996). Intra-amygdala infusion of 7-OH-DPAT following discriminative approach training has suggested the D3DR may be involved in stimulus-reward behaviour (Hitchcott et al., 1997).

The dopaminergic system is believed to be involved with learning and memory. Stimulation of D3DR by 7-OH-DPAT impaired the acquisition, consolidation and retention phases of memory in mice (Ukai et al., 1997). Another study in rats concluded that D2DR and D3DR have opposite effects on memory in a paradigm using scopolamine-induced amnesia with the D2DR facilitating and the D3DR inhibiting memory consolidation (Sigala et al., 1997).

Other behavioural effects attributed to stimulation of D3DR include yawning, hypothermia, decreased sniffing, decreased alcohol consumption and increased penile erection and ejaculatory behaviour (Shafer and Levant, 1998).

1.6 The Dopaminergic System and Disease

Schizophrenia

Schizophrenia is a severe, chronic and debilitating mental disorder that affects approximately 1% of the general population. The symptoms of this disorder are manyfold, including "positive" symptoms such as hallucinations, delusions, disorganized speech and "negative" symptoms such as withdrawal and affective blunting (Andreasen, 1995). In the early 1950's, it was noted that the drug chlorpromazine was able to alleviate schizophrenic symptoms, however, the target of drug action was then unknown. In 1976, Seeman and co-workers recognized that the antipsychotic action of neuroleptics was correlated with their ability to block D2-like receptors leading to the longstanding dopamine theory of schizophrenia (Seeman et al., 1976). This theory has been fundamental in directing research behind the etiology and treatment of this disorder. Some researchers now believe that the complexity of schizophrenia implies that defects in other neurotransmitter systems may also contribute to the disorder. Nevertheless, any satisfactory model of schizophrenia must account for the observation that neuroleptics
with high affinity for D2-like receptors show clinical efficacy in the management of schizophrenic symptoms.

The expression pattern of the D3DR in areas of the brain associated with affective, emotional and cognitive functions suggests that this subtype may be an appropriate target for neuroleptic drug development (Sokoloff et al., 1992b). Further, the selective receptor distribution to limbic areas suggests that drugs selective for the D3DR may not be associated with the undesirable extrapyramidal symptoms observed with non-selective D2 antagonists. A small number of studies have shown an association between polymorphic alleles of the D3DR and schizophrenia. Homozygosity in one allele, known as the Ball restriction fragment length polymorphism, was associated with schizophrenia in two French and British populations with homozygosity at the D3DR (Croq et al., 1992). This finding has been supported by some studies, but not all (reviewed in Levant, 1997).

**Parkinson's Disease**

Parkinson's disease is an idiopathic, slowly progressive, degenerative CNS disorder with characteristic features of akinesia, rigidity and tremor. The onset of Parkinson's is late in life, with symptoms emerging when putamen function declines to 75% of normal as dopaminergic cells of the substantia nigra degenerate (Hagan et al., 1997). A breakthrough in the treatment of Parkinson's disease occurred with the advent of L-dopa which readily crosses the blood-brain barrier and is converted to dopamine in the appropriate neurons (figure 1.1). Unfortunately, over time, decreased drug efficacy and dyskinetic symptoms develop. Second generation drugs for the treatment of Parkinson's include drugs with higher affinity for the D3DR, such as ropinirole and pramipexole, because these selective agents may be associated with a lower incidence of dyskinesias (Hagan et al., 1997).

**1.7 G protein-coupled receptor oligomers**

Until recently, the most widely accepted paradigm of GPCR signaling maintained that a single receptor on the cell surface bound a molecule of agonist which, in turn, caused the activation of an
intracellular G protein leading to a cellular response. It was believed that the stoichiometry of the so-called "ternary complex" was 1:1:1 (agonist: receptor: G protein) (DeLean et al., 1980).

A handful of reports in the early 1980's first proposed the existence of oligomeric GPCR forms. For example, photolabelling of muscarinic receptors followed by SDS-PAGE revealed labelling of species at molecular weights which were consistent in size with receptor monomers, dimers and tetramers (Avissar et al., 1983; Avissar et al., 1982). In recent years, researchers have begun to provide more convincing evidence to support the existence of GPCR dimers. Elegant experiments from Wess's group (Maggio et al., 1993a) constructed α2-adrenergic and m3 muscarinic receptor chimeras with the first five TMs of one receptor paired with the last two TMs of the other. When expressed alone, the chimeras were not functional but when co-expressed the chimeras bound both muscarinic and adrenergic radioligands with affinities similar to that of wild-type receptors. Not only could the co-expressed chimeras bind ligands, but they were also able to functionally couple to phosphatidylinositol hydrolysis, albeit with an E_{max} half that of the wild-type m3 receptor. Combined with earlier observations that functional muscarinic receptors resulted from co-expression of one fragment encoding TM1-5 and another encoding TM6-7 (Maggio et al., 1993b), a model was put forth which proposed that muscarinic receptors consisted of at least two autonomous folding units (Figure 1.4) (Maggio et al., 1993a). Using this model, one can envision the C-terminal portion of one chimera interacting with the N-terminal portion of the other to form a continuous "wild-type" receptor. This exchange of amino-terminal and carboxy-terminal domains is also known as "domain swapping" (Gouldson and Reynolds, 1997).

The idea of receptors being formed from more than one autonomous folding unit has been expanded in experiments using the m3 muscarinic receptor, which have shown that fragments consisting of as few as two TM domains can be inserted stably into the membrane and that co-expression of complementary fragments restored radioligand binding (Schoneberg et al., 1995). Similar experiments with polypeptide fragments of bovine opsin demonstrated that the N-terminal fragments were glycosylated, suggesting that the fragments were inserted into the membrane and were appropriately
Figure 1.4: Model depicting the proposed subunit character of GPCRs. The cylinders represent TM1-5 and TM6-7 and the thick lines represent the extramembranous receptor sequences. The third intracellular loop (ICL3) links the two structural receptor subunits (adapted from Maggio et al. (1993b)).
recognized for transport from the ER to Golgi. Further, co-expression of complementary fragments formed pigments with rhodopsin-like spectral properties (Ridge et al., 1995). Together, these studies support the idea that GPCRs fold and assemble in multiple subunits which then associate to form a functional protein molecule.

The muscarinic/adrenergic chimeras discussed above were also used to show that the interaction between two members of a heterodimer was dependent upon the presence of a long third intracellular loop (Maggio et al., 1996). Functional reconstitution of m3 muscarinic or α2AR binding sites was prevented when 196 amino acids from the third intracellular loop of the chimeras were deleted. A relevant physiological extension of these findings implies that dimerization of the D2S receptor, which lacks 29 amino acids in ICL3, may be compromised compared to dimerization of the D2L. This, however, has been shown not to be the case (Lee et al., unpublished observations).

Functional complementation of co-expressed receptors which are individually defective has also implied that receptors can dimerize (Monnot et al., 1996). While investigating the binding site of the AT1 angiotensin receptor, Monnot and co-workers discovered that the co-expression of two binding deficient proteins with single amino acid mutations in TM3 or TM5 restored angiotensin binding. This implies that intermolecular interactions can occur between two proteins that possess complementary TM domains resulting in the reconstitution of a functional binding pocket.

Another form of functional complementation was discovered with a mutant luteinizing hormone (LH) receptor, truncated after TM5, which was found in a patient with Leydig cell hypoplasia (Osuga et al., 1997). The mutant receptor could not functionally couple to AC and had limited binding capacity. The fact that LH receptors and follicle-stimulating hormone (FSH) receptors have a separate binding domain (the N-terminus) and signalling domain (TMs), led to the construction of a chimera consisting of the FSH binding domain linked to the LH signalling domain. This chimera could not bind LH but rather human chorionic gonadotropin, a FSH receptor ligand. When co-expressed with the truncated LH receptor, stimulation by LH lead to an increase in cAMP, presumably though an intermolecular interaction between the mutant protein which bound LH and the chimera which had the ability to couple
to AC. This functional complementation between the binding domain of one protein and the signalling domain of the other was also shown by the restoration of a cAMP response when the LH receptor mutant was co-expressed with LH receptor mutant lacking the N-terminus (Osuga et al., 1997).

Support for the theory of GPCR dimerization also arose from experiments involving one of the more interesting GPCRs, the thrombin receptor. This receptor is activated via a novel proteolytic mechanism whereby the receptor's amino terminus is cleaved by thrombin to reveal a new amino terminus which acts as a "tethered ligand" which activates the receptor. This tethered activation is most efficacious when acting intramolecularly, however, it can also act intermolecularly between two thrombin receptors (Chen et al., 1994).

That receptors appear to migrate on SDS-PAGE at sizes consistent with multiples of the molecular weight of the monomer has suggested the existence of SDS-resistant dimers of the D1 dopamine (Ng et al., 1994b), D2L dopamine (Ng et al., 1994a; Ng et al., 1996), 5-HT1B (Ng et al., 1993), M2 muscarinic (Parker et al., 1991) and V2 vasopressin (Hebert et al., 1996) receptors. Direct evidence of GPCR dimerization was obtained using co-immunoprecipitation of differentially epitope-tagged β2ARs followed by separation on SDS-PAGE (Hebert et al., 1996). This technique has also been used to show that the metabotropic glutamate 5 receptor (Romano et al., 1996), the H2-histamine receptor (Fukushima et al., 1997) and δ-opioid receptor dimerize (Cvejic and Devi, 1997).

1.8 A role for truncated GPCRs

An intriguing corollary of GPCR dimerization has been the observation that some truncated receptors heterodimerize with their full-length counterparts. Not only do they physically interact, but some truncated receptors inhibit the function of the full-length receptor in a dominant negative fashion. Many experiments have been conducted on truncated receptors which were artificially constructed. However, truncated receptors may arise naturally through point mutations which give rise to a premature stop codon (nonsense mutations) or through alternative splicing mechanisms. The emerging functional role of truncated proteins in GPCR function offers new interpretations to the existence of so-called
pseudogenes which appear to be structurally similar to GPCR genes in sequence but are truncated due to nonsense mutations. Such genes are termed pseudogenes because the premature stop was thought to preempt their translation into protein, however, in light of the fact that many truncated receptors affect full-length receptor function, a new role for pseudogenes may need to be explored. However, many mRNAs that contain premature stop codons are rapidly degraded.

One of the first studies to address the role of truncated receptor proteins demonstrated that mutated proteins which caused retinal degeneration in Drosophila interfered with the maturation of wild-type rhodopsin through the endoplasmic reticulum (Colley et al., 1995). Although not all of the mutated proteins were truncated (some contained missense mutations), each appeared to cause an overproliferation of the endoplasmic reticulum (ER) and a reduction in wild-type rhodopsin expression at the cell membrane. These results were interpreted to show that the defective proteins "complex" with wild-type rhodopsin in the ER thereby preventing transport out of the ER. The researchers did not directly demonstrate that the mutant proteins and rhodopsin physically interacted.

Truncated vasopressin V2 receptors are associated with nephrogenic diabetes insipidus and have been shown, in COS cells, to be unable to bind agonist or couple to G proteins (Schoneberg et al., 1996). Confocal microscopy revealed that the truncated proteins were poorly expressed at the cell surface and were more abundant in the ER whereas the wild-type V2 receptor showed dense staining of both the plasma membrane and ER. These observations suggest that mechanisms exist in the ER to recognize and retain improperly folded receptor proteins to prevent their expression at the cell surface. This study did not address the possible implications of ER retention of truncated proteins on wild-type V2 receptors when co-expressed. However, it was shown that co-expression of a peptide corresponding to TM6-7 of the V2 receptor with the truncated mutant specifically restored binding and coupling to adenylyl cyclase with an increase in cell surface expression.

The function of truncated receptor proteins has also been investigated in receptor families other than GPCRs. For instance, a short isoform of the growth hormone receptor is produced through alternative splicing. The isoform is truncated after the single TM domain and binds growth hormone
with lower affinity and with fewer binding sites (Ross et al., 1997). The variant was not functional on its own however, when co-expressed with the full-length receptor, it could inhibit the function of the full-length receptor. That the variant and full-length receptor formed dimers was confirmed by immunoprecipitation.

A splice variant of the human gonadotropin-releasing hormone (GnRH) receptor results from a 128bp deletion which causes a shift in reading frame, truncating the receptor just after TM5 (Grosse et al., 1997). The variant expressed in COS-7 cells was non-functional and found at a lower cell surface density than the wild-type receptor. Co-expression of the wild-type and variant receptor impaired maximal agonist mediated accumulation of inositol phosphate without changing potency. Immunological studies confirmed that this "dominant negative" effect of the variant on wild-type receptor function was due to impaired trafficking of the wild-type receptor to the cell surface.

The rat prostaglandin EP1 receptor has a splice variant which appears to affect the function of the wild type receptor by a different mechanism (Okuda-Ashitaka et al., 1996). The variant diverges in sequence within TM6 and has a non-homologous TM7, however, it is expressed and is able to bind ligands as well as the wild type receptor but is not coupled to calcium mobilization. Co-expression of the wild-type receptor and variant resulted in a right shift in the concentration-effect curve for stimulation of calcium mobilization. Curiously, expression of the variant also attenuated the increase in cAMP by endogenous EP4 receptors on CHO cells. These findings are most consistent with the hypothesis that the variant EP1 receptor attenuates the function of the wild-type prostaglandin receptors by competing for ligand binding rather than through a dominant negative effect.

A clinically important example of how truncated receptors can affect the function of full-length receptors is a naturally occurring mutation of the human chemokine receptor type 5 (CCR5) which causes the receptor to be truncated after TM4 (Benkirane et al., 1997). The truncated receptor, known as ccr5d32 is preferentially retained in the ER and causes retention of the CCR5 receptor in the ER when the two are co-expressed. CCR5 functions as a co-receptor for the human immunodeficiency virus type 1 (HIV-1) during cell infectivity and when CCR5 is co-expressed with the truncated protein, cell infectivity
by HIV-1 decreases, likely due to the fact that cell surface expression of CCR5 is reduced. These findings have profound clinical implications and provide a molecular mechanism to account for the observation that patients homozygous and heterozygous for the mutant form (CCR5/CCR5d32) progress more slowly to AIDS.

The common theme behind these many examples is that truncated receptors appear to be preferentially retained in the ER, whether it be because they are not recognized for transport to the cell surface or because they are recognized as being misfolded and targeted for digestion, and do not function in the same manner as their wild-type counterpart. Instead, truncated receptors appear to inhibit the function of the full-length receptors by what is known as a dominant negative effect.

1.9 The D3nf, a dopamine D3 receptor splice variant

The most extensively characterized of the D3DR splice variants is known as D3nf. The D3nf was identified by Schmauss in 1993 using PCR and primers designed within the fifth transmembrane spanning domain and carboxyl tail regions of the human D3DR (Schmauss et al., 1993). An unexpected PCR product was found which was approximately 100 bp shorter than the size of the PCR product predicted for amplification of D3DR sequence. This PCR product was used to isolate a full-length cDNA for the open reading frame of the D3nf from parietal and cortical brain tissue. The D3nf cDNA differs from the cDNA for the D3DR by the deletion of 98 bp in the 3' portion of the putative third intracellular loop. The deletion leads to a 1bp shift of the open reading frame, resulting in 58 novel amino acids followed by a premature stop codon. Hence, the D3nf differs from the D3DR in its predicted carboxyl-terminal amino acid sequence and does not contain the D3DR TM6 and TM7 (Figure 1.5).

The 98 bp which are deleted in the D3nf are found within a continuous exon and therefore do not appear to arise from the removal of a cassette intron. It has been hypothesized that the cellular splicing
Figure 1.5: The predicted amino acid sequence of the D3nf. The amino acid residues which differ from the D3DR sequence are shown in black.
machinery recognizes the 98bp as an alternative intron with an atypical 3' splice site (GA:GU instead of AG:GU) (Schmauss, 1996). Further studies by Schmauss's group (Liu et al., 1994), established that D3nf mRNA is as abundant as D3DR mRNA in human cortical tissue and that polyclonal antibodies raised against the unique portion of the D3nf immunoreacts with a 68kD protein in human cortical tissue. This argues that the D3nf mRNA does not result from an error in the cellular RNA splicing machinery and that it is translated into protein. The function of D3nf, however, is not known.

The D3DR has many splice variants (section 1.2) and the role that these receptor variants play in D3DR pharmacology has yet to be understood. It is possible that these variants form at random during the biosynthesis of functionally active D3DR protein. If so, mRNA encoding these variants would be expected to be in low abundance and may not be translated. Since these variants were all discovered using highly sensitive, non-quantitative polymerase chain reaction (PCR) based techniques, mRNA which were identified may have been present at low abundance in the cytoplasm. It is possible, therefore, that these variants represent errors in the RNA splicing machinery and would not be physiologically relevant. This is not likely the case for the D3nf as the mRNA encoding the D3nf has been shown to be as abundant as D3DR mRNA and, further, the message is translated into protein. A more interesting hypothesis regarding the function of D3DR receptor splice variants is that generation of mRNA encoding these variants is physiologically related to mechanisms regulating the density and/or function of D3DR. Further, the formation of these variants may be dependent on cell type or be altered during disease states.
1.10 Spodoptera frugiperda (Sf9) cells and baculovirus infection

One of the most common baculovirus isolates used for recombinant gene expression in insect cells is *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). The genome of the virus is double-stranded, circular and over 130 kb in size. Baculoviruses show both lytic and occluded life cycles that occur over three distinct phases. The early phase includes viral attachment, penetration, uncoating and early gene expression. The initial viral synthesis occurs 0.5 to 6 hours post-infection as the host genes are shut off and normal cellular functions decline. During the late phase, genes which direct replication of viral DNA and viral assembly are expressed. Infected cells stop dividing, increase in diameter and have enlarged nuclei. Extracellular virus begins to be produced 6 to 12 hours after infection and peaks between 18 and 36 hours. In the very late phase 24 to 96 hours post-infection, the polyhedrin and p10 genes are expressed which allows for the production of occluded virus and cell lysis occurs.

In nature, insect larvae become infected by baculovirus through ingestion. The virus infects the cells of the mid-gut and, once extracellular virus is produced during the late phase of infection, the virus particles are released into the hemolymph, thereby promoting the infection of other cells. After several days the insect dies and releases occluded virus. These virions are protected by a crystalline polyhedra which adhere to plant surfaces and allow vertical transmission of the virus to other feeding larvae.

One of the most widely used cell lines for gene expression through recombinant baculoviral infection is Sf9, a clonal isolate derived from the ovarian tissue of the *Spodoptera frugiperda*, a fall armyworm. For recombinant gene expression using the baculoviral genome, the polyhedrin gene, under control of the polyhedrin promoter is replaced with the recombinant cDNA of interest (for details, see Materials and Methods). The polyhedron gene is not required during baculoviral replication in cell culture and the promoter is under very strong transcriptional control during the very late phase of infection (Jarvis, 1991). Recombinant baculoviruses are becoming very popular for use in gene expression over mammalian cells because the strength of the polyhedrin promoter results in abundant recombinant gene expression. Further, insect cells appear to contain many of the protein modification,
processing and transport systems present in mammalian cells. In fact, recombinant gene products produced in insect cells appear to have the antigenic and biological properties of the native product (Jarvis, 1991). One of the greatest drawbacks to the use of these cells is that viral infection may seriously compromise the secretory pathways of the host cells and, indeed, infection results in cell death.
2. **Hypothesis and Objectives**

As introduced above, there are many examples of truncated receptors altering the function of full-length wild-type receptors, in some cases, through hetero-dimerization. These truncated receptors may arise through mutation or though alternative splicing, as is the case for the D3nf. The fact that the D3nf is known to be translated into protein and co-localizes with the D3DR in brain suggests that it may have a physiologically important role.

**Hypothesis:** The truncated D3DR splice variant, D3nf, interacts with the human D3DR to form oligomers and this interaction modulates the pharmacology of the D3DR.

The objectives of this study were to:

I. Generate the cDNA construct for the D3nf.

II. Characterize a heterologous expression system for study of the D3DR and D3nf.

III. Demonstrate oligomers of D3DR and D3nf.

IV. Investigate the i) physical, ii) functional and iii) subcellular/cellular interactions between the D3DR and D3nf.
3. Materials and Methods

3.1 Materials

All enzymes for DNA manipulation were purchased from Pharmacia Biotech (Baie d'Urfe, PQ) except eLONGase which was from Gibco/BRL (Burlington, ON). Primers were synthesized by the Hospital for Sick Children Biotechnology Service Centre (Toronto, ON). Deoxycytidine-5'-triphosphate [α-35S] used in sequencing was obtained from ICN Pharmaceuticals (Irvine, CA).

Spodoptera frugiperda (Sf9) cells were from ATCC (Rockville, MD). All materials for Sf9 cell culture and recombinant baculovirus production including media and supplements were purchased from Gibco/BRL (Burlington, ON). Pre-cast acrylamide gels used for SDS-PAGE were from Novex (San Diego, CA). 9E10 antibody, agarose conjugate and c-myc peptide were from Santa Cruz Biotechnology (Santa Cruz, CA) and the polyclonal M2 antibody, monoclonal M5 antibody, M2 agarose conjugate and FLAG peptide were purchased from Sigma Chemical Co. (St. Louis, MO). The goat anti-mouse antibody coupled to alkaline phosphatase and BCIP/NBT substrates were purchased from Bio-Rad (Burlington, ON). The secondary fluorescent conjugated antibodies were from Calbiochem (San Diago, CA). Miwiol-88 was from Hoechst (Montreal, PQ). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

3.2 General Molecular Techniques

3.2.1 Mini-preparation of plasmid DNA

Unless stated otherwise, all plasmid DNA was prepared by alkaline lysis (Ausubel et al., 1995). Bacterial colonies, or 5 μl of a stock of bacterial culture, were grown overnight at 37°C in an orbital shaker in LB plus the appropriate antibiotic for selection of transformed bacterial colonies with the vector. Two aliquots of this culture were saved: one for short term use and kept at 4°C and the other for long term storage in 30% glycerol at -70°C. The cells in the remaining culture were pelleted by centrifugation at 7000 rpm for 5 minutes. The pellet was resuspended in solution I (50 mM glucose, 25
mM Tris-HCl (pH 8) and 10 mM EDTA (pH 8)). A double volume of solution II (NaOH (0.2N), SDS (1%)) was added and the solution was gently mixed and incubated at room temperature for 2 minutes. 170 µl of potassium acetate (3 M, pH 5.5) was slowly added, mixed and placed on ice for 3 minutes. The protein precipitate was pelleted by centrifugation at 14 000g and the DNA containing supernatant transferred to a fresh eppendorf tube. The DNA was separated from cellular proteins by the addition of equal volumes of phenol and chloroform. The mixture was partitioned by centrifugation at 13000rpm for 5 minutes. The top layer was transferred to a fresh eppendorf tube containing ice-cold 100% ethanol and the DNA was pelleted by centrifugation at 14 000g. The pellet was washed in 70% ethanol, resuspended in TE buffer and stored at -20°C until use.

3.3 Expression vectors

The cDNA encoding the human D3DR in the expression vector pRC/CMV was obtained as a gift from Dr. C. Schmauss (Mt. Sinai School of Medicine, NY). To facilitate immunodetection, the D3DR was tagged with amino acids 411-421 of the human c-myc protein (Evan et al., 1985) and the D3nf was tagged with FLAG, an 8 amino acid synthetic peptide (Table 3.1).

Table 3.1: Characteristics of the epitopes used to immunodetect the D3DR and D3nf.

<table>
<thead>
<tr>
<th>Epitope Name</th>
<th>Protein Sequence</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>EQKLISEEDL</td>
<td>human c-myc protein</td>
</tr>
<tr>
<td>FLAG</td>
<td>DYKDDDDK</td>
<td>synthetic peptide</td>
</tr>
</tbody>
</table>
Table 3.2: The mutagenic and selection primers used to alter the pRC/CMV/D3 vector. Mutated sequences are indicated in bold italics and the sequences encoding the c-myc and FLAG epitopes are underlined.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection Primer</td>
<td>Mutagenic Primers</td>
</tr>
<tr>
<td>BglII-KO</td>
<td>ACGGATCGGGAATATCCCGATCCCCCTATG</td>
</tr>
<tr>
<td>Del-D3nf</td>
<td>GGAGGAGAAGTTGAAAGAGAGAAGACTCGGAA TTTCTTGAGTCAGCTGAGCTGAGTAG CC</td>
</tr>
<tr>
<td>FLAG-D3</td>
<td>CGCGGCCGCGGTGAAACCTTATGGAACCAAACCCCAACCCCAAAATGGTGCCCATTTGCTTGGGGCC</td>
</tr>
<tr>
<td>c-myc-D3</td>
<td>CGCGGCCGCGGTGAAACCTTATGGAACCAAACCCCAACCCCAAAATGGTGCCCATTTGCTTGGGGCC</td>
</tr>
</tbody>
</table>

3.3.1 Site-directed mutagenesis

The Transformer™ site-directed mutagenesis method (Clontech, Palo Alto, CA.) was used to insert the DNA code for the epitope tags after the ATG start codon in the cDNA. This technique introduces the desired mutations (here the epitope code) via a mutagenic primer and selects for the mutated plasmids via a selection primer which eliminates a unique restriction site from the vector. The primers used are shown in Table 3.2. The method was also used to attempt to introduce the 98bp deletion from the D3 cDNA to create cDNA encoding the D3nf.

2 μg of each primer were phosphorylated in Tris-HCl (50 mM, pH 7.5), MgCl₂ (10 mM), DTT (5 mM), ATP (1 mM) by 1 unit of T4 polynucleotide kinase (7 units) for 1 hour at 37°C. The reaction was stopped by incubating for 10 minutes at 65°C. 0.1 μg of pRC/CMV/D3 DNA was denatured in a 100°C water bath for three minutes in a 20 μl reaction containing Tris-HCl (20 mM, pH 7.5), MgCl₂ (10 mM), NaCl (50 mM) and 0.2 μg of the phosphorylated selection primer and the appropriate phosphorylated mutagenic primer. The reaction was chilled in an ice water slurry for 30 minutes to allow primer annealing to the denatured DNA. The mutant DNA strand was synthesized by adding 3 μl
of a solution containing Tris-HCl (100 mM, pH 7.5), dNTPs (5 mM of each), ATP (10 mM), DTT (20 mM), 1 μl of T4 DNA polymerase (7 units) and 1 μl of T4 DNA ligase (6 units) and incubated at 37°C for 2 hours. Synthesis was halted by heating at 70°C for 10 minutes. Parental DNA (without the mutations) was selectively linearized by the addition of 1 μl of BgII (10 units) in 2x One-Phor-All (OPA) and incubated at 37°C for 2h. Linearized DNA is less efficacious at cell transformation resulting in an enriched fraction of cells transformed with the mutated, non-linearized DNA. 10 μl of this DNA was used to transform 100 μl of competent BMH 71-18 mut S E. coli cells (Clontech, Palo Alto, CA) which are mismatch repair deficient and, therefore, unable to correct the introduced mutations. After transformation, the cells were grown overnight at 37°C with shaking in 4 ml of LB with ampicillin (0.1 mg/ml). The DNA was miniprepped by boiling lysis and approximately 2 μg were digested by BgII (10 units) in 2x OPA buffer at 37°C. After 2 hours, another 10 units of BgII was added and the digestion continued for an additional hour. Competent E. coli cells were transformed with 10 μl of this DNA and plated on LB agar plates containing ampicillin (75 μg/ml) for growth overnight at 37°C. Isolated colonies from the plates were grown overnight in LB+ampicillin and miniprepped. Mutated DNA was identified by resistance to BgII digestion as visualized under UV light on a 1% agarose gel/ethidium bromide (0.5 μg/ml). The sequence was confirmed by sequencing in both directions.

3.3.2 Construction of FLAG-D3nf by the Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to create the cDNA encoding the D3nf since site-directed mutagenesis was not successful. The strategy exploited a unique EcoR1 site in the D3DR cDNA located 5' of the point at which the 98 pairs are deleted from the D3nf cDNA, as shown in Figure 3.1. The primers sequences are shown in Table 3.3. PCR was performed using eLONGase enzyme mix, which consists of a mixture of Taq and Pyrococcus species GB-D which allows 3'-5' exonuclease proofreading. 50 μl reactions consisting of 2x Buffer A (with 1 mM MgCl₂), dNTPs (250 μM of each), eLONGase (1 μl), primers (A and B or C and D, 170 ng each) and 0.5 μg pRC/CMV/D3 DNA were pre-
Table 3.3: The PCR primers used to create FLAG-D3nf. The restriction sites used for ligation of the PCR products are indicated in bold italics. The underlined sequence is the code for the FLAG epitope. The arrow indicates the point at which the 98 bp are deleted from the D3 sequence.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Amino-Flag</td>
<td>CGC GGC CGC GGT GAG AAC CTG TTT AAG CTT ATG GAC</td>
</tr>
<tr>
<td></td>
<td>TAC AAG GAC GAC GAC AAG GCA TCT CTG AGT CAG</td>
</tr>
<tr>
<td></td>
<td>CTG AGT AGC CAC CTG</td>
</tr>
<tr>
<td>B. Before-deletion</td>
<td>CTC AGG GAA TTC CGA GTC TTC TCC TCT C</td>
</tr>
<tr>
<td>C. After-deletion</td>
<td>CTC GGA ATT CCC TGA GT ↑ G CCA CTT CGG GAG AAG AAG</td>
</tr>
<tr>
<td></td>
<td>GCA ACC CAA ATG GTG GCC ATT GTG CTT GGG</td>
</tr>
<tr>
<td>D. Carboxy-Xho</td>
<td>GAC CCT CGA GTC AGC AAG ACA GGA TCT TGA GGA AG</td>
</tr>
</tbody>
</table>

denatured at 94°C for 5 minutes and subjected to 35 cycles of denaturing at 94°C for 30s, annealing at 68°C for 30s and extension at 68°C for 1 minute. Reactions were soaked at 68°C for 7 minutes for final product elongation and stored at -20°C until use. A control reaction substituted water for the template DNA. PCR products (4 μl) were run on a 1% agarose/ethidium bromide (0.5 μg/ml) gel for confirmation of product size. The expected size of fragments 1 and 2 was approximately 860 and 240 bp respectively.
Figure 3.1: Scheme of the PCR strategy used to create the FLAG-D3nf from D3DR cDNA as described in section 3.2.2. Two PCR fragments were generated. Fragment 1 consisted of the amino portion of the receptor and was created using primer A, which introduced a *Hind*III site for subcloning and the FLAG code, and primer B, which terminated just before the deletion point in the D3nf and contained the *Eco*RI site. Fragment 2 consisted of the carboxyl portion of the receptor and was created using primer C, which annealed completely to the portion of the gene 3' of the deletion point and contained the *Eco*RI site, and primer D, which introduced a *Xho*I site for subcloning. The PCR fragments were digested with the appropriate restriction enzymes and ligated into the pFastbac1 plasmid.
The PCR products were extracted from the reaction mix using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA). The PCR products and vector, pcDNA3 (1 µg), were digested with the appropriate restriction enzyme for 2 hours (fragment 1 by HindIII and EcoR1, fragment 2 by Xhol and HindIII and pcDNA3 by Xhol and HindIII). Digestion products were run on a 0.4% low melting agarose gel (Seaplaque) and bands were excised using a razor blade under longwave UV light. Gel slices were melted in a 70°C waterbath for 10 minutes. DNA was combined in an eppendorf tube in proportions such that there was twice as much vector as insert fragments 1 and 2 and incubated at 37°C for 5 minutes. To this tube was added ice-cold 1x OPA, ATP (1 mM), dH2O and T4 DNA ligase (6 units) in a final volume of 40 µl. The ligation reaction was allowed to continue for 48h at 15°C. The ligation products were melted at 73°C for 10 minutes before 5 µl of product was introduced to 200 µl of competent E. coli cells for transformation. Cells were plated on LB + ampicillin (75 µg/ml) agar plates for growth overnight at 37°C. Transformed colonies expressing the vector with inserted D3nf cDNA were identified by digestion of miniprep DNA with HindIII and Xhol and running on a 1% agarose gel. The sequence was confirmed by sequencing in both directions.

3.4 Transposition into Baculoviral Genome

The cDNAs for the D3DR and D3nf were subcloned into the HindIII and KpnI site of pFastBacI. The generation of recombinant baclovirus was obtained following the Bac-to-Bac baclovirus expression system (Life Technologies). Competent DH10Bac cells were transformed with 0.5 µg of vector DNA, plated onto LB plates containing kanamycin (50 µg/ml), gentamicin (7 µg/ml), tetracycline (10 µg/ml), X-gal (300 µg/ml) and IPTG (40 µg/ml) and incubated for 24 hours at 37°C, after which plates were stored at 4°C until blue colonies were discernible. The DH10Bac cells contain a baculovirus shuttle vector (bacmid) which has a mini-F replicon, a kanamycin resistance marker, and DNA encoding the lacZα gene. N-terminal to the lacZα gene is the attachment site of the bacterial Tn7 transposon. A helper plasmid facilitates the tranposition of a mini-Tn7 region of pFastBacI to the mini-attTn7 attachment site on the bacmid. Insertion disrupts the lacZα gene allowing recombinant bacmid identification on the selection plates. Putative white colonies (which contain a disrupted lacZα gene and,
therefore, the recombinant bacmid with the D3DR or D3nf cDNA) were restreaked onto fresh selection plates to confirm the white phenotype. Candidate colonies were grown overnight in LB media supplemented with the selection antibiotics as above.

The recombinant bacmid DNA was isolated from the colonies by a modified mini-prep for high molecular weight DNA (GibcoBRL). 1.5 ml of bacterial culture was centrifuged at 14,000g using a desktop centrifuge for 1 minute and the pellet was resuspended in 300 μl of solution I (Tris-HCl (15 mM, pH=8.0) and RNase A (100 μg)), to which was added an equal volume of solution II (NaOH (0.2N), SDS (1%)). The solution was gently mixed and incubated at room temperature for 5 minutes. 300 μl of potassium acetate (3 M, pH 5.5) was slowly added, mixed and placed on ice for 10 minutes. The protein precipitate was pelleted by centrifugation at 14,000g and the DNA containing supernatant transferred to a fresh eppendorf tube. The DNA was precipitated in ice-cold isopropranol and collected by centrifugation at 14,000g. The pellet was washed in 70% ethanol, resuspended in TE buffer and stored at -20°C until use.

PCR was used to verify the insertion of the cDNA into the bacmid. 0.5 μg of bacmid DNA was amplified in a reaction mixture containing 1x PCR buffer, dNTPs (1 mM of each), MgCl₂ (1.5 mM), M13/pUC forward and reverse primer (170 ng of each) and eLONGase (1 μl) in a final volume of 50 μl. Conditions were as follows: pre-denaturing at 93°C (3 minutes) and 35 cycles of denaturing at 94°C (45s), annealing at 55°C (45s) and extension at 72°C (5 minutes). Products were soaked at 72°C for 7 minutes and stored at 4°C. PCR product (4 μl) was visualized on a 1% agarose/ethidium bromide (0.5 μg/ml) gel for size confirmation. Product amplified from bacmid alone was 300 bp and product amplified from the bacmid transposed with pFastBac1 was approximately 2300 bp.

3.5 Initial Amplification of virus

Sf9 cells from a 3 day suspension culture were seeded at a density of 9x10⁵ cells/well in a 12 well plate in Sf-900II SFM containing penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were allowed to attach to the plates at 27°C for 1 hour. Miniprep bacmid DNA (5 μl) and CellFECTIN (6 μl)
were each diluted in 100 μl of Sf-900 II SFM and then combined and incubated at room temperature for 45 minutes. The lipid/DNA complexes were further diluted by Sf-900 II SFM and used to overlay the Sf9 cells. The cells were incubated at 27°C for 5 hours, at which time the transfection mixture was removed and replaced by Sf-900 II SMF containing penicillin (50 units/ml) and streptomycin (50 μg/ml). The virus containing supernant was collected after 72 hours and clarified by centrifugation at 100g for 7 minutes. The virus was stored at 4°C until use.

Further rounds of virus amplification were accomplished by successive infection of monolayer flasks (seeded at a density of 1x10^6 cells/ml from a 3-4 day suspension culture in mid-log phase growth) of increasing size (T-25, T-75, T-125) with the viral supernant of the previous sized flask. The supernatant was cleared of cell debris by centrifugation as above and stored in a foil-covered flask at 4°C until use.

3.6 Sf9 cell culture

Sf9 cells were grown in a monolayer or suspension culture in Grace's Insect Medium supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), qualified heat-inactivated fetal bovine serum (10%), and PLURONIC F-68 (1%). Cells were maintained in a monolayer culture in T-125 flasks at 27°C for less than 30 generations. After 3-4 days, the cells reached 95% confluency, and were split 1:8. For infection, approximately 2x10^5 viable cells/ml were seeded in a 50-100 ml culture. Flasks were kept at 27°C on a orbital shaker set at 135 rpm. After 2-3 days, once cells reached mid-log phase growth (at a density of approximately 1.5-2 x10^6 ml), they were infected with the appropriate recombinant baculovirus at a multiplicity of infection (MOI) of approximately 5. The number and viability of the cells were assessed using a hemocytometer and trypan blue dye exclusion. Cell membranes were harvested after 48h as described below. Cell viability when infecting was >97% and after 48h of viral infection was between 70-100%.

For amplification of viral stocks, a 3 day culture of cells at a density of 1.5-2x10^6 cells/ml in a spinner flask were infected at a MOI of approximately 0.1. Virus was harvested after 48h. The
supernatant was cleared of cells by centrifugation at 100g for 7 minutes and stored in a foil covered flask at 4°C until use.

3.7 Sf9 membrane preparation

Membranes were prepared at 4°C. Infected cells were pelleted by centrifugation at 100g for 7 minutes. Cells were resuspended and washed with PBS, followed by another 7 minute centrifugation. Cells were resuspended in 10 ml of hypotonic lysis buffer (5 mM Tris-HCl (pH 7.4), 2 mM EDTA and the protease inhibitors benzamidine (10 µg/ml), leupeptin (5 µg/ml) and soybean trypsin (10 µg/ml)). The cells were homogenized by a polytron for 60 seconds at a setting of 7.5. The homogenates were centrifuged at 50g for 7 minutes to pellet unbroken cells and nuclei. The supernatant was centrifuged at 27000g for 20 minutes to obtain a P2 pellet which was resuspended in lysis buffer and stored at -70°C until use or used immediately for radioligand binding assays or immunoprecipitation. Protein concentration was determined by the method of Bradford (Bradford, 1976) (Bio-Rad Assay Kit) using bovine serum albumin as the standard.

3.8 Immunoblot analysis

25-50 µg of membrane protein were solublized in SDS loading buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.005% bromophenol blue and 5% β-mercaptoethanol) and incubated at 22°C for 30 minutes. The proteins were electrophoresed in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) on 10 or 12% pre-cast acrylamide gels by application of a constant voltage of 125 V. Once the dye front had migrated off the gel, the proteins were transferred onto nitrocellulose using a wet-dry apparatus at a constant voltage of 30V for 2 hours in transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol, 0.03% SDS). The nitrocellulose was blocked in a solution of 5% milk protein in TTBS (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20 for 1 hour. The blot was then rinsed with TTBS for 10 minutes and incubated overnight at 4°C in a 1:1000 dilution of primary antibody (9E10 antibody for c-myc-D3DR and M5 antibody for FLAG-D3nf). The blot was washed three times
over the course of an hour and incubated for 2-3 hours with a goat antimouse IgG alkaline phosphatase conjugate, diluted 1:1000 in TTBS. The blot was washed again three times during one hour and then developed in 0.1 M Tris-HCl, pH 9.5 containing BCIP/NBT (5-bromo-4-chloro-3-indoly phosphate/nitroblue tetrazolium) substrates. The ladder “SuperBand” (Novex) was used to compared the molecular sizes of the detected proteins.

3.9 Photoaffinity labelling

25-50 µg of membrane protein was resuspended in antagonist binding buffer (see below) with protease inhibitors and incubated in the dark with saturating concentrations (1-5 nM) of [125I]-azidononamapride (Figure 3.2; NINH, custom synthesis program) in a final volume of 1 ml for 1.5 hours at 22°C. The membranes placed in an ice water slurry and exposed to ultraviolet light (370 nm) for 2 minutes. This results in the crosslinking of the reactive azido group to the ligand binding site. Membranes were pelled by centrifugation at 14 000 rpm for 15 minutes and solubilized in SDS loading buffer (see above) and electrophoresed as above. The gel was fixed and dehydrated in a 40% methanol/10% acetic acid solution for 30 minutes, dried on a gel dryer for 1 h and exposed to Kodak XAR film with an intensifying screen at -70°C for 2-7 days. Non-specific binding was defined in the presence of 1 µM (+)-butaclamol.

3.10 Receptor binding assays

Saturation or competition binding experiments were done using P2 membrane proteins prepared fresh from infected SF9 cells or from stock membranes kept at -70°C. The molecular structure of the ligands used in binding assays are shown in Figure 3.2 and 3.3. Binding of [3H]nemonapride or [3H]spiperone (Figure 3.2; 32-6000 pM final concentration) was assayed in duplicate, after 2 hours incubation at 22°C in a final volume of 1 mL of antagonist binding buffer [(50 mM Tris HCl, 5 mM EDTA, 1.5 mM CaCl2, 5 mM MgCl2, 5 mM KCl, 120 mM NaCl (pH 7.4)] with protease inhibitors. Nonspecific binding was defined as binding in the presence of 1 µM (+)butaclamol. Competition
experiments were done in triplicate with increasing concentrations (10^{12} to 10^{-3} M) of unlabelled ligand. The concentration of radioligand used in the assays was approximately equivalent to its K_D. Tubes were incubated for 2 hours at 22°C in a final volume of 1 mL with antagonist binding buffer for antagonists or binding buffer without NaCl for agonists. When dopamine was used, incubation was performed in the dark and the binding buffer contained 0.1% ascorbic acid to prevent oxidation. Each reaction tube contained 5 μg protein which was determined not to contain enough receptor protein to significantly deplete the ligand. Bound ligand was separated from free by rapid filtration through a Brandel 48-well cell harvester onto Whatman GF/C filters and washed with 10 ml of ice-cold 50 mM Tris-HCl (pH 7.4). Filters were placed into glass vials and incubed overnight with 5 ml of scintillation fluid. Tritium was counted using a Beckman LS 6500 scintillation counter at a counting efficiency of 40%.
Figure 3.2: Molecular structures of the radioligands used in this investigation.
Figure 3.3: Molecular structures of the dopaminergic agonists and antagonists used in this investigation.
3.1 Adenylyl cyclase assay

The assay was performed as described previously (Salomon et al., 1974). The assay mix contained 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase, 0.13 μCi of [32P]ATP in a final volume of 50 μl. Cyclase activity in the presence of dopamine (10^{-12} to 10^{-3} M) was determined in triplicate. The reaction was initiated by the addition of 25 μg of freshly prepared membrane protein, allowed to progress for 20 minutes at 27°C and stopped by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP and [3H]cAMP (25 000 cpm). cAMP was isolated by sequential column chromatography using Dowex cation exchange resin and aluminum oxide. Data were analyzed by nonlinear least squares regression using the computer program Prism.

3.12 Immunoprecipitation

P2 membranes from infected Sf9 cells were prepared as above and solublized overnight with gentle agitation in freshly prepared solubilization buffer containing 2% digitonin, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA and protease inhibitors. Non-solublized membranes were pelleted at 27 000 g for 20 minutes. Digitonin was removed from the supernatant by dialysis with Centriprep-30 concentrators (Amicon) against an ice cold buffer S consisting of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4) until the digitonin concentration was reduced below 0.05%. 9E10 or FLAG antibody conjugated to agarose (Santa Cruz, Sigma respectively), washed 5x with buffer S + 0.5% BSA (Sigma), was added to the concentrate and mixed gently overnight at 4°C. The mixture was washed 5x with buffer S. The c-myc-D3DR or FLAG-D3nf was competed from the antibody by the addition of 200 μg/ml of the corresponding c-myc or FLAG peptide with gentle rocking for 30 minutes. The agarose beads were pelleted and the supernatant was added to an equal volume of loading buffer. The mixture was then electrophoresed on SDS-PAGE and Western blotted as described above.

3.13 Immunocytochemistry

Immunofluorescent staining of whole or permeabilized Sf9 cells infected with the D3DR, D3nf or both recombinant baculoviruses was performed in parallel. Control experiments were identical, with
cells infected with wild-type baculovirus. Cells were obtained 48h post-infection with >85% viability as assessed by trypan blue staining. The cells were pelleted and fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.3, for 15 minutes in eppendorf tubes, followed by two washes in PBS. Cells were either resuspended in PBS, for whole cell labelling, or permeabilized with methanol at -20°C for 3 minutes. To reduce non-specific staining, the cells were incubated for 30 minutes in a blocking solution of 1% BSA and 5% heat-inactivated goat serum. Cells were incubated for 60 minutes with 9E10 (mouse) and/or M2 (rabbit) primary antibodies (diluted 1:50 in PBS), followed by three five minute washes. The samples were covered in foil and incubated for 60 minutes with goat anti-mouse FITC-conjugated secondary antibody and/or goat anti-rabbit Texas Red conjugated secondary antibody (diluted 1:500 in PBS), followed by three five minute washes. The cells were resuspended in the mounting media Mowiol-88 with 2.5% 1,4-diazobicyclo-octane to reduce photobleaching. Several drops of the media were placed on a slide, covered by a coverslip and allowed to polymerize overnight. Slides were wrapped in foil and stored at 4°C until use. Visualization of the cells was achieved using a Zeiss LSM516 laser scanning microscope (Carl Zeiss Jenn GmbH) equipped with a krypton-argon laser.

3.14 Data analysis

Binding data were analyzed by nonlinear least squares regression using the computer program Prism (GraphPad Software, San Diego, CA). The program was also used to draw curves which were fitted to the better of one or two binding sites. The significance of the resolution in the two components of an inhibition curve was performed using the F test, where F is the ratio of the deviation between mean squares obtained in the one-site model and that in the two-site model.

Counts in DPM were converted to picomoles/mg protein (X) by the equation:

\[ X = \frac{\text{DPM}}{\text{Cv}/\text{Z}/1000/\text{P}/1000\times1\times10^{12}} \]

where Cv is a conversion factor converting DPM to Ci (2.22x10^{13}), Z is the specific activity of the radioligand (in Ci/mmol), P is the µg amount of protein added.

Saturation binding data were fitted to one-site models according to:
\[ RL = \frac{(B_{\text{max}} \cdot L)}{(K_D + L)} \]

where \( RL \) is the molar concentration of specifically bound ligand, \( B_{\text{max}} \) represents the total concentration of each site, \( K_D \), the concentration of ligand that produces half-maximal saturation of each site, and \( L \) is the molar concentration of free ligand.

Inhibition constants (IC_{50}) for competing ligands were determined by the equation:

\[ Y = \text{Bottom} + \frac{(\text{Top-Bottom})}{(1 + 10^{(x-\log IC_{50})})} \]

where IC_{50} is the concentration of ligand which blocks 50% of the hot ligand bound.

IC_{50} were converted to constants of inhibition (K_i) by the equation of Cheng and Prusoff (Cheng and Prusoff, 1973):

\[ K_i = \frac{IC_{50}}{1+[\text{ligand}]/K_D} \]

Statistical comparisons between the binding parameters determined for the c-myc-D3DR expressed alone or in combination with the FLAG-D3nf were performed using a paired Student t test with a level of significance of \( p<0.05 \). Values for \( K_i \) were compared as pKi as these values are evenly distributed along a logarithmic scale.
4 Results

4.1 Creation of D3DR and D3nf Plasmid Constructs

At the time this project was initiated, antibodies which selectively recognize D3DR or D3nf were not available. To facilitate immunoblot analysis of the D3DR and D3nf, the proteins were epitope-tagged with c-myc and FLAG epitopes, respectively. The sequences of these epitopes are found in Table 3.1. Many groups have introduced epitope tags to the amino terminus of GPCRs and found the tag to have no effect on the function of the receptor (Ng et al., 1994a) (Ng et al., 1993) (Cvejic and Devi, 1997; Fukushima et al., 1997; Hebert et al., 1996; Romano et al., 1996), although one group has reported that the hemagglutinin tag on the δ, κ and μ opioid receptors expressed in Sf9 cells resulted in decreased expression levels but did not affect binding parameters (Massotte et al., 1997). Monoclonal and polyclonal antibodies are available which specifically recognize the short amino acid sequences of the c-myc or FLAG epitopes. The monoclonal 9E10 antibody recognizes the 10 aa sequence of the c-myc epitope and the polyclonal M2 antibody and monoclonal M5 antibody recognize the 8 aa sequence of FLAG.

The c-myc epitope was successfully added to the D3DR cDNA using the Transformer site-directed mutagenesis method, as confirmed by sequencing. This protocol was also used to attempt to create the FLAG-D3nf by simultaneously introducing the epitope code and deleting the 98 bp not found in the D3nf cDNA via two different primers. The deletion primer was a 96-mer, with half of the primer annealing to the sequence before the deletion point and half annealing to the sequence after the deletion point. It was hoped that in this way the primer would "bubble-out" the intervening 98 bp thereby creating the deletion when the new plasmid was synthesised by T7 polymerase. However, this mutagenesis method was not successful in generating the FLAG-D3nf. Many bacterial colonies were screened, however, some contained only the FLAG epitope code and not the 98 bp deletion or only the deletion and not the epitope tag. Of the few plasmids which incorporated both alterations, none were without coding errors. All of the constructs which contained the deletion also had errors in the region surrounding the deletion point. For example, some constructs contained a non-silent base pair mutation at base #862 and
some had a deletion at base #856. These errors may have been introduced in three ways: from inefficient "bubbling-out" of the 98 bp by the primer allowing new bases to be incorporated during new plasmid synthesis, from errors integral to the primer itself made during primer synthesis or from errors in fidelity of the T7 polymerase.

Therefore, an alternate PCR strategy was used to successfully create the FLAG-D3nf as shown in Figure 3.1 and described in Materials and Methods. To confirm that no mutations were introduced into the vectors by T7 polymerase (for c-myc-D3DR) or eLONGase (for FLAG-D3nf), the constructs were sequenced in both directions and compared to the published sequence. In both cases, there was a base pair mutation (T-C) at base #541 which introduced a mutation in codon 181 resulting in a Cys-Arg amino acid change. It was surprising that both plasmids, generated by two different enzymes, contained the same mutation so the parent pRC/CMV/D3 plasmid was sequenced and found to contain the same mutation.

The parent plasmid was then corrected and simultaneously epitope-tagged with c-myc using the mutagenesis method and the primers shown in Table 3.2 (Materials and Methods). PCR was used in the same way as before to create FLAG-D3nf from the corrected pRC/CMV/D3 plasmid.

4.2 Binding characteristics of the c-myc-D3DR and FLAG-D3nf expressed alone in Sf9 cells

To ensure that the pharmacological properties of the c-myc-D3DR in Sf9 cells were comparable to those determined in other heterologous systems, the radiolabelled antagonists [3H]nemonapride and [3H]spiperone were used to assess the saturation binding characteristics of the c-myc-D3DR. As shown in Figure 4.1, the binding of [3H]nemonapride to membranes from c-myc-D3DR infected Sf9 cells was saturable and specific. The low non-specific binding to the c-myc-D3R defined in the presence of excess (+)butaclamol is a characteristic advantage of Sf9 cells. No specific binding of the antagonists was observed in uninfected Sf9 cells or in cells infected with wild-type
Figure 4.1: Saturation isotherm of $[^3\text{H}]$nemonapride binding to Sf9 cell membranes expressing c-myc-D3DR (▲), c-myc-D3DR coexpressed with the FLAG-D3nf (■) or the FLAG-D3nf alone (●). Specific binding is defined as total binding - non-specific binding, defined in the presence of 1 µM (+)butaclamol. Each point is the mean of duplicate determinations in a single representative experiment which was repeated at least 5 times. The data were analyzed as described in Materials and Methods.
Table 4.1: Characterization of [³H]nemonapride and [³H]spiperone binding to Sf9 cell membranes expressing c-myc-D3DR alone or co-expressing c-myc-D3DR and FLAG-D3nf.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>c-myc-D3DR</th>
<th>c-myc-D3DR + FLAG-D3nf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KD (pM)</td>
<td>B_max (pmol/mg protein)</td>
</tr>
<tr>
<td>[³H]spiperone</td>
<td>532±76</td>
<td>17±4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>[³H]nemonapride</td>
<td>204±32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19±4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as the arithmetic mean of the dissociation constant (K_D) or maximal binding capacity (B_max) ± standard error of the mean from five independent trials. Statistical significance was determined by paired student t tests. The B_max was significantly lower when the c-myc-D3DR was co-expressed with the FLAG-D3nf than when expressed alone (a, b, p<0.05). The K_D for [³H]nemonapride was significantly different when the c-myc-D3DR was co-expressed with the FLAG-D3nf than when expressed alone (c, p<0.05).

baculovirus (data not shown). The binding curves for [³H]spiperone and [³H]nemonapride were best-fitted to a single state of the receptor with K_D values of 532±29 and 204±12 pM, respectively (Table 4.1). Infection of suspension cultures of Sf9 cells with recombinant baculovirus at an MOI of approximately 5 routinely resulted in c-myc-D3DR receptor densities of approximately 17-19 pmol/mg protein, 48 hours post-infection.

The primary amino acid sequence of the FLAG-D3nf diverges from that of the c-myc-D3DR after TM5 in the region of ICL3 for 58 amino acids and is prematurely truncated. Hydropobicity analysis suggests that this divergent region contains a span of hydrophobic amino acids which is predicted to cross the membrane forming a non-homologous TM6 (Figure 4.2). No specific binding of [³H]nemonapride, [³H]spiperone or [³H]dopamine was observed in Sf9 cells infected with the FLAG-D3nf (Figure 4.1 and results not shown).
Figure 4.2: Hydropathy plots of the predicted amino acid sequences of the D3DR and D3nf.
4.3 Binding characteristics of c-myc-D3DR co-expressed with FLAG-D3nf in Sf9 cells

Competition and saturation binding experiments were used to compare the pharmacologies of the c-myc-D3DR expressed alone and together with the FLAG-D3nf. These experiments were performed in parallel using the same drug dilutions to minimize variation. The agonists dopamine and 7-OH-DPAT, a selective D3DR ligand, and the antagonists sulpiride and haloperidol were used to compete for \[^3H\]spiperone and \[^3H\]nemonapride binding. The results summarized in Table 4.2 show that the relative affinities \(K_i\) of these ligands for c-myc-D3DR were not significantly altered in the presence of FLAG-D3nf. A representative set of competition curves is shown in Figure 4.3. The majority of the agonist competition curves were best-fitted to a single affinity site, suggesting that c-myc D3DR coupling to G proteins was negligible in Sf9 cells. In certain samples, both high and low affinity sites were detected. The detection of high and low affinity states of the receptor did not appear to be correlated with the density of receptor expression.

The \(K_D\) of \[^3H\]spiperone binding was not significantly altered when c-myc-D3DR was co-expressed with FLAG-D3nf (Table 4.2). However, the \(K_D\) of \[^3H\]nemonapride was significantly

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Ligands</th>
<th>c-myc-D3DR</th>
<th>c-myc-D3DR + FLAG-D3nf</th>
</tr>
</thead>
<tbody>
<tr>
<td>[^3H]spiperone</td>
<td>Dopamine</td>
<td>27.06 ± 14.41</td>
<td>20.85 ± 9.48</td>
</tr>
<tr>
<td></td>
<td>(+)7-OH-DPAT</td>
<td>1.72 ± 0.64</td>
<td>2.07 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>Sulpiride</td>
<td>59.61 ± 11.37</td>
<td>49.63 ± 14.84</td>
</tr>
<tr>
<td></td>
<td>Haloperidol</td>
<td>14.50 ± 1.79</td>
<td>14.39 ± 2.07</td>
</tr>
<tr>
<td>[^3H]nemonapride</td>
<td>Dopamine</td>
<td>58.73 ± 14.57</td>
<td>57.60 ± 11.13</td>
</tr>
<tr>
<td></td>
<td>(+)7-OH-DPAT</td>
<td>7.04 ± 1.12</td>
<td>4.26 ± 0.73</td>
</tr>
<tr>
<td></td>
<td>Sulpiride</td>
<td>82.97 ± 6.67</td>
<td>78.11 ± 14.23</td>
</tr>
<tr>
<td></td>
<td>Haloperidol</td>
<td>54.22 ± 9.27</td>
<td>54.96 ± 10.01</td>
</tr>
</tbody>
</table>

Results are expressed as the arithmetic mean of the inhibition constant \(K_i\) ± standard error of the mean. \(IC_{50}\) values were determined from two to four independent experiments. Calculation of the \(K_i\) is as described in the Material and Methods. No significant differences for the affinity of the different ligands was found between c-myc-D3DR expressed alone or with the FLAG-D3nf (paired student \(t\) test, \(p < 0.05\)).
Figure 4.3: Competition of [3H]spiperone binding to NG cell membranes expressing A. c-myca

Figure 4.4: Competition of [3H]spiperone binding to NG cell membranes expressing 32. c-myca

Least 3 times. The data were analyzed as described in Materials and Methods.

Mean of triplicate determinations in a single representative experiment which was repeated at least 3 times. The data were analyzed as described in Materials and Methods.
reduced. Interestingly, the apparent receptor density recognized by the radioligands ($B_{max}$) was significantly lowered (by 35-37%) when the FLAG-D3nf and c-myc-D3DR were co-expressed. Preliminary experiments demonstrated that the increased viral load on the cells during co-expression of FLAG-D3nf and c-myc-D3DR was able to decrease the amount of c-myc-D3DR synthesized. Therefore, this effect of co-expression was controlled for by co-infecting cells with c-myc-D3DR and an equal MOI of wild-type baculovirus to compare to cells co-infected with c-myc-D3DR and FLAG-D3nf.

To ensure that the effect of co-expression of FLAG-D3nf was specific for c-myc-D3DR, FLAG-D3nf was co-expressed with c-myc-β2AR, another catecholamine receptor. The apparent receptor density recognized by $[^3H]$dihydroalprenolol for membranes co-expressing FLAG-D3nf and c-myc-β2AR was not significantly different from the receptor density detected when the c-myc-β2AR was expressed alone (Table 4.3).

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>c-myc-β2AR</th>
<th>c-myc-β2AR + FLAG-D3nf</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3H]$dihydroalprenol</td>
<td>221±75</td>
<td>217±89</td>
</tr>
<tr>
<td></td>
<td>18±8</td>
<td>23±8</td>
</tr>
</tbody>
</table>

Table 4.3: Characterization of $[^3H]$dihydroalprenol binding to Sf9 cell membranes expressing c-myc-β2AR alone or co-expressing c-myc-β2AR and FLAG-D3nf

Results are expressed as the arithmetic mean of the dissociation constant ($K_D$) or maximal binding capacity ($B_{max}$) ± standard error of the mean from three independent trials. Statistical significance was determined by paired student $t$ tests. There was no significant difference between the binding parameters measured when the c-myc-β2AR was co-expressed with the FLAG-D3nf than when expressed alone ($p<0.05$).
4.4 Coupling of the c-myc-D3DR and FLAG-D3nf in Sf9 cells

Treatment of Sf9 cells with forskolin, an activator of AC, caused an increase in the levels of cAMP. We have previously shown that the D2DR couples to inhibition of AC in Sf9 cells (Ng et al., 1994a). To determine if the D3DR also couples to AC in this system, the effect of dopamine on forskolin-stimulated cAMP production in cells expressing the c-myc-D3DR was tested. Dopamine had no significant effect on the levels of cAMP in cells infected alone or co-infected with the c-myc-D3DR and/or FLAG-D3nf (data not shown).

4.5 Immunoblot and photolabelling analyses of the c-myc-D3DR and FLAG-D3nf expressed alone and together in Sf9 cells

Membranes from Sf9 cells expressing the c-myc-D3DR were analyzed by SDS-PAGE using the anti-c-myc 9E10 monoclonal antibody. Predominant species were observed at approximately 40, 80 and 160 kDa (Figure 4.4A, lane 2). These species are consistent in size with D3DR monomers, dimers and tetramers since the predicted molecular mass of the D3DR with the c-myc epitope is 45 kDa. Similarly, membranes from cells infected with the FLAG-D3nf showed major species at approximately 36, 72 and 144 kDa, consistent with monomers, dimers and tetramers of the FLAG-D3nf based on its predicted size of 38 kDa (Figure 4.4B, lane 2). Membranes co-expressing c-myc-D3DR and FLAG-D3nf show the same predominant species when probed with the c-myc or FLAG antibody as those observed in membranes expressing either protein alone (Figure 4.4A and 4.4B, lanes 3). Membranes infected with wild-type baculovirus did not crossreact with either monoclonal antibody, confirming that these antibodies were specific for the epitopes of interest. The polyclonal M5 antibody, specific for the FLAG epitope, immunodetected many proteins from wild-type Sf9 cells, some of which were approximately the same size as the dimer band (data not shown). For this reason, all Western blots used the monoclonal M2 antibody to detect FLAG.
Figure 4.4: Detection of D3DR and D3nf oligomerization by immunoblotting. A Immunoblot probed with the anti-c-myc (9E10) antibody. 50 μg of total cellular protein from P2 membrane fractions of Sf9 cells harvested 48 hours post-infection were run per lane. Lane 1, membranes from cells infected with wild-type baculovirus. Lane 2, membranes from cells infected with c-myc-D3DR baculovirus. Lane 3, membranes from cells co-infected with c-myc-D3DR and FLAG-D3nf. Lane 4, membranes from cells infected with FLAG-D3nf. Immunoreactive bands in lanes 2 and 3 represent species consisting of the c-myc-D3DR and are observed at ~40, ~80 and ~160 kDa, consistent in size with receptor monomers, dimers and oligomers. B Immunoblot probed with the anti-FLAG (M5) antibody. 50 μg of protein from P2 membrane fractions of Sf9 cells harvested 48 hours post-infection were run per lane. Lane 1, membranes from cells infected with wild-type baculovirus. Lane 2, membranes from cells infected with FLAG-D3nf baculovirus. Lane 3, membranes from cells co-infected with c-myc-D3DR and FLAG-D3nf. Lane 4, membranes from cells infected with c-myc-D3DR baculovirus. Immunoreactive bands in lanes 2 and 3 represent species consisting of the FLAG-D3nf and are observed at ~36, ~72 and ~144 kDa, consistent in size with protein monomers, dimers and tetramers. The results shown are representative of at least three independent experiments.
Figure 4.5: Photoaffinity labelling of Sf9 cell membranes expressing c-myc-D3DR. Autoradiogram showing photolabelling by [\(^{125}\)I]azidonemonapride of Sf9 cell P2 membranes after 4 days exposure. 25 µg membrane protein were run per lane. Species of the same molecular weights are revealed as observed on the immunoblot (Fig. 4A). Lanes 1, membranes from cells infected with c-myc-D3DR baculovirus. Lanes 2, membranes from cells co-infected with c-myc-D3DR and FLAG-D3nf. The lanes following the ones marked 1 and 2 represent non-specific binding defined in the presence of 1 µM (+)butaclamol.
The photoreactive ligand \(^{125}\)Iazidonemonapride was used to bind to Sf9 cell membranes infected with c-myc-D3DR. Three major species were resolved on SDS-PAGE at approximately 40, 80 and 160 kDa (Figure 4.5). That binding to these species was displaced in the presence of excess (+)butaclamol confirms that they represent D3DR proteins. Furthermore, the species revealed via photoaffinity labelling were identical in size to those observed on the immunoblot. This implies that the species represent functional D3DR monomers, dimers and tetramers. Notable is the lesser intensity of dimer band on the photolabel compared to the immunoblot. Membranes from Sf9 cells expressing FLAG-D3nf alone showed no specific binding of \(^{125}\)Iazidonemonapride, consistent with FLAG-D3nf's inability to bind dopaminergic ligands. In membranes co-expressing c-myc-D3DR and FLAG-D3nf, the same species were observed as when the c-myc-D3DR was expressed alone.

4.6 Confocal microscopy

A possible explanation for the apparent decrease in receptor density noted when D3nf is co-expressed with the D3DR is that D3DR/D3nf heterodimers are less efficiently processed from the endoplasmic reticulum resulting in less D3DR at the cell surface. Immunocytochemical studies were performed to test this hypothesis by comparing the expression and subcellular distribution of the c-myc-D3DR and FLAG-D3nf in Sf9 cells. When expressed individually, both the c-myc-D3DR and FLAG-D3nf were found abundantly on the surface of Sf9 cells, as evidenced by the ring of fluorescent staining seen in whole cell preparations (Figure 4.6A, C, E). When expressed together in the same cell, there was no detectable alteration in the overall pattern of surface fluorescence of the cells. In permeabilized cells, both proteins could be seen in the cell's intracellular regions (Figure 4.6B, D, F).
**Figure 4.6: (over)**

**Immunofluorescence staining of Sf9 cells.** Cells expressing c-myc-D3DR were probed with the monoclonal 9E10 antibody and those expressing FLAG-D3nf were detected with the polyclonal M2 antibody. Fluorescence images were obtained with a confocal microscope, using a FITC-linked secondary antibody to detect the 9E10 antibody (green) or a Texas Red-linked secondary antibody to detect the M2 antibody (red). Overlapping signals appear yellow. Images were obtained from non-permeabilized (A,C,E) and permeabilized (B,D,F) cells. The horizontal bar represents 10 μm. The images are representative of three independent experiments and were obtained under identical illuminating conditions.
5. Discussion

5.1 Pharmacology of the dopamine D3 receptor in Sf9 cells

The binding profile of the c-myc-D3DR expressed in Sf9 cells is consistent with reports in other systems. Table 5.1 summarizes results from published reports regarding ligand affinity for the D3DR in various cell lines. In most cases, the binding affinities of the ligands used in this investigation are within an order of magnitude of affinities reported by others, although variations may occur due to differences in assay conditions.

The c-myc-D3DR was unable to couple to AC in Sf9 cells which is in keeping with the contradictory reports of the D3DR's ability (Chio et al., 1994; Griffon et al., 1997) or inability (Burris et al., 1995; Freedman et al., 1994; MacKenzie et al., 1994; Seabrook et al., 1994; Seabrook et al., 1992; Sokoloff et al., 1990; Tang et al., 1994) to couple to this effector (section 1.4), even in cell lines where the D2DR is known to couple efficiently. The inability to couple in Sf9 cells suggests that the appropriate G protein or AC isoform may not be endogenously expressed in these cells. Indeed, it has been shown that the D2L and D4DR, but not D3DR, are able to stimulate AC in stable cell lines expressing type II adenylyl cyclase (Watts and Neve, 1997). However, the D3DR has been shown to couple very robustly to inhibition of cAMP production in cells co-expressing the receptor and type five adenylyl cyclase (ACV), but not types I, II or VI (Robinson and Caron, 1997). This observation has in vivo relevance since mRNA for ACV has been detected in rat brain, including regions which overlap with D3DR distribution, namely the nucleus accumbens, olfactory tubercle and islands of Calleja (Glatt and Synder, 1993).

The other alternative, not exclusive from the above, is that the endogenous G proteins which couple to AC in Sf9 cells are unable to couple to the D3DR. This possibility is supported by the observation that agonist detected high and low affinity sites were rarely noted in competition assays, since high affinity binding is believed to occur when the receptor is coupled to G protein. As well, another study of the rat D3DR in Sf9 cells was also unable to demonstrate two-site binding of agonists
Table 5.1: Affinities of dopaminergic ligands for the dopamine D3 receptor in different heterologous systems as determined by *in vitro* radioligand binding assays, highlighting those ligands used in this investigation.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Species</th>
<th>System</th>
<th>Radioligand</th>
<th>$K_i$ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OH-DPAT</td>
<td>human</td>
<td>CHO</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>1.6±0.41</td>
<td>(Freedman <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>CHO-K1</td>
<td>$[^{3}H]$spiperone</td>
<td>1.6±0.2</td>
<td>(MacKenzie <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>CCL1.3</td>
<td>$[^{3}H]$spiperone</td>
<td>7.1±0.5</td>
<td>(MacKenzie <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>CCL1.3</td>
<td>$[^{3}H]$spiperone</td>
<td>7.1±1.0</td>
<td>(Tang <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>MN9D</td>
<td>$[^{3}H]$spiperone</td>
<td>39±18</td>
<td>(Tang <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>HEK-293</td>
<td>$[^{125}I]$NCQ-298</td>
<td>44±5</td>
<td>(Burris <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>rat</td>
<td>CHO</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>25±3</td>
<td>(Sokoloff <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>CHO</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>3.9±0.8</td>
<td>(Sokoloff <em>et al.</em>, 1992a)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>GH4Cl</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>49</td>
<td>(Seabrook <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>CHO</td>
<td>$[^{3}H]$spiperone</td>
<td>2.5±1.3</td>
<td>(Castro and Strange, 1993)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>Sf9</td>
<td>$[^{125}I]$NCQ-298</td>
<td>265.7±72.5</td>
<td>(Boundy <em>et al.</em>, 1993)</td>
</tr>
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<td>human</td>
<td>NG108-15</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>2.2±0.5</td>
<td>(Pilon <em>et al.</em>, 1994)</td>
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<tr>
<td></td>
<td>human</td>
<td>CHO</td>
<td>$[^{125}I]$iodosulpiride</td>
<td>29±10</td>
<td>(Freedman <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>HEK-293</td>
<td>$[^{125}I]$NCQ-298</td>
<td>1.2±0.1</td>
<td>(Burris <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Haloperidol</td>
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<td>$[^{125}]$NCQ-298</td>
<td>14±1</td>
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However, the lack of high affinity agonist binding could also result from overexpression of the receptor in Sf9 cells. One group has found that the D2L and D2S couple to inhibition of cyclase in Sf9 cells but only show high affinity agonist binding at expression levels below 1 pmol/mg protein (Boundy et al., 1996). In the same way, the D2S receptor in Sf9 cells at levels above 1 pmol/mg protein did not show high affinity binding of agonists, yet still coupled to inhibition of cyclase (Grunewald et al., 1996). However, our group has shown that the D2L in SR cells consistently demonstrates both high and low affinity agonist binding, even at levels greatly above 1 pmol/mg protein (Ng et al., 1994b).

Without a measure of functional response, it is difficult to conclude whether the lack of high and low affinity agonist binding with D3DR in Sf9 cells is due to the absence of an appropriate G protein or reflects the very high levels of receptor expression saturate the G proteins which are endogenously present. Some researchers have used the low abundance of endogenous G proteins to their advantage in order to identify G proteins which couple to their receptor of interest by co-expressing the α and βγ subunits of a G protein heterotrimer in the same cell (or reconstituting receptors with exogenous heterotrimeric G proteins) and measuring the increase in high affinity agonist binding achieved. In this way, putative interacting G proteins are identified by virtue of the resulting increase in agonist binding. Alternatively, researchers may use [35S]GTPγS binding as a measure of G protein activation (GDP/GTP exchange) in response to agonist binding. This co-infection/reconstitution technique has been used to investigate G protein coupling to 5-HT1 receptor subtypes (Clawges et al., 1997), to isoforms of the D2DR (Boundy et al., 1996), to D2S (Grunewald et al., 1996) and to 5-HT1A receptors (Barr et al., 1997).

Little is known concerning the identity of G proteins able to couple to D3DR, however, the fact that effector coupling in certain systems can be abolished by pretreatment with pertussis toxin suggests that the receptor may couple through Gα or Gβ (Chio et al., 1994; Liu et al., 1996; Pilon et al., 1994; Potenza et al., 1994; Seabrook et al., 1994; Werner et al., 1996) (Table 1.2). The low background of endogenous coupling G proteins means that the Sf9 system could be used in future investigations to
identify G proteins which couple to the D3DR. Caution should be used in interpreting such studies because even though a receptor may have the capacity to activate different G proteins, this interaction may not have any physiological relevance if these G proteins are not expressed in the same cell as the receptor in vivo.

5.2 Implications of D3nf non-function on the dopamine D3 receptor pharmacophore

Much work has been devoted to elucidating the identity of the amino acid residues which form the binding pocket of catecholamine receptors, with the aim of assisting rational drug design and understanding receptor function. Pioneering work from Strader's lab in the late 1980's, discovered several of the key amino acids residues involved with ligand binding and activation of the β2AR (Strader et al., 1989; Strader et al., 1988). These included Asp113 in TM3, and 2 serine residues in TM5. As the primary amino acid sequences of many GPCRs are highly conserved, especially in TM regions, it is not surprising that these same aspartate and serine residues are found in all GPCRs whose endogenous ligands are catecholamines. The molecular structure of catecholamines consists of a protonated amine group separated from an aromatic catechol ring by a β-hydroxyethyl chain. Specific structural requirements for binding include an interaction with the amine group by the negative carboxylate side chain of aspartate, hydrogen bonding between the catechol and serine hydroxyl groups and aromatic ring π-π interactions.

No direct evidence has yet identified residues which constitute the D3DR pharmacophore, however, it can be compared to the better studied D2DR since the sequence similarity between the two receptors is very high (Sokoloff et al., 1990). By extension from Strader's work, it has been shown that Asp114 in TM3 and Ser194 and Ser 197 in TM5 are critical for ligand binding to the D2DR (Mansour et al., 1992). Residues in TM6 and TM7 may also be found in the binding crevice, including conserved phenylalanines (389 and 390) in TM6 (Cho et al., 1995) and several residues in TM7 (Fu et al., 1996). By extension, one can propose many reasons why D3nf may not bind dopaminergic ligands: 1) D3nf, being truncated, may not fold properly and hence does not form a binding pocket; 2) D3nf may not be
trafficked to the cell surface to bind ligand; or 3) D3nf, lacking TM6 and TM7, may be missing important residues which impart affinity for ligands. The results in this study are most consistent with the last possibility since the ability of D3nf to form oligomers suggests that TM1-5 fold correctly (see 5.8) and the confocal images show that D3nf is present at the cell surface.

5.3 \(^{[3]H}\)spiperone vs. \(^{[3]H}\)nemonapride detected D3DR binding sites

When the D3DR is expressed alone, there is no difference between the number of binding sites \(B_{max}\) detected by \(^{[3]H}\)nemonapride or \(^{[3]H}\)spiperone. Other studies have noted that there is variability in the number of binding sites recognised by \(^{[3]H}\)spiperone and \(^{[3]H}\)nemonapride in cultured and native D2DRs, with \(^{[3]H}\)nemonapride recognising 1.3-2 times as many sites (Seeman et al., 1992; Terai et al., 1989)(Ng et al., 1996). These results have been interpreted in a model in which butyrophenones (e.g. spiperone) bind D2DR monomers and substituted benzamides (e.g. nemonapride) bind selectively to D2DR dimers. These observations have not been entirely consistent since other reports show that the receptor densities recognised by these compounds may be identical (Malmberg et al., 1996; Vile et al., 1995). Our results with the D3DR differ from that obtained for the D2DR and may imply subtle, but important, differences between the binding pocket of the D3DR and the homologous D2DR. If such a model were appropriate for the D3DR, one might predict that heterodimers consisting of the D3DR and non-functional D3nf would have an altered affinity for the "dimer-selective" nemonapride and/or a diminished number of nemonapride detected binding sites. Interestingly, there was a significant increase in the affinity of nemonapride for membranes co-expressing the D3DR and D3nf compared to those expressing the D3DR alone and the number of nemonapride detected binding sites was lowered, which supports the idea that D3DR/D3nf heterodimers may have a different binding pocket recognised by nemonapride. However, \(^{[3]H}\)spiperone also detected fewer binding sites in the presence of D3nf, yet the affinity remained unchanged. Also, the fact that co-expression of D3nf and D3DR did not alter the affinities of four different dopaminergic ligands for competition of either \(^{[3]H}\)spiperone or
[1H]nemonapride binding, suggests that the D3DR/D3nf heterodimeric binding pocket may not be significantly different from that of a D3DR homodimer (however, see Figure 5.1 described below).

5.4 Possible mechanisms whereby D3nf decreases the apparent density of D3 receptor binding sites

The ER serves an important "quality control" function during the production of secretory proteins. As proteins are nascently synthesized in the ER, they participate in many dynamic folding and unfolding reactions mediated by ER-specific chaperone proteins. It is thought that a protein must attain a transport-competent state before it can exit the ER. If a protein is incompletely folded, misfolded or unassembled, it is retained and targeted for degradation (Sommer and Wolf, 1997). At the cellular level, this prevents potentially non- or misfunctional protein from reaching their target destination. It has been known for some time that certain oligomeric proteins are assembled in the ER before being recognized for transport to cellular destinations (Hurtley and Helenius, 1989). Examples include membrane proteins such as the insulin receptor, nicotinic acetylcholine receptor (nAchR) and T-cell receptor and secretory proteins such as TSH, FSH, LH and IgM.

Several targeting motifs have been discovered which cause retention of proteins in the ER, such as the di-basic motif (KKXX), di-acid motif (DXE) and the DKEL motif of resident ER soluble proteins (Teasdale and Jackson, 1996). Much more is known of the mechanisms which cause retention and retrograde transport of ER resident proteins than the mechanisms involved in the export of proteins bound for the plasma membrane. Originally believed to be a default pathway, the targeting of proteins to the cell surface is now suspected to be much more complex. For example, the nAchR, which exists as an oligomer of five subunits, is known to be assembled in the ER before it is trafficked to the cell surface. An ER resident chaperone, calnexin, has recently been shown to facilitate the assembly of the pentameric form of the nAchR and enhance cell surface expression of the functional channel (Chang et al., 1997).

Several truncated GPCR mutants and splice variants, including mutant forms of rhodopsin (Colley et al., 1995), mutants of the V2 vasopressin receptor (Schoneberg et al., 1995), a splice variant of
the GnRH receptor (Grosse et al., 1997) and a mutant CCR5 receptor (Benkirane et al., 1997), have been shown by confocal microscopy to be poorly or not expressed at the cell surface (section 1.8). These examples illustrate the ability of the ER to retain proteins which are not full-length, and presumably not functional. A model of ER oligomerization would then provide an attractive hypothesis to describe the dominant negative effect of co-expression of truncated receptors on the function of full-length receptors. The truncated receptor, less efficiently or unable to be recognized by trafficking mechanisms, may oligomerize with the full-length receptor and prevent the receptor's exit from ER. Consistent with this idea is a Drosophila model of retinitis pigmentosa where co-expression of mutant rhodopsin interfered with the maturation and biosynthesis of wild-type rhodopsin through retention of wild-type rhodopsin in the ER (Colley et al., 1995). As well, co-expression of a GnRH splice variant impaired signalling via phospholipase C by reducing the amount of full-length receptor at the cell surface (Grosse et al., 1997) and co-expression of the mutant CCR5 receptor reduced HIV infectivity by reducing plasma membrane expression of the wild-type receptor (Benkirane et al., 1997).

In some cases, ER resident mechanisms may not trap truncated receptors. All fragments of the rat m3 muscarinic receptor in which any of the three intracellular or extracellular loops (except the fragment consisting of only one TM) were truncated, were found to be expressed at the cell surface using confocal microscopy (Schoneberg et al., 1995). This would suggest that proper intracellular trafficking of these fragments had occurred, despite the fact that they were truncated.

Our results using confocal microscopy have shown the truncated splice variant D3nf differs from many of the aforementioned examples because it is well expressed at the surface of Sf9 cells. This implies that the protein is properly inserted into the membrane and recognized for trafficking from the ER to the cell surface. Indeed, both the D3DR and D3nf show a similar pattern of staining both on the surface of whole cells and in the interior of permeabilized cells. In Sf9 cells co-expressing the D3DR and D3nf, the staining pattern is unaltered compared to cells expressing either protein alone. These observations suggest that the appropriate signals directing the trafficking of D3DR are contained within
the amino region of the protein, since the D3nf, which is missing TM6-7, is efficiently translocated to the cell surface.

Many of the examples of ER trapping of truncated receptors come from systems where the truncated receptor arose through mutation. Hence, it would be advantageous for the cell to recognize such mutants and target them for degradation. The D3nf is a splice variant and the fact that it is allowed to reach the cell surface may suggest that it has an important role in D3DR biology. Supporting this possibility is the fact that D3nf immunoreactivity has been detected via confocal microscopy in the rat and monkey neocortex and motor cortex (Nimchinsky et al., 1997).

Had the D3nf been retained in the ER, and if co-expression resulted in a similar retention of the D3DR in the ER, this would have conveniently explained the observed decrease in apparent radioligand binding to Sf9 cell membranes expressing both the D3DR and D3nf compared to those expressing D3DR alone. As this was not the case, alternative explanations should be addressed. The decreased D3DR density in the presence of D3nf was specific for the D3DR, as it was not observed with the β2AR. It is possible that the reduction in binding sites noted using the more sensitive radioligand binding assay was unable to be discerned qualitatively by confocal microscopy due to the abundance of receptor expression. Future experiments could quantitatively determine receptor expression using confocal microscopy as has been reported by others (Grosse et al., 1997; Schoneberg et al., 1996; Schoneberg et al., 1995).

Another possible explanation for the decreased radioligand binding is that D3DR/D3nf heterodimers are unable, or have reduced ability, to bind ligand, thereby reducing the number of binding sites at the cell surface. Figure 5.1 is an adaptation of a "domain swapping" model introduced by Gouldson and colleagues (Gouldson et al., 1997), using the D3DR and D3nf. The original model, in Figure 5.1A, was derived using Maggio's chimeric muscarinic/adrenergic receptors (outlined in introduction and Maggio et al., 1993b). Gouldson's model proposes that the chimeras form two
intact binding crevices and active receptors through a "5,6-domain swapped dimer". This means that TM5-6 comprise the putative dimer interface between the two monomers. For Monnot's TM3 or TM5 mutants (Monnot et al., 1996), which, when co-expressed, regained ligand binding but not G protein coupling, the model predicts a "4,5-domain swapped dimer" (Figure 5.1B), where ICL3 is not properly aligned for G protein activation. By extension, it may be that D3DR/D3nf heterodimers are also 4,5-domain swapped as D3nf lacks a homologous TM6 and TM7 (Figure 5.1C). Since TM6 and TM7 are missing, the heterodimer binding pocket may be misformed and unable to bind ligand. However, if this model were complete, one might have expected to see a more dramatic decrease in $B_{\text{max}}$ since the D3DR and D3nf were co-expressed in equal amounts. As well, it would be predicted that the tetramers and dimers observed via photoaffinity labelling of co-expressed membranes would be of lesser intensity compared to membranes expressing D3DR alone, yet they were not (Results: Figure 4.5). One way to determine if the heterodimers are capable of binding ligand would be to immunoprecipitate the heterodimer and subject it to photoaffinity labelling. In that way, one would know if the dimers and tetramers observed on the photolabel represent D3DR homodimers or D3DR/D3nf heterodimers.

An interesting extension of these findings of decreased apparent receptor density in the presence of the D3nf pertains to a strain of D3DR knock-out mice. The D3DR gene in these mice was disrupted by the insertion of an antisense cassette in exon 2, within the region of the gene which corresponds to the putative second intracellular loop (Accili et al., 1996). Since the promoter region and ATG start codon of the gene was left intact, it is possible that a truncated receptor of only three TMs could be transcribed and translated. Since one copy of the D3DR gene was wild-type in the heterozygotes, the researchers expected that these mice would show approximately half the binding of $[^{125}\text{I}]$iodosulpiride in the islands of Calleja. Surprisingly, binding of the radioligand was minimal in heterozygous mice. Such a finding is similar to the results described herein, whereby binding in the presence of the D3nf is reduced. It is possible, then, that the 3 TM truncated protein was transcribed in
Figure 5.1: Model showing the relative orientation of TM domains in receptor dimers. A. The 5,6-domain-swapped dimer of Maggio’s chimeras. The chimeras consist of TM1-5 of one receptor and TM5-6 of another. ICL3 joins two segments and is shown by a loop. The TMs which form a receptor binding pocket are shown in the same colour. B. The 4,5-domain-swapped dimer of Monnot’s mutant. The mutated binding pocket residues are in the dark TM3 and TM5. C. The 4,5-domain-swapped dimer with D3nf. The non-homologous TM6 of D3nf is shown in white. (adapted from Gouldson and Reynolds (1997)).
these mice and exerted a negative effect on binding to the D3DR. Results such as these should serve as a warning to researchers who believe that effective gene-knockout can be obtained by merely truncating a receptor gene. A more satisfying way of attaining receptor gene-knockout would be to completely prevent any receptor-encoded protein from being transcribed, for example, by disrupting the promoter region of the gene or by deleting the entire receptor gene.

5.5 Dopamine D3 receptor oligomers

The c-myc-D3DR formed monomers, dimers and oligomers in Sf9 cells, as detected on immunoblot, based on molecular size. Photoaffinity labelling confirmed that these species consist of functional D3DR protein capable of binding ligand. While this work was underway, another report was published which demonstrated the existence of D3DR oligomers and D3DR/D3nf hetero-oligomers in rat, monkey and human brain tissue (Nimchinsky et al., 1997). Oddly, this group did not observe D3DR oligomers when the receptor was expressed alone in GH3 cells. Only when the D3DR was co-expressed with D3nf were oligomers formed, an observation which led these researchers to propose that the D3nf may function to promote oligomer formation. In Sf9 cells, on the other hand, this does not appear to be the case, since both the D3DR and D3nf form homo-oligomers when expressed alone. Even when the two were co-expressed in equal amounts, there did not appear to be an alteration in the amount of dimers formed. This argues against D3nf promoting dimer formation in Sf9 cells. In fact, the same species were observed on immunoblot and by photoaffinity labelling when the proteins were co-expressed as when they were expressed alone.

This incongruity between Sf9 cells and GH3 cells is most likely specific to GH3 cells themselves. Dimer and oligomer formation of GPCRs have been consistently observed in many cell lines, including Sf9 (Fukushima et al., 1997; Hebert et al., 1996; Ng et al., 1993; Ng et al., 1994a; Ng et al., 1994b; Ng et al., 1996; Parker et al., 1991), COS-7 (Fukushima et al., 1997; Grosse et al., 1997; Schoneberg et al., 1999), HEK-293 (Romano et al., 1996), CHO (Cvejic and Devi,
Moreover, these heterologous systems appear to appropriately model what occurs in vivo since dimers and oligomers have been shown to exist in brain tissue, specifically metabotropic glutamate receptor dimers in rat (Romano et al., 1996), D2DR oligomers in human (Lee et al., unpublished observations), and D3DR oligomers in rat, monkey and human brain (Nimchinsky et al., 1997).

In rat and monkey brain, the D3DR has been shown to exist as monomers, dimers and tetramers, although in human brain the dimeric form was not observed and the tetrameric form was the most abundant (Nimchinsky et al., 1997). The reason for these interspecies differences are not clear. Interestingly, in Sf9 cells, the relative ratio of monomer:dimer:tetramer is not the same on the photolabel as detected by immunoblot (see Results: Figures 4.4 and 4.5). The tetramer band appears to bind more ligand than the dimer, although less than the monomer. The relevance of this finding requires further examination, however, since the tetrameric form of the D3DR is reported to be the predominant form in human brain, this observation suggests that oligomers may play an important functional role in D3DR pharmacology and may, in fact, be the active form of the D3DR.

5.6 D3DR/D3nf hetero-oligomers

Co-immunoprecipitation has been used to show that the D3nf and D3DR form hetero-oligomers in human, monkey and rat brain (Nimchinsky et al., 1997). The present study was unable to successfully immunoprecipitate either the D3DR or D3nf and future studies would be required to directly show a D3DR and D3nf interaction. Further, Western blotting was unable to distinguish a unique molecular weight band which would correspond to the predicted size of the heterodimer (~76 kDa) in membranes expressing both FLAG-D3nf and c-myc-D3DR. This may be a consequence of the broad staining of the homodimer band observed in membranes expressing only D3DR or D3nf. Alternatively, the D3DR/D3nf heterodimer may not survive SDS-PAGE. However, the effect of D3nf co-expression on the number of D3DR binding sites and lack of effect on β2AR ligand binding argues that the FLAG-D3nf exerts a specific effect on the D3DR likely via protein-protein interactions. Coupled with the observation
of D3DR/D3nf heterodimers in other systems (Nimchinsky et al., 1997), this result suggests that D3DR/D3nf hetero-oligomers form in Sf9 cells as well.

5.7 Function of GPCR oligomers

In other receptor families, specifically those receptors with a single TM domain such as tyrosine kinases, dimerization is known to occur in response to agonist binding and is required for signal transduction (Heldin, 1995). For GPCRs, on the other hand, the role that dimerization plays is less established, yet continues to be an area of active investigation. It is apparent, however, that agonist binding is not absolutely required for dimerization, as every report of GPCR dimers has been determined in the absence of agonist. For the β2AR, dimerization may be critical for receptor function because a peptide designed on TM6 of this receptor inhibited both dimer formation and agonist promoted stimulation of AC (Hebert et al., 1996). Further, β2AR agonists may promote the formation of dimers and prevent the disruptive effect of the TM6 peptide whereas inverse agonist promote and stabilize the monomeric form (Hebert et al., 1996). In contrast, agonists appear to decrease the level of δ-opioid receptors dimers (Cvejic and Devi, 1997). Also, increased monomer levels in response to agonist treatment preceded δ-opioid receptor internalization, implying that monomer:dimer interconversions may regulate the process of internalization of this receptor (Cvejic and Devi, 1997). Our group has shown that a TM6 derived peptide of the D1DR had no effect on receptor oligomerization, however, it specifically inhibited D1DR binding and function (George et al., unpublished observations).

This study was unable to examine the functional role of D3DR oligomers on D3DR biology, since coupling to AC was not observed in Sf9 cells. This is unfortunate, because it would be relevant to examine the effect of D3nf co-expression on D3DR effector coupling. As described in the introduction (section 1.8), certain truncated receptor forms are believed to attenuate the function of their full-length counterparts. Since the D3nf decreased the number of D3DR binding sites recognized by radioligands (see below), it would be predicted that D3DR effector coupling would be lowered accordingly in the
presence of D3nf. This hypothesis, of course, assumes that the number of D3DR binding sites recognized by agonists, as well as antagonists, would also decrease in the presence of D3nf.

In order to examine the proposed functional interplay between the D3DR and D3nf in Sf9 cells, other effector pathways would need to be studied. Candidates would be pathways which have already been shown to linked to the D3DR in other cell systems, such as c-fos gene transcription (Pilon et al., 1994), calcium current (Seabrook et al., 1994), mitogenesis and extracellular acidification (Chio et al., 1994).

5.8 Structural implications of D3nf oligomers

The D3nf, which shares TM1-5 with the D3DR and is different in the carboxyl region, was also able to form homo-oligomers as resolved on immunoblot. This is notable as there are no published reports which have directly examined whether any other truncated GPCR is able to form oligomers when expressed alone.

It is not yet known which regions of GPCRs are most important for oligomer formation. As discussed above, TM6 has been implicated in dimer formation of the β2AR but does not appear to be important for D1DR dimerization. In addition, work with the δ-opioid receptor has implicated the importance of the carboxyl tail in dimerization (Cvejic and Devi, 1997). The presence of a long ICL3 is believed to promote dimer formation in Maggio’s adrenergic/muscarinic chimeras (Maggio et al., 1996). Metabotropic glutamate receptors show the least homology to other GPCRs and have what is likely a unique mechanism of dimerization: intermolecular disulfide bonding in the amino terminus (Romano et al., 1996).

The present study has shown that D3nf is able to form oligomers and argues that the presence of TM6-7 or a D3DR-like carboxyl tail is not a prerequisite to the formation of D3nf oligomers. Thus, sufficient information directing oligomer formation is encoded within TM1-5 alone. This observation agrees with Maggio’s model of the multi-subunit autonomous folding nature of GPCRs (see introduction and Maggio et al. (1993a), as it would appear that TM1-5 of D3nf, which are identical in sequence to the D3DR, are able to fold correctly, be inserted in the membrane and form oligomers, independent of the
presence of TM6-7. Although oligomer formation may be a phenomenon common to GPCRs, the above results show that the structural requirements for oligomerization may vary among each receptor subfamily.

5.9 Conclusions

This study has shown that the D3DR forms oligomers in SF9 cells. That the truncated D3nf also forms dimers and oligomers points to TM1-5 containing crucial information directing the oligomerization of dopamine receptors. The amino portion of the receptor may also contain motifs recognized by trafficking proteins which direct dopamine receptors from the ER to the cell surface, since D3nf, in contrast to certain other truncated GPCRs, is well expressed at the cell surface. Our data also show that although D3nf does not have any D3DR-like pharmacology on its own, it may act to decrease capacity of D3DRs on the cell surface to bind ligand. Furthermore, since the D3DR and D3nf have been shown to be expressed in the same brain regions, and even the same neuron, these results imply that the D3nf may play a important role in modulating D3DR density and perhaps function in vivo.
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