Expression, Purification and Crystallisation Studies with the M_2 Muscarinic and H_1 Histamine Receptors.

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The writing of this thesis.

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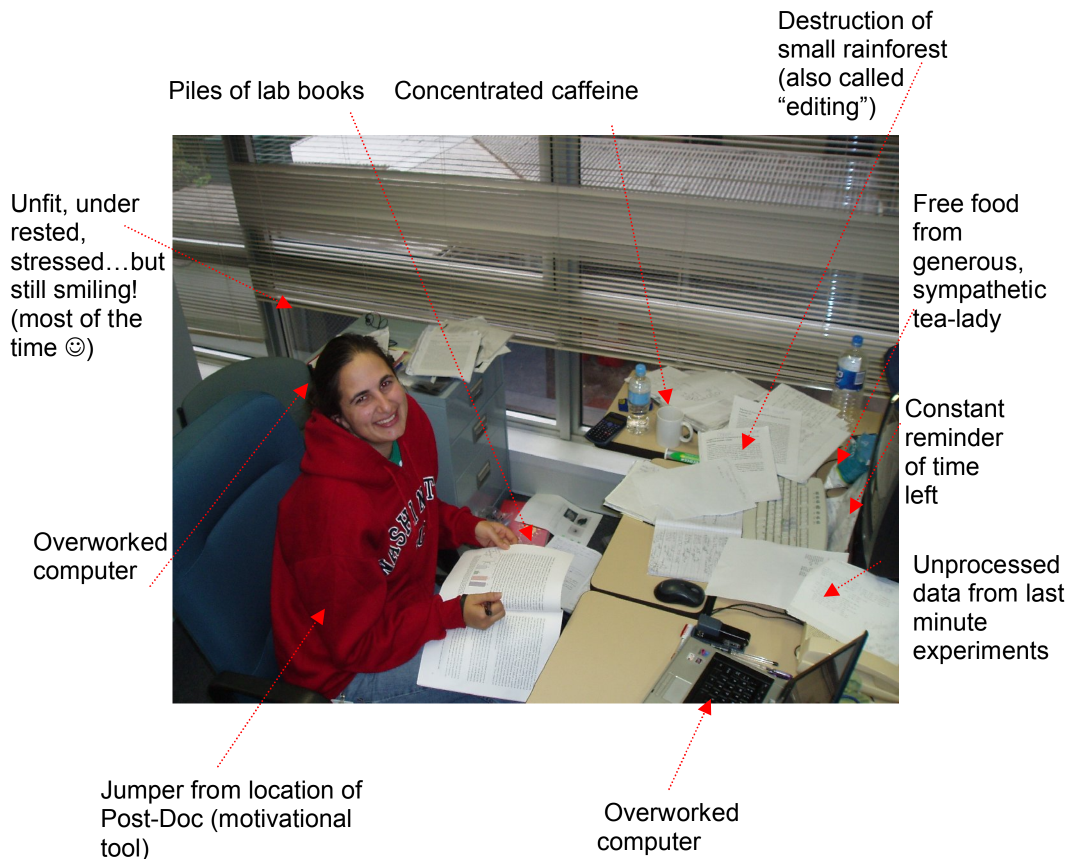

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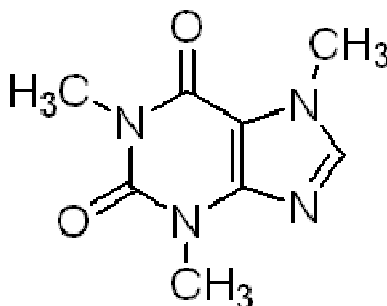

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Abstract and summary of this thesis

This study describes expression, purification and crystallisation trials with three human seven transmembrane receptors (7TMRs).

A variety of Histidine (His) tagged constructs of the M_2 muscarinic receptor (M_2R) and a $5HT_{2A}$ serotonin receptor were prepared in baculovirus. A 10xHis tagged form of the human H_1 histamine receptor was obtained in recombinant baculovirus. M_2R, $5HT_{2A}R$ and H_1R constructs were expressed in *Sf*9 cells. Receptors expressed at between ~15 and 60pmol/mg of total membrane protein with the exception of the $5HT_{2A}R$ construct for which expression could not be conclusively demonstrated by radioligand binding. Two constructs were focused on; a C terminal 6xHis M_2R ($His6_CM_2R$) and the $His10_CH_1R$.

Membrane associated levels of the $His6_CM_2R$ and $His10_CH_1R$ were modulated by expression in the presence of receptor specific ligands. Addition of either atropine ($His6_CM_2R$) or triprolidine ($His10_CH_1R$) to receptor expressing *Sf*9 cells increased membrane associated receptor levels up to 3 fold.

G-protein subunits were purified by IMAC and used in $[^{35}S]$-GTPγS binding assays with the membrane bound $His6_CM_2R$ and $His10_CM_2R$. Addition of the 6xHis tag decreased the ability of the M_2R to activate $G\alpha_{i1}$ but did not render the receptor non-functional. Interestingly, $His10_CH_1R$ was also able to activate $G\alpha_{i1}$ with a 7 fold increase in $[^{35}S]$-GTPγS being observed in the presence of the agonist. This interaction between $His10_CH_1R$ has not been previously demonstrated in a cell-free system.

Solubilisation trials with $His6_CM_2R$ demonstrated n-Dodecyl-β-D-Maltoside (DDM) to be a useful detergent for extraction of the receptor from *Sf*9 membranes. A preliminary purification protocol for the receptor was developed using IMAC and GF-HPLC.

The $His10_CH_1R$ was solubilised using *n*-Octyl-β-D-glucopyranoside (nOG) with an estimated efficiency of 53% as determined by radioligand binding assay. Following IMAC, $His10_CH_1R$ was purified to homogeneity using GF-HPLC. The presence of antagonist throughout the purification was deemed as necessary for final recovery of the receptor but could not be conclusively removed from the receptor, making radioligand binding

measurements difficult. Addition of excess [^{3}H]-ligand gave a functional recovery of the purified receptor of < 5% and a specific activity of ~500pmol/mg. Final yield of the receptor as determined by absorbance measurements was ~1mg from 5L of *Sf*9 cells (~ $2x10^6$ cells/mL).

Two-dimensional crystal trials with the $His10_CH_1R$ were prepared by reconstitution of the receptor into the lipid mixture asolectin. Initially results for the 2D crystals appeared promising with ordered, lipidic areas generating electron diffraction patterns. However, an approximate calculation of the crystal unit cell of the 2D crystals demonstrated it to be too small to contain the receptor.

Three-dimensional trials with the $His10_CH_1R$ were carried out in the *meso* phase of either monoolein or phytantriol. Co-crystallisation trials with $His10_CH_1R$ and $G\alpha_{i1}$ produced clusters of needle-like crystals. These crystals were not formed in the presence of $G\alpha_{i1}$ only. A bunch of the crystals produced an X-ray diffraction pattern similar to that of a powder. Diffraction rings were visible at between 50Å and 3Å but it was not possible to index the diffraction pattern. Work with these crystals is on-going and they will be investigated at the Australian synchrotron later in the year.

Abbreviations commonly used in this thesis.

7TMR – Seven Transmembrane Receptor

AT_1R – Angiotensin 1 Receptor

CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CHO – Chinese Hamster Ovary

CSIRO – Commonwealth Scientific and Industrial Research Organisation

CSIRO MHT – CSIRO Molecular and Health Technologies

DDM – n-Dodecyl-β-D-Maltoside

DTAC – dodecyltrimethylammonium chloride

EC_{50} – half maximum effective concentration

FPLC^{TM} Fast Protein Liquid Chromatography (from Pharmacia)

FRET – Fluorescence Resonance Energy Transfer

GDP – Guanosine DiPhosphate

GF-HPLC – Gel Filtration High Performance Liquid Chromatography

GPCR – G Protein Coupled Receptor

GTP – Guanosine TriPhosphate

H_1R – H_1 Histamine Receptor

HEK – Human Embryonic Kidney

$His10_CH_1R$ – C terminal, 10xHistidine tagged H_1 Histamine Receptor

$His6_CM_2R$ – C terminal, 6xHistidine tagged M_2 Muscarinic Receptor

IMAC – Immobilised Metal Affinity Chromatography

M_2R – M_2 Muscarinic Receptor

MQH_2O – milliQ treated water

NCMLS – Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands

NMR – Nuclear Magnetic Resonance

nOG – *n*-Octyl-β-D-glucopyranoside

PCR – Polymerase Chain Reaction

PIP- phosphatidylinositol bisphosphate

POPC - 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

QNB – 3-quinuclidinyl benzilate

RAMPs - Receptor Activity Modifying Proteins

SARDI – South Australian Research and Development Institute

SDS-PAGE – Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

Abbreviations commonly used in this thesis (cont...).

sMQH_2O – sterile miliQ treated water

SPR – Surface Plasmon Resonance

Throughout the thesis receptor-ligand binding is given in units of pmol/mg. This refers to pico-moles of ligand bound per mg of total cellular protein, unless otherwise stated in the text (for example pmol/mg of total membrane protein).

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed…………………………………………….

Amanda Louise Aloia

Date……………………………………………..