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The effect of opioids on the signalling of the μ-opioid receptor and some variants

By

PREETI MANANDHAR

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Department of Biomedical Sciences

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Declaration of Originality

I, hereby, declare that the work presented in this thesis has not been submitted, either in full or in part, for a degree at this or any other university or institution. To the best of my knowledge, this submission is original, except where due reference is stated otherwise.

Preeti Manandhar

Faculty of Biomedical Sciences

Macquarie University

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List of Abbreviations

AC	Adenylyl Cyclase
β-CNA	β- Chlornaltrexamine
β-FNA	β-Funaltrexamine
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CHO	Chinese Hamster Ovary
CRC	Concentration Response Curve
CRE	cAMP Response element
DAMGO	[D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ERK	Extracellular Signal Regulated Kinase
FSK	Forskolin
GIRK	G Protein-Coupled Inwardly Rectifying Potassium Channel
GPCR	G Protein Coupled Receptor
GRK	G Protein Receptor Kinase
HBSS	Hank's Balanced Salt Solution with HEPES
HEK	Human Embryonic Kidney
hMOR	Human μ- Opioid Receptor
JNK	Jun N-terminal Kinase
LC	Locus Coeruleus

M-6-G	Morphine-6-glucuronide
MAPK	Mitogen Activated Protein Kinase
MOR	μ-Opioid Receptor
MPA	Membrane Potential Assay
mRNA	messenger Ribonucleic acid
PAG	Periaqueductal Grey
PKA	Protein Kinase A
PTX	Pertussis Toxin
RFU	Relative Fluorescence Unit
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
SST	Somatostatin
TM	Transmembrane
WT	Wild Type

ABSTRACT

Opioid analgesics are the most effective medications used for the treatment of moderate to severe nociceptive pain. However, their clinical utility is limited by their adverse effects and prevalence of tolerance and dependence on chronic use. Moreover, the degree of pain relief varies in individuals and studies suggest genetic variation as one of the cause.

In this project, we focus on comparing the effect of pethidine, O-desmethyl tramadol and TRV 130 on signalling of μ-opioid receptor (MOR) and some common polymorphisms of MOR (A118G and C17T). In addition, we sought to compare the efficacy of these opioids with enkephalin analog, DAMGO and commonly studied opioid- morphine in K channel activation assay in AtT20 transfected cells. FLIPR membrane potential assay is used to determine the change in membrane potential caused by different opioids.

The highest concentration tested for pethidine and O-desmethyl tramadol showed a reduced inhibition of forskolin response in CHO cells expressing C17T as compared to WT ($P<0.05$) whereas no significant difference was observed in A118G. TRV 130 showed a significantly reduced inhibition of forskolin response ($P < 0.05$) in the CHO cells expressing A118G and C17T than in WT. Pethidine, O-desmethyl tramadol and TRV 130 activated K channel in AtT20 hMOR cells with pEC_{50} of 5.7 ± 0.1, 6.3 ± 0.1, 8.1 ± 0.1 respectively, as compared to morphine (7.2 ± 0.1) and DAMGO (8.3 ± 0.1). Depleting MOR with an irreversible antagonist, β-CNA, allowed us to calculate a measure of transduction efficiency (tau), which relates receptor occupancy to effect, for both control and depleted cases for DAMGO (67.6, 7.1), morphine (11.1, 1.5) and TRV 130 (5.5, 0.6), demonstrating the lower efficacy of TRV 130 compared to morphine.

All the opioids under study appeared to be lower efficacy agonist than morphine, and their efficacy may be further reduced at common MOR polymorphisms. It remains to be established whether the findings of this project, yields similar clinical effects.

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1

GENERAL INTRODUCTION

1.1 Introduction to opioids

Opioid analgesics are the most effective and commonly prescribed medication for treatment of acute and chronic, moderate to severe pain (Mcquay, 1999). However, the response to opioid therapy varies between individuals, resulting in patients experiencing unwanted side effects and the need to increase dose because of inadequate pain relief (Skorpen *et al.*, 2008). In addition to that, the chronic use of opioid has been reported to cause tolerance and physical dependence in patients in varying degrees (Sees *et al.*, 1993). Numerous parameters may give rise to these individual differences in opioid response, such as pharmacokinetic profile of drug (absorption, distribution, metabolism) and intrinsic efficacy of the drug at the receptor (Knapman *et al.*, 2015a). Variability in these parameters may be contributed by the alteration in specific gene in individuals (Lötsch *et al.*, 2005). Despite the powerful analgesic effect of some of the commonly used opioids, their association with side effects like constipation, respiratory depression, sedation and euphoria, makes the need of intense research to develop a novel opioid with improved analgesia and reduced side effect along with low addiction profile.

1.2 Opioid receptors

The therapeutic opioids like morphine, buprenorphine and fentanyl exert their pharmacological and physiological effects by binding to cell surface receptors termed as

opioid receptor. Opioid receptors were initially reported in 1970s, by radiolabelled opioid ligand binding studies (Pert *et al.*, 1973; Terenius, 1973), after which several other receptors have been proposed. Martin *et* al. (1976) postulated three types of opioid receptors while they studied the effect of morphine and nalorphine like drugs in chronic spinal dogs (Gilbert *et al.*, 1976). They named them μ, κ and σ after the agonists morphine, ketocyclazocine and N-allylnormetazocine respectively. A year later, Lord *et* al. found enkephalins having higher affinity to a receptor other than μ or κ, and named it as δ-receptor (Lord *et al.*, 1977). The research then focused on isolating and purifying opioid receptor proteins from the cell membranes, but none of the studies succeeded in determining the structure of opioid receptors (Simon *et al.*, 1993).

Thereafter, cloning of the opioid receptors started allowing deeper insight into their anatomical distribution, biochemical and pharmacological properties (Satoh *et al.*, 1995). This also led to discovery of a new nociception receptor, also termed as opioid receptor like -1 (Bunzow *et al.*, 1994). However, during 1996 when σ receptor was cloned (Hanner *et al.*, 1996), it was no longer considered an opioid receptor, but rather, a single transmembrane spanning protein, which is the target site for many misused drugs (Monassier *et al.*, 2002). The opioid receptors, along with endogenous opioid ligands are involved in control of pain and for brain's motivation and reward system. Activation of opioid receptor modulates several physiological and autonomic functions including analgesia, hormone release, respiration, blood pressure and gastrointestinal motility (Boom *et al.*, 2012; Brock *et al.*, 2012; Lang *et al.*, 1982; Lewis *et al.*, 1980; Morley, 1981).

The opioid receptors are widely distributed in central nervous system along the pain modulating descending pathway including - periaqueductal grey (PAG), locus coeruleus (LC), rostral ventral medulla and also in the substantia gelatinosa of the dorsal horn (Mansour *et al.*, 1995). The location of opioid receptors at these locations, makes it possible to directly inhibit spinal cord pain transmission by inhibiting spinal neurons (Ahlbeck, 2011). Moreover, the MOR has highest expression in LC, striatum, thalamus and PAG of mammalian brain (Mansour *et al.*, 1995; Peckys *et al.*, 1999).

1.3 μ-opioid receptor (MOR)

The opioid analgesics act by binding to mu-opioid receptor (MOR) which along with other opioid receptors are G-protein coupled receptors (GPCRs). GPCR is one of the largest family of membrane receptors in human genome (Fredriksson *et al.*, 2003). Like all other GPCRs, MOR is a seven transmembrane domain protein, coupled to heterotrimeric G proteins, which mediates intracellular signals (Contet *et al.*, 2004; Minami *et al.*, 1995). Activation of MOR causes dissociation of G protein subunits resulting in decrease in the adenylyl cyclase (AC) mediated cyclic adenosine monophosphate (cAMP) production, inhibition of voltage gated Ca channel and opening of G protein gated inwardly rectifying K channel (GIRK). All these cellular events result in changes in cellular level of cAMP, suppression of neurotransmitter release and membrane hyperpolarisation respectively (Mague *et al.*, 2010; Mestek *et al.*, 1996).

1.4. MOR signalling

Upon binding of the ligand to MOR, G-protein coupled to MOR is activated and promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) associated with G_{α} subunit. This results in conformational change in the G protein complex, thus, leading to dissociation of $G_{\beta\gamma}$ subunit from $G_{\alpha.}$ Activated G_{α} and $G_{\beta\gamma}$ bind to different effector molecules which continue to pass the signal via different second messengers (Tuteja, 2009). Using pertussis toxin which selectively disrupts the ADP-ribosylation of $G_{i/o,}$ the inhibitory mechanism of opioid receptors was found to be signalling via $G_{i/o}$ proteins (Burns, 1988; Mangmool *et al.*, 2011).

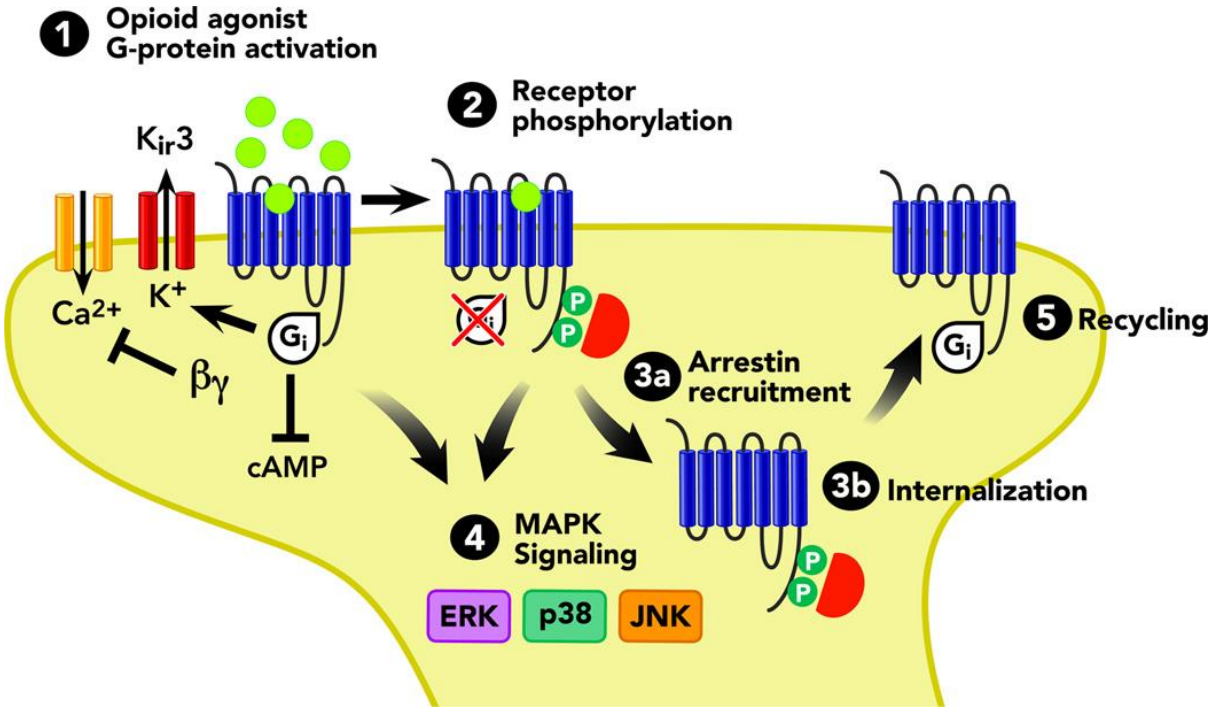

Figure 1. Signalling of opioid receptor. The arrows (→) represents activation and (T) sign represents inhibition or blockage. MAPK= mitogen-activated protein kinase, ERK= extracellular signal regulated kinase, JNK= c-jun N-terminal kinase and (P) = phosphorylation. Figure reproduced from (Al-Hasani *et al.*, 2011).

One of the hallmark of opioid receptor activation is reduction of intracellular cAMP. cAMP is a second messenger involved in the modulation of many biological processes like hormone secretion, neurotransmitter release, memory formation and reproduction (Rall *et al.*, 1958). cAMP levels in cells is catalysed by adenylyl cyclase (AC) which is activated when ligand activates $G_{\alpha s}$. AC converts ATP in the cells to cAMP, which in turn activates Protein Kinase A (PKA). PKA phosphorylates various enzymes and regulates gene expression by activating cAMP response element binding protein (CREB). Another opioid signalling event mediated by $G_{\beta\gamma}$ subunit, which inhibits the neuronal excitability, is the membrane potential hyperpolarisation caused by activation of G-protein coupled inwardly rectifying K channel (GIRK) (Connor *et al.*, 2009; Ikeda *et al.*, 2000). A lot of studies prefer K channel activation pathway to study opioid signalling because of the direct association of G-protein signalling with GIRK activation, which provides a practical way of determining the change in membrane potential. Another cellular event

for opioid receptor signalling is opioid mediated inhibition of voltage gated Ca channel (North, 1986; Surprenant *et al.*, 1990). Modulation of voltage gated calcium channel is believed to be mediated by $G_{\beta\gamma}$ subunit, where $G_{\beta\gamma}$ binds to the channel and blocks its opening (Dolphin, 2003). Both K and Ca pathways are pertussis toxin sensitive, implying the involvement of G_i or G_o proteins (Hescheler *et al.*, 1987; Tatsumi *et al.*, 1990).

The opioid receptors, like other GPCRs, have a very complex signalling mechanism as they show G-protein independent signalling in addition to G protein dependent signalling, which involves interaction with G-protein receptor kinases (GRKs) and β-arrestin (Heuss *et al.*, 2000). Upon continued exposure to an agonist, GRKs are recruited to the membrane which phosphorylate the opioid receptors and associate the receptor with cytoplasmic protein, β-arrestin. This complex uncouples the receptor from G protein and recruits to clarithrin coated pits for endocytosis (internalisation) (Connor *et al.*, 2004). The internalised receptors are either dephosphorylated and recycled back to the membrane for resensitization or recruited to lysosome for degradation (Ferguson, 2001; Qiu *et al.*, 2003). Morphine acts in a different way than other opioids, by not being able to cause efficient receptor internalisation and several contrasting theories have been postulated regarding the mechanism of morphine internalisation (Connor *et al.*, 2004; Koch *et al.*, 1998; Whistler *et al.*, 1998). Studies have suggested DAMGO, etorphine, fentanyl, β-endorphin and methadone showing rapid internalisation of MORs whereas morphine, pentazocine and buprenorphine demonstrating slow and less complete internalisation (Borgland *et al.*, 2003; Celver *et al.*, 2004; Keith *et al.*, 1996). It has been proposed that endocytosis plays a protective role by reducing the development of tolerance of opioids and an *in vivo* study showed MOR endocytosis after exposure to morphine, prevents the development of morphine tolerance (He *et al.*, 2002). These ligand dependent responses to tolerance, which are known as biased agonism has been the area of intense research. An *in vivo* study conducted in mice showed failure to develop tolerance as a result of lack of β-arrestin 2, which prevents chronic morphine induced desensitisation (Bohn *et al.*, 2000). However, another study showed the need of both β-arrestin 1 and 2 for reducing DAMGO induced internalisation (Groer *et al.*, 2011). These findings highlight biased agonism being more pathway specific rather than drug-receptor specific, which provides a promising approach to design a drug, enhancing its therapeutic effect and reducing its unwanted side effects.

GPCR research for last several years have focused on MAPK (mitogen activated protein kinase) signalling of opioid receptors (Al-Hasani *et al.*, 2011; Gutstein *et al.*, 1997). They have discovered that the arrestin-bound complex is not inactive, rather it works as a GPCR signal transducer by actively binding to proteins involved in signal transduction such as Src family kinases and components of ERK1/2 (extracellular signal-regulated kinases) and JNK3 (c-jun N-terminal kinase) MAP kinases (Luttrell *et al.*, 2002). MAPK pathway have found to regulate several cellular pathways such as cellular chemotaxis, apoptosis, cancer metastasis and protein translation (Shenoy *et al.*, 2011). With the discovery of all these new molecular and cellular pathways, opioid receptor pharmacology is bound to see some breakthrough therapies and discovery of some new opioid analgesics, with better analgesia and reduced side effects.

1.5 Single nucleotide polymorphism in MOR

Alteration in μ-opioid receptor gene (OPRM1) sequence such as single nucleotide polymorphism (SNPs) is found to be responsible for significant variability in individual response to opioids. Single nucleotide polymorphisms may be associated with functional changes both in *in-vivo* and *in vitro* characteristics (Lötsch *et al.*, 2005). A number of SNPs of OPRM1 have been reported in humans and most commonly studied are the non-synonymous SNPs such as N40D (A118G), A6V (C17T), S42C, D51N, L85I, R260H (Knapman *et al.*, 2015a). In non-synonymous SNPs, the change in nucleotide sequence leads to change in amino acid which ultimately results in different protein sequence. There may be greater possibility that a non-synonymous SNP in MOR may contribute to the individual differences in the opioid response.

1.5.1 A118G polymorphism

A118G is the most commonly studied MOR SNP in which adenine (A) is substituted by Guanine (G), subsequently resulting in the change in amino acid from asparagine (N) to aspartic acid (D) at position 40. This causes loss of putative glycosylation site in the N-terminal domain of the gene (Beyer *et al.*, 2004). A118G is most common in European (15-30%) and Asian population (40-50%) and comparatively less prevalent in African American and Hispanic groups (1-3%) (Bergen *et al.*, 1997; Gelernter *et al.*, 1999; Haerian *et al.*, 2013). A118G polymorphism have been reported to be responsible for

diverse clinical outcomes like altered pain threshold and difference in opioid response but the association is not consistent (Gelernter *et al.*, 1999; Mura *et al.*, 2013). A study by Zhang et. al (Zhang *et al.*, 2005), showed substitution of A with G at position 118 in OPRM1 caused significant functional changes in mRNA expression in human brain tissues and protein translation. They found mRNA in 118A allele was 1.5-2.5 fold higher as compared to 118G allele in the human autopsy brain tissues. Transfection into Chinese hamster ovary cells cDNA showed 1.5-fold high mRNA expression and 10-fold higher protein yield in 118A. It is still unclear that the difference in the mRNA expression is due to the difference in transfection or the difference in mRNA stability. However, the difference in protein are likely to reflect the real difference in turnover. Nevertheless, the report suggests the reduction in the functional expression of the gene because of the single nucleotide change. According to a study, nucleotide change at 118 position introduces a methylation site at position 117 in OPRM1 gene, which is responsible for inhibiting compensatory up-regulation of MOR mRNA in chronic opioid users (Oertel *et al.*, 2012). However, the study does not take into account the functional consequence of acute opioid exposure on the μ-opioid regulation. Receptor expression was decreased by 90% in 118G allele as compared to the wild type receptor by a study conducted in HEK-293 cells (Kroslak *et al.*, 2007). This study showed that N-linked glycosylation was required for cell-surface receptor expression and the A118G polymorphism causing the loss of putative N-glycosylation site may be the reason for the decrement. All these finding suggest higher doses of opioids may be required in people with 118G allele as compared to 118A allele.

Numerous studies have been performed to investigate the effect of A118G on the cellular signalling of MOR. However, none of them are able to explain the mechanism of how a single change in nucleotide affects the receptor at cellular level. A study in 1998, reported a threefold increase in the potency of β-endorphin for 118G and similar increase in its potency to activate GIRK channels when co-expressed with 118G (Bond *et al.*, 1998). Studies used HEK-293 cells to compare inhibition of AC activity in 118A and 118G variants but no significant changes were observed between the two alleles (Beyer *et al.*, 2004; Kroslak *et al.*, 2007). One major reason for this may be the exposure with opioid agonists for a long time. The receptor undergoes desensitization when exposed to opioid agonists for 10-20 minutes and the above-mentioned studies measures the receptor

activity after an hour incubation with opioid agonists (Connor *et al.*, 2004). Measurement of inhibition of Ca channels have been performed to compare 118A and 118G variants using humanized mouse, HEK-293 cells and acutely transfected cell lines. Although studies conducted in HEK-293 cells found some opioids having more potency in 118G as compared to 118A allele, the studies in humanized mouse and transfected neurons showed different results (Mahmoud *et al.*, 2011; Margas *et al.*, 2007; Ramchandani *et al.*, 2011). The inconsistencies in studies indicate that MOR studies may be dependent on different cell systems being used. Some of the cell lines do not express proteins that are normally expressed in neurons which are involved in mRNA or protein processing or folding (Bailey *et al.*, 2005). Like in rat and bovine tissue, expression of guanylyl cyclase-A (receptor for atrial natriuretic peptide), depends on the degree of N-glycosylation and the expression may not be consistent over different tissues (MüLler *et al.*, 2002).

Particularly, A118G polymorphism is associated with different response to opioid antagonists- naloxone (Hernandez-Avila *et al.*, 2003) and naltrexone (Oslin *et al.*, 2003). Alcohol and other abused drugs are found to activate the release of dopamine from ventral tegmental neurons (Di Chiara *et al.*, 1988). This release of dopamine is slightly boosted by activation of MOR and likewise, studies have suggested the importance of MOR activation as a mechanism of alcohol reward. In a study conducted in alcohol preferring rats, alcohol consumption was decreased on inactivation of MOR (Myers *et al.*, 1999). This association demonstrated a theoretical link to association of A118G polymorphism in the development of addiction of various compounds including alcohol. Some of the studies conducted to test the relationship supported this hypothesis (Arias *et al.*, 2006; Bart *et al.*, 2005; Ray *et al.*, 2004), while some failed to replicate the findings (Crowley *et al.*, 2003; Franke *et al.*, 2001), making the result inconsistent. *In vivo* micro-dialysis study conducted in transgenic mice with 118A and 118G alleles, showed elevated dopamine release in 118G allele after alcohol intake whereas, mice with 118A allele showed no changes over baseline (Ramchandani *et al.*, 2011). Human pharmacogenetics study of alcohol demonstrated people with 118G allele experiencing higher euphoria after standard dose of alcohol as compared to people with 118A allele. The investigation on the response of socially active drinkers to alcohol after a dose of naloxone, showed reduced alcohol induced euphoria in 118G allele as compared to 118A allele. All these reports suggest G allele conveys enhanced reward effect of alcohol than 118A allele and

naltrexone compromises this effect only in 118G allele (Berrettini, 2016). However, the studies in support of the association between A118G SNP and opioid or alcohol dependence, are unable to clarify the nature of their relationship. Nevertheless, the beneficial effect of naltrexone on alcoholic people with A118G polymorphism creates a possibility of a clinically strong alternative of managing addiction causing drugs.

1.5.2 C17T polymorphism

Another common polymorphism in the MOR is A6V or C17T, located at the N-terminal of the MOR. This single nucleotide polymorphism from cytosine to thymine results in the change in amino acid from Alanine to Valine at position 6. The frequency of this allele varies in different ethnic groups from 1% in Caucasian and east Asian cohorts (Rommelspacher *et al.*, 2001; Tan *et al.*, 2003) to nearly 20% in African Americans populations (Crowley *et al.*, 2003) or in Northern Indians (Kapur *et al.*, 2007). Only few studies have been conducted so far to understand the effect of C17T polymorphism as compared to A118G. Although C17T is found to occur in higher frequency in substance abusers (Fillingim *et al.*, 2005), the effect of this polymorphism in the individual variation to opioid response has not been studied in detail. In the study that tested the coupling of human MOR-wild type and human MOR-C17T to signalling pathways in different cell lines, pathway specific changes in the function was observed. The study found the reduction in the ability of the receptor to inhibit AC and to stimulate phosphorylation of ERK1/2. In addition to that, signalling of buprenorphine, a partial MOR agonist, was also found to be compromised in the polymorphic variant (Knapman *et al.*, 2015c).

1.6 Opioids under study

Tramadol is a centrally acting analgesic having low affinity for opioid receptors (Driessen *et al.*, 1993). Tramadol seems to be less effective than combination of aspirin with codeine, in managing moderate acute pain, however, it is as effective as morphine in patients with moderate cancer related pain (Lewis *et al.*, 1997). Despite its controversial effectiveness, its tolerance in short term use, minimal adverse effects at recommended dose and low abuse potential, makes it a common drug of choice for pain management.

Pethidine is another potent opioid analgesic having one fifth to one sixth analgesic activity as compared to Morphine (Woolfe *et al.*, 1944). Pethidine was widely used in labour pain (Bricker *et al.*, 2002) and also for the treatment of post-anaesthetic shivering (Wrench *et al.*, 1997). TRV 130 is a newly discovered ligand at the μ-opioid receptor which has been reported to stimulate only G-protein pathway, unlike morphine which binds to MOR and stimulates both G-protein coupling and β-arrestin recruitment (Dewire *et al.*, 2013). Reports show a broad margin between the effective dose and the dose causing intolerance which suggests TRV 130 can be a safe and more tolerable medicine in acute pain management (Soergel *et al.*, 2014a).

All these opioids under study have been used for managing pain, however the effect of SNPs on the response to these drugs *in vitro* has not been studied yet. Our study will mostly focus on the effect of common SNPs- C17T and A118G on the response to these opioids. The analgesic effect of tramadol is mediated by its active metabolite, O-desmethyl tramadol (Grond *et al.*, 2004), hence we will be performing all the experiments using the metabolite. In addition to that, this project will also determine the effect of pethidine, tramadol and TRV 130 in K channel activation pathway mediated by G-protein signalling. It is likely that this cellular *in vitro* study will continue to unravel greater understanding on the signalling and regulation of opioids.

Hypotheses and Aims

The overall hypothesis of this project is that signalling of opioids under study varies in response to genotypic variation of MOR. The distinct aims of this study are:

1. To compare the effect of pethidine, O-desmethyl tramadol and TRV 130 on wild type MOR and commonly occurring single nucleotide polymorphisms of MOR (A118G and C17T).
2. To determine the efficacy of the opioids under study in K channel activation assay in AtT20 WT transfected cells and compare the effect with enkephalin analog DAMGO or commonly studied MOR agonist, morphine.

Comparing the effect of opioids on signalling of MOR and variants, our research will contribute to a more individualised approach to opioid therapy by maximizing the beneficial effects and minimizing the adverse events.

2

EXPERIMENTAL METHODOLOGY

2.1 Cell Culture

All the experimental works in this project were carried out using cultured cells to understand the molecular mechanism of receptors. Chinese Hamster Ovary (CHO) and AtT20 cells (mouse pituitary adenoma cells) were used for Adenylyl cyclase and GIRK assays respectively. As the cell lines used are genetically modified organisms (GMO), so all the tissue culture procedures were carried out in sterile conditions adhering to the regulations of the Office of Gene Technology Regulator (OGTR) and Biosafety regulations. The approval numbers for biosafety regulation are IBC REF: 5201200023, 5201500367.

2.1.1 CHO Cells

CHO cells are frequently chosen for the Adenylyl cyclase assay because of the unique composition of G-protein coupled receptors. Increase in the level of cAMP after forskolin treatment and effect of opioids in inhibiting this elevation of cAMP in CHO cells has been shown by various studies (Wang *et al.*, 2011). In this project, two variants of MOR were analysed which were constructed as previously described in Knapman et. al 2014a.

2.1.2 AtT20 cells

AtT20 cells are the mouse pituitary adenoma cells and were chosen for this experiment because they constitutively express G-protein coupled inwardly rectifying potassium channels which are not expressed by CHO cells. Additionally, AtT20 cells natively express four of the five known somatostatin (SST) receptors (Patel *et al.*, 1994). When AtT20 cells are treated with somatostatin, the drug binds to somatostatin receptor, activating the G inhibitory protein (G_i), thus activating the GIRK channel. Opening of GIRK channel by somatostatin is taken as a positive control in our assays as absence of response of somatostatin denotes either the blocking of somatostatin receptor by the drugs under study or blockade of the GIRK channel.

2.1.3 Cell growth and passaging

Both CHO and AtT20 cells were cultured in a high glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin (P/S). Both the cell lines were kept in an incubator maintained at 37°C with 5% CO_2. Both the cell lines attached well to the base of the flask and the complete growth medium was changed every 2 to 3 days. For CHO cells, complete growth medium was supplemented with 10µg/ml of Blasticidin and 300µg/ml of Hygromycin whereas for AtT20 cells transfected with hMOR, only 80µg/ml of Hygromycin was added.

The cells were passaged to a new flask at around 80% confluency as the cells are still in their growing phase and passaging was carried out for a maximum of 20 passages. During passaging, the growth media was aspirated and 5ml of phosphate-buffered saline (PBS) was added to rinse off any remaining media from the flask. 2ml of trypsin-EDTA was added and left undisturbed for 2 minutes to detach the cells. The enzymatic reaction of the trypsin-EDTA was neutralized by adding the media, otherwise trypsin can digest the cells, leading to their death.

The suspension of the cells was centrifuged at 1000 rpm for 5 minutes to pellet the cells. The reseeding was done according to the number of cells expected in the flask and a new passage number was recorded after each passaging.

2.1.4 Storage

The cell lines were stored in liquid nitrogen tanks. Freezing the cells involved the same procedure as passaging but suspension was made in freezing media consisting of 75% DMEM, 20% FBS and 5% DMSO. The cell pellet was resuspended in 2 ml of ice cold freezing medium and was transferred to cryo-vial in the volume of 1ml per vial. The cryo-vials were kept in -80°C freezer in Mr. Frosty (a container, filled with isopropanol, which regulates the temperature to drop 1°C per minute). After at least 4 hours of placing in the -80 ºC freezer, the cryo-vials were transferred to the liquid nitrogen tank and the liquid nitrogen register was updated.

2.1.5 Thawing

Thawing was carried out quickly and using good technique to ensure the survival of the cells as the cells are in DMSO and DMSO can be toxic to cells above 4°C. The cells were thawed quickly and added to 75 cm^2 flask containing growth media without antibiotics. After 24 hours, media containing the freezing medium was removed and fresh growth media was added along with the selection antibiotics.

2.1.6 MOR transfection

Flp-In T-Rex CHO cells were generated as explained in Knapman et. al, 2013 (Knapman *et al.*, 2013a). Flp-In system was used to transfect AtT-20 cells and also to integrate human MOR into it as explained in Knapman et. al, 2013b (Knapman *et al.*, 2013b). Flp-In System enables the generation of isogenic stable cell lines by allowing the integration and expression of a gene of interest at a specific genomic location. As we are studying the effect of opioids in the MOR and its variants, the Flp-In system enables the integration of the desired plasmid at the exact same location. In this system, the Flp Recombinase Target (FRT) site is introduced into the genome of a cell line, after which the desired construct is integrated into the genome at the FRT site. Once the cell line with the gene of interest is created, generation of Flp-In cell line expressing variants is rapid and efficient (O'gorman *et al.*, 1991).

The expression of receptors in CHO cells were regulated by addition of tetracycline to the media a day before assay. Tetracycline binds to the Tet repressor protein, undergoes a conformational change and uncouples from Tet operator, which allows expression of the gene of interest. The induction of cells just a day before the assay, excludes the likelihood of non-expressing cells outgrowing the expressing cells (Ward *et al.*, 2011).

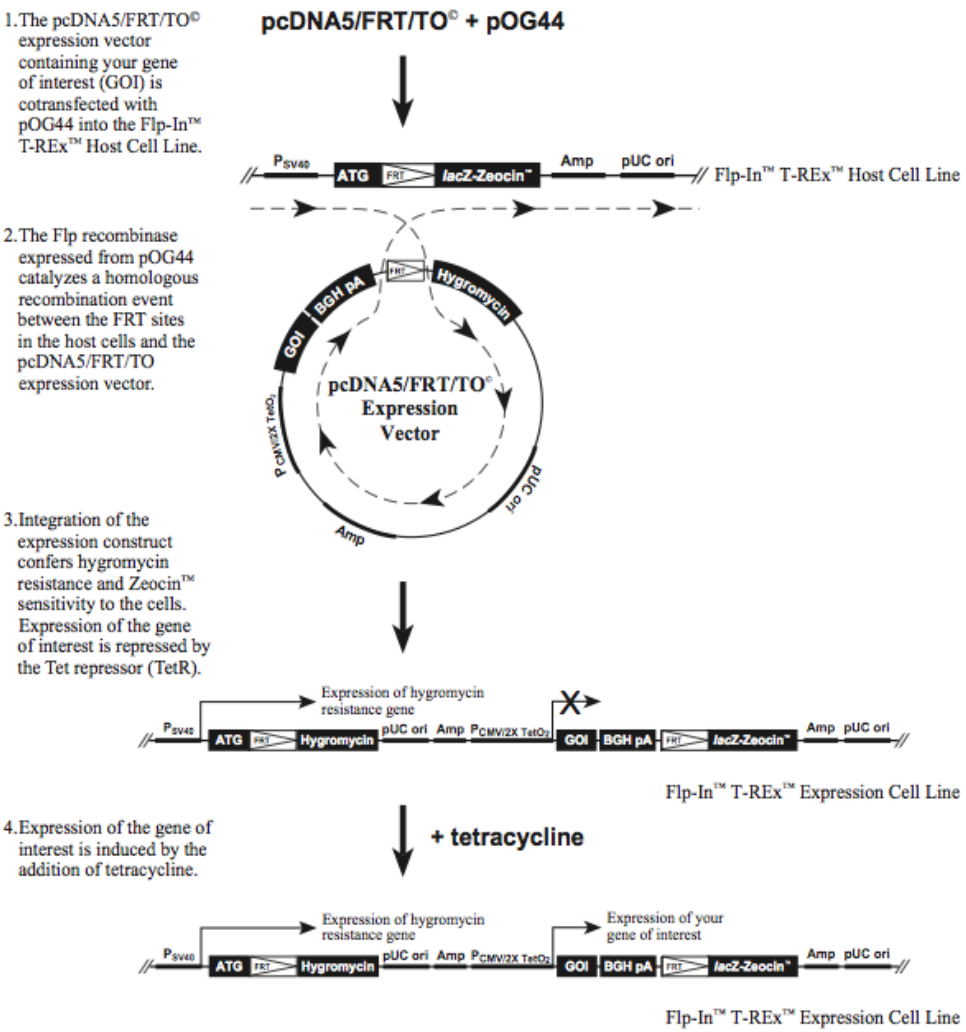

Figure 2. A model depicting the construct of Flp-In T-Rex system. Reproduced from Invitrogen Life Technologies protocol (www.invitrogen.com).

2.2 Membrane Potential Assay to determine the Adenylyl Cyclase Inhibition

The membrane potential assay performed in this project, was initially developed by our group in 2014, which allows real time measurement of membrane potential changes

(Knapman *et al.*, 2013a). The FLIPR Membrane Potential Assay kit from Molecular devices can efficiently detect an increase or decrease of fluorescence as a result of change in the membrane potential. Increase in fluorescence signifies depolarisation of the cells whereas a decrease denotes the hyperpolarisation.

A day prior to assay, cells were detached from the flask using trypsin-EDTA and resuspended in 10ml of Leibovitz's L15 media supplemented with 1% FBS, 100iu Penicillin, 100µg/ml Streptomycin and 15mM glucose. 2µg/ml tetracycline was added to induce the expression of MOR. The cells were plated at a volume of 90µl in black walled, clear bottomed 96 welled microplate (Corning) using 8 channel pipettor. The plate was placed in an incubator overnight at 37ºC in ambient CO_2 (0.04%). Adenylyl cyclase Inhibition assay was performed using blue membrane potential assay dye from Molecular Devices. The dye was reconstituted in Hanks buffered salt solution (pH 7.4 and osmolality 315 ± 5) containing 145mM NaCl, 0.338 mM Na_2HPO_4, 4.17 $NaHCO_3$, 22mM HEPES, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, 1.26 mM $CaCl_2$ and 5.56 mM glucose. The dye was either used at 100% concentration as recommended by the supplier or diluted to 50% using HBSS, as dilution does not show any difference in the signalling (Knapman *et al.*, 2015b). 90µl of reconstituted dye was loaded onto cells and incubated at 37ºC and ambient CO_2 for an hour before assay. Forskolin was added to the drugs or vehicle. The drug with forskolin dilutions were prepared in assay buffer but were made ten times more concentrated so as to get the desired concentration when diluted by the already present L-15 in the cells. The drugs used for this assay were morphine, pethidine, TRV 130 and O-desmethyl tramadol. Fluorescence is measured using Flex Station 3 (Molecular Devices) where cells were excited at a wavelength of 530nm and emission is measured at 565 nm.

The assay parameters are set up as follows:

Read Mode	Fluorescence, Bottom Read
Excitation wavelength	530 nM
Emission wavelength	565 nM
Cut Off	Auto

Readings per well	6
PMT (Photo Multiplier Tube)	Medium
Run time	500 secs
Interval	2 secs

Assay plate parameters:

Initial volume	180µl
Transfers	1
Pipette height	190µl
Volume	20µl
Rate	2
Time point	120 secs

Baseline reading was measured for 2 minutes at an interval of 2 seconds, after which 20µl of Forskolin (FSK) alone, FSK + opioids or blank was added. As forskolin is dissolved in DMSO, dilutions were made in such a way that the final concentration of DMSO in the cells is not more than 0.1%. The assay was carried out for a total of 8 minutes.

2.2.1 Data Analysis

The data collected was saved in .txt format and analysed in Microsoft excel. Changes in the fluorescence of FSK after exposing the cells for 5 minutes with opioids was expressed as percentage of baseline fluorescence after blank subtraction. Concentration response curves (CRCs) were analysed using GraphPad Prism version 6 (La Jolla, California, USA). The equation fitted to the result is the non-linear regression response

vs log concentration- variable slope (four parameters), with the bottom constrained to zero:

$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{(logIC50 - X) * Hillslope}}$$

Data were expressed as mean ± s.e.m. of at least 5 experimental runs made in duplicate, unless otherwise mentioned. Statistical comparisons were made with an unpaired Student's T-test, unless otherwise stated. $P < 0.05$ was considered to be significant. All the channel and receptor nomenclature is consistent and is in accordance with the current NC-IUPHAR approved nomenclature as discussed by Cox et al (Cox *et al.*, 2015).

2.3 Assay to measure GIRK channel activation using Membrane Potential dye

A similar procedure was applied to the GIRK assay as in Adenylyl Cyclase (AC) assay but AtT-20 cells were used for this assay. AtT20 cells unlike CHO cells are not tetracycline inducible. AtT-20 cells were cultured in 75cm^2 flask in DMEM media supplemented with 80ug/ml of Hygromycin. A day prior to assay, cells were detached from the flask with trypsin and resuspended in Leibovitz's L-15 media with 1% FBS, 100u Penicillin, 100µg/ml streptomycin and 15mM glucose. The cells were loaded in 96 well black walled clear bottom plate from Corning and incubated at 37ºC and ambient CO_2. Membrane potential dye (blue) from Molecular devices, reconstituted in HBSS was added to the cells and incubated for an hour. Dilutions of drugs- DAMGO, morphine, TRV 130, O-desmethyl tramadol and pethidine were prepared in HBSS and added to the drug plate from Cornings. Baseline recording was observed every 2 seconds for 2 minutes and after that opioids or vehicle was added. The assay was run for a total of 5 minutes.

2.3.1 Data analysis

The data was exported from the Soft Max Pro connected to the Flex Station 3 as .txt file into the Microsoft excel (2016). GIRK activation was calculated as a percentage decrease in fluorescence from the baseline. Mean of the baseline readings 30 seconds before addition of the drug and also the mean of lowest reading from the peak response and 2 reading either side was calculated. Hence, the percentage difference between the

mean of peak value and mean of baseline reading was generated. In addition to that, we also deducted the change produced by addition of buffer from all experimental data which is usually less than 2%. The CRC hence obtained was plotted in Graph Pad Prism 6 as a mean ± s.e.m. of at least n=5 experimental readings made in duplicates, unless otherwise mentioned. The graph was statistically analysed using unpaired Student's T-test and $P < 0.05$ was considered significant as for cAMP assay.

2.4 Receptor depletion study with β-CNA

β-CNA is irreversible opioid receptor antagonist which binds and alkylates μ, κ and δ opioid receptor and ultimately inactivates the receptor. As it inactivates the bound receptor, lesser number of receptors are available and potency of drug decreases. The extent of this decrease in potency of drugs can be used to estimate the relative level of agonism of the drug under study.

We used AtT20Flp-In wild type cells with hMOR for this experiment which were cultured in similar way as stated earlier. A day prior to assay, cells were plated in a poly D-lysine coated 96 wells plates to ensure maximum attachment of cells on the plate (the procedure for poly D-lysine coating of plates is mentioned in appendix section).

On the day of the assay, 100nM β-CNA was prepared in the cold low potassium HBSS buffer. It was kept at 37°C for few minutes before adding to the cells to ensure less stress to the cells. The β-CNA was exposed to the cells for 20 minutes after which they were washed once with warm HBSS buffer. The membrane potential dye, diluted to 25% with L15, was added to the cells and incubated on Flex Station 3 for an hour. Control cells were also treated similar way, but instead of β-CNA, low potassium HBSS was used. Different concentrations of DAMGO, morphine and TRV 130 were prepared in HBSS buffer mixed with 1% Bovine Serum Albumin (BSA) and added to the drug plate. 100nM somatostatin and 30μM ML-297 were used as negative controls to check specificity of β-CNA for MOR. The baseline reading was collected for 2 minutes after which drugs or vehicle were added and ran for a total of 300 seconds.

2.4.1 Data Analysis

The experiment was performed for n=5 times for both β-CNA treated and control cases for each drug and data collected was exported to Microsoft Excel in the form of .txt format. K activation was measured as percentage decrease in fluorescence from baseline for receptor depleted and no depletion cases. τ and K_a were determined using the Operational model receptor depletion feature in GraphPad Prism 6. The equation fitted to the results is:

$$Y = \frac{Basal + (Effectmax - Basal)}{1 + operate}$$

Where,

$$operate = \left[\frac{10^{logKA} + 10^{X}}{10^{logtau+X}}\right]^{n}$$

2.5 GIRK assay with Somatostatin or ML 297

We performed this assay to check whether the drug is blocking the GIRK channel or if it is antagonizing the somatostatin receptor. For this, AtT20 wild type cells were used which express the native somatostatin receptors. ML 297 is a potent and selective GIRK agonist which activates the GIRK channel via unique mechanism which is different to the classic mode of activation- which is receptor induced activation of Gi/o proteins (Wydeven *et al.*, 2014).

AtT20 Flp-In WT cells were cultured and plated in a similar way as for other AtT20 cell lines as mentioned earlier but the selection antibiotics used for this cell line was zeocin 10µg/ml. The cells were plated in the 96 wells plate as previously described. The membrane potential sensitive blue dye from Molecular devices was diluted to 50%

concentration with low potassium HBSS and 90µl of the dye was added to cells and kept for an hour incubation at 37°C in Flex Station 3.

Baseline readings were measured for 120 seconds at an interval of 2 seconds, after which blank and a range of concentrations of the opioids were added and response was measured for 180 seconds. After another baseline reading till 420 seconds, 100nM somatostatin or 30µM ML 297 was added and the response was measured for another 180 seconds. In order to quantify the GIRK fluorescent signal, the values of SST+ opioids or ML 297 + opioids were expressed as a percentage change produced by SST or ML 297 alone respectively. In our analysis, we incorporated how the higher concentration of the drug was blocking the hyperpolarisation of the cells.

2.6 Drugs and Chemicals

Tissue culture reagents and buffer salts used are from Invitrogen or Sigma unless otherwise stated. Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol acetate (DAMGO) was purchased from Auspep (Tullamarine, Australia). Morphine and β-chlornaltrexamine (β-CNA) is a kind gift from the Department of Pharmacology, University of Sydney. Forskolin is from Ascent Pharmaceuticals (Bristol, UK) and Buprenorphine and pethidine are from National Measurement Institute (Lindfield, Australia). O-desmethyl tramadol is from Toronto Research Chemicals Inc. (TRC, Toronto, Canada), and TRV 130 is from MedChemExpress (MCE, New Jersey, USA). Membrane potential dye is from Molecular devices (Sunnyvale, California, USA).

3

EFFECT OF PETHIDINE ON SIGNALLING OF μ-OPIOID RECEPTOR AND ITS VARIANTS

3.1 Introduction

Pethidine hydrochloride, also known as meperidine, is the synthetic opioid analgesic used for the treatment of mild to moderate pain (Latta *et al.*, 2002). The drug was initially synthesized for use as an anticholinergic agent until later studies discovered its analgesic effects (Eisleb *et al.*, 1939). Initial clinical studies suggested pethidine to be an alternative to morphine, lacking critical side effects of opioids such as respiratory depression, constipation and dependence (Batterman *et al.*, 1944; Latta *et al.*, 2002). During that era, morphine was available only in the form of injectable and with the availability of pethidine in both injectable and tablet forms, it became one of the most prescribed opioid with wide use in the USA, accounting for 60% prescriptions in chronic cases and 22% in acute case (Dimatteo *et al.*, 1987).

Early clinical studies showed pethidine to be as efficacious and potent as morphine, for treating moderate to severe pain, whereas studies conducted later in various settings showed contradictory results. Patients experienced lesser analgesic action and increased side effects with pethidine. A patient controlled analgesia study, which compared three equipotent doses of morphine and pethidine showed both drugs having similar effect of analgesia at rest. However, pethidine provided significantly less analgesia on movement

as compared to morphine, suggesting pethidine to have weak analgesic action for relief of moderate to severe pain (Plummer *et al.*, 1997). Pethidine is metabolized into a toxic metabolite, nor-pethidine, which causes tremor, twitching, agitation and convulsions (Mcquay, 1999). These effects are further intense if multiple doses are required and also in patients with renal failure (Szeto *et al.*, 1977). Initially, pethidine was proposed to have less respiratory depression as compared to other opioids, which favoured its use in labour pain (Alexander *et al.*, 2001; Scott, 1970). In contrast, studies showed pethidine causing higher level of depression than twice the equipotent dose of morphine (Foldes *et al.*, 1965). Pethidine administered during labour pain crossed the placental barrier and resulted in sedation in neonates during breastfeeding (Righard *et al.*, 1990). Studies also showed opposing results to the lesser addictive profile of pethidine as claimed during the earlier developmental phase. A study in 1943, showed continued use of pethidine only causes dependence in patients and does not cause addiction (Weinstein, 1943), whereas, several other case studies reported addiction in patients using pethidine (Rasor *et al.*, 1955). Administration of pethidine also produced a more intense high than any other opioids (Walker *et al.*, 1999).

All these negative reviews about pethidine being less efficacious than morphine, having more intense adverse effects and higher level of addiction in some patients caused a wave of criticism leading to withdraw pethidine from prescribing as a pain analgesic (Pattullo *et al.*, 2011). Despite these reviews, pethidine is still widely used to treat mild to moderate pain especially during labour and the only reason for that may be its short duration of action (Latta *et al.*, 2002). Variability in the effect of different opioids because of the genetic variations has been well documented (Klepstad *et al.*, 2005; Landau *et al.*, 2008; Lötsch *et al.*, 2005), however, we could not find any studies showing the effect of those MOR variants in pethidine. In this project, we have addressed the common polymorphism of MOR- A118G and C17T and their effect on the response of pethidine signalling.

3.2 Results

3.2.1 Inhibition of adenylyl cyclase

Forskolin has been extensively used as a compound to study the molecular basis of Adenylyl Cyclase (AC) activity and cAMP formation, mostly in intact-cell preparations (Seamon *et al.*, 1981). Addition of 300nM of forskolin to CHO cells with transfected human MOR, loaded with membrane potential sensitive dye, showed a rapid decrease in the fluorescence, corresponding to the hyperpolarised state of cells (Knapman *et al.*, 2014b).

We analysed the effect of drugs to inhibit forskolin induced hyperpolarisation in three different cells lines- CHO-MOR WT, CHO-MOR C17T and CHO-MOR A118G. Simultaneous addition of selective MOR agonist, morphine, showed concentration dependent reversal of forskolin stimulated hyperpolarisation in all three cell lines (Figure 3). Morphine showed maximum inhibition of forskolin response in CHO MOR WT cells by 63 ± 8%, with *p*EC50 of 7.1 ± 0.2 (Table 1). There was no significant change in the efficacy of Morphine in CHO MOR A118G and C17T cells as compared to WT cells ($P > 0.05$, Figure 3).

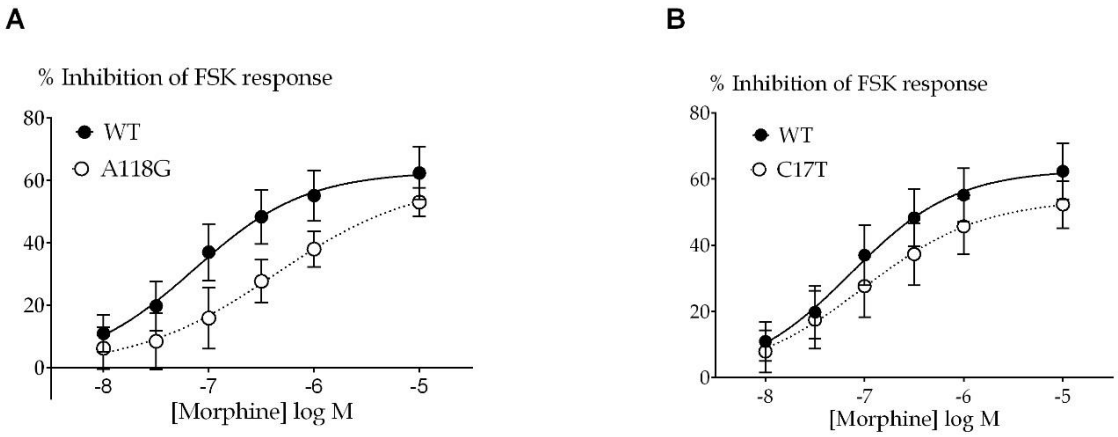

Figure 3. Morphine inhibits adenylyl cyclase in CHO cells expressing MOR WT, A118G and C17T. Inhibition of forskolin stimulated hyperpolarisation of cells by morphine in CHO cells expressing MOR WT and A118G (A) and MOR C17T (B). E_{max} for morphine in CHO WT cells is 63 ± 8 % with pEC_{50} of 7.1 ± 0.2. No significant

difference was observed in both MOR A118G (E_{max} = 59 ± 16 % and pEC_{50} = 6.4 ± 0.5) and MOR C17T (E_{max} = 54 ± 10 and pEC_{50} = 7.0 ± 0.3) as compared to WT cells (P > 0.05). Data represents the mean ± s.e.m of 5 experimental runs carried out in duplicates.

Table 1. The maximum effect and potency of opioids to inhibit the forskolin mediated adenylyl cyclase in CHO cells expressing hMOR-WT, hMOR-A118G and hMOR-C17T.

AC inhibition	**E_{max} (%)**			***p*EC$_{50}$**		
Opioid	**WT**	**A118G**	**C17T**	**WT**	**A118G**	**C17T**
Morphine	63 ± 8	59 ±16	54 ±10	7.1 ±0.2	6.4 ±0.5	7.0 ±0.3
Pethidine	N/A	N/A	N/A	N/A	N/A	N/A

We attempted to determine the efficacy and potency of pethidine in all three cell lines to see if a non-synonymous SNP has any effect in pethidine to inhibit forskolin response. Maximum hyperpolarisation was measured for each concentrations of pethidine. Pethidine showed dose dependent inhibition of forskolin response, and this effect of pethidine is blocked by 1 µM naloxone (data not shown). Figure 4 (A, B, C) illustrates the representative trace of data collected for pethidine CRCs in CHO-MOR-WT, A118G and C17T respectively. Concentrations of pethidine above 3µM did not show a dose dependent change in fluorescence so we ran higher concentrations of pethidine (10µM and 30µM) without forskolin. The result showed transient increase in fluorescence signal (~5%) for A118G and C17T, whereas, lesser change (~1%) in WT cells. The representative curve for higher concentrations are shown in figure 4 (D, E, F) for CHO-MOR-WT, A118G and C17T respectively.

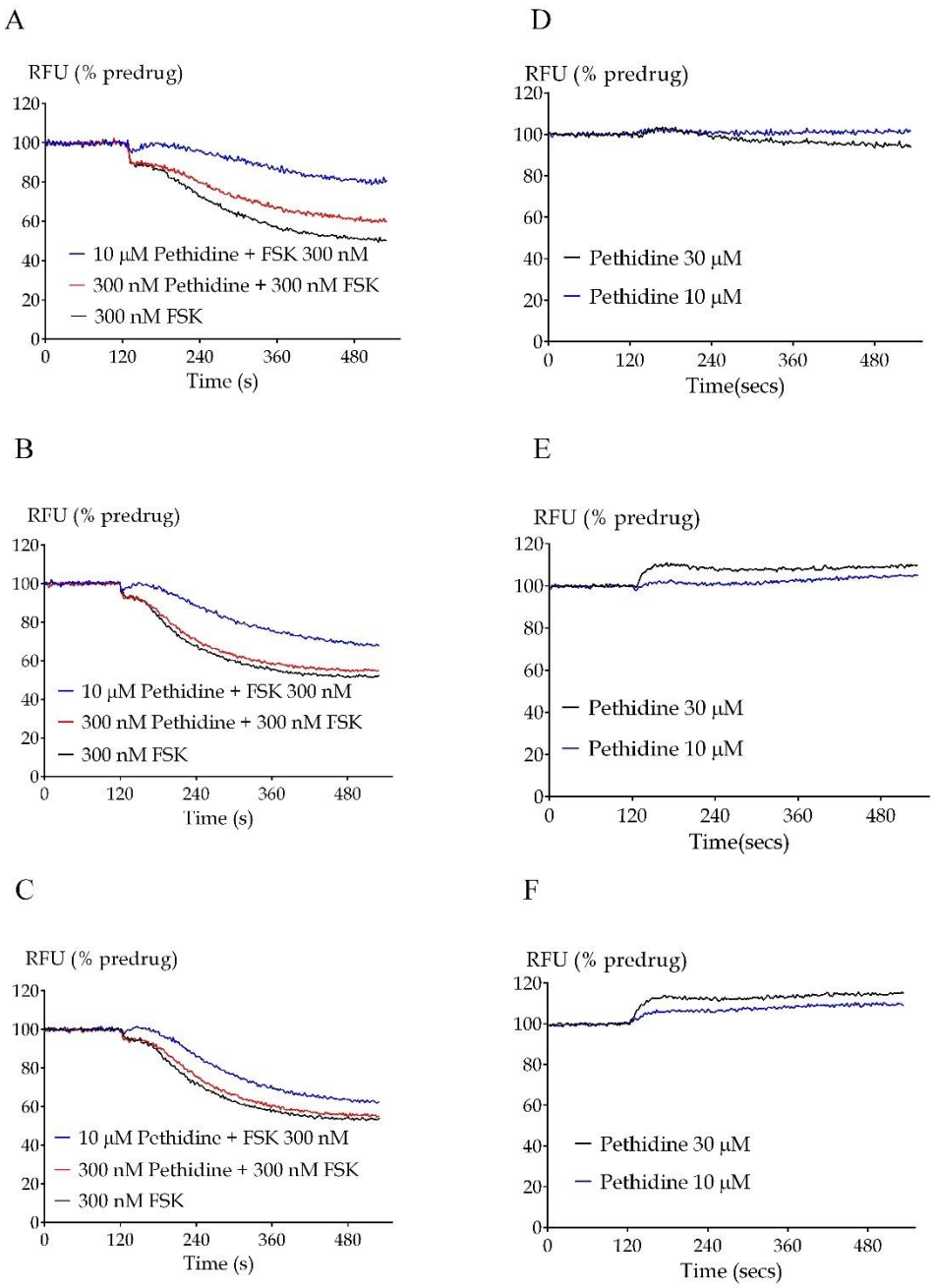

Figure 4. Example traces for different concentration of pethidine in membrane potential assay in CHO cells. Traces of (A, D) CHO-MOR WT, (B, E) CHO-MOR A118G and (C, F) CHO-MOR C17T response to varying concentrations of Pethidine. Dose dependent membrane potential hyperpolarisation as shown by WT (A), A118G (B) and C17T (C). 30µM and 10µM pethidine producing an increase in fluorescence with less

increase in WT (D) as compared to A118G (E) and C17T (F). The data presented here is the representative trace of a single experimental run.

The peak increase in fluorescence which signifies depolarisation caused by the higher concentration of pethidine i.e. 30µM in three different cell line is portrayed in a column graph as shown in figure 5.

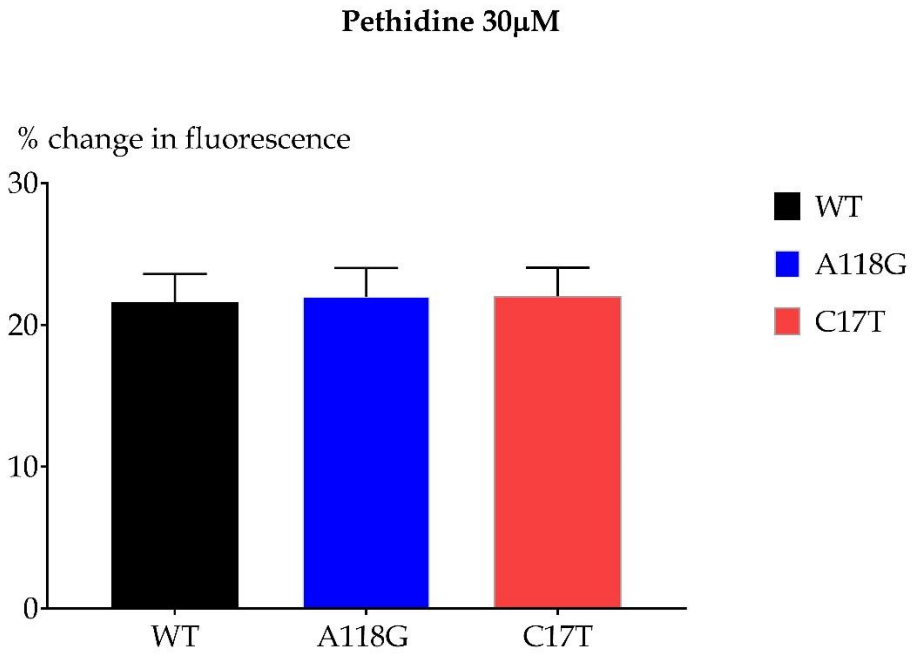

Figure 5. The peak change in fluorescence caused by 30µM pethidine in CHO-WT, CHO-A118G and CHO-C17T cells. The highest concentration of pethidine showed similar increase in all three cell lines. The data presented here is for n =3 experimental runs carried out in duplicates.

We only used concentrations (≤ 3 µM) which were not giving any change in fluorescence by itself. However, we were unable to estimate an accurate EC_{50} and E_{max} values as the concentrations ≤ 3µM did not produce a plateauing response as shown in figure 6. Nevertheless, comparing the highest concentration tested (3µM) on all three cell lines statistically using unpaired t-test, we observed significant decrease in the forskolin response in C17T than WT ($P < 0.05$), whereas no difference was observed between A118G and WT ($P > 0.05$).

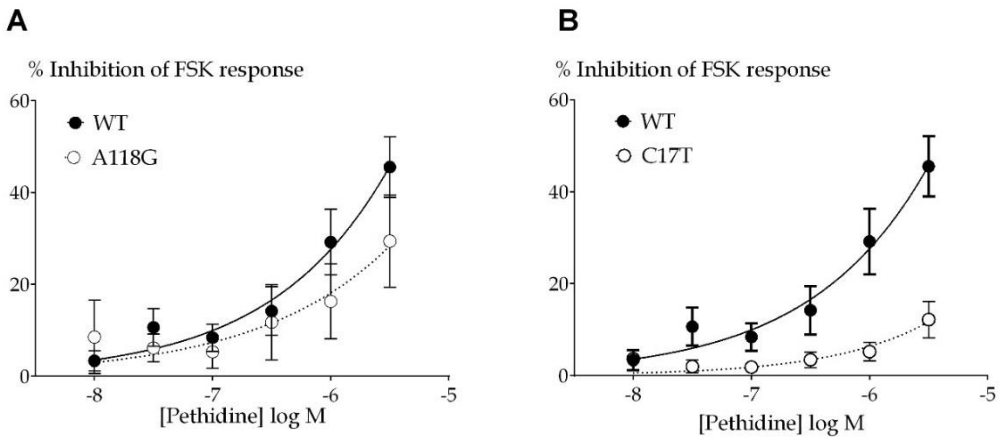

Figure 6. Inhibition of Forskolin response caused by different concentration of Pethidine in CHO cells expressing MOR-WT, MOR-A118G and MOR-C17T. Adenylyl Cyclase inhibition was determined as explained in methods section. (A) Pethidine induced inhibition of forskolin stimulated hyperpolarization of CHO cells expressing MOR-WT and MOR-A118G. (B) Forskolin induced hyperpolarisation of CHO cells expressing MOR-WT and MOR-C17T caused by pethidine. E_{max} and pEC_{50} of pethidine in different cell lines could not be calculated because pethidine response did not reach a plateauing response at concentration tested. The curves represent the mean ± s.e.m. of n= 5 experimental runs carried out in duplicates.

3.2.2 GIRK channel activation

The activation of MOR by agonist causes the dissociation of $G_{\beta\gamma}$ subunit from G protein complex, which in turn activates GIRK channels, resulting in hyperpolarisation of cells which express them. As AtT20 cells endogenously express GIRK channel, we ran GIRK activation assay for DAMGO, an enkephalin analog and pethidine in AtT20 cells expressing hMOR-WT. Application of different concentration of opioids in AtT20 cells loaded with membrane potential sensitive dye resulted in a dose dependent hyperpolarisation of cells (Figure 7).

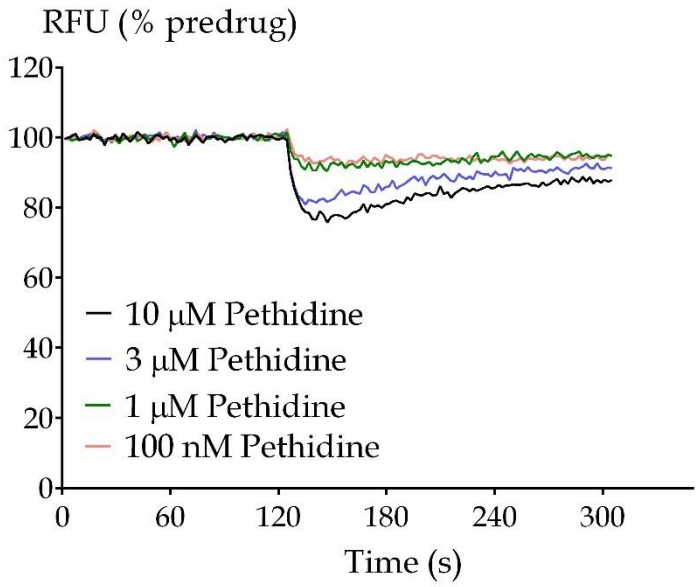

Figure 7. Example traces of pethidine causing hyperpolarisation of AtT20 cells expressing hMOR. Baseline readings were taken for 2 minutes at an interval of 2 seconds, after which increasing concentrations of pethidine was added to AtT20-hMOR cells, which dose dependently decreased the fluorescent signal as shown in the figure. The curve represents a trace of a single experimental run after blank correction.

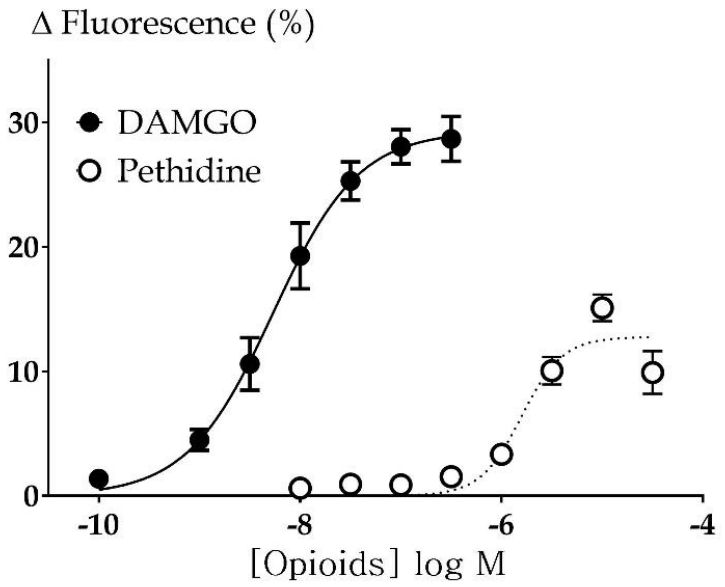

Figure 8. **CRC for pethidine and DAMGO induced hyperpolarisation of AtT20 cells.** GIRK activation assay was determined as described in the methods section. DAMGO showed maximum decrease in fluorescence at 29 ±1% and Pethidine at 13 ±1% whereas pEC_{50} was 8.3 ± 0.1 and 5.7 ±0.1 for DAMGO and pethidine respectively. Data represents the mean ± s.e.m. values of n=5 experimental runs performed in duplicates.

The CRC for different concentrations of DAMGO and pethidine was plotted as shown in the figure 8. DAMGO, which is μ-opioid selective peptide agonist, activated GIRK channel with E_{max} 29 ± 1% and pEC_{50} value of 8.3 ± 0.1. Pethidine showed maximum change in fluorescence at 13 ± 1% and pEC_{50} value of 5.8 ± 0.1 demonstrating lower affinity agonism.

3.3 Discussion

Using CHO cells, we investigated whether the A118G and C17T polymorphism alters the pethidine response in inhibiting the forskolin induced adenylyl cyclase activation. We were unable to calculate an accurate value for determining the efficacy and potency of pethidine as concentrations above 10μM showed change in fluorescence by itself (without forskolin), more in the polymorphic alleles. The highest concentration of pethidine, used to plot the CRC, showed significantly reduced forskolin response in C17T as compared

to WT. These findings suggest the possible effect of SNPs in response of pethidine in MOR signalling. Several studies have been undertaken to determine the functional consequences of the A118G SNP at both the cellular and clinical levels. A recent study showed reduction in the efficacy of buprenorphine in inhibiting AC and activating GIRK channel in A118G (Knapman *et al.*, 2014b). Also, A118G has been reported to affect the binding affinity of some ligands such as- β-endorphin and morphine-6-glucuronide. The binding affinity of β-endorphin was found to be increased in 118G allele in an *in vitro* study (Bond *et al.*, 1998, Lötsch *et al.*, 2002) but the result was not found to be consistent (Befort *et al.*, 2001; Beyer *et al.*, 2004). Moreover, the decrease in binding affinity of morphine in 118G allele may account for the need of higher dose of morphine in people with 118G variant (Lötsch *et al.*, 2002, Skarke *et al.*, 2003)However, C17T polymorphism are found to affect the signalling of MOR as several opioids like morphine, fentanyl, oxycodone, methadone, DAMGO, buprenorphine, showed decreased efficacy and potency to inhibit AC in CHO-MOR-C17T cells as compared to CHO-MOR-WT cells (Knapman *et al.*, 2015c). Studies conducted in post-mortem human brain and animal models proposes loss of putative n-glycosylation site in N-terminal of MOR caused by A118G polymorphism, to reduce receptor expression of MOR-A118G (Zhang *et al.*, 2005; Oertel *et al.*, 2012). However, C17T reducing the opioid mediated signalling of MOR suggests some other mechanism underlying the effect of SNP in signalling, as C17T has no effect in the glycosylation process. Moreover, the use of Flp-In technology, ensures the receptor construct to be inserted in the same location in the genome in both the cells lines. This suggests that genetic variation in MOR in CHO cells, is solely responsible for change in the effect of various opioids in MOR signalling.

In the present K channel activation study, DAMGO showed maximum efficacy at concentration of 5.4 nM and pethidine at 1.6 µM suggesting consistency in the result of pethidine rarely being superior in terms of efficacy to other opioids and endogenous opioid peptides (Mcquay, 1999). Pethidine shows high effectiveness in the treatment of mild pain especially when administered via intravenous (Stambaugh *et al.*, 1976) or intradural route (Paech *et al.*, 1994), as pethidine given orally shows low bioavailability (Mather *et al.*, 1976). In addition, need of administration of higher doses for treatment of chronic pain, exacerbates its toxicity (Kaiko *et al.*, 1983). Short duration of action and

lesser toxicity when given by intradural route may be the reason for pethidine being widely used in labour pain (Latta *et al.*, 2002).

In conclusion, common polymorphisms of MOR show varying effect on the response of pethidine. Additionally, pethidine demonstrates lower potency in activating K channels. The use of pethidine in labour pain despite its lesser efficacy than other opioids suggests more trial studies are needed both at the cellular and clinical levels. Deeper understanding of signalling of pethidine in MOR and variants will help to better define the proper usage of pethidine in controlling both acute and chronic pain with lesser toxicity.

4

EFFECT OF GENOTYPE VARIATION ON TRAMADOL SIGNALLING

4.1 Introduction

Tramadol is a synthetic centrally acting and clinically effective opioid (Lewis *et al.*, 1997; Hullet *et al*. 2006) with studies showing comparable analgesic efficacy and potency to morphine and pentazocine in acute therapeutic cases (Raffa *et al.*, 1992). Studies showed lower affinity of tramadol to μ-receptor than morphine (Raffa *et al.*, 1992), which is believed to be caused due to substitution of the phenolic group in morphine by a methyl group in tramadol (Hennies *et al.*, 1988). The affinity of active metabolite of tramadol – O-desmethyl tramadol is 300 times higher than the parent compound, yet is still lesser than morphine's (Dayer *et al.*, 1994; Raffa *et al.*, 1992). Moreover, tramadol is considered to have a dual action of pain relief- showing its analgesic effect by acting on opioid receptors and also on central nervous system as reuptake inhibitor of norepinephrine and serotonin. This dual action of analgesia is due to two enantiomers of tramadol: its active metabolite- O-desmethyl tramadol acts as the selective MOR agonist (Grond *et al.*, 2004) and the (-) tramadol is responsible for inhibition of nor-epinephrine reuptake (Paar *et al.*, 1992). (+) tramadol has greater affinity at MOR but is mainly responsible for the inhibition of serotonin reuptake (Grond *et al.*, 2004).

Tramadol is gaining popularity among physicians for treating mild to moderate pain as they consider it as an alternative to other opioids having more severe side effects (Kaye,

2015). It is the second most prescribed medication for treatment of pain in Australia after codeine (Hollingworth *et al.,* 2015). Tramadol's efficacy differs in accordance to the mode of administration of the drug, like intravenous or intramuscular administration of tramadol works similar to pethidine, in patients with moderate to severe post-operative pain (Grond *et al.*, 2004). 15mg of tramadol hydrochloride administered intravenously showed equivalent analgesic efficacy as 5mg of morphine sulphate (Houmes *et al.*, 1992). However, when intensity of pain was taken into consideration, morphine showed better analgesia. Several contrasting results has been reported for efficacy of tramadol when given via epidural route. A study performed in children undergoing urological surgery showed preference to epidural tramadol than morphine considering the side effects and efficacy (Demiraran *et al.*, 2005). Patients after knee replacement surgery, assessed by visual analog scale (VSA), experienced less effective pain relief with tramadol than morphine, both administered epidurally (Grace *et al.,* 1995). In contrast, Baraka et. al, reported no significant difference in the efficacy of tramadol and morphine when given epidurally for post-operative pain, but with less pronounced respiratory depression in tramadol (Baraka *et al.*, 1993). The preference of tramadol as a pain analgesia is also because of its tolerability and lesser adverse effects. In comparison to equivalent doses of other opioids, tramadol shows low potential for respiratory depression, addiction and constipation (Lanier *et al.*, 2010; Reeves *et al.*, 2008; Scott *et al.*, 2000). Tramadol is well tolerated in short term use with main reported adverse reactions as nausea, vomiting, sweating, itching, constipation and headache (Gong *et al.*, 2014).

The possible reason behind studies showing enhanced efficacy and greater tolerability profile of tramadol (Giorgi, 2012) may be its mechanism of action, which is the result of combined effect of MOR activation with serotonin and noradrenaline (NA) reuptake (Hui-Chen *et al.,* 2004). Tramadol is metabolised into its active constituent by the catalytic effect of CYP450 (Wu *et al.*, 2002). Its analgesic properties are dependent on metabolism by CYP2D6 polymorphic enzyme into O-desmethyl tramadol (Grond *et al.*, 2004). The activity of CYP2D6 shows marked individual variability and based on this, individuals are divided into three categories- poor metabolisers (PM), extensive metabolisers (EM) and ultra-extensive metabolisers (UEM) (Wang *et al.,* 2006). The involvement of CYP2D6 enzyme for its metabolism suggests change in efficacy and potency of tramadol because of genetic variation (García-Quetglas *et al.,* 2007), which

has been well documented in the literature. At least 74 variants of human CYP2D6 are known (Lassen *et al.*, 2015). The prevalence of PM in Asian population is 0-1% and in Caucasians is 7-10%, whereas the frequency of UM in Asian is 1-2% and around 29% in African population (Bradford, 2002; Ji *et al.*, 2002; Sachse *et al.*, 1997). UM allele has found to enhance the toxicity of tramadol whereas individuals with PM allele experiences lesser efficacy of tramadol (Depriest *et al.*, 2015). (Cavallari *et al.*, 2011). A comparative study conducted in Malaysian patients with CYP450 allele showed higher adverse events in PM than UM and UM having more than UEM (Gan *et al.*, 2007). PM showed lower response to tramadol administered as a post-operative analgesia than UM (Stamer *et al.*, 2003). All these findings suggest the impact of genetic variation in pharmacokinetic profile of tramadol. However, not much studies have been performed to show the effect of sequence variation in MOR for analgesic properties of tramadol.

A clinical trial study conducted to investigate the effect of MOR-A118G on efficacy of tramadol/acetaminophen combination drug (Ultracet), showed decreased response in the efficacy of the drug (Liu *et al.*, 2012). Hence, in this project, we are determining the effect of O-desmethyl tramadol alone in the signalling of hMOR-A118G and also in C17T while comparing the efficacy and potency of tramadol with some commonly used MOR agonist- Morphine and DAMGO.

4.2 Results

4.2.1 Inhibition of Adenylyl Cyclase inhibition in different variants of MOR

Similar experiment was run to determine the inhibition of forskolin mediated adenylyl cyclase activation as for pethidine (chapter 1). The cells were treated with varying concentrations of active metabolite of tramadol i.e. O-desmethyl tramadol, which exerts the analgesic action.

The raw traces from a single experimental run are shown in figure 9. Change in fluorescence as a measure of AC inhibition were determined for each concentration and CRC was plotted. O-desmethyl tramadol mediated inhibition of AC activation was blocked by 1µM MOR antagonist, naloxone (data not shown). We ran concentrations ≥ 10µM by itself, without addition of forskolin. Both 30µM and 10µM showed slight

increase in fluorescence (~10%) in WT cells but no change in other polymorphisms as shown in figure 9 (D, E, F).

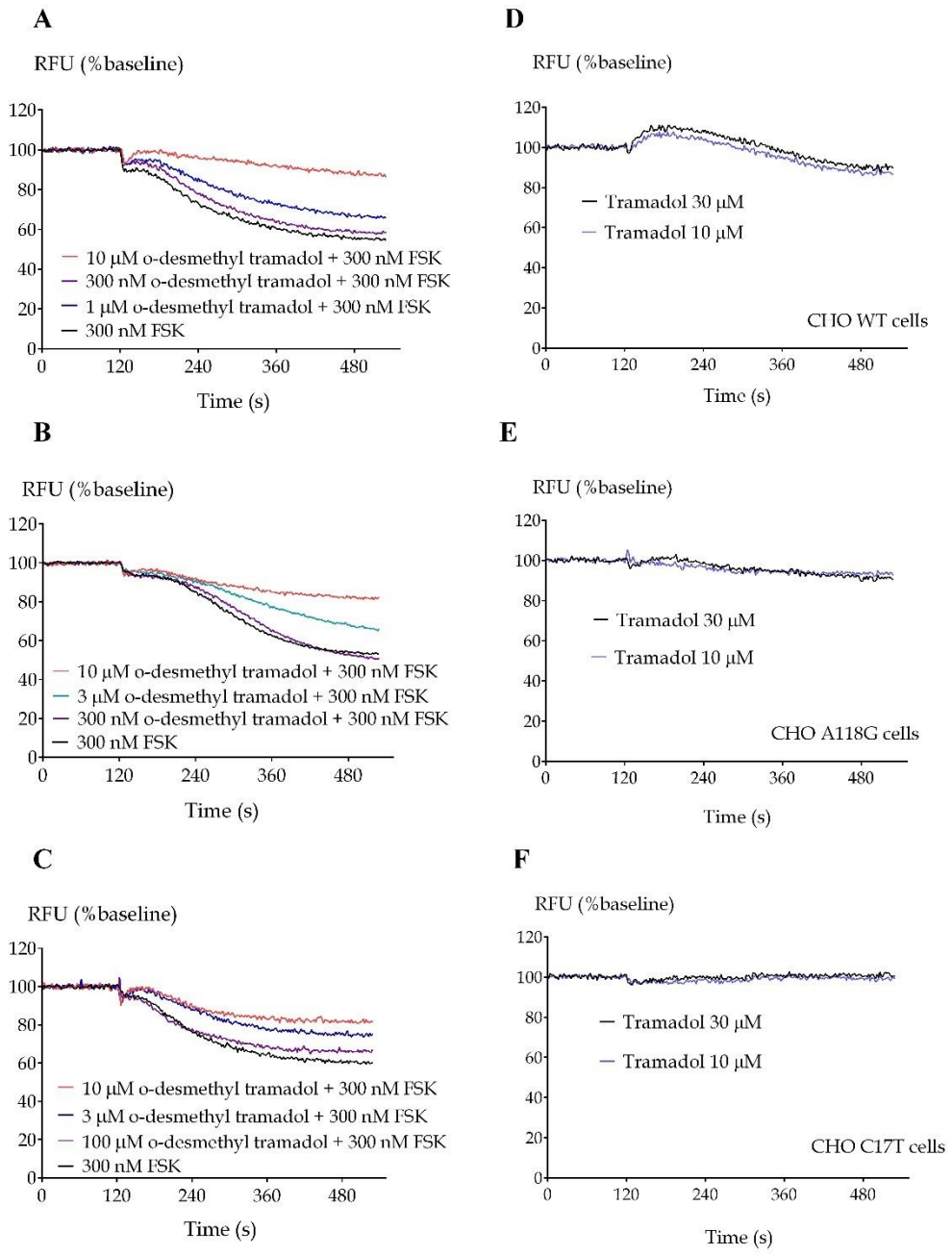

Figure 9. Example traces for different concentration of O-desmethyl tramadol in membrane potential assay in CHO cells. Normalized traces showing dose dependent change in the fluorescence in CHO cells expressing MOR- WT (A), A118G (B) and C17T (C). The concentration 30µM and 10µM without addition of forskolin caused increase in

fluorescence in CHO-hMOR-WT (D), corresponding to depolarisation. Not much changes were observed for same concentrations in A118G (E) and C17T (F). Data presented here are from a single experimental run.

The peak increase in fluorescence which signifies depolarisation caused by the higher concentration of o-desmethyl tramadol i.e. 30µM in three different cell line is portrayed in a column graph as shown in figure 10.

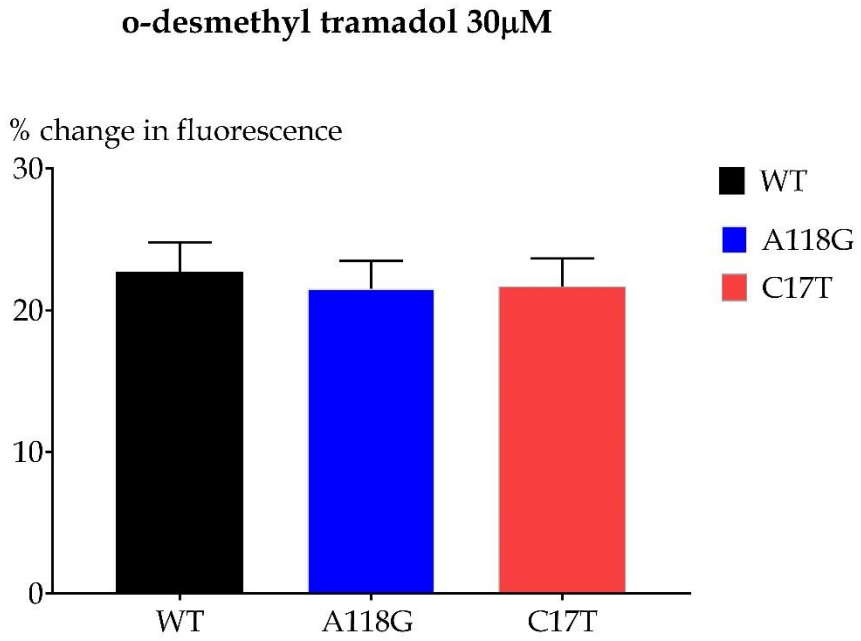

Figure 10. The peak change in fluorescence caused by 30µM o-desmethyl tramadol in CHO-WT, CHO-A118G and CHO-C17T cells. The highest concentration of o-desmethyl tramadol showed similar increase in A118G and C17T whereas a slight increase peak effect in WT cells. The data presented here is for n =3 experimental runs carried out in duplicates.

Hence, we only took concentrations ≤ 10µM to obtain concentration response curve, which did not show a plateauing response in CHO cells with A118G and C17T. Thus, we were unable to determine an absolute E*max* and pEC_{50} values for O-desmethyl tramadol. However, comparing effect at the highest concentration studied, i.e. 10µM, inhibition of forskolin response was significantly different in C17T (unpaired T test; $P < 0.05$) as compared to WT, whereas, no difference was observed for A118G (unpaired T test; $P > 0.05$).

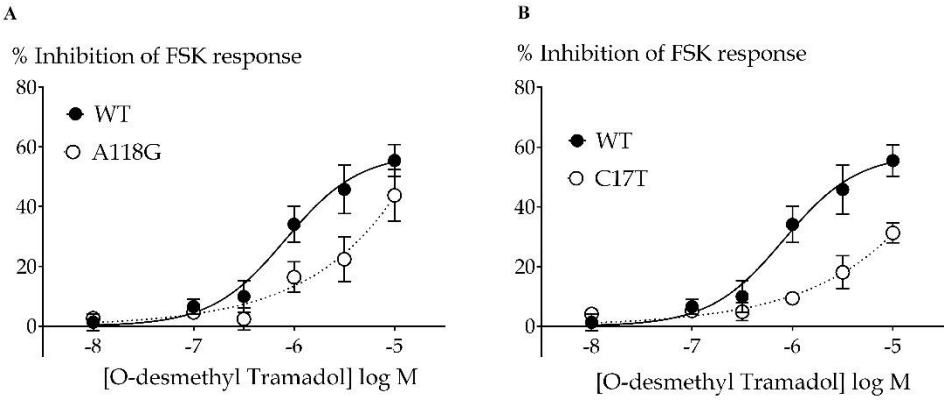

Figure 11. o-desmethyl tramadol inhibiting the forskolin induced adenylyl cyclase in CHO cells expressing different polymorphisms. Adenylyl cyclase inhibition was determined as explained in methods section. (A) O-desmethyl tramadol inhibition of forskolin response in CHO cells transfected with MOR-WT and MOR-A118G. The highest concentration taken for O-desmethyl tramadol showed no significantly different effect in A118G as WT (B) O-desmethyl tramadol inhibition of forskolin response in CHO cells transfected with MOR-WT and MOR-C17T. The highest concentration taken for O-desmethyl tramadol showed significantly less effect in C17T than WT ($P < 0.05$). Data presented here are the mean ± s.e.m. value of the n= 7 experimental runs performed in duplicates.

4.2.2 Activation of GIRK channels

We ran an assay to determine the extent of activation of GIRK channel by O-desmethyl tramadol as compared to DAMGO and morphine. In ATt20 cells transfected with hMOR, the application of opioids, resulted in the concentration dependent decrease in fluorescence from the baseline, corresponding to the hyperpolarisation (Figure 12).

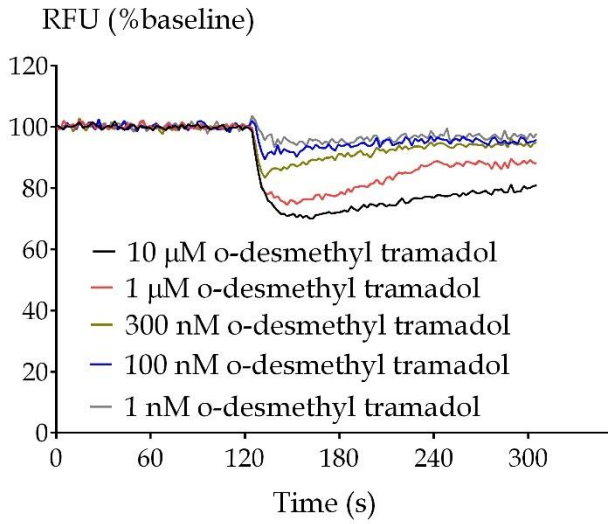

Figure 12. Example traces for o-desmethyl tramadol mediated activation of K channel. Addition of different concentrations of o-desmethyl tramadol resulted in the dose dependent membrane potential hyperpolarisation. The figure shows the data of a single experimental run, normalised to baseline.

The data obtained was analysed as percentage decrease from baseline and CRC is plotted as shown in figure 12. E_{max} and pEC_{50} values for DAMGO and morphine are same as shown in the previous chapter as we ran all the opioids simultaneously (CRC for DAMGO and morphine also shown in figure below). O-desmethyl tramadol showed maximum decrease in fluorescence signal by 24 ± 2% and with pEC_{50} of 6.3 ± 0.1.

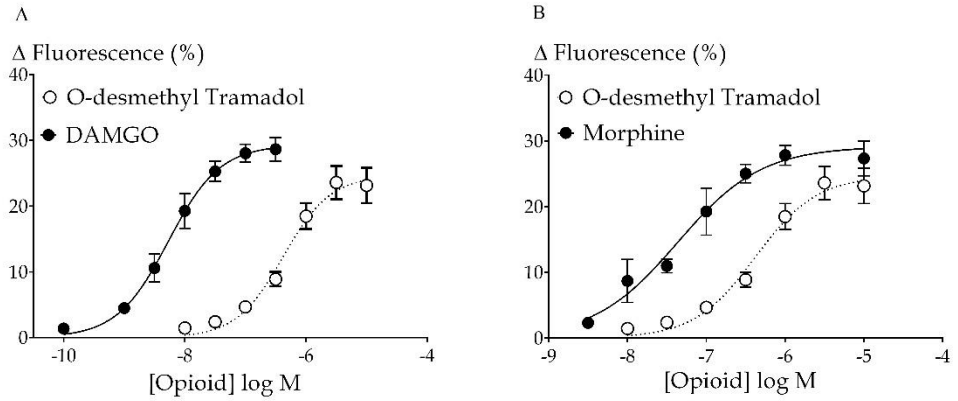

Figure 13. DAMGO, morphine and O-desmethyl tramadol mediated activation of GIRK channel. GIRK channel activation assay performed as described in methods section. (A) DAMGO and O-desmethyl tramadol stimulated activation of GIRK channel. Emax value of DAMGO is 29 ± 1% and pEC50 is 8.2 ± 0.1 and for O-desmethyl tramadol is 24± 2% and pEC50 is 6.3 ± 0.1. (B) The response for Morphine and O-desmethyl tramadol to activate GIRK channel. Emax of morphine is 29 ± 3 % and EC50 value of 7.3 ± 0.1. The data presented here is the mean + s.e.m. value of 5 experimental runs made in duplicates.

4.3 Discussion

Clinical studies showed tramadol causing difference in efficacy and rate of adverse events in individuals with genetic variation in CYP450 enzyme (Ingelman-Sundberg *et al.*, 1999; Samer *et al.*, 2013). We hypothesized the possibility of genetic variation in MOR having a variable tramadol response, which we addressed by running fluorescence based assay in CHO cells expressing WT, A118G and C17T. However, we were unable to calculate an absolute value for efficacy and potency as concentrations of O-desmethyl tramadol (≥10 μM) showed response by itself in CHO-MOR WT cells. Assessing the highest concentration of O-desmethyl tramadol (10 μM) tested for AC inhibition in CHO cells, C17T variant conferred a less efficacious signalling of MOR in response to o-desmethyl tramadol, suggesting the possible effect of genotype variation.

The fluorescence based cAMP assay in CHO cells, used in this project, is a real time based assay and follows the natural MOR activation signalling, however, it is unable to

clearly define the detailed mechanism of association between AC inhibition of cAMP and hyperpolarisation of the cell. It is believed that hyperpolarisation is the result of K^+ ions movement though the K channel (Knapman *et al.*, 2013a), but CHO cells do not express much of endogenous K channel (Yu *et al.*, 1998). Hence, use of CHO cells to study opioid mediated inhibition of adenylyl cyclase raises a question to the mechanism underlying hyperpolarisation. Additionally, higher concentrations of O-desmethyl tramadol showed a change in fluorescence by itself which affected the results obtained for inhibition of FSK stimulated AC activation, therefore we were unable to clearly show plateauing response of O-desmethyl tramadol. There are several other cAMP assays such as ligand binding, reporter gene assays, time-resolved FRET (fluorescence resonance energy transfer) and genetically encoded FRET probes (Hill *et al.*, 2010; Thomsen *et al.*, 2005), however, a majority of them pose some limitations. A study conducted in 2013, used real time kinetic BRET (Bioluminescence Resonance Energy Transfer) CAMYEL (cAMP sensor using YFP-Epac-RLuc) assay, to reveal the molecular mechanism of allosteric modulation of cannabinoid receptor (Cawston *et al.*, 2013). The response of CAMYEL sensor when exposed to increase or decrease in cAMP, is directly evaluated as change in BRET signal. This technique of measuring cAMP makes BRET assay more suitable to study the kinetics of intracellular cAMP (Jiang *et al.*, 2007). We will be pursuing further studies with this type of assay to clarify the results of our present assay.

We can also make use of a novel label free, real time and cell based assay- cellular dielectric spectroscopy. CellKey^{TM,} an electrical impedance based biosensor, detects change in impedance as a result of change in shape, size, volume of cells and cellular alterations (Peters *et al.*, 2007). This assay can be beneficial to study $G_{i/o}$ mediated change in cAMP level. The cells are seeded into the 96-welled plate with an electrode and the change in cellular level is detected by an electrode as change in impedance. Different signalling pathway (G_s, $G_{i/o}$) shows different pattern of change in impedance making it possible to directly measure cAMP change (Miyano *et al.*, 2014; Peters *et al.*, 2007).

In this project, we also performed K channel activation assay in AtT20 hMOR cells to compare the signalling of O-desmethyl tramadol with DAMGO and morphine. O-desmethyl tramadol (420 nM) activated K channel with much lesser efficacy and was less potent as compared to DAMGO (5.4 nM) and morphine (43.5 nM), which is in good

agreement with the literature. The GTPγS binding assay which measures the receptor mediated G-protein activation by opioids showed efficacy of opioids following the rank order of DAMGO > Morphine > O-desmethyl tramadol (Gillen *et al.*, 2000). The same study also suggested different contribution of tramadol vs its metabolite in humans and mice. This assumption was based on the finding that analgesic effect of tramadol was completely antagonized by naloxone in mouse or rat flicked test (Carlsson *et al.*, 1987) whereas, in human, naloxone only showed partial antagonism, showing lesser influence of opioid mechanism in human (Collart *et al.*, 1993).

Reduced efficacy as compared to morphine, its severe side effects (eg. Serotonin syndrome in moderately high doses) and need of CYP2D6 for metabolism which is more liable to have genetic variation, may be the reason for suggesting lesser use of tramadol. A work-alike drug that has been developed and marketed as being an advance over tramadol is tapentadol (Guay, 2009). Tapentadol is also a synthetic centrally acting opioid analgesic having similar dual mechanism of action as tramadol- MOR agonist and nor-epinephrine reuptake inhibitor (Hartrick *et al.*, 2012). In contrast to tramadol, lesser 5-HT reuptake and absence of racemic mixture as well as activation independent to metabolism, makes tapentadol a preferred opioid over tramadol with lesser side effects and enhanced tolerability (Barbosa *et al.*, 2016). Unfortunately, tapentadol was not available to compare with O-desmethyl tramadol in this project.

In conclusion, O-desmethyl tramadol appears to be less potent drug and its efficacy may be further reduced at common MOR polymorphisms. Detailed study is suggested with different assays which directly measures the change in cAMP as a result of opioid activation in MOR.

5

SIGNALLING OF TRV 130 ON μ-OPIOID RECEPTOR AND VARIANTS

5.1 Introduction

Several novel opioids have been discovered in past few decades such as multi target drugs like tapentadol (Tzschentke *et al.*, 2007) and ultra-short acting analgesic- GI 87084B (James *et al.*, 1991). Although the newly discovered opioids are effective in management of pain in different settings they pose some drawbacks including dependence and analgesic tolerance exemplified by the requirement for larger doses to maintain pain relief in the absence of disease state worsening. Development of new opioids with lesser adverse effects such as respiratory depression and nausea has been an area of intense research in opioid pharmacology.

A β-arrestin 2 knockout mice showed increased analgesic action of morphine with less constipation and respiratory depression than wild type mice (Bohn *et al.*, 1999; Raehal *et al.*, 2005). This study hypothesized the involvement of β-arrestin for adverse events associated with opioids. Several studies suggested the use of biased ligand to investigate this association (Violin *et al.*, 2007), which led to synthesis of TRV 130, a G-protein biased, MOR ligand with similar G-protein coupling efficacy as morphine but with reduced β-arrestin recruitment (Soergel *et al.*, 2014b). TRV 130, also known as oliceridine, recently received FDA breakthrough therapy designation for treatment of acute to moderate pain (Chen *et al.*, 2016).

Phase 1 clinical trial study conducted in healthy volunteers, compared the safety and tolerability of TRV 130 with morphine, which showed TRV 130 having greater analgesic effect with faster onset and similar duration of action. Also, TRV 130 was better tolerated with less respiratory depression (Soergel *et al.*, 2014a; Soergel *et al.*, 2014b). Phase 2-clinical trial studies are still ongoing and the report, till date, shows TRV 130 to be a promising opioid in treatment of moderate to severe pain. A randomized study to investigate the tolerability and efficacy in treating acute pain in patients after bunionectomy, demonstrated TRV 130 providing enhanced analgesia for longer duration as compared to morphine (Viscusi *et al.*, 2016).

In the current study, we investigated possible variation in TRV 130 response as a result of some common single nucleotide polymorphisms of the MOR. In addition to that, the study also addresses K channel activation by TRV 130 and compare the efficacy and potency with DAMGO and morphine. Overall, this study will help to put some light on the cellular mechanism of TRV 130.

5.2 Results

5.2.1 TRV 130 stimulated inhibition of Adenylyl Cyclase

Inhibition of the forskolin response on exposure to DAMGO and morphine has been shown in previous chapters. As the responses of some opioids have been affected by SNPs, we next determined if the common SNPs- A118G and C17T also have an impact on TRV 130 signalling.

We ran an assay in CHO cells transfected with MOR-WT, A118G and C17T as has been explained in previous chapters. Upon addition of different concentrations of TRV 130 with 300nM forskolin, it showed a concentration dependent decrease in fluorescence corresponding to inhibition of AC activation, which was blocked by 1µM naloxone (data not shown). However, higher concentration of TRV 130 showed an increase in fluorescence probably corresponding to depolarisation of the cells. 30µM TRV 130 showed ~10% increase in WT cells, ~15% in A118G and ~30% in C17T cells whereas, 10µM showed lesser increment but followed the same pattern as shown in figure 14 (D, E, F).

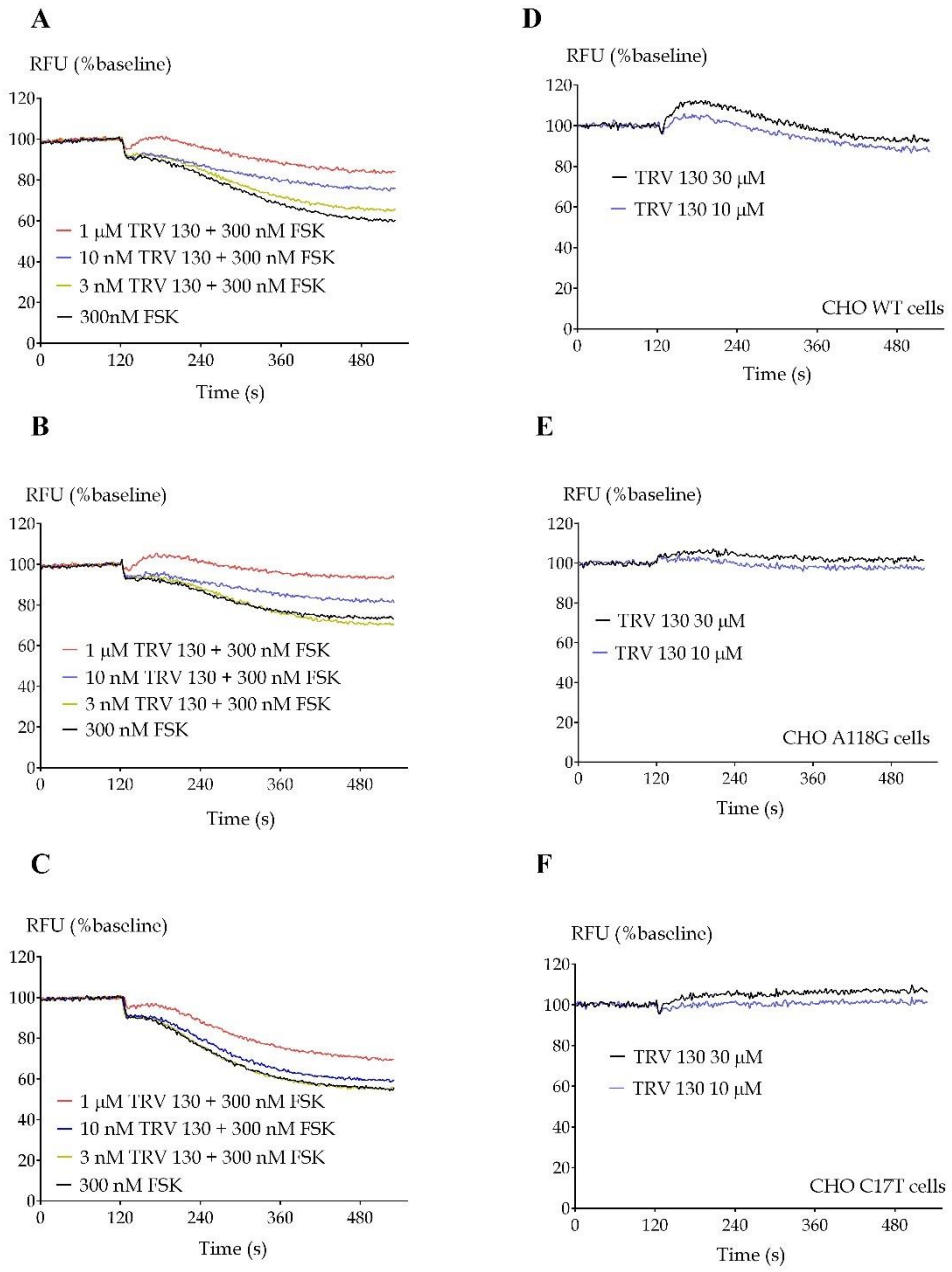

Figure 14. Example traces for different concentration of TRV 130 in membrane potential assay in CHO cells. Normalised traces of different concentrations of TRV 130 showing dose dependent change in forskolin response in CHO-hMOR-WT (A), A118G (B) and C17T (C). Raw traces showing increase in fluorescence signal, upon addition of 30µM and 10µM concentration of TRV 130 with forskolin in CHO cells expressing

hMOR–WT, (D), A118G (E) and C17T (F). Data shown here is the raw data from the Flex station after baseline reduction of a single experimental run.

The peak increase in fluorescence which signifies depolarisation caused by the higher concentration of TRV 130 i.e. 30µM in three different cell line is portrayed in a column graph as shown in figure 5.

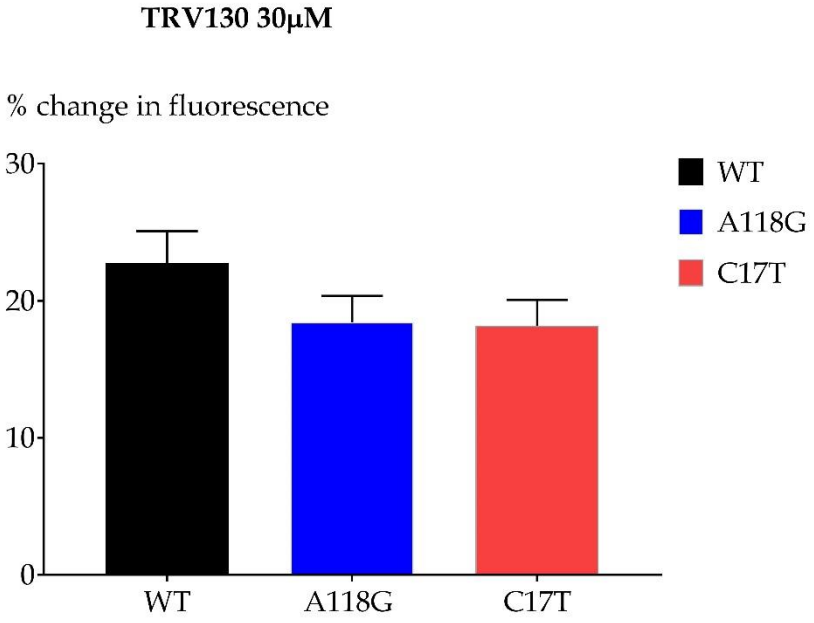

Figure 15. The peak change in fluorescence caused by 30µM TRV 130 in CHO-WT, CHO-A118G and CHO-C17T cells. The highest concentration of TRV 130 showed approximately 5% more change in fluorescence in CHO-WT cells as compared to A118G and C17T. The data presented here is for n =3 experimental runs carried out in duplicates.

We only took concentrations less than 10µM to obtain the CRC (Figure 16) which were not showing response by itself. The efficacy of TRV 130 to inhibit forskolin response was found to be significantly decreased ($P < 0.05$) in cells expressing polymorphism with E_{max} = 33 ± 7 % for A118G and 18 ± 3% for C17T as compared to WT (E_{max} = 45 ± 3). However, the potency was similar for all the alleles as shown in table 2.

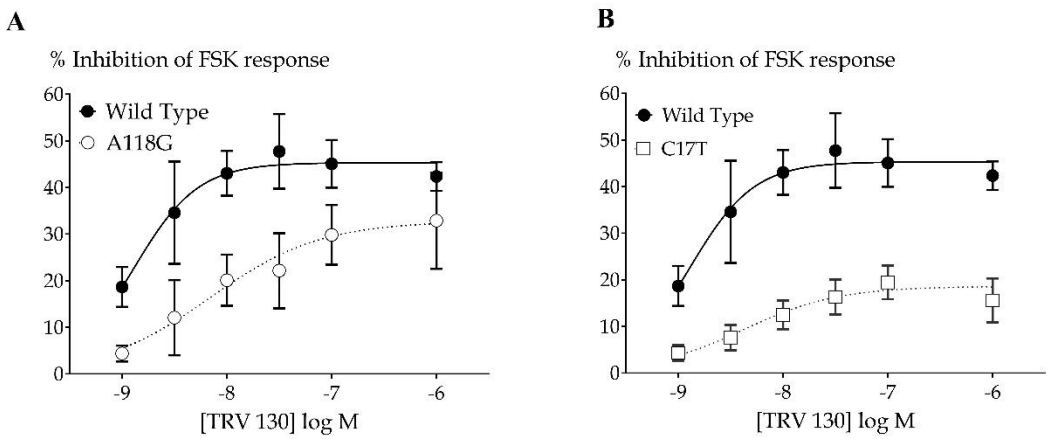

Figure 16. TRV 130 causes hyperpolarisation of CHO cells expressing hMOR-WT, A118G and C17T in a concentration dependent manner. (A) TRV induced inhibition of forskolin stimulated Adenylyl cyclase response was significantly different between MOR-WT and MOR-A118G (unpaired t-test, $P<0.05$). (B) TRV 130 showed significantly different response in inhibiting forskolin response in hMOR C17T cells as compared to hMOR WT (unpaired t-test, $P<0.05$). E_{max} and pEC_{50} values are listed in Table 2. Data presented here are the mean ± s.e.m. value of five experimental runs performed in duplicates.

Table 2. List of efficacy and potency of TRV 130 in CHO cells expressing different polymorphisms- hMOR WT, A118G and C17T, to inhibit the forskolin mediated adenylyl cyclase.

EFFECT OF TRV 130					
hMOR–WT		**hMOR-A118G**		**hMOR-C17T**	
Emax (%)	**p*EC*50**	**Emax (%)**	**p*EC*50**	**Emax (%)**	**p*EC*50**
45 ± 3	8.9 ± 0.1	33 ± 7	8.2 ± 0.3	18 ± 3	8.4 ± 0.2

5.2.2 GIRK channel activation by TRV 130

We next determined the efficacy and potency of TRV 130 to activate K channel in AtT20 hMOR-WT cells and compared the values with DAMGO and morphine. TRV 130 caused dose dependent decrease in fluorescence corresponding to hyperpolarisation as shown in figure 17.

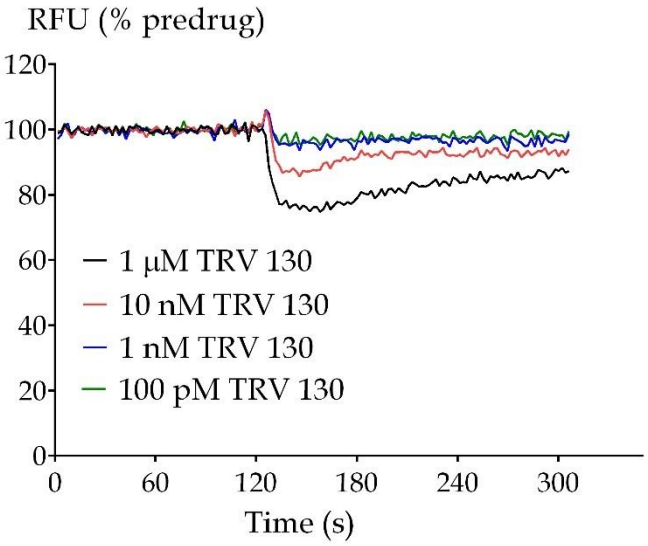

Figure 17. Example trace of GIRK channel activation by TRV 130. Raw traces of normalised data for TRV 130 showing concentration-dependent decrease in fluorescence corresponding to hyperpolarisation of cells. The curve here represents the traces from a single experimental run after baseline reduction.

TRV 130 caused a decrease in fluorescence with E_{max} = 26 ± 2% and pEC50 of 8.1 ± 0.1. The Emax and pEC_{50} values for DAMGO and morphine are the same as for previous two chapter and are enlisted in table 3. According to our results, TRV 130 appears to be more potent than morphine in causing the hyperpolarisation of cells as shown in figure.

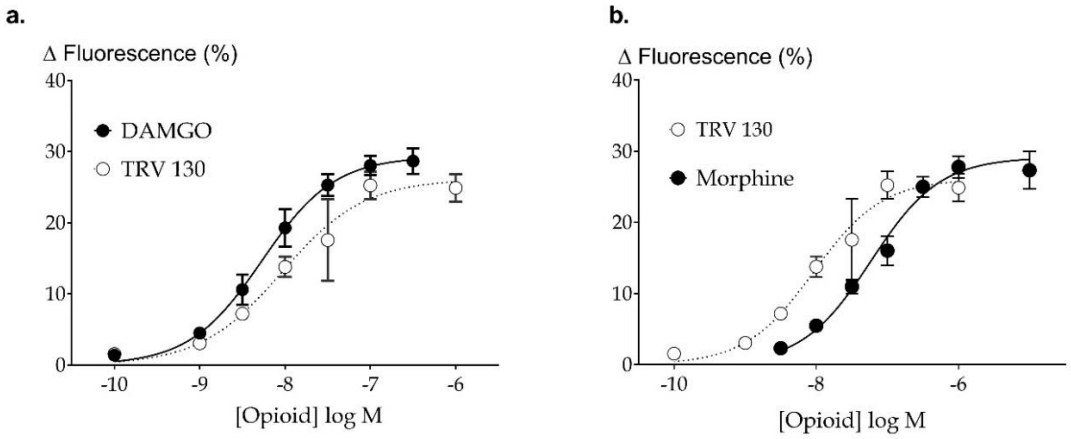

Figure 18. Opioid activation of GIRK in AtT20 cells expressing MOR-WT. Activation of GIRK upon addition of opioids was determined as described in methods. DAMGO activated GIRK showing maximum response at 29 ± 2% and pEC_{50} = 8.3 ± 0.1. TRV 130 showed maximum response at 26 ± 2% and pEC_{50} of 8.1 ± 0.1 and morphine showed maximum efficacy at 29 ± 1 and potency of 7.2 ± 0.1 (Table 3). Data represents the mean ± s.e.m. values of n=5 experimental runs performed in duplicates.

Table 3. List of efficacy and potency of DAMGO, morphine and TRV 130 in activating GIRK channels as performed in AtT20 WT cells transfected with hMOR.

DRUGS	E_{max} (%)	pEC_{50}
DAMGO	29 ± 1.5	8.3 ± 0.1
Morphine	29 ± 1.5	7.2 ± 0.1
TRV- 130	26 ± 1.8	8.1 ± 0.1

5.2.3 Receptor depletion study

The pharmacological potency of an agonist at the receptor is determined not only by the affinity of that agonist to bind to the receptor to show its intrinsic activity but also by the receptor density (Ariens *et al.*, 1960; Stephenson, 1956). This suggests the greater the number of functional receptors present, the lower the concentration of agonist required to show action. Our result showed the potency of TRV 130 to be similar to DAMGO and

higher than morphine. Hence, we next determined change in potency of all these three opioids after reduction in receptor reserve by treating cells with β- chlornaltrexamine (β-CNA). β-CNA is an irreversible antagonist of opioid (Portoghese *et al.*, 1978) which binds to all three opioid receptors and decreases the number of receptors available for agonist to bind.

The receptor depletion assay was performed as mentioned in methods section and CRC was plotted for both depleted and not depleted condition for all three drugs which are shown in figure below. The $Effect_{max}$ and tau value were determined using the operational model of receptor depletion in GraphPad Prism 6 for receptor depletion and no depletion case and are enlisted in the table below (Table 4). Tau value is inverse of the proportion of receptors needed to give 50 % response (Graph Pad prism, curve fitting guide). We also ran SST and ML 297 after treating cells with β-CNA, which showed no change in the response in control and receptor depleted cases, suggesting no effect of β-CNA in GIRK channel.

Table 4. List of $Effect_{max}$ and Tau value for DAMGO, morphine and TRV 130 as determined by plotting the change in response of all three drugs in control cases and receptor depleted cases.

DRUGS	**$Effect_{max}$**	**Tau (control)**	**Tau (receptor depleted)**
DAMGO	29 ± 1	67.6	7.1
Morphine	29 ± 1	11.1	1.5
TRV 130	31 ± 3	5.5	0.6

This shows that DAMGO needs to occupy 1.5 % receptors in no receptor depletion and 14 % on depleted case to show 50% effect. Morphine needs 9 % occupancy on control cases and 68 % on receptor depleted case. TRV 130 needs to occupy 18% receptors normally to show 50% response but in receptor depleted cases, it needs to occupy more than 100%, suggesting TRV 130 as a low efficacy agonist.

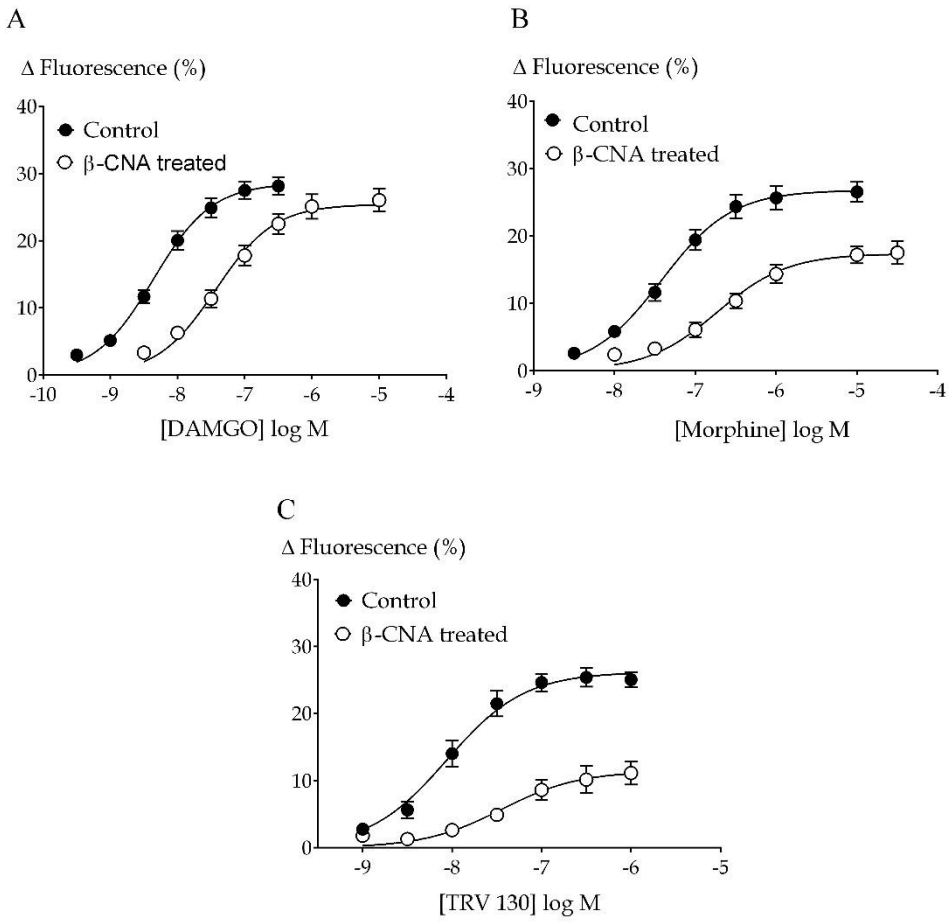

Figure 19. **Concentration response curve of DAMGO, morphine and TRV 130 for both receptor depletion and no depletion cases.** The cells were treated either with low K+ HBSS or with β-CNA for 20 minutes, after which they were washed and dye was added. After an hour incubation in the dye, GIRK activation assay was performed for both the control (symbolised as black) and the treated cells (symbolised as white). The concentration response curve was obtained using operational model of receptor depletion in GraphPad prism 6. The tau value for (A) DAMGO is 67.6, 7.1, (B) for morphine is (11.1, 1.5) and (C) for TRV 130 is (5.5, 0.6), for control and receptor depleted cases respectively. The data represents the mean ± s.e.m. of 6 experimental runs performed in duplicates.

5.2.4 Assay incorporating TRV 130 and somatostatin in AtT20 wild type cells

In both cAMP and K channel activation assay, concentrations ≥ 10μM of TRV 130 produced an increase in fluorescence, corresponding to depolarisation of cells. This raised

a possibility of TRV 130 either interfering with GIRK channel or with some receptor present. Hence, we ran an assay in AtT20 WT cells which expresses endogenous somatostatin receptors as detailed in methods section. As shown in figure 20 A, concentrations ≤ 10 µM did not show any response by itself but after subsequent addition of somatostatin, produced dose dependent hyperpolarisation. However, 30 µM TRV 130 seemed to depolarise the cells by itself and also inhibit the somatostatin mediated hyperpolarisation. We plotted concentration response curve of TRV 130 as percentage change of TRV 130 + somatostatin normalized to the change caused by somatostatin alone as shown in Figure 20 B.

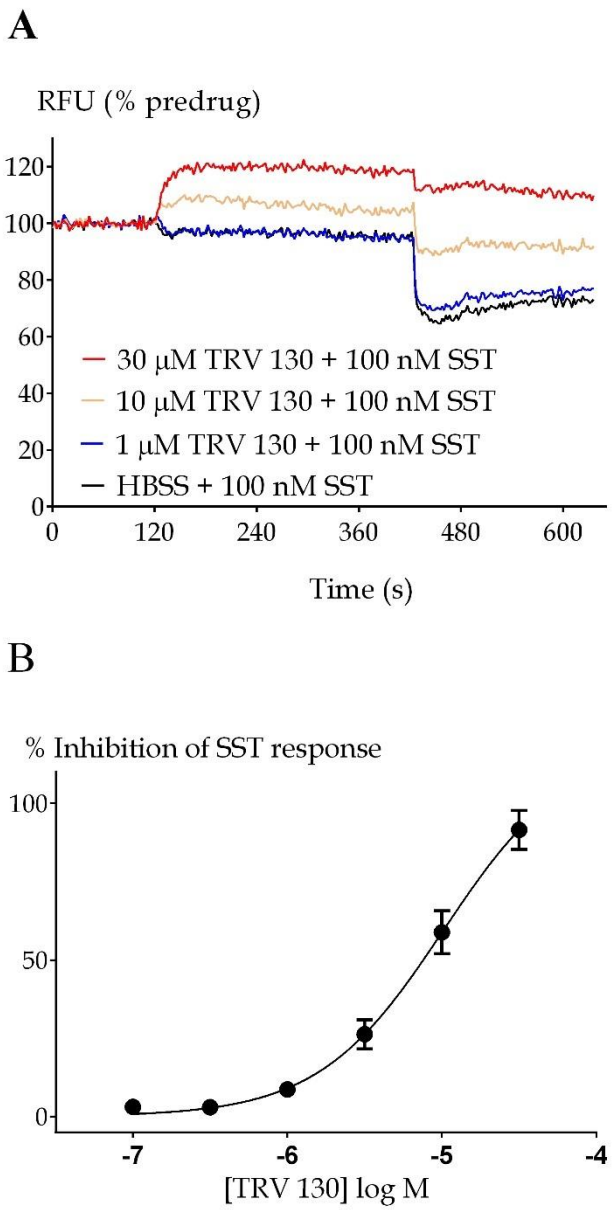

Figure 20. **TRV 130 and SST example traces and CRC in AtT20 WT cells.** (A) Raw traces of membrane potential assay in AtT20 WT cells for TRV 130 and somatostatin. After 2 minutes of baseline reading, various concentrations of TRV 130 are added and ran till 420 seconds after which somatostatin (100nM) is added to the cells. 30μM red) TRV 130 showed an increase in fluorescence whereas all the concentrations ≤ 10 μM showing no response. However, after addition of somatostatin, all concentrations showed a decrease in fluorescence except 30μM, which seemed to inhibit the hyperpolarisation caused by somatostatin. The change in fluorescence caused by TRV 130+ somatostatin is

plotted as percentage change compared to change caused by somatostatin alone. The data is the normalized representation of a single experimental run made in duplicate. (B) GIRK activation assay was performed in AtT20 wild type cells with intact somatostatin receptors. Change in fluorescence caused by TRV 130 + somatostatin is expressed as percentage change caused by somatostatin alone. Data presented here is representative of n=5 experimental runs performed in duplicates.

5.2.5 TRV 130 interference with GIRK channel

The inhibition of somatostatin mediated hyperpolarisation by 30 µM and dose dependent change by concentrations ≤ 10µM suggests the possibility TRV 130 interfering with K channel. Hence, we performed similar experiment as for somatostatin but with addition of a direct GIRK activator- ML 297 (Kaufmann *et al.*, 2013). After 120 seconds of baseline reading, TRV 130 was added and ran for 5 minutes, after which 30µM ML-297 was introduced. Raw data (Figure 21 A) shows increase in fluorescence caused by 30µM and 10µM of TRV 130, however, on addition of 30µM ML-297, all concentrations of TRV 130 showed dose dependent decrease in fluorescence corresponding to hyperpolarisation. Concentration response curve was plotted as percentage inhibition caused by TRV 130 + ML-297 and normalized to ML-297 alone (figure 21 B).

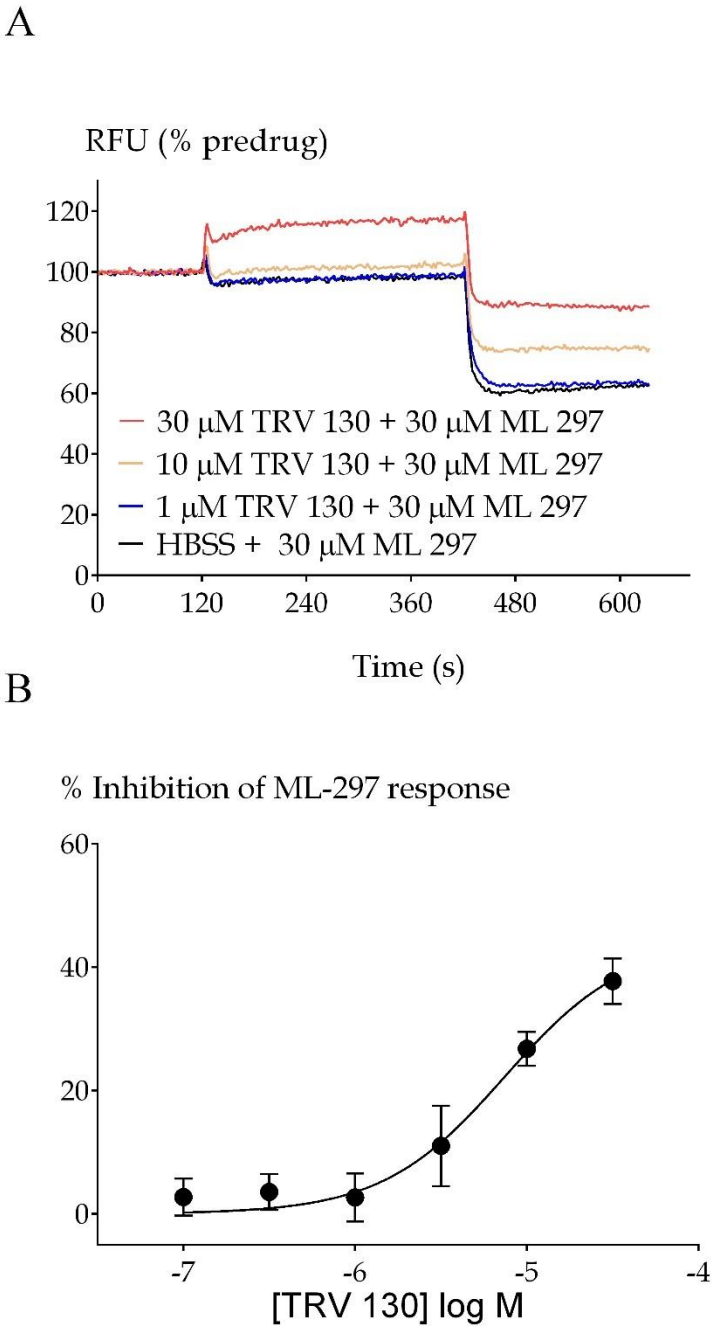

Figure 21. GIRK channel activation assay in AtT20 wild type cells for simultaneous addition of TRV 130 and ML 297. A. Representative traces of experimental run for GIRK channel assay where different concentrations of TRV 130 is added after baseline reading for 2 minutes. After running TRV 130 for 5 minutes, ML 297 is simultaneously added to cells at 420 seconds and the experiment is run for a total of 10 minutes. Higher concentrations – 30μM (red) and 10μM (yellow) showed increase in fluorescence but

after addition of ML 297, both the concentrations are showing a drop-in fluorescence after addition of ML 297. **B.** The change caused by TRV 130+ ML 297 is expressed as percentage change produced by ML 297 alone. Data presented here is the mean value of five experimental runs performed in duplicates.

5.3 Discussion

In this study, we used real time fluorescent based membrane potential assay to investigate the effect of a novel opioid, TRV -130, in G protein-coupled receptor signalling. In membrane potential assay, TRV 130 showed reduced efficacy in inhibiting AC in cells expressing μ-opioid polymorphisms as compared to wild type, suggesting significant variability in TRV 130 response, owing to genetic difference. If the finding observed here has a relevant clinical outcome, then it would result in inadequate pain relief and increased risk of toxicity. So, further studies are recommended to investigate if TRV 130 shows similar variability in clinical settings as well. The pre-clinical trial study suggested TRV 130 as a solution to conventional opioids, whose clinical effectiveness is limited by the adverse events they are associated with. However, study of morphine signalling in same polymorphisms did not show significant difference in its efficacy to inhibit the adenylyl cyclase as shown in chapter 1 of this thesis.

TRV 130 activated K channel with similar efficacy as DAMGO and morphine but it showed more potency than morphine. However, depleting MOR with β-CNA showed TRV 130 having half the efficacy as morphine and much less efficacy than DAMGO. This shows the importance of studying receptor reserve to analyse the actual potency of a drug. An *in vivo* study conducted in mice to address the effect of morphine in receptor depleted case showed morphine still exerting its analgesic action after treatment with β-FNA (beta-fulnaltrexamine), specific MOR antagonist. They showed binding of morphine with other opioid receptor – κ and δ, when less number of MOR are present (Takemori *et al.*, 1987). Use of β- CNA which is irreversible antagonist of all three opioid receptors and use of AtT20 WT cells with only hMOR transfected, shows involvement of no other opioid receptors. This also suggests that higher efficacy of morphine than TRV 130 is not by binding to other opioid receptor as in the earlier study. Several studies have been conducted with β-CNA to study the tolerance of opioids. An *in vivo* study in

the rat locus coeruleus showed, intrinsic efficacy of morphine metabolite is lesser than DAMGO when the MOR are depleted with β-CNA (Christie *et al.,* 1987). The study further suggested the mechanism of tolerance is associated with MOR and/or their coupling with K channel. The MOR effectors, we have considered in this project to check the efficacy of drug is transmembrane K ion conductance. A study suggests stoichiometric interaction of the receptor and effector molecule to be responsible for decrease in potency of drug with reduction in receptor reserve (Chavkin *et al.*, 1984). However, it is difficult to specify the correlation of receptor reserve with efficacy and potency of a drug, unless we completely understand the mechanism of receptor transduction.

It may be possible that TRV 130 is not really a biased agonist and may be its lower efficacy to β-arrestin may be mistaken for its biasness. As in general, a drug couples better with G protein than to β-arrestin (Kelly, 2013). So, lower efficacy of TRV 130 than morphine, may possibly account to lower efficacy of TRV 130 in β-arrestin signalling, which may not be readily detectable in β-arrestin assays.

The concentrations ≥10μM showed increase in fluorescence corresponding to depolarisation in both AC and GIRK activation assay, we hypothesized the possible interaction of TRV 130 either with some other receptors or with K channel itself. 30μM TRV 130 seemed to inhibit the hyperpolarisation caused by somatostatin and ML 297, possibly indicating inhibition of K channel at high concentration.

In conclusion, this current study shows TRV 130 as a lower efficacy opioid than morphine and its efficacy may be further reduced at common MOR polymorphisms. The higher concentration of TRV 130 inhibiting the hyperpolarisation mediated by somatostatin and ML-297 suggest possible interference with K channel at higher concentration. As we did not study about the effect of TRV 130 in β-arrestin pathway, so, the efficacy of TRV 130 in G protein signalling over β-arrestin pathway is yet to be compared with other ligands in the context of similar receptor reserve. In addition to that, more studies are required to address the clinical effect of TRV 130 in response to MOR polymorphisms.

6

GENERAL DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS

Opioids are widely used for treatment of pain, despite their clinical effect, limited by adverse events, tolerance and physical dependence associated with them. Our present study demonstrated a single nucleotide change in MOR having a profound effect in an opioid's signalling which also implies the influence of genetic variability in the individual response to opioids. Various studies have addressed the effect of common SNPs of MOR, with respect to their analgesic effect, internalisation, desensitization and abuse potential of opioids (Beyer *et al.*, 2004). Most of those studies have concluded either reduction or change in efficacy of opioids as a result of genetic variation (Knapman *et al.*, 2014b; 2015c). Even β-endorphin, which was found to bind to MOR in AV-12 cells with 3 times more potency in 118G allele than in 118A allele (Bond *et al.*, 1998), showed reduction in efficacy and potency in CHO cells expressing C17T than in WT cells (Knapman *et al.*, 2014a).

The use of fluorescent based membrane potential assay is a rapid real time based study having proven advantages over other GPCR assays in determining the inhibition of adenylyl cyclase, GIRK activation and desensitization of receptors (Knapman *et al.*, 2013a). One of the limitation of the assay is the variability in the data which can be because of levels of receptor expression. In case of adenylyl cyclase inhibition study in CHO tetracycline induced cells, the variability may be resulted as a difference in time of induction of cells with antibiotic before assay. We ran similar assay for morphine (as

shown in chapter 1) and compared the finding with data from experiments performed by our group earlier (Knapman *et al.*, 2014b). Although the core finding of morphine showing no significant difference among MOR variants is same, the value for efficacy and potency is inconsistent. As our laboratory is not equipped for radio-ligand binding, we could not determine the receptor expression, and so we assume the difference in receptor expression may be the reason behind this inconsistency.

The present work highlighted the significance of common SNPs of MOR in the signalling of pethidine, O-desmethyl tramadol and TRV 130. To our knowledge, this present study provides the first evidence in CHO cells that the drugs under study are affected by genetic variation in MOR. Though we could not statistically quantify the difference of pethidine and O-desmethyl tramadol for different variants, the highest concentration for both drugs tested, showed reduced efficacy in the polymorphic variants as compared to wild type. It is noteworthy that our assay is not suitable to study the actual effect of drugs which interfere with fluorescence signal before reaching maximum effect. The assay has no issues with highly potent drugs, however, higher concentrations of low potency drugs need to be used to show maximal response, and they result in depolarisation (as seen for pethidine and tramadol).

Despite the effect of polymorphism in signalling of opioids, only few studies have been conducted to address their effect in adverse events. A study checked the effect of A118G in respiratory depression profile of an active metabolite of morphine- morphine-6-glucuronide (M6G) and showed polymorphism does not make any difference to adverse events associated with opioids (Romberg *et al.*, 2005). Although the study was limited with small sample size, the result can still be taken as a preliminary step to perform more work to study the effect of genetic variation on severe side effect profile of other opioids.

An interesting finding in this project was TRV 130 showing potency higher than morphine to activate G protein whereas it appeared to be half as efficacious as morphine when receptors were depleted by β-CNA. Similar results were also observed for methadone in earlier studies where it displayed full agonism in guanosine 5'-O-[gamma-thio] triphosphate (GTP gamma S) binding studies and lower efficacy in MOR signalling via K channel activation (Matsui *et al.*, 2010; Rodriguez-Martin *et al.*, 2008). This dual

nature of methadone has been well documented in the literature and has been proposed to be caused due to methadone directly inhibiting the GIRK channel (Matsui *et al.*, 2010). The higher concentration tested for TRV 130 in both AC and K channel activation assay seemed to inhibit the GIRK channel which was further supported by inhibition of hyperpolarisation caused by SST and ML 297. Thus, it is expected, but not proven that a higher concentration of TRV 130 blocks the K channel. Hence, for further study into the cellular mechanism, we aim to perform calcium mobilization assay in CHO cells transfected with somatostatin receptors. If TRV 130 is not interacting with somatostatin receptor, it should not show any response.

Tolerance is a well- researched but equally complex mechanism associated with opioid analgesia. It is related to loss of analgesic efficacy after chronic use of opioids (Kieffer *et al.*, 2002). During past few decades, many facets of tolerance related to opioids have appeared. A study investigating tolerance of morphine, etonitazene (a potent and highly efficacious μ-opioid) and buprenorphine, came up with the most appealing theory which concluded that the ability of a drug to develop tolerance is inversely proportional to its efficacy (Walker *et al.*, 2001). If there is some possibility of the link between tolerance and efficacy as shown by this theory, then our finding might be interpreted as TRV 130 having higher tolerance profile than morphine. Although, our current project does not imply to β-arrestin pathway but pre-clinical study suggests TRV 130 not stimulating β-recruitment. If we assume the claim of clinical trial to be true, then, another theory of tolerance which inversely links tolerance with degree of internalisation, may also interpret TRV 130 having higher tolerance as compared to morphine (Borgland, 2001; He *et al.*, 2002; Kieffer *et al.*, 2002). It is notable that investigations on TRV 130 has not yet extended beyond acute signalling. Furthermore, functional studies to examine the effect of TRV130 on other MOR signalling pathways such as phosphorylation by ERK1/2, internalisation and desensitisation are required to provide valuable insight into most effective form of TRV 130 therapy.

We cannot draw a valid conclusion about the possible efficacy and potency of an opioid based on study performed on just one effector molecule using a single cell line. Variability of cell line in terms of availability of G-proteins, effector molecules and regulatory proteins has been explained by the microarray analysis performed in four cell

lines frequently used for GPCR studies (HEK-293, AtT20, BV2 and N18) (Atwood *et al.*, 2011). This also suggests opioids activating different G proteins in CHO cells and AtT20 cells which may be responsible for buprenorphine showing different efficacy between MOR variants in CHO cells and difference in potency in AtT20 cells (Knapman *et al.*, 2014a; b). Hence, studies of genotype variation of the opioids (studied under current project), should also be performed in AtT20 cells to investigate the effect on K channel activation.

As opioids with different efficacies for activating MOR, have different functional outcomes, it remains to be established whether the findings observed here has a relevant outcome in the clinical settings. A translational study is necessary to determine if clinical effects of pethidine, O-desmethyl tramadol and TRV 130 are influenced by MOR genotypes. As per our findings, pethidine and tramadol are less potent and less efficacious as compared to morphine, and further reduced response in people with MOR variants, may result in inadequate pain relief and increased toxicity. Hence, more studies are suggested to investigate the effect of these opioids on other SNPs of MOR so as to make considerations in prescribing these opioids in people with different polymorphisms. Although association of A118G polymorphism and addiction is not yet established, studies have shown higher prevalence of A118G polymorphism in opioid addicts (Arias *et al.*, 2006; Szeto *et al.*, 2001). If the result of our study finds clinical relevance, then the drugs under study should be well considered before prescribing to patients with these variants. There has been an emerging development in the field of pain analgesics with the discovery of a couple of new opioids like BU08028 (Ding *et al.*, 2016) and PMZ21 (Manglik *et al.*, 2016) within last few months. Both of these new opioids claim to be safer than the conventional opioids with less abusing profile, however, their effect on genetic variation is yet to be studied.

The main findings of our research are pethidine and o-desmethyl tramadol are less efficacious opioids and their efficacy is further reduced at common MOR polymorphisms. Furthermore, TRV 130 was found to be lesser efficacious than morphine, although it showed more potency in activating the GIRK channel. In conclusion, further studies need to be performed to investigate the effect of genotype variation on signalling of all the opioids used, which will help to individualise opioid therapies. More research into

polymorphisms and molecular mechanism of opioids will make some breakthrough therapies within reach, that could greatly increase the efficacy of clinically relevant analgesic drugs.

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Appendices

RECIPES, MATERIALS and EQUIPMENTS

1. Hank's Balanced Salt Solution with HEPES (HBSS)

Low Potassium HBSS

S.No.	Chemicals	Molecular Weight	Final concentration (mM)	Amount
1	NaCl	58.44	145	4.2g
2	HEPES	238.31	22	2.6g
3	Na_2HPO_4	141.96	0.338	24mg
4	$NaHCO_3$	84.01	4.17	175mg
5	KH_2PO_4	136.09	0.441	30mg
6	$MgSO_4$	120.37	0.407	24.5mg
7	$MgCl_2$	95.21	0.493	123µL of 2M solution
8	Glucose	180.2	5.55	500mg
9	$CaCl_2$	110.98	1.26	630µL of 1M solution
10	Milli-Q water			Quantity sufficient to 500mL

The pH is adjusted to 7.4 and osmolarity to 300-330 osm/L.

The solution is filtered through a 0.22 µm filter under the laminar flow hood for sterilisation and stored at 4ºC.

2. MATERIALS

3.1 DRUGS

Name	Product Code	Supplier
β-CNA		*
DAMGO	2283	Auspep
O-desmethyl tramadol		Toronto Research Chemicals Inc
Morphine		*
Naloxone hydrochloride		*
Pethidine		National Measurement Institute
Somatostatin	2076	Auspep
TRV 130		MedChemExpress

* Gift from Department of Pharmacology, University of Sydney

2.2 CHEMICALS

Name	Product Code	Supplier
Calcium Chloride (CaCl2)	190464K	AUS Tritium (VWR)
Dimethyl Sulfoxide	D45040 or D2650	Sigma-Aldrich®
Di-sodium Hydrogen orthophosphate (Na2HPO4)	SA026	Chem-Supply
D- (+)-Glucose	G7021	Sigma-Aldrich®
DMEM	D6429	Sigma-Aldrich®
Fetal Bovine Serum (FBS)	12003C	Sigma-Aldrich®
FLIPR®Membrane Potential Blue Assay Kit	R8034	Molecular Devices
HEPES	H4034	Sigma-Aldrich®
Hygromycin B 100mg/ml	Ant-hm-5	InvivoGen
Leibovitz's L-15 Medium	11415-064	Gibco®
Magnesium chloride (MgCl2)	M8266	Sigma-Aldrich®
Magnesium Sulphate (MgSO4)	M7506	Sigma-Aldrich®
PBS Tablet	09-8912	Medicago
Penicillin(10,000U/mL)-Streptomycin (10,000µg/mL	15140-122	Gibco®
Poly-D-Lysine	P6407/P0899	Sigma-Aldrich®
Phosphate Buffered Saline (PBS)	20012-027	Gibco®
Potassium Chloride (KCl)	PA054	Chem-Supply
Potassium dihydrogen phosphate (KH2PO4)	26936.260	AnalaR Normapur
Sodium Bicarbonate (NaHCO3)	S6297	Sigma-Aldrich®
Sodium Chloride (NaCl)	SA046	Sigma-Aldrich®
Sodium Hydroxide (NaOH)	221465	Sigma-Aldrich®
Tetracycline Hydrochloride	T9823	Sigma-Aldrich®
Trypsin-EDTA Solution 0.25%	T4049	Sigma-Aldrich®
Zeocin ™ 100mg/mL	Ant-zn-1	InvivoGen

2.3 EQUIPMENTS

Name	Supplier
Benchtop 314 Incubator (Ambient CO2)	Lab-Line

Centrifuge 5430	Eppendorf
Flex Station® 3 Multi-Mode Microplate Reader	Molecular Devices
HeraCell™ 150i CO2 Incubators	Thermo Scientific™
Magnetic Stirrer with Heating MR Hei-Standard	Heidolph
Microscope Olympus CKX41	Olympus
Mr Frosty™ freezing container	Thermo Scientific™
Pipettes (including automated multi-channel)	Gilson® and Eppendorf
Water Bath- Constant temperature (NBCT2)	Labec

LIST OF SUPPLIERS

Auspep	Melbourne, Victoria, Australia
Chem-Supply	Gillman, South Australia, Australia
Corning Life Sciences	Clayton, Victoria, Australia
Eppendorf	North Ryde, New South Wales, Australia
GenScript	Piscataway, New Jersey, USA
Gibco®	Mulgrave, Victoria, Australia
GraphPad Software	La Jolla, California, USA
Invitrogen™ (Life Technologies)	Mulgrave, Victoria, Australia
InvivoGen	San Diego, California, USA
Labec	Marrickville, New South Wales, Australia
MedChem Express	New Jersey, USA
National Measurement Institute	Linfield, New South Wales, Australia
Olympus Life Sciences	Notting Hill, Victoria, Australia
Sigma Aldrich®	Castle Hill, New South Wales, Australia
Thermo Fisher Scientific	Scoresby, Victoria, Australia
Toronto Research Chemicals Inc.	Toronto, Canada

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