

# Posttranscriptional Regulation of Mu Opioid Receptor (MOR-1): Implications of Alternative Splicing in the Neuropathogenesis of Morphine Tolerance. Literature Review and Research Approach



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## Abstract

It is no surprise that the progressive increase in the use, misuse and abuse of prototypical narcotic analgesic, morphine, has so heightened awareness within scientific and clinical communities that researchers are clamoring to discover new and divergent approaches to disrupt negative opioid receptor-mediated outcomes and to improve drug efficacy toward beneficial effects. Beyond the traditional pharmacologic approaches investigating cellular changes and receptor binding strategies, the wealth of genetic information coming from the Human Genome Project provokes deeper insight to be sought at the more significant, mechanistically relevant, posttranslational and posttranscriptional levels. Enthusiasm for the contributions of alternatively spliced variants has been slow, except for the widely accepted cytochrome P450 metabolic enzymes. Nonetheless, a steadily growing repository of recently identified opioid receptor alternatively spliced variants (ASVs) places an even greater demand on scientists to elucidate the signal transduction pathways and molecular mechanisms employing these variants. The hope of discovering functional signatures for these variants is increasing and it appears that their clinical utility as new and relevant molecular targets for improving opioid pharmacotherapy may gain momentum.

As the field of pharmacogenomics grows, the central role of the mu opioid receptor (MOR-1) gene (OPRM1) and its variants are being explored in greater depth. MOR-1 belongs to a family of 7-transmembrane (7TM) g-protein coupled receptors (GPCR). It serves as the essential target for pain-mediating drugs, the chief of which is morphine. The medical decision to prescribe morphine for the management of moderate-to-severe chronic pain is complicated by the existence of polymorphisms in drug response to opioid pharmacotherapy. Under acute or chronic conditions, morphine induces pharmacological tolerance, avoidable only by abstaining from its use. Over the past three decades, the enigmatic development of morphine tolerance has been variably attributed to reversible modifications in receptor binding, signal transduction and neuropeptide mechanisms, but none of these theories explain the sustained tolerance that progresses to physical dependence and in some cases, opioid addiction. The current review advances surveys existing knowledge and peers ahead to anticipate a role for alternatively spliced mu opioid receptor variants in the development and termination of morphine tolerance, withdrawal and addiction.

**Keywords:** Mu opioid receptor; MOR-1; Alternative splice variant; ASV; Morphine; Naloxone; Gene expression; Post-transcription; Tolerance; Neuron; Neuroblastoma; SH-SY5Y

**Abbreviations:** Mu opioid receptor; MOR-1; Alternative splice variant; ASV; Morphine; Naloxone; Gene expression; Post-transcription; Tolerance; Neuron; Neuroblastoma; SH-SY5Y

## Introduction

The National Institute on Drug Addiction has recently rendered drug addiction as 'a chronic disease characterized by drug seeking and use that is compulsive, or difficult to control despite harmful consequences' [1]. Physical, biochemical and

physiological changes in the brain over time may be accompanied by behavioral and emotional cue of the systematic degradation on brain function with ensuing modifications of the brain reward circuit in chronic users [2-4]. The predictability of man's responses to morphine intake is highly variable, giving

rise to inter-individual and intra-individual differences whose context in the current opioid crisis is not clearly understood [4-5]. Hence, it is important to adequately dissect the extent to which mu opioid receptor (MOR-1) alternatively spliced variants (ASVs) are involved in the biological processes and physiological mechanisms underlying the development of morphine tolerance, addiction and withdrawal [6-8]. It is also unclear to what extent the multiplicity [9] of MOR-1 ASVs govern human inter- and intra-individual differences in drug response. Close evaluation of the molecular influences of MOR-1 ASVs may reveal relevant targets for further preclinical research and future clinical intervention. The current paper reviews existing information concerning the mu opioid receptor and alternative splicing at the intersection of much needed clinical interventions to curb the vast extent to which individuals become addicted or display maladaptive behaviors following chronic opioid use.

## Background

**Opioid class of pharmaceutical Agents:** Opioids, one of most frequently prescribed pain medications, accounted for roughly 257 million prescriptions in 2009, up 48% from 2000. Nearly 4 million long-acting or extended-release opioids are prescribed every year (FDA 2010; CARES Alliance 2010). Therapeutic uses of opioid drugs include analgesic, antitussive (cough suppressant) and anti-diarrheal applications. Morphine, a mainstay of opioid pharmacotherapy, was recently identified on the 2008 list of 'Top 20 Molecules in U.S. Markets' as having \$277 million in sales (GPhA, 2009). It is officially used in preoperative medical and short-term sedation procedures, and as an analgesic (WHO 2007). Morphine is listed as an essential medicine for adults (WHO 2009a) and children (WHO 2009b) by the World Health Organization. Due to its strong analgesic properties, morphine is the drug-of-choice for moderate-to-severe pain and is recommended for freedom from pain (e.g., cancer pain, pain of myocardial infarction, surgical pain) consistent with the World Health Organization (WHO) patient bill of rights and its introduction of the 'pain ladder' (WHO 1986). Due to its hemodynamic action's morphine sulfate is also used to relieve dyspnea of acute left ventricular failure and dyspnea of pulmonary edema.

**Statement of the Problem:** Morphine exerts its pain-relieving effects primarily through the mu opioid receptor (MOR-1), which are ubiquitous throughout the central (chiefly) and peripheral nervous system. Maintenance of the specialized state of neurons through regulation of gene expression is essential for achieving optimal efficacy of opioid response systems (e.g., OPRM1). Neurons mediate normal cellular communication and responses, and their ability to function is compromised in disease. Moreover, neuronal failure to recognize and respond to changes in the cellular environment may adversely impact human MOR-1 (hMOR-1) responsiveness. Conversely, diminished hMOR-1 physiology may negatively influence the capacity of the nerve cell to adequately respond to persistent stressful stimuli. A growing number of studies indicate an increasing propensity for

variable responsiveness to opioids acting through MOR-1. Among the 70-90% of cancer patients requiring individualized opioid therapy for intense chronic pain, their response to morphine is highly variable, necessitating dose escalation with an increased risk of developing tolerance. Recently, variable pain sensitivity in the general population has been linked to common variants of the  $\mu$ -opioid receptor. As is true of all human pathologies, the ultimate source of genetic variation may lie in the differences in DNA sequences and the proteins expressed. Central to these molecular extremes is the RNA molecule, whose successful posttranscriptional modification facilitates the expression of relevant genes and the production of functional proteins to meet the body's physiologic needs. It remains uncertain whether recent findings of alternative splicing of hMOR-1 are implicated in the highly subjective variability in drug response, in the development of tolerance to opioid pharmacotherapy [10], or whether these effects might be mitigated through manipulation of hMOR-1 alternatively spliced variants.

## Methods

The literature review was developed based upon electronic searches of digital scientific and public health databases, including: ScienceDirect, IUPHAR, PubMed (MEDLINE), PubMed Central, Medscape, and Biomed Central Open Access. Using the terms such as: mu opioid receptor, alternative splicing, alternative splice variant, tolerance, and gene expression, keyword searches were conducted with English language restrictions. Government reports and relevant textbooks were also consulted. All resources were manually assessed for relevancy and adequacy in developing the manuscript.

## Discussion

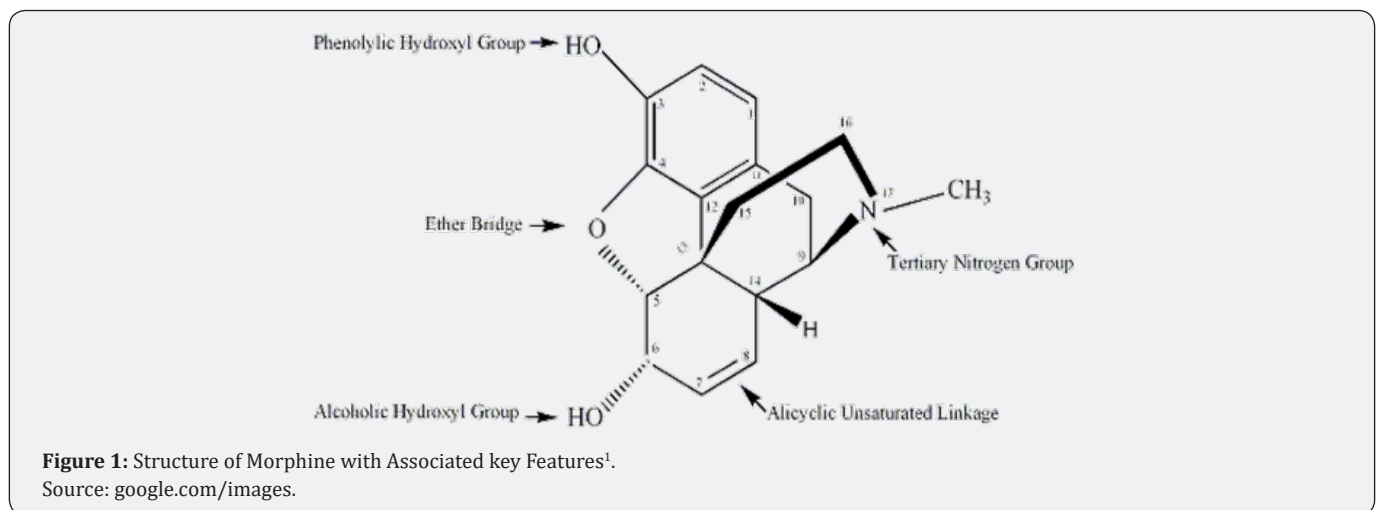
### Significance of the Problem

Pain is a universal phenomenon (WHO 1986; Tao 2010). Regrettably, in the United States, a 400% increase in the number of rehabilitation admissions for treatment of opioid pain reliever abuse, misuse and addiction has also been observed between 1998 and 2008 (SAMHSA 2010; CARES Alliance 2010). Between 2004 and 2008, the number of emergency room visits for non-medical use of opioid analgesics soared by 111% (CDC 2010a; CARES Alliance 2010). Nearly 40% of all poisoning deaths in 2006 were attributed to opioid analgesics (CDC 2010b; CARES Alliance 2010). More than 5 million persons >12 years old self-reported personal non-medical use of morphine (USDHHS, 2009). Due to its euphoric effects, morphine is one of the most abused opiate drugs.

**History of Morphine:** Morphine ( $C_{17}H_{19}NO_3$ ) (Figure 1) is today recognized as a naturally occurring principle of the opium poppy plant (*Papaver somniferum* L.) (<http://apps.who.int/tdr/publications/tdrnews/pdf/TDRnews-issue-79.pdf>). Slang terms for identification of morphine include 'dreamer', 'Emsel', 'firstline', 'God's drug', 'Miss Emma', 'Mister blue', 'Hows', 'Unkie' (ONDCP 2008), 'white stuff', 'M' and 'morf'. In the addiction

realm, morphine is sought out for its euphoric effects (Hamid et al., 2005). The medicinal qualities of opium were apparently discovered centuries before the isolation of morphine in the chemical laboratory. Opium is believed to have been discovered between 4000-2000BC in the Mediterranean territories. It was listed as one of 7000 remedies in the Egyptian papyri around 1500BC, however, poisoning by opium was not described until the 1st century AD. In 1655, Acosta, a Portuguese physician described withdrawal sickness related to opium. Later in 1701, John Jones, a British physician, cautioned against discomforting symptoms of continued opium use. Morphine (originally German 'morphium') was identified as the main ingredient in the opium poppy in 1805, followed by its purification in 1806 by Friedrich Wilhelm Adam Sertürner, a German pharmacist (apothecary). Sertürner's discovery brought him honors with the Institut de France and earned him the title 'Father of Alkaloid Chemistry' (Herbert et al., 2000). His discovery is said to have marked the beginning of the "modern era of narcotics" (Blakemore and White 2002). The isolation of morphine was the first such isolation of a 'natural product', and its isolation was acclaimed as a "seminal event in the development of pharmacology as an independent discipline" (Huxtable and Schwartz 2001; Brownstein 1973). The name 'morphium' was changed to 'morphine' by Joseph Louis Gay-Lussac, a French scientist. History records that the introduction of the habit of smoking opium in the United States came with the immigration of Chinese laborers between 1850-

1865, and this is perhaps the earliest record of cultural influence on opium habituation (Kipnis and Davidoff). It is said that physicians lauded morphine as "God's own medicine" in the 19th century, and by the testimony of 360 physicians, administering it for about 54 different diseases including: anemia, angina pectoris, diabetes, nymphomania, ovarian neuralgia, tetanus, vaginismus, vomiting during pregnancy, (Kane 1880; Brecher 1972) and less commonly, as a substitute for alcohol (WHO 1964). The invention of the hypodermic needle by Dr. Francis Rynd also significantly contributed to the "more severe variety of compulsive drug abuse seen at the turn of the 20th century". Despite his genius of inventing this device for administering morphine to patients with neuralgia, Rynd's personal use of the hypodermic needle sadly resulted in self-inflicted addiction in both him and his wife. History records that Rynd's wife was the first person to die of a narcotic overdose (Dickinson 2010). Reportedly, the wounds of several hundred thousand soldiers were anesthetized with morphine during the American Civil War. On medical discharge, these soldiers were given morphine as a prophylaxis for future pain relief; however, many of them later became addicted to morphine. Later, the introduction of several prime pieces of legislation began the effort to restrict the use of opioids in medical practice, one the earliest of which was the Harrison Act (1914) and the latest, the Children's health Act (2000). Sir Robert Robinson won the Nobel Prize in 1947 for deciphering morphine's structure (Robinson 2010).



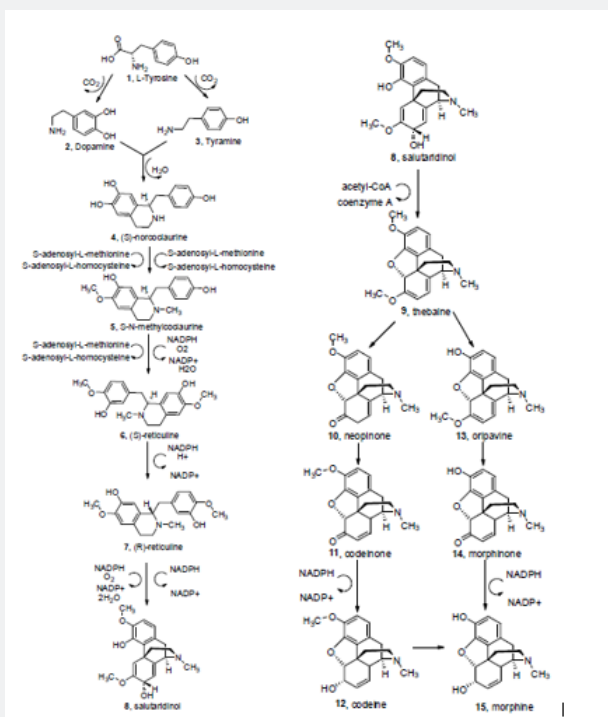
**Natural and Synthetic Sources of Opium:** The opium poppy plant is an herbaceous annual or biennial found in the Southeast Asia, Middle East, Latin America, and South America that grows between 50 and 150cm in height. It has slightly branched stems; large, erect, and oblong leaves; petals 4-8cm long that can be white, pink, purple and violet in color. A few days after flowering, the petals drop off, leaving bulbous green capsules atop the stalks, which are the seed pods. Active principles present in opium include: morphia (1-10%), narcotia, codeia (0.7-2.5%), narcea, meconine, thebia or paramorphia (0.5-1.5%), pseudo-morphia, meconic acid, brown acid extractive, sulfuric acid, resin, fat, oil,

gummy matter, caoutchouc, albumn, odorous principle, volatile oil, and lignin (Ohnwyn 1854; Wood 1867). Other sources suggest that the pod fluid contains 4-21% morphine and 1-25% codeine (Wood 1867). Given the ban on opium growth in the United States, except for medical purposes, intercontinental distribution of opium is primarily due to trade with Southeast Asia, the Middle East, and Latin and South America. Opium is the coagulated, milky juice from the unripe seeds of the poppy plant that contains 40 (Robinson 1947) different atoms and 20-24 of these are pharmacologically active constituents (called opiates). Therapeutic emphasis has surrounded morphine,

codeine, noscapine (narcotine) papaverine, as well as thebaine. Other opium alkaloids, like narceine have not been shown to exhibit and medicinal properties [11]. Morphine is classified as an alkaloid that bears characteristically potent pain-relieving (analgesic) capacity. The term alkaloid refers to nitrogenous basic substances that occur naturally throughout the plant kingdom. Synthetic formulations of morphine-like substances have been derived pharmaceutically to curb the strong effects of opiates. Because of their further capacity to produce numbness, analgesia, CNS depression, or stupor-like symptoms, opioids (natural and synthetic collectively) are considered to be narcotic substances, or the common pharmacological designation as narcotic analgesics (Holland and Adams, 2007). Examples of opiates in common use around the world include codeine (Tylenol #3), dihydrocodine, diacetyl morphine (heroin), ethyl morphine, hydrocodone (Lortab, Vicodin), morphine (MS Contin, Kadian), hydromorphone (Dilaudid), and oxycodone (Oxycontin, Percodan, Percocet). Synthetic opioids include: dextropropoxyphene, duphenoxylate, and methadone (Dolophine) (INCB 2007). Other opioids include: opium, fentanyl (Sublimaze, Actiq), propoxyphene (Darvon, Darvocet, Wygesic), levorphanol (Levodromeran), buprenorphine (Suboxone, Subutex), butorphanol (Stadol), nalbuphine (Nubain, not scheduled), tramadol (Ultram, not scheduled), and meperidine (Demerol) [12]. Synthetic opioids can be subdivided further based on their chemical structure into one of several groups: phenanthrenes (e.g., morphine, codeine, thebaine), phenylhepylamines, phenypiperidines (e.g., fentanyl, alfentanil, sufentanil), morphinans, and benzomorphans.

Benzylisoquinolones (e.g. papaverine-1%, noscapine-5-10%) also found in opium (Ebadi 2008) are not regulated under the Controlled Substances Act (CSA) due to the absence of significant central nervous system effects (DEA 2010).

**Morphine Biosynthesis:** The biosynthesis of morphine from its endogenous precursor, tyrosine, is presented in Figure 2, as adopted from Wong (2008) and other referenced peer-reviewed publications (PMN 2008a, 2008b; Blakemore and White, 2002). Commencing from "L-Tyrosine [1], an amino acid from the shikimate pathway, goes through decarboxylation, condensation, and methylation, all enzymes catalyzed to produce S-N-methylcoclaurine [5]. The compound is then hydroxylated with NADPH and O<sub>2</sub> and methylated with SAM (S-adenosylmethionine) to form (S)-reticuline [6] which is later converted to (R)-reticuline [7] through oxidation and then reduction. (R)-reticuline marks the beginning of the pathway solely used for morphine biosynthesis as it is hydroxylated with NADPH and O<sub>2</sub> followed by reduction, also with NADPH, to form salutaridinol [8]. Salutaridinol is then acetylated with acetyl-CoA to form thebaine [9], which is the precursor of various opioids such as neopinone [10], codeinone [11], oripavine [13], and morphinone [14]. The enzymes that are catalyzed to create these less potent opioids are not yet known but demethylation occurs; there are theories that these demethylations are not enzyme catalyzed. After codeinone and morphinone are produced, reduction with NADPH forms codeine [12] and morphine [15] respectively, while codeine is further demethylated to form morphine.



**Figure 2:** Morphine biosynthesis<sup>2</sup>.

2Wong A (2008) [23] Synthetic opium: The occurrence, bioactivity, biosynthesis, and synthesis of oxycodone.



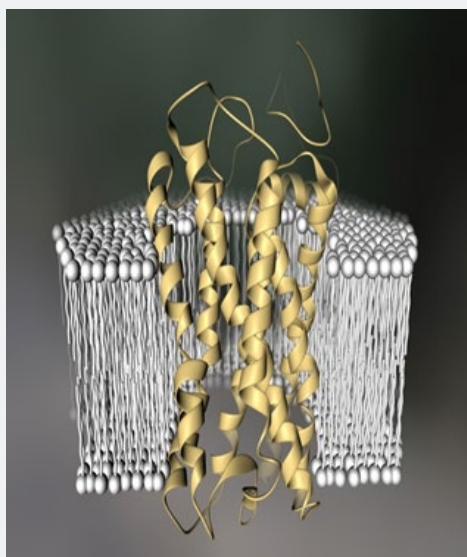
### Structure-Activity Basis of Morphine Action

Based on their structural similarities, opiate alkaloids can be subdivided into two main classes: phenanthrenes and the benzylisoquinolones. The pharmacological actions of phenanthrenes are generally central nervous system (CNS) suppression as well as stimulation, while the benzoylisoquinones are more inductive of antispasmodic action on smooth muscles. The medical profession has capitalized on the antispasmodic effects of morphine as an appropriate remedy for diarrhea. However, there are also a few severe and undesirable effects for which resolution is still sought. The depressant actions of morphine are the most potent of its useful properties and are responsible for the development of increased tolerance in painful states, sleepiness/drowsiness, lower perception of external stimuli, and a heightened sense of well-being (euphoria). The respiratory depression and addiction produced by morphine are also centrally mediated [11; Way and Adler, 1961]. The molecular structure of the morphine atom is very sturdy (rigid), possibly contributing both to its difficulty in crossing the blood-brain barrier and low clearance via the kidneys, as well as to its high binding affinity at the mu opioid receptor surface. The architecture of the morphine molecule is very complex and includes several peripheral functional groups: a tertiary nitrogen (N) group at the 17 position, an alicyclic unsaturated linkage between the 7 and 8 positions, an alcoholic hydroxyl group at the 6 carbon position, an ether bridge, a phenolic hydroxyl group at the 3 position, a methyl group at the 17N position [11]. Hence, all morphine-like analgesics possess (Small et al., 1938; Eddy et al., 1957):

- a) "A tertiary nitrogen, with the group on the nitrogen being relatively small;
- b) A central carbon atom, of which none of the valences is connected with hydrogen;

- c) A phenyl group or a group isosteric with phenyl, which is connected to the central carbon atom; and
- d) A two-carbon chain separating the central atom from the nitrogen for maximal activity."

Other sources propose a special combination of functional substructures required for mu receptor binding and activation that seems to fit well the metabolites of morphine (e.g., M6G, C6G), namely: the nitrogen atom, the quaternary carbon-13 group separated from the nitrogen by an ethyl chain, and an -OH or ester at carbon-3. Derivatives of morphine contain various substitutions on the 3- and 6-carbon atoms as well as on the 17 nitrogen (N) position. Both natural and synthetic opioids generally contain substitutions of ester (-OCX<sub>2</sub>), hydroxyl (-OH), keto (=O), methyl (-CH<sub>3</sub>), or multiple carbon groups. The structure of morphine has also been modified to include: a single bond between carbon 7 and 8, an -OH group added to 14, no oxygen between 4 and 5, and a bridge between 6 and 14 to synthesize semi-synthetic derivatives (Armstrong and Cozza, 2003). Esterification of the phenolic and/or alcoholic hydroxyl groups, and etherification of the phenolic hydroxyl group, are some of the modifications of the morphine skeleton that have produced enhanced activity (and thus, increased toxicity and addiction potential) of the molecule. Morphine undergoes both Phase I and Phase II metabolism. Phase I processes are undertaken by CYP2D6 (chiefly) and CYP3A4. Phase II metabolism occurs via uridine diphosphate glucuronosyl transferase (UGT) enzymes. Glucuronidation is the primary route of morphine metabolism (ONDCP 2008). Oxidative metabolism of morphine by the cytochrome P450 (CYP450) system occurs at the 3 ("O"), 6 ("N") and 17 (also "N") positions. The 3- and 6-positions are the primary sites for Phase II conjugation with glucuronic acid and the required UGT enzymes [13].



**Figure 3:** The Opiate receptor: A class A (Rhodopsin-like) 7 Transmembrane (7TM) G-protein Coupled Receptor (GPCR)<sup>3</sup>.

<sup>3</sup>Source: google.com/images.

## Morphine Pharmacology

Morphine is the prototypical alkaloid analgesic that is derived from the opium poppy plant (*Papaver somniferum*). It is renowned for its proven efficacy in providing relief from various forms of physiological and psychological pain (Zech et al., 1995; Turkoski et al., 2005; WHO 2007; Rang et al., 2007) [14], relieving stress and insomnia, cough suppression and inducing a sense of well-being (euphoria) (Hamid et al., 2005; Rang et al., 2007) [15] in a manner that is proven to be more expedient than the implementation of behavioral or lifestyle modifications (Geppetti and Benemei, 2009). In addition, its high bioavailability (16-60%), rapid onset of action (20-30min), extended duration of action (4-24hr) and low organ toxicity (Forbes 2007) make it particularly attractive for palliative care (Haanpaa et al., 2009), surgical sedation, supraspinal/spinal/peripheral analgesia, nociceptive pain (Rang et al., 2007) [14], and more recently, neuropathic pain. However, the propensity for morphine to induce tolerance, bradycardia (respiratory depression), constipation (gastrointestinal immotility), itching (Rang et al., 2007), immunosuppression (Vallejo et al., 2004), physical dependence and a host of other undesirable adaptive changes (Rang et al., 2007) reportedly accounts for the reluctance of the medical community to prescribe it as first-line pharmacotherapy for chronic, non-malignant pain (Portenoy and Russell, 1996). Identification of mechanisms to regulate opioid receptor activities [6] may provide useful targets for intervention of undesired side-effects of these drugs (Figure 3).

Morphine elicits its pharmacological effects primarily through specific binding to the inhibitory G-protein-linked, mu opioid receptor subtype 1 (MOR-1) [16,17] of interneurons within the central nervous system. Opioid receptors are widely distributed (Gray et al., 2006) in the central neuroaxis (neocortex, thalamus, nucleus accumbens, hippocampus, amygdala) as well as in the peripheral nervous system (PNS) (myenteric neurons and vas deferens) (<http://www.genecards.org>). These receptors commonly bind endogenous opiate peptides, the enkephalins, with high affinity (Urch 2007). Morphine interacts with the heptahelical, transmembrane domains (7TM) of G-proteins, whose primary function is signal transduction [18,19,6]. The receptor-ligand interaction triggers the dissociation of the  $\beta\gamma$  subunit and association of the  $\alpha$ -subunit with GTP-activated adenylyl cyclase (Raynor et al., 1993; Manour et al., 1995). Subsequent inhibition of adenylyl cyclase reduces intracellular cyclic adenosine monophosphate (cAMP) stores (Dhawan et al., 1996) by restricting protein phosphorylation activities required for signaling. Without functional activation (phosphorylation) of signaling cascades by cytosolic kinases, cellular function is diminished or eventually abolished [6]. Additional cellular actions of morphine include changes in voltage-gated ion channels that are directly coupled to G-proteins. Morphine facilitates the opening of potassium channels, leading to increased potassium ion conductance associated neuronal hyperpolarization and secondary reductions in cellular excitability. Moreover, there

is a concomitant blockade of calcium channels which inhibits vesicular transport toward the nerve terminus and prevents neurotransmitter release (e.g., 5-HT, enkephalin, norepinephrine) (Brownstein 1993) [19]. The cumulative suppressive effect of morphine action suppresses firing of inhibitory interneurons (Rang et al., 2007). Hence, all three core mechanisms of opioid action (i.e., receptor mechanisms, opioid peptides, and second messengers) are implicated in the development of morphine tolerance as a consequence of their natural progression.

## Mu Opioid Receptor Pharmacology

**G-Protein Coupled Receptors and Opioid Receptor Morphology:** The opioid receptors belong to a superfamily of guanosine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) that constitute ~3% of the human genome. In humans, the mu opioid receptor gene subtype 1 (OPRM1) is localized on chromosome 6q24-25 and follows an intronic pattern of organization. These constitutively active receptors share extensive (~70%) sequence homology and are characterized by the presence of 7 extracellular and intracellular transmembrane (7TM) domains. The opioid receptor is capable of both monomeric and heteromeric dimerization, the latter being necessary for its activation. Ligands approach and engage the receptor from the extracellular space, and receptor activation results in coupling to heterotrimeric subunits ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits) of G-proteins on the intracellular face of the membrane. The primary function of opioid receptors is signal transduction via intracellular inhibitory G-proteins (Gi/GO), which are relatively resistant to tolerance and desensitization. These discoveries have led to the expansion of the opioid class of receptors to include multiple variant forms ( $\mu$ ,  $\delta$ ,  $\kappa$ ) and subclasses ( $\mu$ 1,  $\mu$ 2,  $\mu$ 3,  $\mu$ 4;  $\delta$ 1,  $\delta$ 2;  $\kappa$ 1,  $\kappa$ 2,  $\kappa$ 3). The opioid receptors are widely distributed mainly in the central nervous system where they produce many physiological and behavioral effects including pain perception, motor control, mood and autonomic functions.

### Mechanisms of Mu Opioid Receptor (MOR-1) Expression:

A gene is said to be expressed decoding of its constituent DNA produces changes in the characteristics and physiology of the resident cell. The modulatory or disruptive effects of exogenous as well as endogenous compounds tend to alter gene expression by influencing transcriptional control of the gene (Wassarman 2002; Alfaras-Melanis et al., 2010; Rhodes and Crabbe, 2005). Gene expression can also be altered via regulation of protein activity, which is regulated by protein phosphorylation (Hardie 1999; Secko 2011). Proper functioning of cellular phosphorylation mechanisms is vital to the maintenance of cellular homeostasis. Phosphorylation occurring at serine and threonine residues in the carboxy-terminal domain (C-terminus) of agonist-bound receptors plays a role in membrane targeting by G-protein coupled receptor kinases (GRKs) [18]. At the receptor level, phosphorylation of the mu opioid receptor by Src protein is thought to account for the transition from an early inhibitory signal to a stimulatory signal following long term opioid therapy [10].

Some authors have also shown that phosphorylation of certain signaling pathways modulates alternative splicing of certain genes (Shultz et al., 2010). It is therefore of interest that, the mu opioid receptor subtype 1 (MOR-1), in its active state, is dimerized within the plasma membrane (George et al., 2000; Gomes et al., 2000 [21]), which contributes to receptor heterogeneity. There are at least six phosphorylation sites within MOR-1 (Y168, S268, Y338, S365, T372, S377) (<http://www.phosphosite.org>). Recent studies have demonstrated that this MOR-1 dimerization is essential for phosphorylation [22]. The functional consequence of chemical exposure can be directly determined by measurement of the transcriptional gene expression, which is synonymous with messenger ribonucleic acid (mRNA) abundance. As the central molecule in the progression of deoxyribonucleic acid (DNA) transcription that results in protein synthesis, messenger RNA (mRNA) is the preferred biomarker in genetic evaluations (Pollock 2002). Quantitative real-time polymerase chain reaction (qRT-PCR) is the technology of choice for quantifying mRNA levels as a direct indicator of gene expression [23]. It has recently been suggested but not definitively shown that cell-specific and tissue-specific expression of certain MOR-1 isoforms (MOR-1C, MOR-1D and MOR-1E) may significantly modulate tolerance development [22].

### Conceptual framework

**Neuroadaptation theory:** Morphine is tolerogenic following both acute and chronic administration of the drug. A state of opioid tolerance has been characterized in animals by the apparent insensitivity to narcotics which is accompanied by increased sensitivity to narcotic antagonists in the absence of changes in opiate receptor binding (Takemori 1975). Several theories have been proposed relating mechanisms of neuroadaptation to the regulation of narcotic action, among them RNA inhibition and antagonism of protein synthesis [24-28]. Inhibition of protein synthesis is thought to contribute to the development of tolerance and subsequent dependence to narcotics and is nonspecific across the narcotic class of drugs (Hitzelmann et al., 1979). It is of note that these nonspecific responses are not limited to narcotics but are consistent regardless of the specific nature of the agent inducing cellular damage [29]. Such adaptation may be triggered by physical, biological, psychological, or chemical (e.g., drugs) stressors, resulting in shifts in homeostasis [30,31]. Adaptation theories have been proposed for decades in attempts to explain the neurochemical, genetic, and behavioral changes that occur after longterm drug administration. Among them are theories posited by Hans Selye, Clifton K. Himmelsbach and Koob and LeMoal (1997). Selye [29] proposed a progressive, three-stage general adaptation syndrome in response to stressful stimuli. The alarm stage is characterized by early arousal of the CNS and body defense mechanisms and precedes the second stage of resistance or adaptation. On prolonged exposure, exhaustion and dysregulation of compensatory mechanisms (acquired adaptations) and homeostasis ensues, which may give rise to disease and death.

Himmelsbach (1941) studied the chemical activities within the brain associated with tolerance, dependence, and withdrawal. His published observations of human subjects on the morphine abstinence syndrome bring to bear the progressive nature of the development of drug dependence, with tolerance to the drug being the key factor leading to more intense and adverse effects. Himmelsbach was the first researcher to point out the variability in patient responses to addictive drugs, as well as to suggest a role for adaptive homeostatic mechanisms in the pathophysiology of the nervous system and the development of morphine tolerance [32-34] applied these concepts of neuroadaptation to drug dependence and changes that occur in brain chemistry on repeated administration of highly abused drugs. Imbalances in endogenous adaptive mechanisms aimed at regulating or opposing the effects of drugs may result in tolerance and withdrawal, both of which were proposed to result from neuroadaptive changes within the system [34]. The subsequent vulnerability of both the dopamine reward system and the endogenous opioid system has therefore been evaluated relative to individual susceptibility to the adverse effects of narcotic agents [35].

**Significance of Adaptation:** Brain neurons adapt functionally on narcotic exposure [3], even the first dose, and these changes are progressive [36], occurring at an uneven pace [3]. Morphine is tolerogenic following both acute and chronic administration of the drug. A state of opioid tolerance has been characterized in animals by the apparent insensitivity to narcotics which is accompanied by increased sensitivity to narcotic antagonists in the absence of changes in opiate receptor binding (Takemori 1975). Others have also proposed mechanisms (e.g., RNA inhibition and antagonism of protein synthesis) relating neuroadaptation to the regulation of narcotic action [24-28]. Inhibition of protein synthesis is thought to contribute to the development of tolerance and subsequent dependence to narcotics and is nonspecific across the narcotic class of drugs (Hitzelmann et al., 1979). It is of note that these nonspecific responses are not limited to narcotics but are consistent regardless of the specific nature of the agent inducing cellular damage [29]. Such adaptation may be triggered by physical, biological, psychological, or chemical (e.g., drugs) stressors, resulting in shifts in homeostasis [30,31].

**Gate Theory:** The gate theory was postulated in 1965 by Melzack and Wall to explain the differences in conduction of electrical stimuli between mid-sized, lightly myelinated A-delta fibers versus nociceptive transmission through smaller, unmyelinated C-fibers within nervous tissue. Pre-synaptic (and/or post-synaptic) inhibition and facilitation of electrical input to the dorsal horn of the spinal cord were thought to be governed by a balance in activity between A- and C- fibers. Dominant activity via C-fibers would result in pre-synaptic facilitation, thereby opening the gate for central transmission of pain. The gate is closed when A-delta fiber activity predominates, thereby reducing stimulation of the spinal cord, which results in reduction or abrogation of the painful stimulus [37,38]. Based on the gate theory, central nervous system depressing drugs (e.g., narcotics)



that have a sedating effect and work at sites involved in pain perception (e.g., cerebral cortex and thalamus) would be capable of pharmacologically altering the physiology of these brain regions, thereby modulating pain perception (Siefele 1974). While this theory provides extensive basis for a neuroanatomical understanding of the mediation of pain via central and peripheral receptors and may be quantitatively be assessed, the gate theory still does not address the inter- and intra-individual variability in patient response to opioid drugs.

**Epidemiology of Pain:** Pain is an ‘unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage’ that is highly subjective (IASP 2011). The sting of pain affects us all, irrespective of age, race, color or creed. Pain is both recognized nationally as an urgent medical condition (IASP 2011) and internationally as a priority health care need (WHO 2010). Pain manifests in one or more parts of the body as an unpleasant acute, chronic or episodic sensation that may result from actual tissue damage or whose initiating stimuli likely produce physical damage (IASP 2011). Chronic pain is ranked third globally among all health problems (NIH 1982; WHO 1986; Tao 2010) and painful experiences accompany at least 5 (diabetes, heart, cancer, osteoporosis, obesity) of the top 10 chronic diseases (AAPM 2010, CARES Alliance 2010). In the United States, most persons seeking medical intervention do so because of pain (Loeser 2000) and its debilitating effects (Austin et al., 1996) have produced significant economic impacts in excess of \$100 billion per year (NIH 2010; CARES Alliance 2010). That pain should be classified as a disease has recently been proposed and justified on the basis that pain relief is allusive and there is no known cure. According to a 2006 survey, 51 percent of chronic pain sufferers felt that they had little or no control over their pain (CARES Alliance 2010). These same fears are shared by medical professionals for whom prescription of morphine, the prototypical opioid drug, presents specific challenges due to its selective anatomical effects as well as the variability of response to morphine within and between patients (Forbes 2007; Mercadante & Portenoy, 2001). Inter-individual differences in pain response are well-documented (Ikeda et al, 2005). Women, children and elderly subpopulations are disparately impacted by pain (IASP 2011). Pain affects more women than men globally and women are more likely to experience recurrent, severe, chronic pain. Infant, toddlers and children commonly experience a more profound impact of pain than adults and many children become susceptible to chronic pain conditions in adulthood (WHO 2009a, 2009b). A growing proportion of persons  $\geq 12$  years old reported at least one non-medical/recreational usage of prescription pain relievers in 2008 (FDA 2010, CARES Alliance 2010). In both developing and industrialized countries, use of pain medications among the elderly has increased significantly (IASP 2011). Thirty-three percent (33%) of the adult patients being treated for cancer and 66% of advanced malignant cases experience pain (IASP 2011).

**Definition of Tolerance:** That all patients on sustained opioid pharmacotherapy will develop tolerance and physical dependence to the drug (Eddy et al., 1965) [4] is an inevitable and indisputable fact that every prescriber must consider whatever the pain-related indication. The American Psychiatric Association’s Diagnostic & Statistical Manual of Mental Disorders (DSM-IIIIR) is the authority by which the United States (U.S.) healthcare system classifies diagnoses of all known psychiatric disorders, including those related to substance abuse and substance dependence. Based upon this standard, tolerance is defined either as “a need for markedly increased amounts of the substance to achieve intoxication or the desired effect” or “markedly diminished effect with continued use of the same amount of the substance” (APA 1987). This etiology-based classification of drug dependence and definition of pharmacological tolerance are also used to address the issue of opiate addiction in the International Classification of Disease Version 10 (ICD-10), which is the international standard diagnostic classification used in epidemiology, health management, and clinical diagnostic applications (WHO 1992).

**Development of Morphine Tolerance:** All patients taking morphine for prolonged periods will develop tolerance to the drug (Mercadante & Portenoy, 2001). Pharmacologic tolerance is the markedly diminished responsiveness an individual exhibits after chronic exposure to a drug, or conversely, the apparent need for increasingly larger dosages of a drug to achieve a fixed level of the same desired clinical effect (as seen in analgesic tolerance) (WHO 1992; Mellar & Pasternak, 2005; APA 2010). Factors implicated in opioid tolerance development (Horner & Zadina, 2004; Dumas & Pollock, 2008; Marie et al., 2006; Farooqui et al., 2006) include: reduction in receptor number (down-regulation); impaired receptor-effector coupling (receptor/G-protein uncoupling); modified activity of effector systems regulated by opiates (e.g., adenylyl cyclase); modified gene expression (reduced or increased expression of specific genes); neural network adaptations (altered activity in regulatory pathways); and behavioral adaptations (“associative” tolerance; opponent process models). Tolerance can develop to a single dose (Kornetsky and Bain, 1968) or repeated administration of morphine. Studies in mice lacking the mu-opioid receptor have shown that this receptor is essential for the development of morphine-induced analgesia, reward effect and withdrawal symptoms (Matthes et al., 1996). To date, several hypotheses have been proposed to explain the development of opioid tolerance. The development of tolerance may be attributed to receptor desensitization, involving phosphorylation of opioid receptors by kinases; uncoupling of opioid receptors from G-proteins by arrestins; and opioid receptor trafficking that leads to receptor internalization, recycling and degradation (Marie et al., 2006). It has also been demonstrated that superactivation of the cAMP pathway leads to the development of opioid-induced tolerance. Most recently, it has been suggested that enhanced opioid metabolism in painful states (e.g., disease progression, neuropathic pain, cancer) (Anand et al., 2010) may cause tolerance. It is also noteworthy that the same therapeutic approaches (e.g.,



opioid dose escalation, opioid rotation) employed to mediate worsening painful states are among the mechanisms that further exacerbate opioid tolerance development (Anand et al., 2010).

### Recent Advances in Opioid Pharmacology

Since the discovery of an endogenous opioid system [39,40], 3 types of endogenous opioid receptors ( $\mu$ ,  $\delta$ ; and  $\kappa$ ); have been identified (Gilbert & Martin, 1976; Martin et al., 1976). Building on these findings, various researchers have characterized multiple endogenous opioid receptor ligands (opiate peptides: enkephalin, dynorphins, endorphins) (Hughes et al., 1975; Li & Chung, 1976; Lord et al., 1977; Wuster et al., 1978; Schulz et al., 1979; Tachibana et al., 1982) [39-41]. Subsequent cDNA cloning of the opiate peptide precursor proteins (Nakanishi et al., 1979; Noda et al., 1982; Kakidani et al., 1982) served as a model for other efforts to clone and identify the complementary DNA (cDNA) of  $\delta$ -opioid receptor protein from NG108-15 and COS cells (Kieffer et al., 1992) [42]. An evolution of cloning opioid receptors ensued in the years following. Opioid receptors were cloned for several species (rat, mouse, guinea-pig); sequencing of the rat  $\mu$ -opioid receptor; namely: rat,  $\mu$  (Kieffer et al., 1992; Chen et al., 1993; Fukuda et al., 1993; Minami et al., 1994; Bunzow et al., 1995) [40-43]; rat,  $\kappa$  (Chen et al., 1993; Li et al., 1993; Minami et al., 1993; Nishi et al., 1993) [40]; rat,  $\delta$  (Fukuda et al., 1993; Fukuda et al., 1994; Abood et al., 1994; Bunzow et al., 1994; Chen et al., 1994; Wick et al., 1994; Lachowicz et al., 1995) [43]; mouse,  $\kappa$  (Yasuda et al., 1993; Nishi et al., 1994). mouse,  $\delta$  (Augustin et al., 1995); mouse,  $\mu$  (Min et al., 1994); and guinea-pig,  $\kappa$  (Xie et al., 1994). Subsequent and current efforts have been directed toward characterization of these receptors and newly discovered alternatively spliced variants of opioid receptors.

**Molecular Genetics of Opioid Receptors:** The OPRM1 gene, which is the only gene known to be responsible for encoding the mu opioid receptor, is the primary site of action for endogenous opioid peptides (e.g., beta-endorphin and enkephalin) and morphine-like substances [44]. It is physically located on chromosome 6 on cytogenic band number 6q24-q25, contains 4 exons and spans more than 50 kilobases (50 kb). The exons are spaced at variable distances from each other: exon1-exon2 (28 kb), exon2-exon3 (0.8 kb) and exon3-4 ( $\approx$ 20 kb). Exon 1 encodes the amino (N) terminus and the first transmembrane (TM) domain; exon 2 encodes TM domains 2, 3 and 4 as well as their corresponding intracellular loops; exon 3 encodes TM domains 5, 6 and 7; and exon 4 constitutes all of the amino acids that make up the carboxyl (COOH) (C)-terminus. Exon 1 appears to be involved with morphine analgesia while exon 2 mediates morphine-6-glucuronide analgesia [45,46,10]. Alternative splice variants and certain transcription factors under investigation in this research work are localized or act in the region of exon 3, exon 4 and exon 5.

**Splicing of Mu Opioid Receptors:** Three isoforms of the opioid receptor ( $\mu$ ,  $\kappa$  and  $\delta$ ) and three variants of the mu opioid receptor- $\mu$ 1,  $\mu$ 2 and  $\mu$ 3 have presently been identified. These variants occur as a result of splicing of the mu opioid receptor,

which involves the excision of non-coding intronic regions and fusion of coding exon regions (Borsodi et al., 2010). Splicing occurs co-transcriptionally (Proudfoot et al., 2002) and is thought to be completed in the nucleus before mRNA export (Pessa et al., 2008). After transcription and RNA processing, the mature mRNA has an open reading frame (ORF) that encodes a human mu opioid receptor protein of 400 amino acids (aa). Each splice variant may exhibit different agonist-induced activation, signal transduction and protein expression patterns. The transcription of  $\mu$ 1 is tightly regulated by various transcription factors (e.g., STAT3, NRSF1, NRSF2, STAT1, STAT1- $\beta$ , STAT1- $\alpha$ , PPAR- $\gamma$ 1, ppar- $\gamma$ 2, RREB-1, MEF-2a and others) within the promoter region or OPRM1 (<http://www.genecards.org>).

**Alternative Splicing of the Mu Opioid Receptor (MOR-1) Gene (OPRM1):** Recent literature points to the identification of at least 10 alternatively-spliced isoforms of the MOR-1 variant of the human mu opioid receptor gene (OPRM1) [44-52] but are not yet fully characterized (Pasternak and Pan, 2009). Specialized (sensory) neurons throughout the body mediate pain sensation by regulating human MOR-1 gene (OPRM1) expression. Compared to traditional splicing events, alternative splicing may alter gene function [53]; switches substrate specificity (Christmas et al., 2001; Bauman et al., 2009); and, though essential for life, can lead to disease – e.g., cancer, in cases of aberrant splicing events (Faustino 2003; Buratti et al., 2006). In alternative splicing, one or more exons may be selectively excised and less frequently, an intron or partial intron may be found. MOR-1 is a vital part of the opioid receptor system that primarily acts through the nervous system to mediate pain perception and transmission throughout the body. Since then, immunohistochemical studies have been used to identify such variants in mice, rats, and humans (Mansour et al., 1995, 1996, 1997; [52]. Several new MOR isoforms have also been shown to modulate binding of the human mu opioid receptor [54]. Consistent with other Class A GPCR family members, these receptors exhibit similar 7-transmembrane in humans, rats and mice ( $\approx$ 15, [47,50]). The N-terminus is extracellular, and the C-terminus is intracellularly oriented. Alternative splicing patterns vary largely in the C-terminus downstream of N-terminus exons 1-3 [4]. As MOR-1 plays a central role in anti-nociception, brain reward systems, opiate addiction and homeostasis [52], nerve cell differentiation [54], and brain development [12], it is reasonable to question the influence of alternatively spliced variants of this receptor on MOR-1-mediated effects. The neuroadaptive effects of morphine on mu opioid receptors in the nervous system are largely responsible for both its beneficial and strongly addictive properties [55]. Thus, it is also reasonable to consider the impact of MOR-1 ligands on the regulation of the expression of these variants. Opioids interact with the central reward circuitry, giving rise to alterations in the limbic system which are associated with opioid misuse and abuse behaviors. In the brain, repeated opioid exposure can induce neuroadaptation and plasticity [55]. While there is also evidence to suggest that the neurochemical dysregulation contributes to addiction states

follows the development of opioid tolerance [56], there is no evidence to date to correlate these changes with the expression of MOR-1 alternatively spliced variants. The recent suggestion that a less efficient post-endocytic sorting mechanism may contribute to decreased recyclability of the variant receptors due to changes that occur during alternative splicing is still speculative (Tanowitz et al., 2008). The literature is sparse concerning the physiological significance and pharmacological implications of OPRM1 alternatively spliced variants [19,45-48,54,57]. Initial studies have suggested that decreased hMOR-1 functionality through altered gene expression may both increase the rate and severity of chronic illness [58]; Freye and Latasch, 2003; MacAuley et al, 2005). However, it is unknown whether alterations at the level of the MOR-1 receptor are amplified at the level of OPRM1 alternatively spliced variant proteins.

#### Common Approaches to the Study of Morphine Tolerance:

Approaches to studying opioid tolerance have typically focused on disruption of the two mechanisms by which GPCRs perform their chief function of signal transduction: extracellular ligand binding to the receptor; and intracellular removal of inhibitory constraints on spontaneous receptor activity. Key pathways: neuroactive ligand-receptor interaction, longterm potentiation, GnRH signaling pathway, MAPK signaling pathway\*, gap junctions (Li et al., 2008).

**Contemporary Opioid Pharmacogenomics:** In keeping with the notion that the multiplicity of opioid receptors explains its selectivity, it is possible to consider that this feature may also account for the variability in responses to drugs interacting with the receptor (Tremblay and Hamett, 2010; Roopra et al., 2001) [59,60]. Contemporary opioid pharmacology has embraced results published under the Human Genome Project which showed for the first time that >90% of the human genome undergoes alternative splicing (Keren et al., 2010). Alternative splicing is a major posttranscriptional mechanism through which a single gene produces different gene isoforms from pre-mRNA, resulting in a transcriptome that is enhanced and the production of a diverse protein pool [53]. Hence, a new hypothesis was put forth that the  $\mu$ -opioid receptor undergoes alternative splicing [48]. These data now serve as a catalyst for new research into the family of transcription factors (transcriptomics) and associated gene products (proteomics) that regulate the mu opioid receptor.

#### Technological Advances in the Study of Morphine Tolerance

**Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR):** The process of gene expression analysis typically involves four (4) biochemical steps: RNA isolation; removal of contaminating DNA; first-strand complementary DNA (cDNA) synthesis; and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). PCR technology is essential for the accurate execution of the latter two steps. The polymerase chain reaction (PCR) is a sensitive technology which was discovered by Kary Mullis in

1983 for the original purpose of improving DNA quantification. Over the past 25 years, advances in PCR technology - such as the introduction of quantitative real-time reverse transcription PCR (i.e., real-time RT-qPCR) and the emergence of sophisticated instrumentation to detect vanishingly small quantities of nucleic acids - have advanced the field of gene expression analysis. The term 'gene expression' is synonymous with 'messenger ribonucleic acid (mRNA) levels'. Real-time RT-qPCR precisely and reliably measures gene expression levels along specific nucleic acid sequences throughout the course of the entire reaction. The evident superiority of RT-qPCR surpasses older technologies (e.g., Northern blot, RNase protection assays) and affirms its designation as the "gold standard" or method-of-choice for analyzing gene expression of modest numbers of genes. Four key attributes are fundamental to the utility of qRT-PCR, which represents a technological advancement of conventional PCR techniques to facilitate real-time quantification of DNA amplification. First, nanogram quantities of mRNA can be readily detected by RT-qPCR [61]. Secondly, the sensitivity and effectiveness of qRT-PCR technology is unsurpassed in its ability to discriminate between MOR-1 alternatively spliced variant mRNA levels in developmentally regulated SH-SY5Y cells [62]. Thirdly, RT-qPCR successfully amplified distinct nucleic acid sequences of most of the variants tested (historic exception: MOR-1B4 - possibly due to design error) [63]. Finally, robust qRT-PCR study designs are essential tools for elucidating the functional implications of novel MOR-1 variants in morphine tolerance [64]. The biological significance of RT-qPCR to modern biology and biomedical sciences is irrefutable. In the aftermath of discoveries made in the Human Genome Project, scientists have begun to explore more intensely the molecular underpinnings of sickness, disease, and drug interactions in the body. It is hoped that the answers to illusive medical conditions, such as cancer and drug tolerance, can be elucidated at the molecular level to shed greater insight into the nature of these conditions as well as the mechanisms by which they occur. The development of morphine tolerance is one such condition for which scientists continue to supersede reasonable interventions with the elimination of the causes of this condition. For centuries, morphine has been utilized as the prototypical analgesic drug in the treatment of chronic and intractable pain. Morphine exerts its pain-relieving effects primarily through the mu opioid receptor (MOR-1). With the recent advances of receptor polymorphisms and gene splicing, several variant forms of MOR-1 have recently been identified. Through advances in polymerase chain reaction (PCR) technology, the fields of molecular genetics and pharmacology have begun to converge and so enable a deeper understanding of the mechanistic basis of opioid-related diseases, like opioid dependence, addiction and withdrawal, which are chronic outcome of morphine tolerance. Our goal is to employ advanced molecular genetics techniques, including real time RT-qPCR, to address the pharmacological problem of morphine tolerance [65].

## The Human Genome Project

The extent of human genetic variation is illuminated at the molecular level by the provision of a near-complete sequence of the human genome in 2001. Individuals are only 0.1% (i.e., ~1 out of 1000 DNA bases) dissimilar. The molecular mechanisms of phenotypic and genotypic differences that exist in this segment of the genome may explain the effect of genetic variation on human physiology and disease.

**Human Genetic Variation:** The identification of DNA as the material genes are made of (1944), followed by the elucidation of the structure of the molecule (1953), were pivotal to the emergence of molecular analyses of genetic variation. More than 200 genes cause or are associated with neurological disease (NIH 2010). Knowing the molecular mechanisms by which genes directly influence health status (i.e., presence or absence of disease) will facilitate targeted therapeutic interventions to prevent or alleviate these conditions [66].

**Sources of Genetic Variation:** One mechanism by which genetic variation occurs is through the process of alternative splicing, a common modulatory mechanism for enhancing the diversity of the transcriptome and proteome from bacteria to humans. Studies of the genomic architecture indicate that >90% (Keren et al., 2010) of protein-coding human genes (142,634) undergo alternative splicing [67]. The pre-mRNA splicing process is a vital regulatory phase of the gene expression pathway that orchestrates the excision of non-coding introns and the subsequent ligation of relevant coding exons to form mature mRNA [65]. Alternative splicing is the discriminatory inclusion of different exons in mature mRNA, resulting in the production of different gene isoforms from a single gene, and by extension, different protein isoforms [53].

**Gene Expression Pathway:** Molecular biology dogma: DNA RNA Protein, is often called the gene expression pathway in which DNA is transcribed to messenger RNA (mRNA) through a constitutive splicing process. Mature mRNA is translated to protein. The term 'gene expression' is synonymous with 'messenger ribonucleic acid (mRNA) levels'. Protein function is determined by the expression of the gene that encodes its amino acid sequence. The expression and repression of certain genes is essential for effective cellular differentiation and appropriate function [68,69].

**Empirical Review:** Several studies suggest that evaluation of changes in mRNA levels may be relevant to defining the paradigm of morphine tolerance development. A study examining mRNA regulation of the rat peripheral nervous system in morphine-tolerant rats (10mg/kg SC) showed by qRT-PCR analysis that MOR was down-regulated by 62% ( $p < 0.05$ ) after 4 days with a 280% recovery of mRNA levels on acute (24hr) withdrawal of morphine (Meuser et al., 2003). In cell-based studies, longterm agonism of various immortalized cell lines and neurons resulting in MOR down-regulation has been observed (Yamamoto et al., 2008). The cumulative effect, manifested as down-regulation (Binyaminy et al.,

2008), is reasoned to be a consequence of sequential sequestration of MOR (Keith et al., 1998) after its autophosphorylation (Law et al., 2000 [66]; Harrison et al., 1998), endocytic internalization [67] and proteolytic degradation (Harrison et al., 1998). However, whether there is a truly integrated effect of these actions remains controversial as it has recently been suggested that morphine does not induce internalization (Whistler et al., 1999). Other opposing studies have also documented no conclusive finding that chronic morphine treatment that induces tolerance also alters MOR mRNA expression and refute a link between MOR expression and receptor activation (Brotsky et al., 1995). However, more recent investigations have suggested that the suppression of MOR1 reporter activity following chronic morphine treatment requires upregulation of the expression of miRNA23b, a novel trans-acting factor that binds to the K-box motif in the MOR1 3'-untranslated region (UTR) [58]. Associations between the functional roles of alternative splicing relative to gene expression are less common in the literature (Adams 2008) [49]. One study examining the ability of alternative exons in the N-terminus of HIV-1 virus to promote or inhibit the nuclear degradation of surrounding mRNA, researchers showed that these changes regulate HIV-1 gene expression (Krummheuer et al., 2001). Others have shown that alternative splicing of SC35 regulates its mRNA stability (Sureau et al., 1994, 2001). It has also been suggested that alternative splicing pathways may have some chemotherapeutic utility (Mercatante and Kolce, 2000). Relative to opioids, however, the question of the functional role of MOR-1 alternatively spliced variants has not yet been addressed, and any correlation to morphine tolerance has also not been demonstrated. As exploration of the molecular influences of prolonged morphine treatment continue, we propose yet another approach to resolving the problem of morphine tolerance development. Ultimately, gene expression and the production of a diverse pool of functional or non-functional proteins are controlled by a mechanism within the splicing process termed alternative splicing. In the aftermath of the Human Genome Project, the scientific community has begun to appreciate the contribution of alternative splicing to the pathology of disease such as cancer (Stoss et al., 2000; Cogan et al., 1997; Faustino and Cooper, 2003). Disruption of alternative splicing can alter normal cell and tissue functions (Stamm et al., 2005). Thus we can appreciate that mechanisms that govern protein synthesis, such as alternative splicing, contribute tangibly to these outcomes. The mu opioid receptor subtype 1 (MOR-1) is extensively alternatively spliced in humans, rats, mice and various other species [70-73].

## Proposed Research Approach

The following model approach provides an example of a small pilot assessment utilized to establish baseline responses, to develop standard operating procedures, and to configure a workable study design for a more elaborate future research project.

**Study Rationale:** The mu opioid receptor (MOR) is primary target of opioid drugs administered for the mediation of pain.



Morphine is the prototypical drug within this class that acts chiefly through the MOR subtype 1 (MOR-1). It has recently been demonstrated that the expression of the mu opioid receptor (MOR-1) is regulated by neuronal response silencing element (NRSE), a silencing element resident within the C-terminal of the mu opioid receptor gene (OPRM1) (Andria and Simon, 2001). Subsequent studies suggested that these changes in MOR-1 gene expression may be mediated through the PI3K/Akt/CREB signal transduction pathway (Westbrook et al., 2005). The pharmacological identification of novel MOR-1 alternatively spliced variants [50,52] raises questions as to the physiological role of these variants in drug response mechanisms. To this end, it has been further demonstrated that patient sensitivity to opioid medications may be governed by particular variants of MOR (Tremblay & Hamet, 2010). Moreover, it remains controversial whether MOR-1 alternatively spliced variants contribute to the clinically observed variability in opioid therapy response [74,75]. The proposed investigation was conducted to determine if morphine-induced modulation of alternative splicing of MOR-1 is responsible for the divergent sensitivities to morphine observed in clinical settings. Data from this study will increase our understanding of the potential contribution of MOR-1 alternatively spliced variants in opioid system physiology and pharmacotherapy.

**Research Hypothesis:** The overall goal of the proposed research is to test the hypothesis that chronic morphine alters posttranscriptional and posttranslational indices of MOR-1 regulation. The specific hypothesis tested in this study is that morphine inhibits mechanisms involved in the posttranscriptional regulation of MOR-1 in human neuroblastoma SH-SY5Y cells.

**Specific Aims:** This study will investigate the relationship between chronic morphine exposure and MOR-1 alternative splicing, signal transduction, transcription and phosphorylation. To achieve this goal, the following specific aims will be investigated:

- a) Aim 1: To identify the presence of MOR-1 alternatively spliced variants in naïve and differentiated human neuroblastoma SH-SY5Y cells.
- b) Aim 2: To study the effects of chronic opioids on the posttranscriptional expression of MOR-1 variants in naïve and differentiated human neuroblastoma SH-SY5Y cells exposed to a MOR-1-specific agonist, morphine or DAMGO, or naloxone (opioid antagonist) for 24 hours.
- c) Aim 3: To evaluate the effects of chronic opioids on the posttranscriptional expression of MOR-1 in differentiated human neuroblastoma SH-SY5Y cells exposed to morphine, DAMGO, or naloxone for 48 or 72 hours.
- d) Aim 4: To examine in vitro the posttranslational effect (i.e., phosphorylation) of chronic (24 or 48hrs) opioids on signaling proteins and nuclear transcription factors after morphine (10 $\mu$ M), DAMGO (10 $\mu$ M), or naloxone (10 $\mu$ M) treatment of human neuroblastoma SH-SY5Y cells.

- e) Aim 5: To examine morphine-induced posttranslational effects on proteins involved in G-protein coupled receptor (GPCR) signal transduction and nuclear transcription in differentiated human neuroblastoma SH-SY5Y cells after 48hr exposures.

### Experimental Design

**Study Design:** A hypothesis-driven, experimental design was selected for the evaluation of alternative splicing in an all-trans retinoic acid (RA)-induced, neuronally-differentiated, cell model of morphine tolerance [76-80].

**Cellular Model:** Differentiated human neuroblastoma cells (SH-SY5Y) are commonly used in basic neuroscience research as a model of normal neuronal function [68]; Prince and Oreland, 1997). Preliminary studies in our laboratory indicate that this cell line measurably expresses the mu opioid receptor and that MOR-1 gene expression is higher in differentiated SH-SY5Y cells than in control (undifferentiated) cells [81]. Our laboratory has also found higher expression of certain MOR-1 isoforms in RA-differentiated cells when compared to controls (differentiated, untreated) and to endogenous housekeeping genes, such as  $\beta$ -actin. Given the above and the obvious advantage that individual cells provide clear insight into many phenomena that are not complicated by systemic interactions, we selected the differentiated SH-SY5Y cells as our model to further investigate the role of chronic opioids on various MOR-1 related indices. Tolerance is defined a state of diminished responsiveness to increasing concentrations of a drug, evidenced by a gradual loss of effectiveness of a drug following longterm administration. Repeated administration of morphine in animal models is an established mechanism through which tolerance may be induced in the laboratory setting. Cellular studies in Chinese hamster (CHO) cells and other cell lines have previously been used to investigate in vitro morphine tolerance [69,70]. We will induce morphine tolerance in our model cell line by chronically exposing differentiated SH-SY5Y cells to morphine for 24 hours under experimental conditions. Naloxone, a non-selective, competitive opioid receptor antagonist, which has been shown to reverse morphine-induced effects in animal studies (Kalyuzhnyi, 1996), will be evaluated for its own contribution to actions mediated through the mu opioid receptor [82-85].

**Gene Expression Analysis:** A combination of molecular approaches were used to identify relevant target genes, namely, PCR array and real-time quantitative polymerase chain reaction (RT-qPCR), the latter of which is the recognized 'gold standard' for genetic analysis. Immature human neuroblastoma cell cultures were grown to confluence, differentiated into neuronal cells then treated with various opioid-related compounds to directly assess the effects at the MOR-1. Aseptic techniques were applied throughout all phases of experimentation. Detailed procedures are chronologically presented below.

**Functional Assessment of Posttranslational Modification of MOR-1 Protein:** Phosphorylation is one of several key



posttranscriptional modifications of proteins that are necessary for protein function. It has been suggested that neuroblastoma cells assume a senescent phenotype with early disruption of the G1/S phase of the cell cycle by anti-mitogenic signals (e.g., regulation of cyclin production and binding to the cdks, subunit phosphorylation, cdk inhibition). Where senescence dominates there is a coincident interruption of the differentiation program (Wainwright et al., 2001) which can have negative consequences for downstream RNA splicing events. Moreover, the dysregulation of phosphorylation and of signal transduction are key mechanisms of transcriptional repression [86-90].

**Functional Assessment of Transcriptional Regulation by Chronic Opioids:** Within the nuclear compartment, basal transcription co-repressors (e.g., Sin3A, coREST, REST and histone deacetylases (HDACs)) suppress DNA transcription. In addition, the nuclear binding of Htt to CBP, TBP, Sp1, the CREB-TAF4 complex, or the TAFII130 complex directly represses transcription, with the direct effect of altered gene expression. Activation of CREB, a master transcription factor, is necessary for initiating gene expression. Signals transduced through the mu opioid receptor converge on CREB through several different pathways [91,92]. We investigate two transcription factors, CREB and Elk1, whose activation of gene expression occurs through different signal transduction pathways. It is our assumption that the combination of these (and possibly other) effects create an environment amenable to the downregulation of the expression of the mu opioid receptor as well as its alternatively spliced variants. Functional dysregulation of the phosphorylation machinery may explain the loss of resilience of the mu opioid receptor in morphine tolerance [93-96].

### Analytical Approach

**Experiment 1:** Undifferentiated Human Neuroblastoma (SH-SY5Y) Cell Study

The measurement outcome (dependent variable) of these experiments is “gene expression”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Categories of treatments (n=4) used in the experiments included morphine, DAMGO, naloxone, or control (media alone), each of which was administered to triplicate plates as independent experiments. MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR1-B4, MOR1-B5, MOR1-K1 and  $\beta$ -ACT genotypes (n=8) were evaluated in each sample for each treatment at one time-point (24hr). Plated cells were randomly allocated to the respective treatment groups at or near confluency. A total of 12 samples (experimental units) were prepared for analysis [97,98].

**Experiment 2:** Differentiated Human Neuroblastoma (SH-SY5Y) Cell Study

The measurement outcome (dependent variable) of these experiments is “gene expression”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Categories of treatments (n=4) used in the experiments included

morphine, DAMGO, naloxone, or control (media alone), each of which was administered to triplicate plates as independent experiments. MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR1-B4, MOR1-B5, MOR1-K1 and  $\beta$ -ACT genotypes (n=8) were evaluated in each sample for each treatment at one of three time-points (n=3), 24hr, 48hr and 72hr. Plated cells were randomly allocated to the respective treatment groups at or near confluency. A total of 36 samples (experimental units) were prepared for analysis [99-105].

### Experiment 3: Phosflow Experimental Design

This experiment tested the hypothesis that chronic morphine alters the level of serine-residue phosphorylation in differentiated human neuroblastoma (SH-SY5Y) cells using a newly introduced Phosflow® technique. The measurement outcome (dependent variable) of these experiments is “level of phosphorylation”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Treatments used in the experiment included morphine, DAMGO, naloxone, or control (media alone), each of which was administered to triplicate plates as independent experiments. Akt, Elk1, PKA and CREB genotypes were evaluated for each treatment at one of two time-points, 24hr and 48hr. Plated cells were randomly allocated to the respective treatment groups. A total of 24 samples (experimental units) as well as relevant assay setup controls were prepared for analysis [105-108].

### Experiment 4: RT2 PCR Array of Neurodegeneration Genes

This experiment examined the effects of chronic morphine on neurodevelopmental genes in differentiated human neuroblastoma (SH-SY5Y) cells using the Huntington Disease RT2 PCR Array (96-well format) Plate. The measurement outcome (dependent variable) of these experiments is “gene expression”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Treatments used in the experiment included morphine or control (media alone), each of which was administered to one plate as independent experiments. Genes spotted on each well were evaluated for a single time-points (48hr). Triplicate plates of cells were grown, treated, harvested and pooled for RNA isolation by column analysis.

### Experiment 5: RT2 PCR Array of Transcription Factors

This experiment examined the effects of chronic morphine on transcription factors in differentiated human neuroblastoma (SH-SY5Y) cells using the Transcription Factors RT2 PCR Array (96-well format) Plate. The measurement outcome (dependent variable) of these experiments is “gene expression”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Treatments used in the experiment included morphine or control (media alone), each of which was administered to one plate as independent experiments. Genes spotted on each well were evaluated for a single time-points (48hr). Triplicate plates of cells were grown, treated, harvested and pooled for RNA isolation by column analysis [108-111].

## Impact

**Potential Clinical Impact:** Morphine is the drug-of-choice for moderate to severe pain. It is a myth that long term use of morphine for pain can lead to addiction. However, its clinical use is overshadowed by the risk of tolerance development following chronic therapy. Many physicians use precautionary pharmacotherapeutic procedures to avoid tolerance development, as it may serve as a gateway to physical dependence, withdrawal and addiction in cases of opiate overuse or abuse. In the latter scenario, overdose may precipitate severe respiratory depression followed by the inadvertent death of the abuser, due pharmacologically to the absence of a ceiling concentration for morphine [4,46]. The development of morphine tolerance is associated with responses mediated through the mu opioid receptor, subtype 1 (MOR-1), for which several human alternatively spliced variants have recently been identified. To date, the relative contribution of the MOR-1 splice variants to the overall function of the OPRM1 gene and to the development of morphine tolerance has not been elucidated. Research from our laboratory suggests a potential regulatory role for MOR-1 alternatively spliced variants in morphine tolerance. Our data shows that several MOR-1 alternatively-spliced variants (i.e., MOR-1A1, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B4, MOR-1B5, MOR-1K1 and MOR-1Y1) are expressed in human neuroblastoma SH-SY5Y cells, suggesting that this cell-line is a novel model for understanding the biology of these variant receptors and specific molecular mechanisms underlying their effects [112-116]. As we currently explore the novel hypothesis that neuronally-derived alternative splice variants of the MOR-1 gene mediate the induction of morphine tolerance through potentially compensatory disinhibition of master transcription factors, it is further expected that this research will introduce new molecular targets needed to improve patient-specific interventions for morphine (and opioid) tolerance.

## Conclusion

A wealth of information remains to be gathered concerning the functional nature of opioid receptor alternatively spliced variants. Scientists are still in the formative stages of identifying and cloning larger quantities that would be conducive to further experimentation. The approach proposed in this paper foregoes cloning to prefer retinoic-acid induction of mu opioid receptor expression in the natural cell environment. Under these conditions, and with minimal handling, the potential to discover ASV contributions that may be aligned with the diverse array of morphine effects in human cells appears promising. ASVs may someday become useful in elucidating specific regions of OPRM1 that are responsible for beneficial, adverse and side effects of morphine, thereby enabling pharmacologists to identify better molecular targets for clinical and pharmacotherapeutic intervention.

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## Conflict of Interest

The author knows of no financial interest or any conflict of interest relative to this article.

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