Antimuscarinic drugs exert β-arrestin-biased agonism at the muscarinic acetylcholine type 1 receptor

by

Shayan Amiri

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ABSTRACT

The M1 muscarinic acetylcholine receptor (M_1R) is highly expressed in the nervous system, and has been studied to discover new therapeutic drugs for a range of neurological and psychiatric disorders. Studies from our lab and others also highlighted the fact that antagonists of M_1R could be therapeutic agents for neuropathies. However, the mechanism(s) of action through which M_1R antagonists exert their therapeutic effects are not well understood. Previous studies indicate that both pirenzepine (PZ), a selective M_1R antagonist, and muscarinic toxin 7 (MT7), a negative M_1R allosteric modulator, act via M_1R to promote neuritogenesis in rodent primary dorsal root ganglia (DRG) sensory neurons, in part, through β-arrestin-dependent activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). Furthermore, these antagonists reverse nerve degeneration in a variety of rodent models of peripheral neuropathy through multiple complementary pathways. To understand the therapeutic effects and mechanism of M_1R antagonist-induced ERK1/2 phosphorylation, we tested the hypothesis that PZ and MT7 possess β-arrestin-biased agonism at M_1R to drive activation of ERK and enhance neurite outgrowth. Treatment for up to 30 min with PZ and MT7 dose-dependently recruited β-arrestin2 to M_1R (analyzed using nano-BRET) and increased ERK phosphorylation in both HEK293 cells and DRG neurons. These novel pharmacological effects occurred in the absence of activation of G protein signaling or receptor internalization. PZ promoted M_1R phosphorylation at six specific serine/threonine residues (Thr^{230}, Ser^{251}, Thr^{254}, Ser^{321}, Thr^{354}, Ser^{356}) and deletion mutation of these sites suppressed PZ and MT7 induction of β-arrestin binding to M_1R and inhibited ERK activation. β-arrestin-biased activity of PZ and MT7 involved the mobilization of casein kinase 2 (CK2) and this occurred in the absence of Gαq or G protein receptor kinase (GRK) activity. Pharmacological or siRNA-based inhibition of CK2 blocked PZ-induction of β-arrestin association, ERK activation and neurite

outgrowth in DRG neurons. In conclusion, PZ/MT7 activated M_1R toward the β-arrestin signaling pathway in both HEK293 cells and DRG neurons to augment ERK activation and neurite outgrowth via engagement of CK2. Results of this work provide novel findings with far reaching consequences on our understanding of the signaling mechanism of antimuscarinic drugs.

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DEDICATION

To Woman, Life, Freedom…

and to those who lost their precious lives,

received prison sentence,

experienced torture and sexual violence

forced to immigrate,

lost their family and friends

but, did not stop fighting for equality, freedom, and human rights.

To Woman, Life, Freedom…

Table of Contents

List of Abbreviations, Symbols and Nomenclature

Abbreviation	Definition
A_1AR	Adenosine A1 receptor
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AGKOS	Agarose gel keratinocyte outgrowth system
AKAP	$G\alpha_q$-A kinase anchoring protein
AMPK	AMP-activated protein kinase
AP2	Adaptor protein 2
AT_1R	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
BDNF	Brain- derived neurotrophic factor
BK	Big conductance
CaMKKβ	Ca^{2+}/calmodulin-dependent protein kinase kinase β
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium-sensing receptor
Ca_V2	Ca^{2+} voltage-gated channel
CCP	Clathrin-coated pits
ChaT	Choline acetyltransferase
CHO	Chinese hamster ovary
CIPN	Chemotherapy-induced peripheral neuropathy
CK	Casein kinase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease

CREB	cAMP response element-binding protein
CRF	Corticotrophin-releasing factor
CXCR4	C-X-C chemokine receptor type 4
D_1R	Dopamine-1 receptor
D_2R	Dopamine-2 receptor
DAG	Diacylglycerol
DOR	δ-opioid receptor
DRG	Dorsal root ganglion
DSPN	Distal symmetric polyneuropathy
EAE	Experimental autoimmune encephalomyelitis
ECL	Extracellular loop
ECM	Extracellular matrix
ERK1/2	Extracellular signal-related kinase 1 and 2
FAK	Focal adhesion kinase
FZD	Frizzled receptors
$GABA_B$	γ-aminobutyric acid receptor B
GDP	Guanosine diphosphate
GH	Growth hormone
GIP	Calcitonin, gastric inhibitory polypeptides
GIRK	G protein-gated inward rectifier K^+
GLP	Glucagon-like peptide
GPCR	G protein-coupled receptor
GRHR	Gonadotropin-releasing hormone receptors
GRK	G protein-coupled receptor kinase
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine triphosphate
H_1R	Histamine H1 receptor

H_4R	Histamine H4 receptor
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenocortical
IBS	Irritable bowel syndrome
ICL	Intracellular loop
IGF-1	Insulin-like growth factor I
IK	Intermediate conductance
IP_3	Inositol-1,4,5- triphosphate
IUPHAR	International Union of Pharmacology Committee
JNK	c-Jun N-terminal kinase
K2P	Two-pore domain potassium
KC	Keratinocytes
K_{Ca}	Ca^{2+} activated potassium channel
Kir	Inwardly rectifying potassium
LGIC	Ligand-gated ion channels
LTD	Long-term depression
LTP	Long-term potentiation
M_1R	Muscarinic acetylcholine type 1 receptor
M_2R	Muscarinic acetylcholine type 2 receptor
M_3R	Muscarinic acetylcholine type 3 receptor
M_4R	Muscarinic acetylcholine type 4 receptor
M_5R	Muscarinic acetylcholine type 5 receptor
mAChRs	Muscarinic acetylcholine receptors
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MAPK	Mitogen-activated protein kinase

MCH	Melanin-concentrating hormone
mGluRs	Metabotropic glutamate receptors
MMP3	Matrix metalloprotease 3
MOR	μ opioid receptor
MS	Multiple sclerosis
MT7	Muscarinic toxin 7
nAChRs	Nicotinic acetylcholine receptors
NAM	Negative allosteric modulators
NGF	Nerve growth factor
NMF	Natural moisturizing factor
NMJ	Neuromuscular junction
NNCS	Non-neuronal cholinergic system
NT-4	Neurotrophin 4
OAB	Overactive bladder
OPC	Oligodendrocyte progenitor cells
PAM	Positive allosteric modulators
pChAT	Peripheral form of choline acetyltransferase
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3’ kinase
PIP_2	Phosphoinositol-1,4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
PSC	Perisynaptic Schwann cells
PSM	Peptide-spectrum matches
PV	Pemphigus vulgaris

PZ	Pirenzepine
SK	Small conductance
SMO	Smoothened receptor
STAM1	Signal-transducing adaptor molecule 1
TASK	Two-pore domain, acid-sensitive K^+ channel
TM	Transmembrane
TREK	TWIK-related K^+ channel
TRHR	Thyrotropin-releasing hormone receptor
TrkB	Tropomyosin-related kinase B receptor
TRPC	Transient receptor potential channel
TRPM	Transient receptor potential melastatin
TWIK	Tandem of P-domains in a weak inward rectifying K^+ channel
VAChT	Vesicular acetylcholine transporter
VFTM	Venus flytrap module
VIP	Secretin and vasoactive intestinal peptide
VTA	Ventral tegmental area
β_2AR	β2-adrenergic receptor

List of Figures and Tables

Figures

Supplementary figures

Tables

Chapter 1:

Background and literature review

1.1 Overview of cholinergic system

Acetylcholine (ACh) is synthesized by choline acetyltransferase (ChAT) and is hydrolyzed by acetylcholinesterase (AChE) in different biological systems. Cholinergic receptors, including nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), are responsible for the biological effects of ACh. These two classes of receptors have significant structural and functional differences. Having high affinity to nicotine, the nAChRs are the well-characterized members of the pentameric ligand-gated ion channels (LGIC) family, which are distributed in neuronal and non-neuronal cells and contribute to the regulation of several physiological responses such as cognitive functions, food intake, and anxiety. Nicotine binds to the α subunit of the receptor and activates the opening of a non-specific cation channel consisted of different combinations of other subunits such as β subunit (Dani and Bertrand, 2007). The muscarinic effects of ACh are mediated through five mAChR subtypes (M1-M5) expressed in almost all cell types in the body (Caulfield, 1993; Caulfield and Birdsall, 1998). Unlike nAChRs, the mAChRs are seven transmembrane G-protein coupled receptors (GPCR) and are divided into two distinct functional classes according to their ability in the activation of diverse heterotrimeric G proteins. Generally, the muscarinic M2 and M4 receptors couple to G proteins of the $G\alpha_i$ family, which inhibit adenylate cyclase (AC) activity and the M1, M3, and M5 receptors couple to G proteins of the $G\alpha_q$ family, which activate phospholipase C (PLC) (Caulfield, 1993; Caulfield and Birdsall, 1998). However, these receptors are able to bind to more than one G protein subtype, and

M_1R binds to $G\alpha_i$ and $G\alpha_s$ families as well (Avet et al., 2022). The muscarinic actions of ACh on peripheral tissues (such as decrease in the heart rate, increase in glandular secretion and smooth muscle stimulation) are mediated by mAChRs (Caulfield, 1993). In the central nervous system (CNS), mAChRs are responsible for the regulation of a wide range of behavioral, cognitive, autonomic, sensory, and motor functions (Bubser et al., 2012; Thiele, 2013; Thomsen et al., 2018; Wess et al., 2007). Ample evidence indicates that dysregulated signaling through distinct mAChR subtypes plays a critical role in the pathobiology of many human disorders such as Alzheimer's disease (AD) (Flynn et al., 1995), epilepsy (Friedman et al., 2007), schizophrenia (Crook et al., 2000), cancer (Shah et al., 2009) and metabolic disorders like type 2 diabetes and obesity (Wess et al., 2007). It is important to note that involvement of cholinergic system in the pathogenesis of human disorders is not limited to the nervous system. Increasing lines of research indicate non-neuronal cholinergic system (NNCS) is dysregulated in several human diseases and contributes to their pathophysiology (Beckmann and Lips, 2013; Wessler and Kirkpatrick, 2008). In the current work, I briefly introduce mAChR subtypes (with a focus on M_1R), discuss the role of ACh in neuronal damage-repair process and axonopathies, and finally review the evidence for the therapeutic effects of muscarinic antagonists for peripheral neuropathies.

1.1.1 General introduction for G Protein-Coupled Receptors (GPCRs)

GPCRs are seven transmembrane-spanning proteins, which are known as one of the largest protein families encoded by the human genome (Southan et al., 2016). Mostly located on the cell membrane, GPCRs play crucial roles in transducing extracellular signals (in response to peptides, ions, neurotransmitters, pheromones, odorants and photons) into key physiological responses (Katritch et al., 2013; Pierce et al., 2002). GPCRs are implicated in a vast majority of physiological processes and large number of diseases such as metabolic disorders, neurological disorders,

cancers and many others. GPCRs are easy to access proteins with diversified downstream signaling pathways, which make them interesting candidates for drug development. Indeed, GPCRs are the target of a large proportion (almost 30%) of drugs in the market (Overington et al., 2006; Sriram and Insel, 2018). However, it should be noted that GPCRs have extreme diversity in their cellular function, tissue distribution/expression, and endogenous/exogenous ligands. These features have led to slow progress in drug discovery projects due to poor efficacy or off-target adverse effects. Currently, drug discovery programs aim to expand the list of targeted GPCRs to find drugs, which are more selective with better efficacy and less off-target effects. To do this, the focus of GPCR research is to discover allosteric and/or functionally selective modulators, which are able to activate biased downstream signaling toward specific G protein($G\alpha_q$/$G\alpha_s$/Ga_i)-activated or β-arrestin-activated pathways (Katritch et al., 2013; Stallaert et al., 2011; Valant et al., 2012).

1.1.2 Classification of GPCRs

GPCRs are a diverse, complex group of integral membrane proteins, but they have a common basic structure. They share 7 α-helical hydrophobic transmembrane (TM 1-7) domains, bridged by 3 intracellular (ICL) and extracellular (ECL) loops, and connected to extracellular N-terminus and an intracellular C-terminus (Venkatakrishnan et al., 2013). International Union of Pharmacology Committee on Receptor Nomenclature and Classification (NC-IUPHAR) classified GPCRs into subfamilies based on phylogenetic features, and divided them into 5 subfamilies including A, B, C, Frizzled and other receptors (Foord et al., 2005; Lagerström and Schiöth, 2008). Please see **Figure 1.**

1.1.2.1 Family A/Rhodopsin GPCRs

Family A/Rhodopsin is considered the largest and most intensely studied family of GPCRs with almost 700 members including light activated and olfactory receptors (Yang et al., 2021). In recent years, researchers have determined several crystal structures of class A GPCRs bound with different ligands, peptides, G proteins and antibodies (Haga et al., 2012; Hanson et al., 2012; Thal et al., 2016). These valuable studies provided novel insights into the structural and functional diversity of class A GPCRs. One interesting fact about this family is that they have a wide range of endogenous ligands. They are divided into 4 groups (α, β, γ and δ) based on this ligand diversity. Endogenous ligands for α-group GPCRs are amines (such as muscarinic, adrenergic, dopamine and serotonin receptors) while β-group GPCRs in class A are activated by peptides (such as the oxytocin receptor). GPCRs in γ-group are activated by both peptides and lipid-like ligands (angiotensin and opioid receptors), while the δ-group receptors prefer purines and proteases as their ligands (Lagerström and Schiöth, 2008). Despite the ligand diversity for family A GPCRs, all ligands are known to bind in a ligand binding pocket in the extracellular side of the TM bundle with key residues in TM3, TM6 and TM7 (Hulme, 2013; Venkatakrishnan et al., 2013)

1.1.2.2 Family B/Adhesion and Secretin GPCRs

Family B GPCRs, the second largest family in humans, is comprised of 15 Secretin-like receptors and 33 Adhesion receptors. The Secretin-like receptors are marked with an extracellular hormone-binding domain, a conserved cysteine residue sequence in the N-terminus, and extracellular loops, which enables them to bind several hormones such as glucagon, corticotrophin-releasing factor (CRF), calcitonin, gastric inhibitory polypeptides (GIP), glucagon-like peptide (GLP), secretin and vasoactive intestinal peptide (VIP) (de Graaf et al., 2017; Yang et al., 2021). One of the main differences between Adhesion receptors and Secretin-like receptors is N-terminal architecture, which has multiple domains contributing to the specificity of receptor ligand binding interactions

(de Graaf et al., 2017; Lagerström and Schiöth, 2008). In addition, unlike Secretin-like receptors, Adhesion receptors bind extracellular matrix molecules rather than peptide hormones (Lagerström and Schiöth, 2008). Glucagon and the CRF-1 receptors were among the first studied GPCRs in class B for their crystal structures (Hollenstein et al., 2013; Siu et al., 2013). Despite some similarities in the intracellular positions, class B GPCRs show obvious differences with class A GPCRs at the extracellular domains (Sexton and Wootten, 2013). Another difference between the 2 classes is that class B GPCRs lack the conserved amino acid motifs (such as DRY and NPxxY), which are seen in class A GPCRs (Siu et al., 2013).

1.1.2.3 Family C/ Glutamate Receptors

The γ-aminobutyric acid receptor B ($GABA_B$), the metabotropic glutamate receptors (mGluRs), and the calcium-sensing receptor (CaSR) are among the 22 members of family C/ Glutamate GPCRs in humans. These receptors possess a unique distinct extracellular domain inclusive of the Venus flytrap module (VFTM), which is responsible for ligand recognition and binding. Indeed, most GPCRs in this family bind their endogenous orthosteric ligand within their N-terminal region (Cao et al., 2009). Also, family C GPCRs exist constitutively in the form of homo- or heterodimers, which is necessary for their structural and functional activities such as trafficking and signaling (Comps-Agrar et al., 2011; Kniazeff et al., 2011). Among class C GPCRs, mGlu1R and mGlu5R crystal structures were studied in recent years (Doré et al., 2014; Wu et al., 2014).

1.1.2.4 Frizzled/ Taste 2 GPCRs

This family of GPCRs (also known as F family) consists of the smoothened receptor (SMO) and 10 frizzled receptors (FZD 1-10). The frizzled receptors are involved in Wnt signaling and play a major role in embryonic development and adult organogenesis. Smoothened receptor binds small

organic compounds and contributes to Hedgehog signaling, which is critical for maintaining homeostasis of cellular differentiation. (Bansal et al., 2023; Schulte and Kozielewicz, 2020; Wang and Malbon, 2004). Taste 2 receptors (TAS2Rs), the receptors modulating taste perception of humans, show structural similarity with class A GPCRs, but their low sequence homology (<20%) with the existing types of GPCRs isolates them to a novel category of class T GPCRs (Zhang et al., 2024). Both FZD and SMO receptors have an extracellular cysteine-rich domain, where Wnt glycoproteins bind to FZD receptors, but its role is not clear for SMO receptor (Wang et al., 2013). Recently, crystal structures of several members of F family GPCRs have been studied. These studies provided detailed insights into the structure and function of these receptors (Wang et al., 2014; Wang et al., 2013).

Receptor-ligand complexes count (from low to high)

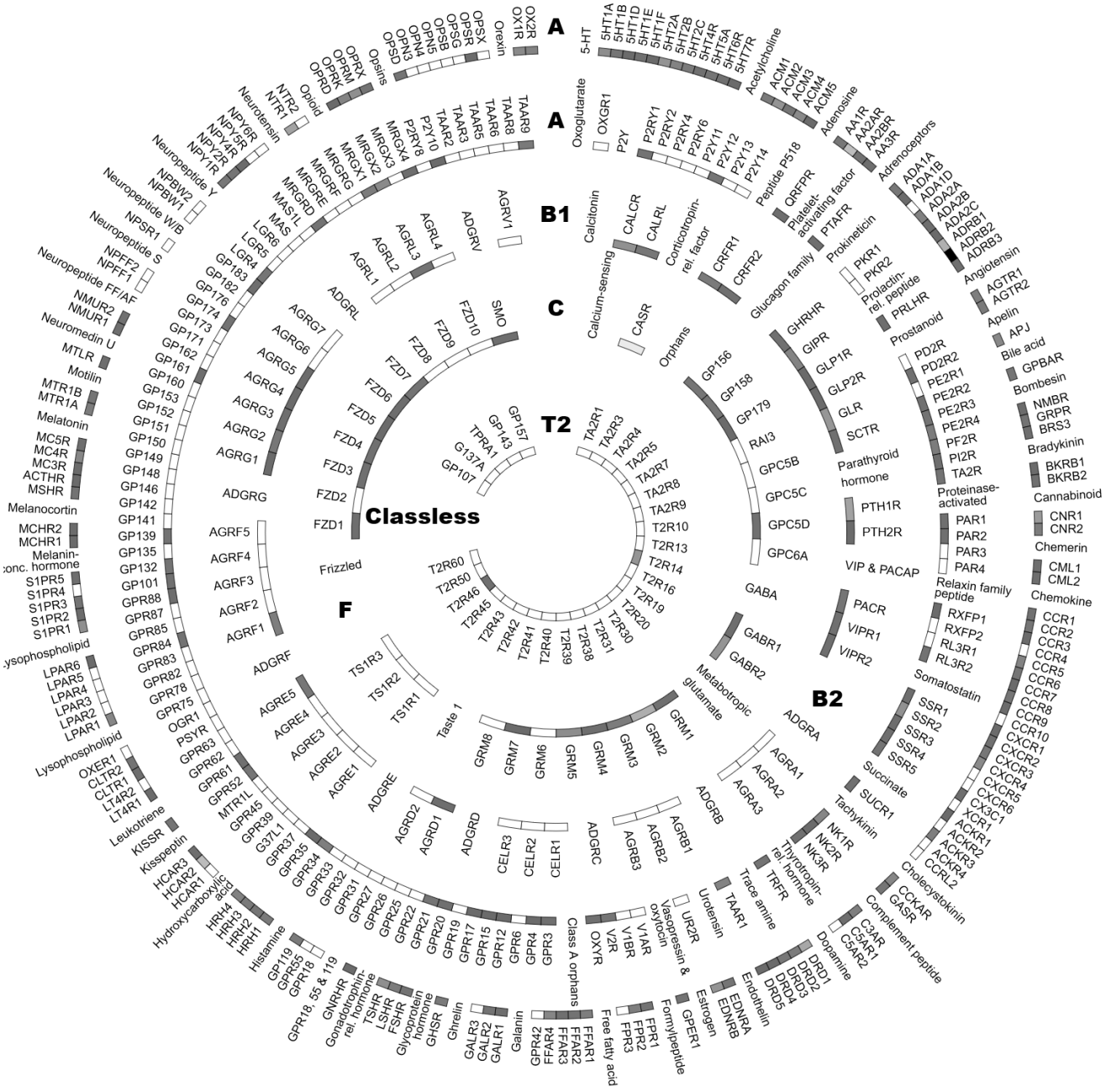


Figure 1. GPCR classification. The GPCRome wheel plot shows non-odorant human GPCRome. The outer to inner layers list receptors by their class, as follows: class A with known physiological ligands, class B (B1/Secretin and B2/Adhesion families), class C, class T2 and class F. Plot represents GPCRs named according to their UniProt gene name and are organized according to the GPCR database. Figure is extracted from https://gpcrdb.org/structure/statistics.

1.1.3 GPCR activation

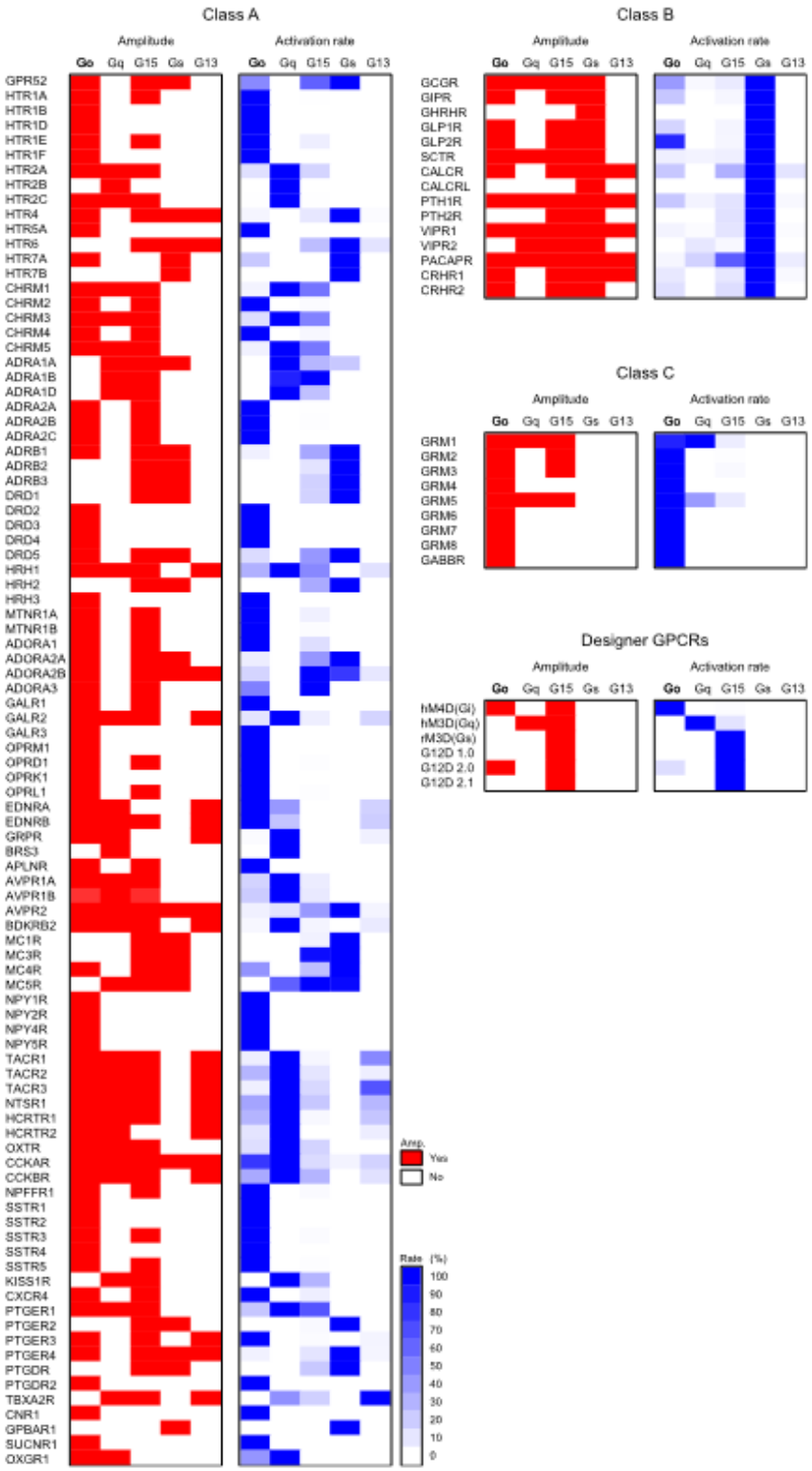
1.1.3.1 G protein signaling

In classic pharmacology, activation of GPCRs occur when orthosteric ligands bind to the GPCR orthosteric site (where the endogenous ligand binds). Orthosteric ligands are divided into 3 main categories including 1) agonists (activating GPCR), 2) inverse agonists (inactivating GPCRs by reducing constitutive activity), and 3) antagonists (neutral compounds that bind to the orthosteric site and have no effect). While the majority of natural ligands are agonists, some are antagonists (such as melanocortin antagonists). Activation of GPCRs results in the outward movement of TM V and VI helices, forming a cavity on the cytoplasmic side of the receptor, which acts as a docking site for the heterotrimeric G proteins (Farrens et al., 1996; Gurevich and Gurevich, 2019). A diverse range of extracellular ligands are capable of activating GPCRs in several different ways. A classic GPCR signaling pathway is where ligand-activated GPCR engage intracellular G proteins (Wang et al., 2018). G proteins are heterotrimeric proteins that are made of alpha (α), beta (β) and gamma (γ) subunits (Gilman, 1987). As mentioned before, GPCR activation is accompanied by conformational changes leading to TM helices rearrangement, which provides space for binding many effector proteins, such as G proteins (Pierce et al., 2002). The ligand-activated GPCRs act as a guanine exchange factor to boost the catalytic exchange of GDP for GTP at the α subunit of G proteins. Once activated, the heterotrimeric G protein dissociates into the α-GTP-bound subunit and the βγ complex, where each is capable of regulating distinct effectors and triggering several signaling pathways (Gilman, 1987; Milligan and Kostenis, 2006). While classic G protein-dependent signaling is accepted worldwide, there are some exceptions such as activation

of cell signaling without heterotrimeric G protein dissociation (Frank et al., 2005) and GPCR-G protein pre-assembly (Challiss and Wess, 2011; Qin et al., 2011).

G proteins classification is based on their α subunit, which are classically divided into 4 major families $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$. Each of these G protein families activates a specific intracellular signaling pathways (Gilman, 1987; Wess, 1998). Recruitment of $G\alpha_s$ by GPCR results in enzymatic activity of the ACs, which convert ATP to cyclic AMP (cAMP), and activates protein kinase A (PKA). However, the activation of $G\alpha_{i/o}$ blocks ACs activation and cAMP signaling. Recruitment of $G\alpha_{q/11}$ by GPCRs leads to activation of PLC, which causes hydrolysis of phosphoinositol-1,4,5-biphosphate (PIP_2) into inositol-1,4,5- triphosphate (IP_3) and diacylglycerol (DAG). Each of these secondary messengers activates separate signaling pathways by increasing intracellular Ca^{2+} levels and stimulation of protein kinase C (PKC) (Felder, 1995). Lastly, $G\alpha_{12/13}$ G proteins bind and activate Rho-specific guanine nucleotide exchange factors and initiate a range of effects such as cytoskeletal rearrangements (Milligan and Kostenis, 2006; Wess, 1998). The effects of G protein activation are not limited to α-subunits and the βγ complex also plays a major role in cellular signaling processes (Bondar and Lazar, 2014; Khan et al., 2013). Indeed, the βγ complex contributes to the activation of 1) extracellular signal-related kinase 1 and 2 (pERK1/2), 2) G protein-gated inward rectifier K^+ (GIRK) channels, and 3) PLC and phosphatidylinositol 3' kinase (PI3K) (Gutkind, 2000; Whorton and MacKinnon, 2013). Most GPCRs couple to more than one G protein and trigger several signaling pathways. This effect depends on several factors such as cell-type, dose and nature of drugs (Defea, 2008; Liu et al., 2015; Wisler et al., 2018). **Figure 2** shows the G protein coupling selectivity for different classes of GPCRs.

Figure 2. GPCRs and G protein coupling selectivity. Subfamilies of GPCRs and their corresponding known binding G proteins are shown. Graph is derived from Masuho *et al.* (Masuho et al., 2023).



1.1.3.2 G protein independent signaling

In addition to G protein-dependent signaling, recent studies on GPCRs discovered several new modes of GPCR activation, which shed light on the complex behavior of GPCRs. Among these new modes of activation, β-arrestins were studied extensively (Pyne and Pyne, 2011; Shukla et al., 2013; Shukla et al., 2014; Wang et al., 2018; Zheng et al., 2012). Mammals express 4 arrestin subtypes, which bind activated phosphorylated GPCRs. Among 4 arrestin subtypes, arrestin1 and 4 are expressed in photoreceptors in the retina, and arrestin 2 and 3 (also known as β-arrestin 1 and 2 or non-visual arrestins) are responsible for serving all other GPCRs (Gurevich, 2024). Classically, following GPCR activation, G protein signaling is terminated via receptor phosphorylation at the cytoplasmic loops and C-tail by G protein-coupled receptor kinases (GRKs). This promotes binding of β-arrestins to the receptor and uncoupling of the receptor from G proteins, a process known as desensitization. Recruitment and binding of β-arrestins leads to initiation of receptor internalization via clathrin-coated pits (CCP) (Gurevich and Gurevich, 2024; Rajagopal et al., 2010). Classically, activated GPCRs couple to 1) GRKs, 2) heterotrimeric G proteins, and 3) β-arrestins, where the latter two serve as signal transducers. Indeed, β-arrestins not only are known as mediators of GPCR desensitization, but also as multifunctional adaptor proteins capable of initiating several signaling pathways, particularly mitogen-activated protein kinases (MAPKs) and c-Jun N-terminal kinases (JNKs) (Defea, 2008; Gurevich and Gurevich, 2024; McDonald et al., 2000).

1.1.4 Biased signaling

Initially, GPCR signaling was thought to be governed mainly by intracellular G proteins. In the classic two-state model, binding of full or partial agonists stabilize the active state of GPCRs, while inverse agonists stabilize the inactive state, and antagonists block both agonist/inverse agonist-

induced activities (Stallaert et al., 2011). However, in recent decades, many studies showed that the two-state paradigm was unable to explain some drug responses in GPCRs, thus a new concept of biased signaling was emerged in pharmacology (Azzi et al., 2003; Violin et al., 2014). Biased signaling, also known as functional selectively or biased agonism, refers to the ability of each ligand for a distinct receptor to induce differential efficiency for diverse signaling responses. Indeed, different ligands for a particular GPCR are able to uniquely induce conformational changes in the receptor, which leads to activation of one or several signaling pathways. For example, when a ligand selectively activates either the G protein or the β-arrestin pathway, it is called a G protein biased or β-arrestin biased ligand (Violin et al., 2014; Wootten et al., 2018). An example for β-arrestin biased signaling is angiotensin II type 1 receptor (AT_1R). Once activated by angiotensin II, activation of the G protein pathway results in vasoconstriction and increased blood pressure, while β-arrestin signaling is associated with beneficial effects such as antiapoptotic and cytoprotection effects. TRV120027 is a AT_1R ligand that selectively activates β-arrestin signaling mediated cardiomyocyte contractility and cardiac output, while blocking G protein signaling mediated vascular contraction. Thus, TRV120027 is a β-arrestin biased ligand for AT_1R with potential for treating acute heart failure (Ahn et al., 2009; Ryba et al., 2017; Violin et al., 2010). An example for G protein biased agonist is TRV130 (Oliceridine), which activates the μ-opioid receptor, and is the first FDA approved biased agonist. In some GPCRs, β-arrestin signaling is associated with unwanted/adverse effects, while G protein signaling mediates beneficial and therapeutic effects. In the case of μ-opioid receptor, adverse effects of morphine (such as pain tolerance, constipation, and respiratory arrest) are mediated by the β-arrestin pathway, while therapeutic effects such as analgesia are mediated by the G protein pathway. TRV130, has lower

adverse effects on gastrointestinal tract and respiratory function while having analgesic effects (Daksla et al., 2023; DeWire et al., 2013).

Generally, biased responses occur in 3 different levels in biological systems; ligands, receptors, and transducers. Biased receptors are GPCRs that have been modified in a way that their ability to bind specific ligands/transducers are altered. This can be the result of mutations, different splicing, and epigenetic regulations. An example of a biased receptor is substitution of Ala293 in the alpha-1 adrenergic receptor which leads to constitutive G protein activity (Kjelsberg et al., 1992; Smith et al., 2018). System bias (also known as apparent biased agonism) occurs when receptors and transducers have differential expression in different cell types and tissues. For example, some dopamine-2 receptor (D_2R) agonists induce different responses in the striatum and prefrontal cortex due to different expression levels of arrestins and GRKs in these 2 brains structures (Smith et al., 2018; Urs et al., 2016). **Figure 3** shows schematic concept for G protein, and β-arrestin biased signaling.

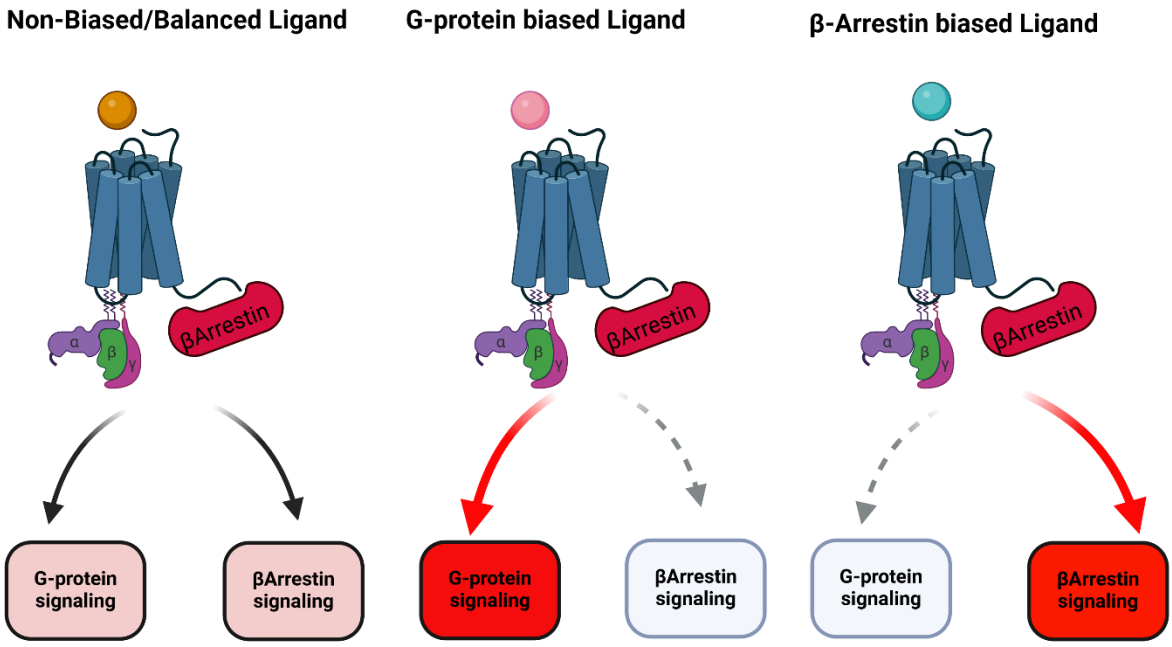


Figure 3. **Biased signaling at GPCR.** Schematic presentation of ligands activity at GPCRs. A balanced ligand activates both arrestin and G protein pathways. A G protein-biased ligand activates G protein signaling without triggering arrestin pathway, and an arrestin-biased ligand activates the arrestin pathway without triggering G protein signaling.

1.1.4.1 β-arrestin biased signaling

Ligand-stabilized receptor conformations drive the selection of β-arrestins or G protein interactions and these selections can be discerned within G protein subunit families. For example, oxytocin analogs differentiate between individual $G_{i/o}$ family members (Busnelli et al., 2012). Ligand bias between subunit members of G protein families was reported for agonists for μ-opioid receptors ($G\alpha_{i1}$, $G\alpha_{oA}$) (Saidak et al., 2006), and dopamine receptors ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$) (Möller et al., 2017). Bias between G_{α} and $G_{\beta\gamma}$ subunit activation also was reported. For example, the small molecule agonist GUE1654 for $G_{i/o}$-coupled oxoeicosanoid receptors caused inhibition

of $G_{\beta\gamma}$ otherwise allowing activation of G_{α} dependent pathways (Blättermann et al., 2012). As mentioned above, certain ligands for a distinct GPCR are capable of inducing conformational changes in the receptor, which results in selective activation of the β-arrestin pathway. To date, there are several ligands, which have been reported as β-arrestin biased ligands for several GPCRs (Morales et al., 2024). Indeed, ligands induce specific alterations in the GPCR secondary and tertiary structure, and facilitate the recruitment of a range of proteins, which are capable of inducing post-translational modifications in the C-terminus or intracellular loops of GPCRs. Among these modifications, GPCR phosphorylation and ubiquitination induce biased signaling through facilitating the recruitment of selective transducers (Patwardhan et al., 2021; Shenoy and Lefkowitz, 2011).

1.1.4.2 β-arrestin biased signaling and GPCR phosphorylation

Phosphorylation of GPCRs is mainly regulated by GRKs. In addition to GPCR phosphorylation, GRKs have several functions in cells. GRKs only phosphorylate serine/threonine residues in the activated receptor, and facilitate binding of arrestins to phosphorylated GPCRs with higher affinity than unphosphorylated receptors (Gurevich and Gurevich, 2019). There are 7 GRK genes, which are conserved among mammals, giving rise to GRK1-7, which are soluble proteins. GRK1 and 7, also known as visual GRKs, are expressed in the retinal rod cells and their expression is restricted to the visual system. These GRKs have a prenylated C-termini, which allows their constitutive membrane localization (Penn et al., 2000). Another group of GRKs are the cytosolic GRK2 and GRK3, which bind to Gβγ via their pleckstrin homology (PH) domain allowing localization to the vicinity of the plasma membrane (Koch et al., 1993; Touhara et al., 1994). Unlike the latter 2 groups, GRKs 4, 5 and 6 are mostly attached to the membrane via palmitoylation and/or lipid-binding motifs in most cells. Interestingly, activation of GRKs 4, 5 or 6 does require

binding to agonist-activated GPCRs, and they can be activated by a variety of molecules (such as phospholipids) (Kunapuli et al., 1994). Also, GRKs 4, 5 and 6 phosphorylate inactive GPCRs (Baameur et al., 2010; Li et al., 2015a). GRK4 expression is restricted to myometrium, brain, testes, kidney and uterus, while GRKs 2, 3, 5, and 6 are ubiquitously expressed in all cells (Gurevich et al., 2012).

Although the majority of GPCRs are phosphorylated by GRKs, there are other kinases that phosphorylate and regulate GPCR activity and function. Among the first kinases shown to phosphorylate GPCRs were protein kinase A (PKA) and protein kinase C (PKC) (Benovic et al., 1985). Casein kinases (CK) such as CK1 and CK2 phosphorylate a range of GPCRs (Tobin, 2002). These proteins comprise a small part of the whole kinome, but are responsible for the phosphorylation of 40-50% of non-redundant phosphosites in humans (Jiang et al., 2018; Venerando et al., 2014). CK1 phosphorylates adrenergic and muscarinic receptors, and in the case of muscarinic acetylcholine M3 receptor (M_3R), CK1 phosphorylation activates the ERK1/2 pathway (Budd et al., 2001; Tobin, 2008). Similarly, CK2 phosphorylates thyrotropin-releasing hormone receptor (TRHR), and muscarinic receptors (Hanyaloglu et al., 2001; Torrecilla et al., 2007). Early studies on CK2 showed that this kinase mostly phosphorylates receptors at their ICL3 region, which is associated with arrestin binding but not receptor internalization and ERK1/2 activation. However, recent studies showed that receptor phosphorylation (for example M_1R) by CK2 results in β-arrestin recruitment and ERK1/2 activation (Jung et al., 2017; Tobin, 2008). Also, CK2 was reported to negatively regulate insulin release through M_3R both *in vitro* and *in vivo* (Rossi et al., 2015). CK2 activity also negatively affects $G\alpha_s$ signaling associated with dopamine D_1 (D_1R) and adenosine A2A receptors (Rebholz et al., 2009).

It is important to note that phosphorylation of GPCRs by GRKs or CKs plays a key role in GPCR-arrestin interaction. Ample evidence indicates that each ligand has the potential to induce unique patterns of GPCR phosphorylation, which is associated with recruitment of specific GRK or CK enzymes (Smith et al., 2018; Tobin, 2008). Intensity and pattern of receptor phosphorylation determines the strength of GPCR-arrestin interaction and subsequent activation of downstream pathways (Butcher et al., 2011; Cahill et al., 2017; Zidar et al., 2009). This ability of ligands to induce specific phosphorylation patterns has been termed as the barcode hypothesis, which explains how phosphorylation of different patterns of residues at GPCR results in different arrestin related signaling pathways (Tobin, 2008). It is clear that specific patterns of phosphorylation change GPCR-arrestin interaction, which then activates a certain signaling pathway (Bouzo-Lorenzo et al., 2016; Jung et al., 2017; Lee et al., 2016). Despite the fact that the majority of literature support the concept of a barcode hypothesis, it remains unclear how different patterns of phosphorylation affect arrestin behavior and conformations.

1.1.4.3 Downstream consequences of biased β-arrestin signaling.

First, it should be noted that β-arrestin-associated signaling occurs either via free arrestins or the GPCR-arrestin complex. In this section I focus on the GPCR-arrestin complex-related signaling events, and free arrestin signaling has been discussed elsewhere (Gurevich and Gurevich, 2024). In recent years, there has been remarkable progress in our understanding of the structural details of GPCR–β-arrestin interactions and how various conformations of receptor-bound β-arrestins regulate receptor signaling and desensitization. A large body of studies identified two distinct elements in receptors as key for their interaction with β-arrestins. These are the phosphorylated

carboxyl terminus (also referred to as the receptor 'tail') and the cytoplasmic surface of the GPCR (also referred to as the receptor 'core') (Ranjan et al., 2017). In some GPCRs like vasopressin receptor (V_2R), partially bound arrestin (tail mode) is enough for arrestin-mediated receptor desensitization, internalization, and ERK activation (Kumari et al., 2017). However, full β-arrestin engagement (core) is required for arrestin-mediated G protein-independent signaling pathways, such as the activation of ERK for most GPCRs (Chen and Tesmer, 2022)

As stated earlier, arrestins act as a scaffold for ERK and JNK. MAPK cascades are conserved in all eukaryotes, starting with MAPK kinase kinases (MAP3Ks), which phosphorylate the kinases of MAPK kinases (MAP2Ks), and the latter activate effector MAPKs (Johnson, 2011). Since arrestins often contribute to ERK1/2 activation by GPCRs, ERK1/2 activation is considered as a hallmark of GPCR signaling through arrestins. Although GPCRs are capable of activation of ERK1/2 by $G\alpha_s$, $G\alpha_q$, and $G\beta\gamma$-mediated mechanisms, and ERK1/2 activation was reported in arrestin knockout cells, it is clear that GPCR-induced ERK1/2 is sensitive to the presence of arrestins (Burghi et al., 2023; Coffa et al., 2011; Luttrell et al., 2018; Naor et al., 2000; O'Hayre et al., 2017; Shenoy et al., 2006). All visual and non-visual arrestins bind to all 3 members of the ERK1/2 activation cascade, which are cRaf (Qu et al., 2021), MEK1 (Meng et al., 2009), and ERK1/2 (Perry-Hauser et al., 2022). However, the GPCR-arrestin complex has a higher affinity for ERK (Luttrell et al., 2001). Interestingly, binding of arrestins to GPCRs is necessary for their ability to activate ERK1/2. GPCRs induce a specific conformational change in arrestins, which enables them to activate ERK1/2, and this effect is persistent even when arrestins dissociate from GPCRs (Eichel et al., 2018; Eichel et al., 2016). GPCR-arrestin complex signaling is not restricted to the ERK1/2 pathway. Arrestins bound to GPCRs are able to activate the Src pathway in the case of β2AR and D_1R (Kaya et al., 2020; Luttrell et al., 1999). Src is a non-receptor cytoplasmic

tyrosine kinase in which its activation occurs upon stimulation of cell membrane receptors, such as integrins (Guarino, 2010).

Another protein that arrestin-GPCR complex activates is signal-transducing adaptor molecule 1 (STAM1). Upon activation of the C-X-C chemokine receptor type 4 (CXCR4), β-arrestin 1 recruits STAM1 resulting in the activation of focal adhesion kinase (FAK) and CXCR4-induced chemotaxis (Zhuo et al., 2020). Thus, β-arrestin biased signaling has 3 branches including ERK1/2, Src, and FAK activation. Among these branches, ERK1/2 is the most studied one with few studies published on the Src and FAK. With regard to the peripheral nervous system (PNS), *in vitro* studies showed the development of peripheral neurons required neurotrophins and consequently ERK1/2 activation, which led to differentiation and neurite outgrowth (Atwal et al., 2000; Kolkova et al., 2000; Markus et al., 2002; Newbern et al., 2011). However, Newbern *et al*., showed that ERK1/2 did not play a crucial role in the prenatal development of dorsal root ganglia (DRG) and motor neurons under *in vivo* conditions. In contrast, ERK1/2 signaling was necessary for PNS myelination and Schwann cell development (Newbern et al., 2011). They also found that cutaneous innervation mediated by DRG neurons was ERK1/2 dependent. Other studies showed that reduction in ERK phosphorylation during PNS development via B-RAF deletion had no effect on DRG survival but ERK1/2 phosphorylation played an essential role in the survival of sensory neurons in adulthood (O'Brien et al., 2015; Zhong et al., 2007). Similarly, ERK activation was a necessity for the axotomy-induced growth cone formation (Chierzi et al., 2005; Wiklund et al., 2002). Some studies also showed an increase in ERK phosphorylation following sciatic nerve injury (Agthong et al., 2006; Sheu et al., 2000; Yamazaki et al., 2009). It was reported that phospho-ERK-vimentin complex was transported to the nucleus and increased the expression of genes (such as *Elk1*) associated with regeneration (Perlson et al., 2005; Perlson et al., 2006).

Results from our lab showed that treating DRG neurons with muscarinic antagonist, muscarinic toxin 7 (MT7), increased ERK phosphorylation and neurite outgrowth via cAMP response element-binding protein (CREB) activation (Sabbir and Fernyhough, 2018). There are similar studies in the literature that showed activation of a GPCR-β-arrestin-ERK pathway increased neuronal survival. Emery *et al*., showed that activation of mGluR1 was associated with neuronal survival via sustained β-arrestin-1 and ERK1/2 signaling (Emery et al., 2010). Interestingly, in another study, β-arrestin2-ERK1/2 biased agonism at β-adrenergic receptor by propranol and carvedilol increased survival of hippocampal neurons (Tzingounis et al., 2010). These results highlighted the importance of ERK1/2 signaling in neuronal survival in both CNS and PNS.

1.2 Muscarinic acetylcholine receptors

1.2.1 An overview of muscarinic acetylcholine receptors

More than a century ago, Henry Hallett Dale reported the differences between muscarinic and nicotinic actions of ACh, which today we consider as ACh effects through muscarinic and nicotinic receptors. In 1951, Riker and Wescoe reported the first evidence on the existence of mAChR subtypes by showing the cardiospecific effects of gallamine (Riker and Wescoe, 1951). The introduction of pirenzepine (PZ) in the early 80s led to the identification of three subtypes of mAChRs (Birdsall et al., 1983; Hammer et al., 1980). However, progress in our understanding of the function and role of distinct mAChRs subtypes has been achieved by the cloning of five genes encoding mammalian muscarinic receptors in the 80s and 90s (Bonner et al., 1987; Bonner et al., 1988; Kubo et al., 1986a; Kubo et al., 1986b; Peralta et al., 1987). According to NC-IUPHAR, mAChRs were classified as M1, M2, M3, M4, and M5 (Caulfield and Birdsall, 1998). These

receptors are members of class I seven transmembrane helix GPCRs, which are distributed in almost all tissues and mediate the hormonal/neuronal effects of ACh. In general, mAChRs are involved in numerous behavioral, sensory, and motor functions in the CNS, while their eminent role in the periphery is to regulate heart rate, glandular secretion and smooth muscle contraction (Eglen, 2006; Wess et al., 2007). Considering the key physiological effects mediated by mAChRs, numerous attempts have been made to develop and design pharmacologic agents that selectively modulate distinct subtypes of mAChRs. The majority of muscarinic ligands bind to the orthosteric binding site (endogenous ligand binding site), which is a highly conserved region placed within mAChRs (TM3 and TM5-TM7) (Hulme et al., 2003). Except Asp^{105}, which is conserved in many GPCRs of Class I and recognizes the polar head of ACh, Thr^{231}, Thr^{234}, Tyr^{148}, Tyr^{506}, Tyr^{529}, and Tyr^{533} are key residues involved in mAChRs function (Hulme et al., 1999; Leach et al., 2012). Due to the high homology among mAChRs, the majority of developed derivatives exhibit no *intra* subtype selectivity and show several adverse effects restricting their clinical use (Felder et al., 2000). Besides orthosteric site, several allosteric sites have been identified in mAChRs, which are capable of modulating the receptor function following ligand binding. Allosteric agonists are capable of stabilizing a unique receptor conformation, which alters (increase/decrease) the receptor response to orthosteric ligands (Presland, 2005; Wess, 2005). Intriguingly, recent advances in molecular pharmacology resulted in the development of several allosteric compounds that displayed *intra* subtype selectivity for mAChRs (Conn et al., 2009a; Conn et al., 2009b).

As described above, mAChRs signal through heterotrimeric G proteins and trigger several second messengers in various tissues. The preference for the coupling between the mAChRs and Gα subunit is determined within the ICL3. Interestingly, the amino acid residues in the ICL3 are similar between group I and group II, but the homology is low among these two different groups

(Kostenis et al., 1999; Liu et al., 1996). In general, group I mAChRs mobilize IP_3 and DAG through the activation of PLCβ, leading to the elevation of intracellular Ca^{2+} levels. Also, other cellular messengers (such as nitric oxide, NO) are activated via M1, M3, and M5 receptors. On the other hand, Group II mAChRs inhibit AC activity, and prolong the opening of potassium channels and transient receptor potential cation (TRPC) channels (Eglen, 2005). Prolonged activation of mAChRs leads to their desensitization, which is associated with a decrease in their function. Receptor desensitization occurs through different mechanisms, which are; 1) uncoupling between G protein and the receptor, 2) internalization of receptors (decrease in cell surface number) and, 3) decreased transcription of the receptor (Koenig and Edwardson, 1996; van Koppen and Kaiser, 2003). The phosphorylation of the mAChRs by GRKs is required for the receptor internalization. Receptor kinases recognize the ligand-bound form of the receptor and phosphorylate serine and threonine amino acids in the ICL3 and C-terminus of mAChRs (Moro et al., 1993). Intriguingly, the phosphorylation of GPCRs by GRK2 and GRK3 results in the receptor internalization, while receptor phosphorylation by GRK5 and GRK6 leads to β-arrestin biased signaling and ERK1/2 phosphorylation (Reiter and Lefkowitz, 2006). It is now evident that mAChRs couple to multiple cellular effector pathways suggesting their capacity for biased agonism (Antony et al., 2009). Signaling via multiple pathways is reliant on the agonist properties and also the complex interaction of both the agonist and the receptor (Ehlert, 2008; Thomas et al., 2008).

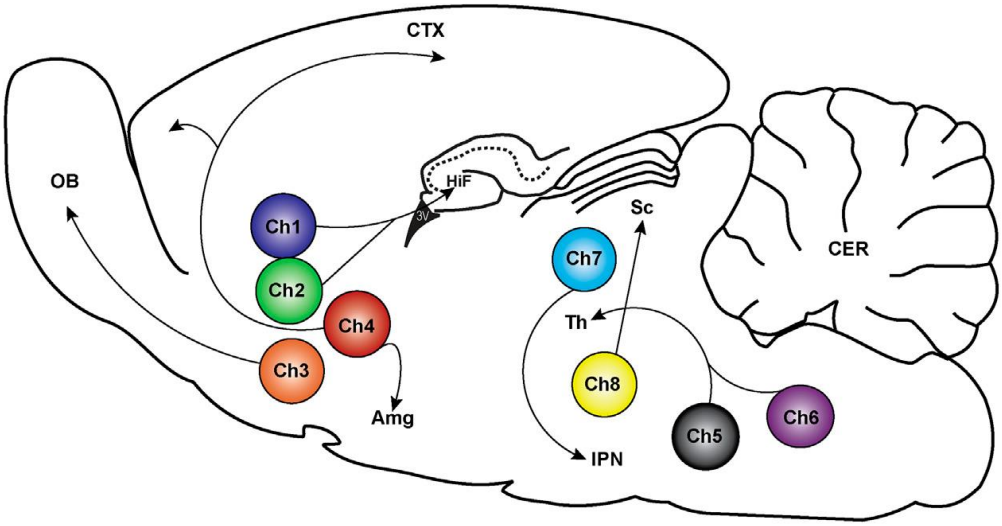


Figure 4 Location of cholinergic neurons and their projections in the rodent brain. Cholinergic neurons exist in eight distinct clusters labeled Ch1-Ch8 (van der Westhuizen et al., 2020). Ch1 and 2 send projections to the hippocampus (HiF), medial cortex, and thalamic nuclei (Th). Ch3 to the olfactory bulb (OB), Ch4 to amygdala (Amg), Ch5 and 6 to the thalamus (Th), Ch7 to the interpeduncular nucleus (IPN), and Ch8 to the superior colliculus (Sc).

1.2.2 Muscarinic M1 receptor (M_1R)

M_1R is encoded by the *CHRM1* gene and is located on 11q12-13 in humans (Bonner, 1989). These receptors are highly expressed in the CNS, namely forebrain areas such as cerebral cortex, hippocampus, and striatum (**Figure 4**) (Levey, 1993; Levey et al., 1991; Wess, 2004). Thus, it is expected that M_1Rs largely mediate ACh actions in the forebrain regions. Using M_1R knockout mice ($M_1R^{-/-}$) and pharmacological approaches, several studies revealed that M_1Rs are involved in the regulation of a wide range of behavioral, cognitive, motor, physiological and sensory functions in the CNS (Bymaster et al., 2003; Dennis et al., 2016; Kurimoto et al., 2018; Thomsen et al.,

2018; Wess, 2004). A pioneer study by Hamilton and colleagues showed that $M_1R^{-/-}$ mice were resistant to proconvulsant effects of pilocarpine due to M_1R-dependent dysregulation of the M current (Hamilton et al., 1997). Results from the latter study and others suggest that M_1R play an important role in the control of hyperexcitability and seizure via regulating M current and TRPC channels in the brain (Carver and Shapiro, 2019; Miller et al., 2017). The M current is a type of non-inactivating potassium current regulated by the M-channels, a group of voltage-gated K^+ channels, which control phasic- and tonic-firing of neurons (discussed in 1.3.2) (Marrion, 1997). Ablation of M_1R gene has been shown to increase locomotor activity of animals in several behavioral tests possibly due to an increase in dopamine release in the striatum (Bradley et al., 2020; Crans et al., 2022; Gerber et al., 2001; Miyakawa et al., 2001). These results suggest that M_1R antagonists may have therapeutic effects for Parkinson's disease through increasing dopamine release from the striatum. Interestingly, early studies using $M_1R^{-/-}$ mice revealed that these animals showed no cognitive deficits in some hippocampus-dependent learning and memory tasks (Miyakawa et al., 2001). However, M_1R ablation was associated with deficits in the eight-arm radial maze test, auditory-cued and context testing, and non-matching-to-sample working memory task (Anagnostaras et al., 2003; Miyakawa et al., 2001). Further studies showed M_1R play a key role in regulating long-term potentiation (LTP), long-term depression (LTD), neuronal differentiation, synaptic plasticity, and auditory cortical function in the hippocampus and cortical areas (Dennis et al., 2016; Kamsler et al., 2010; Shideler and Yan, 2010; Shinoe et al., 2005; VanDeMark et al., 2009; Vijayraghavan et al., 2018). These findings indicate that M_1R is physiologically linked to numerous executive functions of the brain. In this context, selective M_1R activation has been the focus of scientists as a therapeutic approach to treat AD, schizophrenia-related cognitive deficits and age-associated memory impairment (**Table. 1**) (Jiang et al., 2014).

Ablation of M_1R also is associated with electrophysiological deficits in sympathetic ganglion neurons due to dysregulation of M current and N- and P/Q-type Ca^{2+} channels (Carver and Shapiro, 2019; Hamilton et al., 1997; Shapiro et al., 1999). Recently, our lab and others showed that genetic/pharmacologic manipulations of M_1R in DRG sensory neurons might have potential therapeutic benefits against peripheral neuropathies and axonopathies (Calcutt et al., 2017; Deshmukh et al., 2013; Jolivalt et al., 2020; Sabbir and Fernyhough, 2018; Saleh et al., 2020).

Table 1. M_1R-based drugs used in clinical studies for AD. List of M_1R ligands used for the treatment of AD. Table was adopted from Jiang *et al*. (Jiang et al., 2014) with slight modifications.

M_1R orthosteric agonists	
Compound	**Effects**
Xanomeline	Improves cognition and reduces psychotic symptoms in both clinical and preclinical studies
Talsaclidine	Enhances non-amyloidogenic processing of APP and decreases Aβ level in the CSF of AD patients.
AF102B	Reverses cognitive impairments at low dose and reduces CSF Aβ in AD patients. The first drug ever shown to have such an effect in human patients.
AF267B	Improves cognitive function, decreases Aβ, hyperphosphorylated tau, and BACE1 levels in AD mice
WAY-132983	Enhances performance memory and cognition in animal models.
M_1R positive allosteric modulators	
Compound	**Effects**
Brucine	The first reported M_1-PAM. Potentiates ACh affinity to M_1R.
VU0029767	Increases ACh affinity to M_1R

VU0090157	Increases ACh affinity to M_1R
BQCA	Reverses learning impairment in an AD mouse model.
AC-42	The first confirmed allosteric agonist of M_1R
TBPB	Highly selective bitopic agonist for M_1R subtype. Shifts APP processing toward the non-amyloidogenic pathway in cells and appears to have antipsychotic-like effects.
77-LH-28-1	Highly selective and highly efficient agonist of M_1 mAChR. Promotes several physiological functions related to cognition.
AC-260584	Orally bioavailable with favorable antipsychotic and cognitive enhancing effect.
VU0184670	Highly selective to M_1R and excellent pharmacokinetic profile. Also potentiates NMDA receptor-mediated current in hippocampal neurons
VU0357017	Highly selective to M_1R and excellent pharmacokinetic profile. Reverses cognitive deficits induced by mAChR antagonist in animals

1.2.3 Muscarinic M2 receptor (M_2R)

Muscarinic M_2R is encoded by *CHRM2* gene and is localized on the long arm of chromosome 7 (7q31-35) in humans. These receptors are abundantly expressed in both nervous system and peripheral tissues, namely in the heart and smooth muscles (Levey, 1993; Stengel et al., 2000). The M2 and M4 mAChRs are both $G\alpha_i$-coupled receptors and share similar physiological and pharmacological properties, particularly in the CNS (Wess, 2004). However, M2 and M3 mAChRs are co-expressed in several peripheral tissues (mainly smooth muscles) and mediate the effects of ACh (Eglen, 2012; Ehlert, 2003). Regarding the role of M_2R in the nervous system, evidence indicates M_2R are involved in the muscarinic agonist-induced akinesia and tremor (Gomeza et al., 1999a), hypothalamic-pituitary-adrenocortical (HPA) axis activity (Hemrick-Luecke et al., 2002), regulation of hypothermic responses (Gomeza et al., 1999a), and

cognitive function, namely learning and memory (Seeger et al., 2004; Tzavara et al., 2003). Focusing on the latter, using pharmacological (such as SCH 57790 and BIBN-99) or gene ablation ($M_2R^{-/-}$ mice) approaches, previous studies showed that the M_2R played a critical role in the regulation of learning, memory and hippocampal synaptic plasticity (Carey et al., 2001; Rowe et al., 2003; Seeger et al., 2004). However, while complete ablation/antagonism of M_2R is associated with behavioral deficits, partial antagonism of M_2R has been suggested as a better pharmacological approach for cognition enhancement in diseases such as AD. Interestingly, clinical and experimental studies revealed that the *CHRM2* gene was associated with alcohol dependency, bipolar and major depressive disorders (Cannon et al., 2011; Comings et al., 2002; Hemrick-Luecke et al., 2002; Wang et al., 2004). In addition, results of the previous studies suggest that central M_2R (namely spinal cord) contributes to the analgesic effects of muscarinic agonists (Wess et al., 2003). Intriguingly, the role of M_2R in mediating the analgesic effects of muscarinic agonists is not restricted to the CNS. Studies on the skin and skin saphenous nerve of $M_2R^{-/-}$ mice showed that muscarine treatment did not induce peripheral antinociception effects (Bernardini et al., 2002). Acetylcholine, as a neurotransmitter, regulates its own release through activation of inhibitory auto-receptors, M_2R (in the hippocampus and cerebral cortex) and muscarinic M4 receptor (M_4R) (striatum) on cholinergic synapses (Zhang et al., 2002a). In addition, both M_2R and M_4R are considered as muscarinic heteroreceptors, which inhibit norepinephrine release from peripheral sympathetic nerves (Fuder and Muscholl, 1995).

In the peripheral tissues, the M_2R is abundantly expressed in the myocardium and is responsible for ACh-induced negative chronotropic and inotropic effects. Studies using M_2R knockout mice showed that administration of muscarinic agonists did not induce bradycardia in the animals (Gomeza et al., 1999a; Stengel et al., 2000). It has been reported that M_2R is voltage

sensitive and alterations in membrane potential affects the conformation of the receptor orthosteric binding site (Ben-Chaim et al., 2003; Harvey and Belevych, 2003; Navarro-Polanco et al., 2011). In smooth muscle, M_2R is the predominantly expressed mAChR along with smaller numbers of M_3R (Eglen et al., 1996). Also, studies on the $M_2R^{-/-}$ and $M_3R^{-/-}$ mice showed that treating isolated smooth muscle (from trachea and bladder) with muscarinic agonists had less effects on the muscle contractions when compared to wild type counterparts (Matsui et al., 2000; Stengel and Cohen, 2002). These findings suggest the role of M_2R and M_3R in muscle contraction as M_2R/M_3R double knockout mice showed a completely abolished contraction response to muscarinic agonists (Matsui et al., 2002).

Muscarinic M_2R was purified and cloned by the early studies on the GPCRs and since then, M_2R has been considered as an appropriate model system to study GPCR pharmacology and biology (Kubo et al., 1986b; Peterson et al., 1984). Focusing on the allosteric modulation of mAChRs signaling, studies on the inactive structures of M_2R and M_3R demonstrated a large extracellular vestibule, which was responsible for binding to allosteric ligands (Bock et al., 2012; Gregory et al., 2007; Kruse et al., 2013). Further studies showed that two amino acid residues in the M_2R were responsible for the allosteric activity of pharmacological compounds such as alcuronium and gallamine (Gregory et al., 2010). Despite attempts to design highly selective and biased bitopic ligands for mAChR subtypes, designed compounds showed less affinity to the receptors (Lane et al., 2013). However, the antagonist THRX-160209 possesses considerable affinity for M_2R with high selectivity (Steinfeld et al., 2007).

1.2.4 Muscarinic M3 receptor (M_3R)

M_3R is encoded by the *CHRM3* gene and is localized on the long arm of chromosome 1 (1q43) in humans. These receptors are expressed in both nervous system and peripheral tissues, namely in the smooth muscles and salivary glands (Wess et al., 2007). In the CNS, M_3R is expressed in the hypothalamus and regulates appetite and body metabolism (Gautam et al., 2006a; Yamada et al., 2001b). Previous studies revealed that $M_3R^{-/-}$ mice exhibited increased locomotor activity, energy consumption, and core body temperature (Yamada et al., 2001b). The hypophagia phenotype in $M_3R^{-/-}$ mice has been attributed to a decrease in melanin-concentrating hormone (MCH), which controls the hypothalamic feeding centers (Gautam et al., 2006a; Schwartz et al., 2000). A recent study revealed that stimulation of cholinergic neurons in the dorsomedial hypothalamus was associated with increased food intake and administration of 4-DAMP (a selective M_3R antagonist) reversed this effect (Jeong et al., 2017). Interestingly, using Cre/loxP technology, Guatam and colleagues generated a mutant mouse whose neurons and glial cells lacked M_3R (brain-$MR^{-/-}$). Unlike $M_3R^{-/-}$ mice, the brain-$M_3R^{-/-}$ mice showed no alterations in food intake, glucose and insulin levels and metabolism, but exhibited a dwarf-like appearance and a decrease in the growth hormone (GH) and insulin-like growth factor I (IGF-I) in the serum (Gautam et al., 2009). These results suggest neuronal M_3R contribute to the proliferation of the anterior pituitary and release of growth hormone. Although M_1R and M_2R are known to mediate cholinergic-induced hippocampal learning and memory, studies on $M_3R^{-/-}$ mice showed that *Chrm3* ablation was associated with deficits in learning and memory due to fear conditioning (Fedoce et al., 2016; Poulin et al., 2010). Results of these studies suggest that lack of phosphorylation-dependent signaling through M_3R (namely arrestin-biased signaling) is associated with cognitive impairment in $M_3R^{-/-}$ mice.

As noted above, M_2R and M_3R are co-expressed in several peripheral tissues (mainly smooth muscles) and mediate the effects of ACh. Using selective pharmacological agents for mAChRs subtypes, previous studies showed that M_3R played a key role in the smooth muscle contraction (Caulfield, 1993; Eglen et al., 1996). In agreement with pharmacological studies, smooth muscle tissues derived from $M_3R^{-/-}$ mice exhibited a decrease in contractile responses to mAChRs agonist treatments in a tissue-specific manner ranging from almost 40% in trachea to ~90% in bladder (Matsui et al., 2002; Stengel et al., 2002). Considering the key role of M_3R in smooth muscle contraction, numerous pharmacological studies have been conducted to target M_3R as a therapeutic target for pathological conditions such as overactive bladder (OAB), irritable bowel syndrome (IBS), and chronic obstructive pulmonary disease (COPD) (Peretto et al., 2009). For example, Darifenacin, a selective M_3R antagonist, is an FDA approved drug that inhibits bladder responsiveness and reduces OAB (Haab et al., 2004; Zinner, 2007). Solifenacin, also a selective M_3R antagonist, has a longer half-life than darifenacin and these two drugs are the frontline therapies for the OAB (Cardozo et al., 2004). Different mAChR subtypes are expressed in the airways and pulmonary system and among which, M_3R and M_2R mediate the effects of ACh (Coulson and Fryer, 2003). *In vivo* studies on $M_3R^{-/-}$ mice showed that vagal stimulation or methacholine treatment failed to induce bronco-constriction suggesting the important role of M_3R in ACh-induced broncho-constriction (Fisher et al., 2004). Intriguingly, the same study showed a significant increase in the bronchoconstrictor responses following vagal stimulation in $M_2R^{-/-}$ mice. Authors of this study suggested that pulmonary prejunctional M2 autoreceptors inhibited ACh release from parasympathetic nerve endings (Coulson and Fryer, 2003). It is known that inflammatory factors play a major role in the pathobiology of COPD (Chung and Adcock, 2008). In this context, it has been shown that M_3R is required for thromboxane A2-induced bronco-

constriction (Allen et al., 2006). Based on these studies, muscarinic antagonists such as ipratropium and mainly tiotropium have been used for the treatment of COPD (Moulton and Fryer, 2011).

Xerostomia (oral dryness), is a pathological condition associated with decreased salivation and is considered as one of main symptoms of Sjögren's syndrome (Maleki-Fischbach et al., 2024). Evidence indicates that stimulation of glandular mAChRs causes saliva secretion (Turner and Sugiya, 2002). Studies on $M_1R^{-/-}$ and $M_3R^{-/-}$ mice showed that these two mAChRs mediate the salivary fluid secretion. Accordingly, targeting M_1R and M_3R with muscarinic agonists (such as pilocarpine and cevimeline) had a beneficial use in patients suffering from xerostomia (Fox et al., 2001; Gautam et al., 2004; Nakamura et al., 2004). In addition, M_3R contributes to bone mass regulation by reducing bone resorption and increasing bone formation via the parasympathetic system (Shi et al., 2010).

One of the important roles of M_3R is to mediate ACh-induced insulin secretion. Many hormones and neurotransmitters regulate glucose-stimulated insulin secretion (GSIS) through GPCRs expressed by β-cells in the pancreas (Ahrén, 2000; Gilon and Henquin, 2001). Using Cre/loxP technology, Guatam and colleagues generated a mutant mouse whose pancreatic β-cells lacked M_3R (β-$MR^{-/-}$). Studies on β-$MR^{-/-}$ mice showed a significant decrease in GSIS and impairment in glucose tolerance suggesting the role of M_3R in insulin release (Gautam et al., 2006b). Further, authors of this study generated a transgenic mouse that overexpressed a modified M_3R in β-cells (β-M3-Tg1 mice). Unlike β-$MR^{-/-}$ mice, the β-M3-Tg1 mice exhibited reduced levels of blood glucose and elevated levels of serum insulin suggesting that M_3R on pancreatic β-cells play an important role in glucose homeostasis through regulating insulin release (Gautam et al., 2006b).

Numerous attempts have been made to develop and design pharmacologic agents that selectively modulate mAChRs and the structure and dynamics of these receptors have been recently discovered (Haga et al., 2012; Kruse et al., 2012; Thal et al., 2016; Vuckovic et al., 2019). There are compounds that act as allosteric ligands of M_3R and recent studies have tried to use these drugs for therapeutic purposes (Bock et al., 2018). For example, a recent study by Zhu and colleagues showed that treating wild-type mice with VU0119498 (a positive allosteric modulator at M_3R) increased serum insulin levels and reduced blood glucose, while the same effect was absent in β-$MR^{-/-}$ mice due to lack of M_3R. In addition, a recent study reported that pilocarpine was either an agonist (through β-arrestin-biased signaling) or antagonist of M_3R depending on the cell type and expression levels of receptor (Pronin et al., 2017).

1.2.5 Muscarinic M4 receptor (M_4R)

M_4R is encoded by *CHRM4* gene and is localized on the long arm of chromosome 11 (11p11.2) in humans. These receptors are expressed in both CNS (mainly striatum) and the peripheral nervous system (mainly prejunctional nerve endings) (Levey, 1993; Trendelenburg et al., 2003). As mentioned above, the M_2R and M_4R are both $G\alpha_i$-coupled receptors and share similar physiological and pharmacological properties, particularly in the CNS. For example, both of these receptors mediate the inhibitory effects of ACh as auto-receptors and heteroreceptors (controlling parasympathetic and sympathetic transmission). Also, activation of M_4R auto-receptors in the midbrain reduces dopaminergic activity in the ventral tegmental area (VTA) and results in the attenuation of reinforcing effects of addictive drugs (Tzavara et al., 2004). Using $M_4R^{-/-}$ mice, studies reveal M_4R was involved in the regulation of muscarinic agonist-induced analgesia at the spinal and supraspinal level (Gomeza et al., 1999b). In agreement with KO mice studies, previous

research using M_4R selective agonists (CMI-936 and CMI-1145) showed these compounds were capable of inducing analgesia in mice (Ellis et al., 1999).

Results obtained from the studies on $M_4R^{-/-}$ mice showed that M_4R facilitates striatal dopamine release (Gomeza et al., 1999b; Zhang et al., 2002b). Indeed, balanced interaction between mAChRs and dopaminergic system in the striatum is necessary for the regulation of locomotor activity and stimulation of striatal mAChRs facilitates dopaminergic activity (Di Chiara et al., 1994; Raiteri et al., 1984). In the striatum, striatal projection neurons express both D_1R and M_4R and regulate locomotion (Ince et al., 1997). Studies on $M_4R^{-/-}$ mice showed striatal M_4R has an inhibitory role on D_1R-induced locomotion (Gomeza et al., 1999b). These findings on the regulatory role of M_4R on D_1R-dependent locomotion may be useful to design therapeutic compounds against Parkinson's disease. In another study, wild-type and $M_4R^{-/-}$ mice were treated with haloperidol (a D_2R antagonist), an antipsychotic drug known to induce catalepsy, and scopolamine, a muscarinic antagonist. Haloperidol treatment induced cataleptic behaviors in both wild-type and $M_4R^{-/-}$ mice, and scopolamine treatment only succeeded to reverse cataleptic behaviors of wild-type, but not $M_4R^{-/-}$ mice (Karasawa et al., 2003). These results agree with previous research that selective M_4R antagonists may be useful in the treatment of Parkinson's disease. Further studies on $M_4R^{-/-}$ mice revealed that these animals exhibited severe impairment in the prepulse inhibition test (a test used in the diagnosis of psychosis) following treatment with phencyclidine (Felder et al., 2001). Related to the role of M_4R in psychosis, xanomeline (a mixed M1/M4 agonist) possesses antipsychotic properties with few side effects (Mirza et al., 2003). In recent decades, several allosteric ligands have been developed for M_4R (Bock et al., 2018). For instance, LY2033298 and VU10010 are selective M_4R agonists and were examined for their antipsychotic properties in animal models (Nawaratne et al., 2010).

1.2.6 Muscarinic M5 receptor (M_5R)

The last identified member of mAChRs is M_5R, which is encoded by *CHRM5* gene located on chromosome 15 (15q14) in humans. These receptors are mainly expressed in the CNS and are involved in the regulation of dopaminergic system activity (Wess, 2004). Both pharmacological and genetic studies showed that dopaminergic neurons of the substantia nigra pars compacta (SNc) express M_5R, and their stimulation facilitates striatal dopamine release (Felder et al., 2000; Yamada et al., 2001a). Also, M_5R is expressed in the VTA, which consists of dopaminergic neurons that project to the nucleus accumbens (Nac) and limbic regions (Koob et al., 1998; Vilaró et al., 1990). It is well known that VTA and the mesolimbic dopaminergic pathway are highly involved in the regulation of reward and behaviors associated with addiction (Koob et al., 1998). In this context, studies on $M_5R^{-/-}$ mice showed that morphine-induced reward seeking behavior was impaired in these animals when assessed in the conditioned place preference (CPP) test. These behavioral abnormalities were associated with a decrease in Fos-B expression and reduced dopamine release in the Nac. Interestingly, slight withdrawal symptoms were observed in these animals following the administration of naloxone (Basile et al., 2002). Further studies revealed that M_5R mediated behaviors associated with cocaine withdrawal and reinforcement, thus, M_5R selective antagonists may be useful for the treatment of addiction disorders (Fink-Jensen et al., 2003). Although there are few selective ligands for M_5R, amiodarone is a selective allosteric modulator of M_5R (Stahl and Ellis, 2010). Collectively, pharmacological studies with novel compounds and evidence from transgenic mice demonstrated that each mAChR subtype has specific and defined function and regulates a wide range of ACh actions in both CNS and peripheral tissues. Several effects of mAChRs on cellular functions are mediated through ion channels. The communication of mAChRs and ion channels is highly complex, and in the next

section, regulation of ion channels by mAChRs (namely M_1R) will be discussed. A summary of the behavioral and physiological characteristics of mAChRs KO mice are presented in **Figure 5**.

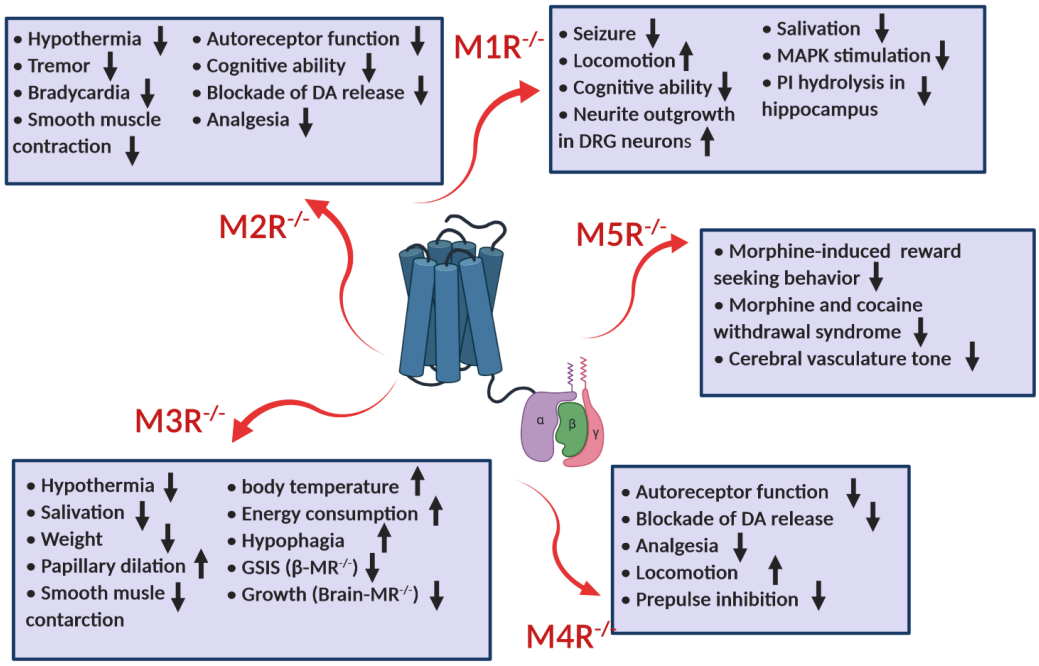


Figure 5 A summary of the impact of the ablation of the mAChR subtype genes in mice. Genetic knockout of each muscarinic receptor is associated with specific physiological, behavioral, and biochemical responses in mice. Arrows indicates increase/decrease in the effects.

1.3 M1 muscarinic acetylcholine receptor

1.3.1 M_1R signaling

1.3.1.1 M_1R activation and trafficking

Acetylcholine (ACh), as the endogenous ligand for M_1R, is a signaling biomolecule present in a wide range of prokaryotic and eukaryotic cells (e.g bacteria, algae, yeast, and plants) for about

3 billion years, and it is now clear that ACh mediates multiple biological functions in neuronal and non-neuronal cells (Horiuchi et al., 2003; Wessler et al., 1999). Although M_1R has been identified decades ago, the crystal structure and detailed characterization of its orthosteric and allosteric binding sites have only just recently been discovered (Hammer et al., 1980; Thal et al., 2016). Previous studies used Alanine substitution mutation to identify key residues in the TM domains of M_1R. Amino acid residues in the TM3 (Asp^{105}, Tyr^{106}, Ser^{109}, Asn^{110}), TM5 (Thr^{189}, Thr^{192}, Ala^{196}), TM6 (Tyr^{381}, Asn^{382}), and Tyr^{404} in TM7 comprise the ACh binding pocket and are critical for ACh binding to M_1R (Allman et al., 2000; Lu et al., 1997; Lu and Hulme, 1999; Lu et al., 2001; Ward et al., 1999). As mentioned above, the high sequence conservation of the orthosteric binding site among mAChRs has urged current research toward targeting allosteric sites on M_1R. There are several selective M_1R allosteric ligands that have been synthesized in recent years for the treatment of neurological and psychiatric disorders. For example, AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrochloride) is a highly selective M_1R bitopic ligand that binds to the upper region of the TM1 and TM7 domains and directly activates M_1R (Langmead et al., 2006; Spalding et al., 2006). Intriguingly, M_1R Ago-PAMs (such as AC-42 and 77-lH-28-1) are able to activate the M_1R in the absence of the orthosteric agonist (Conn et al., 2009a).VU0090157 and VU0029767, as M_1R positive allosteric modulators (PAMs), indirectly modulate M_1R via enhancing responses to ACh (Marlo et al., 2009).

Achieving M_1R selectivity has been an important goal in the development of therapeutic ligands. However, the majority of PAMs for M_1R have several central and/or peripheral side effects, making them inappropriate for clinical applications. The most concerning side effects of M_1R PAMs or orthosteric selective ligands is epileptogenesis (Bradley et al., 2020; Davoren et al., 2016). Thus, there is a need for focusing on the development of selective ligands with minimal

side effects and maximum efficacy. Interestingly, Bradley and colleagues demonstrated that M_1R-induced β-arrestin biased signaling was associated with improved learning and memory, and alleviated the M_1R-associated adverse effects such as anxiety-like behaviors, hyperlocomotion, and seizures. Authors of this study used phospho-deficient and chemo-genetically modified M_1R, and showed that M_1R phosphorylation and the β-arrestin pathway (but not G protein pathway) played a critical role in attenuation of adverse effects (Bradley et al., 2020). Results of this study highlighted the importance of M_1R phosphorylation patterns, biased agonism, and the β-arrestin pathway.

Despite the ability of M_1R to bind all 4 groups of G proteins, M_1R primarily recruits $G\alpha_{q/11}$ when exposed to non-biased ligands such as ACh and carbachol. However, as mentioned above, each ligand has its own signature on GPCR based upon the barcode hypothesis. In this regard, M_1R ligands can be divided into several groups such as non-biased, G protein biased (could be multiple or individual G proteins), β-arrestin biased, and even biased toward a particular transducer such as PLD/PLC (see **Table 2**). Classically, binding of ACh to M_1R leads to $G\alpha_{q/11}$ coupling, PLC activation, generation of IP_3 and DAG, and consequently activation of PKC and Ca^{2+} signaling pathways (**Figure 6**). Also, $G\alpha_q$-PLC is associated with cAMP activation through IP_3-calcium release and calmodulin (Felder et al., 1989; van der Westhuizen et al., 2020). Upon activation, β-arrestins (mostly β-arrestin 2) bind to M_1R to uncouple G proteins, arrest signaling, and initiate receptor internalization. Internalization of M_1R is both G protein and β-arrestin dependent with an important role of ICL3 for β-arrestin recruitment. Binding β-arrestins to M_1R makes a scaffold that recruits members of the clathrin-dependent endocytic machinery and initiates the internalization of the receptor (Oakley et al., 1999; Yeatman et al., 2014). β-arrestins also cluster receptors into CCPs by bridging between CCP protein adaptor protein 2 (AP2) and receptor. Following this,

dynamin initiates the scission of vesicles from the plasma membrane and formation of early endosomal compartments (Jean-Alphonse and Hanyaloglu, 2011). Carbachol-induced internalization of M_1R involves formation of early and recycling endosomes in 10 minutes. Prolonged carbachol treatment resulted in increased M_1R degradation by lysosomes (Yeatman et al., 2014). GPCR internalization and subcellular trafficking via endocytic machinery is ligand-dependent. For example, PZ, a selective orthosteric antagonist for M_1R, induces receptor dimerization and oligomerization and increases the receptor expression on the cell surface *in vitro* (Ilien et al., 2009; Pediani et al., 2016; Zhou et al., 2024).

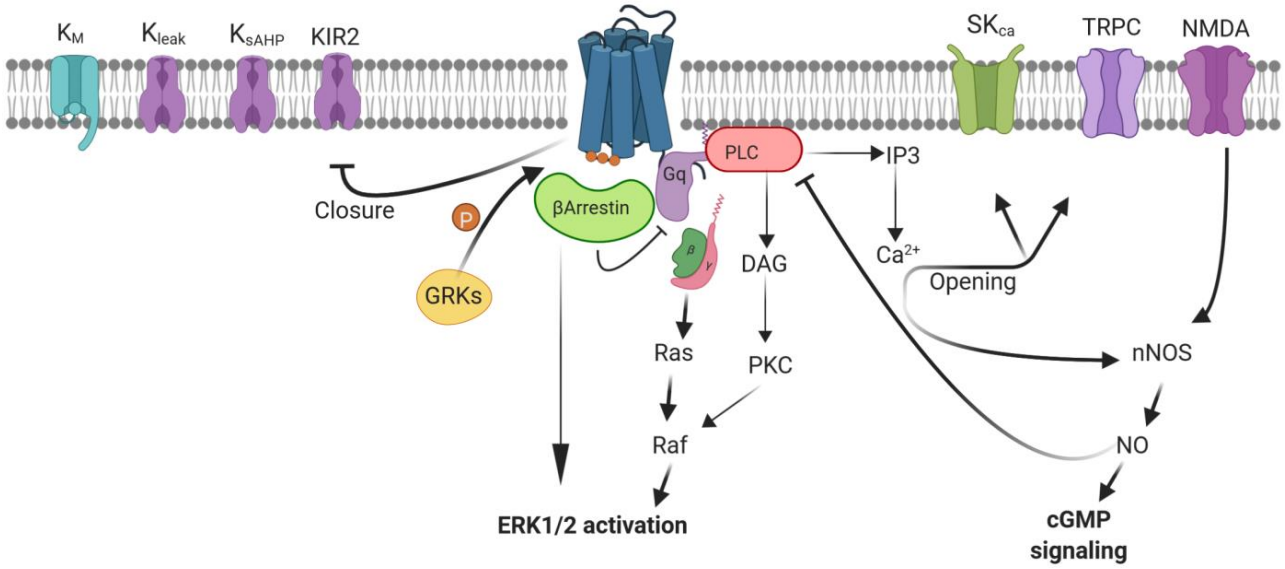


Figure 6 Schematic M_1R signaling and regulation of ion channels. Activation of M_1R (in)directly engages several signaling pathways. GRKs (G protein-coupled receptor kinase) and CKs (casein kinases) phosphorylate the receptor after ligand binding. $G\alpha_q$ signaling results in the generation of IP3 (Inositol trisphosphate) and DAG (diacylglycerol) by PLC (Phospholipase C). PIP_2 hydrolysis by PLC leads to closure of potassium channels. Beta-arrestin binding to the M_1R uncouple G protein activity and activates arrestin pathway by ERK1/2 phosphorylation. K_M (potassium M-channels), K_{leak} (potassium leak channels), K_{sAHP} (Potassium slow after hyper polarization channel), Kir2 (inward-rectifier potassium ion channel), SK_{ca} (small conductance calcium-activated potassium channels), TRPC (transient receptor potential cation channels), NMDA (N-Methyl- d-aspartate receptor), , PKC (Protein kinase C), ERK (extracellular signal-regulated kinase), NO (Nitric oxide), nNOS (neuronal nitric oxide synthase), cGMP (cyclic guanosine monophosphate).

1.3.1.2 M_1R phosphorylation and signaling

Similar to all other GPCRs, M_1R is a subject of phosphorylation by different kinases, which regulate the function and behavior of the receptor. There is only a limited set of studies focused on M_1R phosphorylation in the literature. Both GRKs and CKs have been reported to phosphorylate M_1R. Interestingly, unlike the majority of GPCRs, there is no specific GRK responsible for M_1R phosphorylation in response to agonist ACh. In fact, all non-visual GRKs contribute to the phosphorylation of the receptor to some extent (Drube et al., 2022). GPCR phosphorylation depends on a variety of factors such as type of cell and ligand. For example, treating CHO cells overexpressing M_1R with carbachol was reported to involve GRK2/3 and β-arrestin 2 recruitment to the receptor, while treating HEK293 cells overexpressing M_1R with ACh engaged all non-visual GRKs at the same time (Drube et al., 2022; Yeatman et al., 2014). Additionally, GRK2 directly interacts with $G\alpha_q$ to block IP_3 signaling events (Willets et al., 2005). The work by Butcher *at al*. showed that ACh treatment phosphorylated M_1R at 14 residues at ICL3 and 2 residues at the C-terminus. Authors of this work concluded that Ser^{228} experienced the highest phosphorylation level in response to ACh, and considered it as the hallmark of M_1R activation *in vivo*, where they observed high phosphorylation level of Ser^{228} in response to medication (xanomeline) or memory acquisition in the hippocampus (Butcher et al., 2016). Another study showed that Ser^{228} and Ser^{273} phosphorylation was linked to β-arrestin 2 activation and ERK phosphorylation.

As mentioned above, different ligands have the potential to activate one or more signaling pathways on a distinct receptor. In the case of M_1R, there are several pathways that were linked to this receptor upon activation by different ligands (Jung et al., 2017; van der Westhuizen et al., 2020). Previous studies indicate that M_1R binds to a variety of G proteins (Avet et al., 2022). M_1R binds to the members of the $G\alpha_{q/11}$ class: Gαq, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15}$ (Nelic et al., 2024).

Interestingly, previous studies showed that M_1R directly engaged $G\alpha_{12/13}$ and activated Rho proteins, which contributed to cytoskeleton formation and membrane trafficking (Borroto-Escuela et al., 2011; Sabbir et al., 2018; Suzuki et al., 2009). M_1R also recruits $G\alpha_s$ and increases cAMP in response to several agonists in different cell lines (Burford and Nahorski, 1996; Russell et al., 1994).The primary signaling pathway for M_1R is the $G\alpha_q$-IP_3-calcium-PKC pathway. Another signaling route that M_1R is linked with is the $G\alpha_q$-A kinase anchoring protein (AKAP) 79-PKC-potassium channel pathway, through which the M_1R-AKAP79-PKC complex regulates neuronal excitability by activating potassium channels and M-currents (Carver and Shapiro, 2019; Hoshi et al., 2010). Another pathway is linked to kinases such as PI3K and Src, where M_1R-induced ERK1/2 activation by carbachol was blocked by PI3K and Src inhibitors. Thus, M_1R activation is linked to the $G\alpha_q$-PI3K-Src-ERK1/2 pathway (Rosenblum et al., 2000).

1.3.2 Regulation of ion channels by M_1R

mAChRs do not act as ion channels, however, their effects on cellular functions are mediated by a range of endogenous ion channels. Regardless of some exceptions (e.g. arrestin-mediated signaling pathways), the majority of mAChRs effects are transcribed by ion channels through G proteins or effectors associated with the G proteins. Since ion channels are regulated by a downstream product of mAChRs signaling (e.g. $G\alpha_q$ activation), it is not easy to determine which mAChR (M1, M3 or M5) has activated the $G\alpha_q$ protein (Brown, 2018; Dascal, 2001). However, there are examples of subtype-specific regulation of ion channels in neurons. For instance, while both M_2R and M_4R are expressed on sympathetic ganglia as heteroreceptors, it is M_4R (and not M_2R) that inhibits Ca_V2 (a Ca^{2+} voltage-gated channel) (Bernheim et al., 1992) . Another example is the connection between M_1R and potassium voltage-gated channels (K_M or M-channels), which has been heavily studied and is considered as a model system (Hille et al., 2014). In brief, M-

channels require PIP_2 to maintain the open state conformation and PIP_2 hydrolysis following M_1R activation leads to M-channel closure (Brown and Adams, 1980; Winks et al., 2005). Many factors (such as agonist concentration, cell type, agonist type) should be considered when studying the connection between mAChRs and ion channels. For example, unlike primary neurons, NG108-15 neuroblastoma-glioma hybrid cells release a large amount of intracellular Ca^{2+} following the activation of M_1R and stimulate Ca^{2+}-activated potassium channels. Activation of these channels along with M-channel closure is associated with a greater sensitivity of downstream response to receptor activation in NG108-15 cell when compared to primary neurons, which release relatively less intracellular Ca^{2+} (Delmas et al., 2002; Fukuda et al., 1988; Robbins et al., 1993). Since the focus of current work is M_1R, the modulation of ion channels (mainly potassium channels) by M_1R will be discussed in the following sub-sections. In general, the activation of $G\alpha_q$-coupled mAChRs (including M_1R) is usually associated with excitatory physiological responses in cells through the inhibition of potassium channels, chloride channels, and activation of cation channels.

1.3.2.1 M_1R-mediated inhibition of potassium leak currents

Two-pore domain potassium (K2P) channels (encoded by *KCNK* genes) cause leak potassium currents and are involved in the regulation of resting membrane potential and excitability of neurons (Zinner, 2007). Based on their structure and function, K2P channels are divided into six main families, among which TWIK (tandem of P-domains in a weak inward rectifying K^+ channel), TREK (TWIK-related K^+ channel), and TASK (two-pore domain, acid-sensitive K^+ channel) are well-studied members modulated by M_1R (Brown, 2018; Goldstein et al., 2005; Goldstein et al., 2001). Since these receptors are not voltage-dependent, the open state is not affected by membrane potential. Following M_1R stimulation, leak channels are inhibited (no outward leakage of K^+) and facilitate cell depolarization through inducing an action potential or

increasing firing capacity (Galligan et al., 1989). TREK 1 and TREK2 (encoded by *KCNK2* and *KCNK10*, respectively) are active in the presence of PIP_2 and are closed following PIP_2 hydrolysis (Lindner et al., 2011; Lopes et al., 2005). It is possible that closure of TREK channels by M_1R is associated with PLC-induced PIP_2 depletion because this effect is reversed by adding PIP_2 to cells (Bista et al., 2015; Rivas-Ramírez et al., 2015). Also, M_1R-induced endocytosis of TASK1 (encoded by *KCNK3*) is another mechanism, by which M_1R increases the excitability of neurons (Matsuoka and Inoue, 2017). K2P channels are widely expressed in DRG sensory neurons and play an important role in their excitation and TREK and TRESK (TWIK-related spinal cord potassium channel, encoded by *KCNK18*) channels are responsible for more than 95 % of the background potassium conductance (Cadaveira-Mosquera et al., 2012; Kang and Kim, 2006).

1.3.2.2 M_1R-mediated inhibition of M-channels

Kv7 potassium channel subunits (Kv7.1-Kv7.5) are encoded by *KCNQ* genes and (Kv7.2–Kv7.5) are expressed in neurons. Among these subunits, Kv7.2 and Kv7.3 (encoded by *KCNQ2* and *KCNQ3*, respectively) are the main subunits of the slow voltage-gated potassium channels (M-channels), which regulate neuronal excitation. As mentioned earlier, activation of $G\alpha_q$-coupled mAChRs (such as M_1R) closes M-channels resulting in cholinergic excitation. M-channel closure is mediated by PLC-induced PIP_2 hydrolysis and depletion (Brown and Passmore, 2009). The subunit composition of M-channels plays an important role in their regulation by muscarinic receptor because their sensitivity to PIP_2 levels varies. The affinity of Kv7.3 subunit to PIP_2 is almost 100-fold more than the other subunits, and M-channels containing Kv7.3 require a high level of muscarinic receptor occupation and activation for closure (Hernandez et al., 2009). For example, C-fiber sensory neurons abundantly express Kv7.5 as the predominant subunit, while medium and large size DRG neurons express hetero-multidimer M-channels consisting of Kv7.2

and Kv7.3 subunits (King and Scherer, 2012; Passmore et al., 2003). M_1R is responsible for the M-channel closure in sympathetic ganglion neurons (Bernheim et al., 1992; Hamilton et al., 1997; Marrion et al., 1989), striatal (Shen et al., 2005) and hippocampal pyramidal neurons (Rouse et al., 2000). Interestingly, DAG-activated PKC is able to inhibit M-channels following M_1R activation (Kosenko et al., 2012). Also, in non-neuronal cells, M-channels can be inhibited by a mechanism that involves intracellular Ca^{2+} mediated channel-attached calmodulin (Gamper and Shapiro, 2003).

1.3.2.3 M_1R-mediated inhibition of inwardly rectifying potassium (Kir) channels

Inwardly rectifying potassium (Kir) channels play an important role in maintaining the resting membrane potential of the cells, potassium hemostasis and the regulation of the electrical activity of several types of cells including neurons. All members of Kir family require PIP_2 for their function and are sensitive to PIP_2 hydrolysis and depletion (Reimann and Ashcroft, 1999). The activity of Kir channels is regulated by different modulators including PKC, $G\alpha_q$ -coupled receptors, pH, magnesium ions and the $\beta\gamma$-subunits of G proteins (Du et al., 2004). Evidence indicates that M_1R stimulation is associated with the inhibition of Kir2 channels (Kir2.1-Kir2.3 encoded by *KCNJ2*, *KCNJ12*, and *KCNJ4*, respectively), mainly via PIP_2 hydrolysis and depletion (Brown, 2018). *In vitro* studies on teh tsA201 mammalian cell line revealed that M_1R stimulation inhibited all Kir2 channels through Ca^{2+}-dependent activation of the small GTPase Rho and PIP_2 depletion (Rossignol and Jones, 2006). M_1R-meidated Kir2 inhibition increases dendritic excitability in striatopallidal neurons (Shen et al., 2007) and enhances temporal summation of excitatory synaptic potentials of pyramidal neurons in prefrontal cortex (Carr and Surmeier, 2007). The latter research showed that under *in vivo* conditions, PIP_2 depletion played the major role in Kir2 inhibition and PKC or intracellular Ca^{2+} alteration has no effect on Kir2 activity (Carr and

Surmeier, 2007). Another Kir subfamily is Kir3 channels (Kir3.1-Kir3.4 encoded by *KCNJ3*, *KCNJ6*, *KCNJ9*, and *KCNJ5*, respectively). Ample evidence showed that Gβγ dimers released from $G\alpha_i$-coupled receptors directly engaged with Kir3 channels and induced activation. Because Kir3 channels are activated by Gβγ, these receptors are called G protein-coupled inwardly-rectifying potassium channels (GIRKs) (Lüscher and Slesinger, 2010). However, $G\alpha_q$-coupled receptor activation is associated with GIRKs inhibition (Lei et al., 2001). Hill and Peralta showed that stimulation of M_1R inhibited GIRK1 and GIRK4 through a mechanism that involves PKC and intracellular Ca^{2+} mobilization (Hill and Peralta, 2001). Another study showed $GABA_B$-induced GIRK activation was inhibited by stimulation of M_1R/M_3R-induced PIP_2 depletion in hippocampal neurons (Sohn et al., 2007).

1.3.2.4 M_1R-mediated inhibition of calcium-activated potassium channels

Ca^{2+} activated potassium (K_{Ca}) channels are sensitive to intracellular Ca^{2+} levels and membrane potential alterations. These receptors are widely distributed throughout the CNS and contribute to neuronal excitability and neurotransmitter release. Based on their conductance properties, K_{Ca} channels are divided into three subfamilies 1) SK (small conductance), IK (intermediate conductance) and BK (big conductance) channels (Vergara et al., 1998). There are inconsistencies between the studies whether M_1R has an inhibitory effect on K_{Ca} channels (Dasari and Gulledge, 2011; Rouse et al., 2000). Also, the mechanism of K_{Ca} channels inhibition is not clear and a number of studies support the role of PIP_2 depletion in M_1R-mediated K_{Ca} channel inhibition (Andrade et al., 2012; Zhang et al., 2014a). Related to this, selective M_1R activation inhibits SK channels in CA1 hippocampal pyramidal neurons through PKC activation (Buchanan et al., 2010). A study by Giessel and Sabatini showed that M_1R enhanced synaptic potentials and Ca^{2+} influx in dendritic spines by inhibiting postsynaptic SK channels via a mechanism that

involves CK2 (Giessel and Sabatini, 2010). Similarly, a recent study revealed that M_1R-mediated SK channel inhibition boosted synaptic weight via PKA and CK2 contribution and SK channels were involved in the activity-dependent plasticity of membrane excitability in L2/3 pyramidal neurons of the mouse primary somatosensory cortex (Gill and Hansel, 2020). Interestingly, Zivkovic and colleagues reported that M_1R modulated the endotoxemia-induced loss of synaptic plasticity through the inhibition of SK channels. Authors of this work showed that treating mice with the selective allosteric M_1R agonist (TBPB) or SK channel inhibitor (apamin) restored deficits in synaptic plasticity induced by endotoxemia (Zivkovic et al., 2015).

1.3.2.5 M_1R-mediated activation of cation channels

$G\alpha_q$-coupled muscarinic receptors are capable of activating Ca^{2+}-dependent non-selective cation channels. Activation of these receptors is associated with a slow depolarization condition accompanied by long-lasting action potential discharges or burst discharges (Constanti et al., 1993; Rahman and Berger, 2011; Shen and North, 1992). Many of these biological processes are mediated through the activation of TRPC channels (Strübing et al., 2001; Yan et al., 2009; Zhang et al., 2011b). Also, stimulation of M_1R triggers the formation of a multiprotein complex centered on TRPC6 channels (Kim and Saffen, 2005). Based on this evidence, it is believed that M_1R activation excites neocortical pyramidal neurons through TRPC channels that generate Ca^{2+}-dependent (after depolarizing potentials or ADPs) (Gulledge et al., 2009; Yan et al., 2009). However, Dasari *et al*. showed that TRPC5 and TRPC6 channels were not necessary for M_1R-induced excitation of layer 5 pyramidal neurons in the mouse medial prefrontal cortex (Dasari et al., 2013). M_1R-mediated effects on ion channels (e.g. M- channels) are not similar in all tissues. M_1R activation is associated with the inhibition of M-channels and GIRK channels in CA1 pyramidal neurons, similar to the effects observed in the peripheral tissues. However, the same

study found that M_1R activation led to the activation of M-channels and GIRK channels in the dentate gyrus granule cells in the hippocampus. Authors of this work showed that this phenomenon was regulated by Ca^{2+}-mediated stimulation of PIP_2 synthesis following M_1R stimulation accompanied by simultaneous activation of TRPC channels by PLC (Carver and Shapiro, 2019). Another subfamily of TRP channels are transient receptor potential melastatin (TRPM) channels, which are non-selective cation channels (TRPM1-8), and are involved in membrane depolarization by allowing the entry of sodium, magnesium and Ca^{2+} (Zholos, 2010).

1.3.2.6 M_1R-mediated regulation of TRPM channels

Upon activation of $G\alpha_q$ coupled GPCRs, PIP_2 hydrolysis by PLCβ results in PIP_2 depletion, which is necessary for the activity of many members of the TRP channels family. Evidence indicates the regulation of TRPM channels by M_1R. A recent report by Chauhan and colleagues showed that PIP_2 contributed to M_1R-mediated regulation of TRPM3 and antagonizing M_1R by PZ increased PIP_2 levels leading to TRPM3 activity in DRG sensory neurons (Chauhan et al., 2025). Another study showed that activation of M_1R depleted the PIP_2 pool in HEK293 cells leading to the inhibition of Ca^{2+} currents by TRPM4 (Nilius et al., 2006). TRPM5 is a sodium permeable channel involved in the taste transduction process. Activation of M_1R by ACh in HEK293 cells increased intracellular Ca^{2+} levels and TRPM5 activation. Interestingly, continuous exposure to Ca^{2+} desensitized TRPM5, but PIP_2 reversed channel desensitization (Liu and Liman, 2003). Xie *et al*., showed that PIP_2 was a strong regulator of both TRPM6 and 7. Application of carbachol depleted PIP_2 via M_1R activation and blocked TRPM6 and TRPM7 currents in HEK293 cells (Xie et al., 2011). Lastly, TRPM8 inhibition was reported in response to M_1R-associated PIP_2 depletion in DRG sensory neurons (Liu et al., 2019a). Also, a similar

effect was observed in a study on liver cells, that revealed that M_1R activation negatively regulated TRPM8 (Youhanna et al., 2025).

Table 2 Biased profile of orthosteric, allosteric and bitopic ligands at M_1R.

Experiments were conducted in recombinant Chinese hamster ovary (CHO) or Human embryonic kidney (HEK) cells stably expressing either the rat (rM1) or human (hM1) mAChRs, OC-033 mouse tumor cell lines or Sprague-Dawley rat dorsal root ganglia (DRG) neurons. Abbreviations: cAMP, cyclic adenosine monophosphate; IP3, inositol trisphosphate; ERK, extracellular signal-regulated kinase.

Ligand	Pharmacology	Bias profile	References
Acetylcholine	Agonist. ↑IP_3, ↑ cAMP, pERK1/2, β-arrestin pathways. (CHO-hM1 or rM1 cells	Non-biased	(Gurwitz et al., 1994; Keov et al., 2014; van der Westhuizen et al., 2018)
Carbachol	Agonist. ↑IP, Ca^{2+}, ↑cAMP, β-arrestin pathways. (CHO-hM1 or rM1; HEKhM1 OC-033-rM1, HEKCRISPR/Cas9-Δβ-arrestin, rat DRG cells)	Non-biased	(Davis et al., 2010a; Davis et al., 2009; Gurwitz et al., 1994; Sabbir and

			Fernyhough, 2018)
Oxotremorine-M	Agonist. $G\alpha_q$, IP_3, ↑cAMP, β-arrestin pathway. (CHO-hM1 or rM1 cells)	Non-biased	(Davis et al., 2010a; Davis et al., 2010b; Gurwitz et al., 1994; Thomas et al., 2008)
cis-dioxolane	Agonist. ↑cAMP pathways	Biased cAMP> IP3	(Gurwitz et al., 1994)
Pilocarpine	Agonist. IP_3, Ca^{2+}, ↑cAMP, β-arrestin pathway (CHO-hM1 or rM1 cells)	Non-biased	(Davis et al., 2009; Gurwitz et al., 1994; Thomas et al., 2008)
Arecoline	Agonist. $G\alpha_q$, IP_3, ↑cAMP, β-arrestin pathway (CHO-hM1 or rM1 cells)	Non-biased	(Gurwitz et al., 1994; Thomas et al., 2008)
Xanomeline	Agonist, IP_3, β-arrestin pathways (CHO-hM1 cells)	Non-biased	(Davis et al., 2009)

McN-A-343	Agonist. IP_3, ↑cAMP (CHO-hM1/rM1 cells)	Non-biased	(Gurwitz et al., 1994)
AC-42	Bitopic agonist. $G\alpha_q$, IP_3, ↑cAMP, β-arrestin pathways	Biased without efficacy for $G\alpha_{i/o}$	(Davis et al., 2009; Thomas et al., 2008)
77-LH-28-1	Bitopic agonist. $G\alpha_q$, IP_3, ↑cAMP (CHO-hM1 cells)	Biased without efficacy for $G\alpha_{i/o}$	(Thomas et al., 2008)
AC-260584	Bitopic agonist.IP_3, and Ca^{2+} (CHO-hM1 and HEK-hM1cells)	Biased G protein over β-arrestin	(Davis et al., 2010a; Davis et al., 2009)
TBPB	Bitopic agonist. Ca^{2+} and ERK1/2 (CHO-hM1 and HEK-hM1 cells)	Biased G protein over β-arrestin	(Davis et al., 2010b; Keov et al., 2014)
VU0357017	Bitopic agonist. Ca^{2+}, ERK1/2 (CHO-rM1 cells)	Biased G protein over β-arrestin	(Digby et al., 2012)
VU0364572	Bitopic agonist. Ca^{2+}, IP_3 ERK1/2 (CHO-rM1 cells)	Biased G protein over β-arrestin	(Digby et al., 2012)
BQCA	PAM. ↑ signaling on IP_3, ERK1/2, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_{12}$, ↑cAMP, β-arrestin pathways (CHO-hM1)	Non-biased	(Canals et al., 2012; Yeatman et al., 2014)

MIPS1645	PAM. ↑ signaling on IP_3, ERK1/2 and β-arrestin pathways (CHO-hM1)	Non-biased	(van der Westhuizen et al., 2018)
MIPS1780	PAM. ↑ signaling on IP_3, ERK1/2 and β-arrestin pathways (CHO-hM1)	Non-biased	(van der Westhuizen et al., 2018)
VU6004256	PAM. ↑ signaling on Ca^{2+} β-arrestin pathways. (CHO-hM1)	Biased Ca^{2+} and β-arrestin without internalization	(Rook et al., 2017)
VU0029767	PAM. ↑signaling on IP_3 (CHO-rM1)	Biased PLC >PLD	(Marlo et al., 2009)
VU0405645	PAM. ↑signaling on IP_3 (CHO-rM1)	Biased PLC >PLD	(Moran et al., 2019)
Pirenzepine	Antagonist. ERK1/2, β-arrestin, CREB pathways. (OC-033-rM1, HEK-CRISPR/ Cas9-Δβ-arrestin, rat DRG cells)	Biased β-arrestins over G proteins	(Sabbir and Fernyhough, 2018)
Muscarinic toxin 7	Antagonist. ERK1/2, β-arrestin, CREB pathways. (OC-033-rM1, HEK-CRISPR/ Cas9-Δβ-arrestin, rat DRG cells)	Biased β-arrestins over G proteins	(Sabbir and Fernyhough, 2018)

1.4 Acetylcholine impact on neuronal plasticity, differentiation and neurite outgrowth

1.4.1 Overview of the impact of ACh on neuroplasticity

It is well known that multiple factors are involved in the process of nervous system development and, among which epigenetic factors, growth factors, adhesion molecules and neurotransmitters play the major role in cell survival, fiber elongation, differentiation, proliferation, and plasticity (Jessell, 1988; Lipton and Kater, 1989; Roth and Sweatt, 2011; Snider, 1994). In the developing nervous system, neurotransmitters are not involved in synaptic neurotransmission and instead contribute to developmental processes such as neuronal differentiation, proliferation and outgrowth (Lipton and Kater, 1989). Among neurotransmitters, ACh is released from growing axons and regulate neuronal growth, proliferation, differentiation, and plasticity of vertebrates and invertebrates (Abreu-Villaça et al., 2011; Biagioni et al., 2000; Lauder and Schambra, 1999). Based on the neuron phenotype, the effects of ACh can be inhibitory or stimulatory. Also, ACh is considered as a chemoattractant in both central and the peripheral nervous system of amphibian and mammalian species (Kater et al., 1994; Kuffler, 1996; Owen and Bird, 1995; Zheng et al., 1994). In this review, having a focus on DRG neurons, the effects of ACh on neurite outgrowth, plasticity, and differentiation are discussed.

1.4.2 Acetylcholine and neuronal proliferation and differentiation

The cholinergic system is active during early stages of nervous system development. In rats, ChAT immunoreactive neurons emerge after the differentiation of progenitor cells, while ChAT positive cells appear in the germinative zones of the forebrain in mice during the second trimester from embryonic day (E) 12.5 to E18.5 (Lauder and Schambra, 1999; Schambra et al., 1989; Semba and Fibiger, 1988). In addition, the expression of mAChRs starts at the same time in

the forebrain and hindbrain during embryonic development (Gremo et al., 1987; Schlumpf et al., 1991). Several lines of research show that cholinergic neurons produce ACh during their migration and release ACh from their growth cones, neurites, and soma (Allen and Brown, 1996; Antonov et al., 1999; Hume et al., 1983; Tatsumi et al., 1995; Young and Poo, 1983). Released ACh from these neurons is involved in shaping neuronal networks, axonal navigation, neurite outgrowth and growth cone morphology (Owen and Bird, 1995; Rüdiger and Bolz, 2008; Zheng et al., 1994). Neurotransmitters stimulate/inhibit the proliferation of neurons through stimulation of GPCRs and cognate second messenger associated signaling (Weiss et al., 1998). Stimulation of proliferation is mostly associated with the activation of $G\alpha_i$- or $G\alpha_q$-coupled (but not $G\alpha_s$-coupled) receptors. Stimulation of $G\alpha_s$-coupled receptor and increased cAMP-signaling usually inhibits cell proliferation (Lauder and Schambra, 1999). In this regard, ACh is considered as a neurotransmitter that promotes cellular proliferation. In relation to this, previous studies showed that transfected fibroblasts with human M1, M3, and M5 genes exhibited proliferation following carbachol administration *in vitro* (Ashkenazi et al., 1989; McKenzie et al., 1992).

The pivotal growth-promoting impact of ACh was reported by studies that showed ChAT knockout in *C. elegans* triggered severe growth defects (Rand and Russell, 1984). Another study on embryonic P19 carcinoma cells, as an *in vitro* model system for neurogenesis, showed that treating progenitor cells with muscarine induced proliferation via stimulation of $G\alpha_q$-coupled muscarinic receptors and increased intracellular Ca^{2+} mobilization (Resende et al., 2008a; Resende et al., 2008b). Moreover, activation of mAChRs stimulated the proliferation of multipotent precursor and neuronal progenitor cells in the ventricular and subventricular zones of embryonic rat cortex (Ma et al., 2004; Ma et al., 2000). Further research revealed mAChRs-induced proliferation was mediated through ERK1/2 phosphorylation and the PI3K pathway (Li et al.,

2001). To confirm these findings, treating progenitor cells with atropine inhibited the proliferation and differentiation of these cells *in vitro* (Zhou et al., 2004). Neurogenesis occurs in the hippocampal formation of adult brain and newborn neurons express M_1R and M_4R. Inducing a lesion in septal cholinergic nuclei (which innervate hippocampus) inhibits proliferation and survival of dentate gyrus neurons and impairs spatial memory (Mohapel et al., 2005). In agreement with this, treating mice with donepezil, a selective AChE inhibitor, increased neuron survival and scopolamine administration suppressed donezepil-induced effects in the hippocampal dentate gyrus by blocking the CREB signaling (Kotani et al., 2008). Interestingly, the proliferative effect of ACh is not restricted to neuronal lineage cells and ACh induced proliferation in the glial lineage cells, such as astrocyte and oligodendrocyte lineages (Ashkenazi et al., 1989; Cohen et al., 1996; Guizzetti et al., 1996)

1.4.3 Acetylcholine and neurite outgrowth and plasticity

ACh has an inhibitory effect on neurite outgrowth in rat retinal ganglion cells (Lipton et al., 1988) and chick sympathetic neurons (Small et al., 1995), while preventing serotonin-mediated neurite outgrowth inhibition in *Helisoma* (McCobb et al., 1988). Evidence indicates that both nAChRs and mAChRs mediate the effects of ACh on neurite outgrowth (Abreu-Villaça et al., 2011). Each cell type expresses a unique pattern of nAChRs and mAChRs, thus the effects of ACh on different cell types may vary. For example, ACh administration to primary neurons isolated from the thalamus of E14 mice reduced axon length in a dose dependent manner and mAChRs played the major role in neurite outgrowth suppression (Rüdiger and Bolz, 2008). However, stimulation of M_1R by carbachol resulted in accelerated axonal outgrowth in neocortical pyramidal neurons (but not cerebellar granule cells) via increased Ca^{2+} mobilization, PKC activation, and ERK1/2 phosphorylation (VanDeMark et al., 2009). Also, the inhibitory effect of ACh on neurite

outgrowth and motility was reported in cultured embryonic mouse spinal cord neurons (Owen and Bird, 1995).

Since ACh suppresses neurite outgrowth, it is expected that ACh metabolism by AChE prevents the suppressing effects of ACh on neurite outgrowth. Interestingly, AChE not only prevent ACh-induced inhibition of neurite outgrowth, but guides neurites towards a target by a non-enzymatic mechanism through recruitment of adhesive molecules (Paraoanu and Layer, 2008). Treating different types of cells (*in vitro*) with specific AChE inhibitors, that do not inhibit enzymatic active-site (such as BW254c51, propidium, and fasciculin), reduces neurite outgrowth. Intriguingly, treating cell cultures with AChE inhibitors, which target enzymatic active-site (such as echothiophate and galanthamine) had no effect on neurite outgrowth (Dupree and Bigbee, 1994; Layer et al., 1993; Olivera et al., 2003). These findings suggest that peripheral anionic site of AChE enzyme is responsible for the non-catalytic function of AChE, In agreement with this, several studies using primary cultured cells or cell lines demonstrated that overexpressing AChE-S (synaptic variant of AChE) increased neurite outgrowth, while suppression of enzyme by antisense oligonucleotides led to a reduction (Bigbee et al., 2000; Karpel et al., 1996; Sternfeld et al., 1998). Also, addition of AChE to the medium of hippocampal neurons increased neurite outgrowth and synapse formation (Olivera et al., 2003). A summary of the effects of cholinergic drugs on neuronal behaviors are listed in **Table 3**.

Table 3. A summary of the effects of cholinergic drugs on the neuronal proliferation, differentiation, and outgrowth.

Treatment	Mechanism	Effects	References
Proliferation and Differentiation			
Carbachol	Increased DNA synthesis through M_1R and M_4R involvement	Increased the proliferation of Chinese hamster ovary (CHO) cells, primary astrocytes	(Ashkenazi et al., 1989)
Carbachol	Increased PIP_2 hydrolysis through M_1R	Increased proliferation of Chinese hamster lung fibroblast (CCL39) cell line	(McKenzie et al., 1992)
Muscarine	Increasing free cytosolic Ca^{2+} levels through M_1R and M_3R	Increased the proliferation of P19 Neural progenitor cells	(Resende et al., 2008a; Resende et al., 2008b)
Nicotine	Increasing free cytosolic Ca^{2+} levels through nAChRs	Increased the proliferation of P19 Neural progenitor cells	(Resende et al., 2008a; Resende et al., 2008b)
ACh and Carbachol	Different mechanisms including the activation of MAP kinase phosphorylation and Ca^{2+} signaling through M_2R/M_4R activation	Increased proliferation and differentiation of neural progenitor cells	(Ma et al., 2004; Ma et al., 2000)

Carbachol	Activation of PI3K and ERK1/2 through mAChRs	Increased neural stem cell proliferation	(Li et al., 2001)
ACh	Increased Ca^{2+} signaling through M_2R	Increased neural stem cell proliferation and differentiation	(Zhou et al., 2004)
Physostigmine	Increased ACh levels in the hippocampus and increased neurogenesis through M_1R/M_4R	Enhanced neurogenesis and neuron survival in the hippocampus following brain lesions	(Mohapel et al., 2005)
Donepezil	Increased CREB phosphorylation by increasing ACh in the hippocampus	Enhanced the proliferation and differentiation of newly born neurons in the hippocampus	(Kotani et al., 2008)
Carbachol	Increased *c-fos* expression through mAChRs-mediated increase in PKC activity	Increased oligodendrocyte precursor cells proliferation	(Cohen et al., 1996)
Carbachol	Increased DNA synthesis through M_3R mediated increase in PKC activity	Increased the proliferation of primary astrocytes	(Guizzetti et al., 1996)
Neural outgrowth			
α-Tubocurarine Mecamylamine	Blocked nAChRs	Increased sprouting or regenerate neuronal processes in retinal ganglion cells	(Lipton et al., 1988)
ACh, Carbachol, Oxotremorine	Stimulation of mAChRs/nAChRs	Inhibited neural outgrowth in chick sympathetic neurons	(Small et al., 1995)

ACh, Oxotremorine, Nicotine	Stimulation of both nAChRs and mAChRs	Inhibited neurite outgrowth and induced morphological changes in developing thalamic neurons. Effects reversed by the administration of nAChRs and mAChRs antagonists.	(Rüdiger and Bolz, 2008)
ACh	Stimulation of cholinergic receptors, mecamylamine (but not atropine) reversed the effects	Decreased growth cone motility and inhibited neural outgrowth in mouse spinal cord neuron *in vitro*	(Owen and Bird, 1995)
BW 284C51 (AChE inhibitor), Ethopropazine (BChE inhibitor)	Decreased in neural outgrowth through both (non)enzymatic mechanism	Decreased neural outgrowth in chicken retina ganglion cells	(Layer et al., 1993)
AChE	Decreasing ACh levels.	Increased neurite elongation, synapse formation, and surface expression of AMPA receptors in hippocampal neurons	(Olivera et al., 2003)
BW 284C51	AChE inhibition and cytoskeletal abnormalities in the cell body	Decreased neurite outgrowth and neuronal migration of cultured DRG neurons	(Dupree and Bigbee, 1994)
ACh, DMPP	Increased Ca^{2+} influx and intracellular Ca^{2+} levels.	caused pronounced filopodial elongation in neurons from the buccal ganglion of the pond snail *Helisoma trivolvis*	(Zhong et al., 2013)
Carbachol, Muscarine	Involvement of both nAChRs and mAChRs in neural outgrowth	Increased neurofilament expression and neurite outgrowth in DRG neurons from chick embryo	(Tata et al., 2003)

Carbachol	Increased PKC and ERK1/2 activity through M_1R activation	Increased axon length of pyramidal neurons from the neocortex but not cerebellar granule neurons	(VanDeMark et al., 2009)

1.4.4 Acetylcholine effects on DRG sensory neurons

Dorsal root ganglia are a complex structure of the peripheral nervous system, which transduce peripheral messages and convey them to the CNS through the spinal cord. These structures consist of different types of sensory neurons and non-neuronal cells (such as fibroblastic, Schwann, and satellite cells) and appear in the early stages of nervous system development (Carr and Simpson, 1978; Pannese, 1974). Several lines of research demonstrated that DRG sensory neurons are ChAT immunoreactive and they are able to synthesize and release ACh (Biagioni et al., 2000). The peripheral form of ChAT (pChAT) is an alternative splice variant of ChAT responsible for ACh synthesis in the peripheral cholinergic system and DRG sensory neurons, and is considered as the most reliable marker for the visualization of cholinergic neurons (Bellier and Kimura, 2007, 2011). In addition, nAChRs are expressed on the soma of DRG neurons in chick, amphibia, and rat (Boyd et al., 1991; Morita and Katayama, 1984; Sucher et al., 1990), and mAChRs are also present in DRG neurons and glial cells of a wide range of species including amphibia and rat (Morita and Katayama, 1984; Tata et al., 1999; Tata et al., 2000b). The M_2R subtype is the predominant mAChR in DRG neurons of rat and chick (Bernardini et al., 1999; Bernardini et al., 2002; Tata et al., 1999). Treating DRG neurons (isolated from bullfrog) with ACh induced a long-lasting depolarization in the soma of neurons, which was mediated by a decrease in membrane potassium conductance (Morita and Katayama, 1984). Treating DRG neurons with muscarine and oxotremorine increased intracellular Ca^{2+} levels, which was

suppressed by atropine administration (but not mecamylamine) indicating the involvement of mAChRs. Using different antagonists against mAChRs subtypes, Tata *et al*., showed Ca^{2+} mobilization in DRG neurons was mainly regulated by M_1R/M_3R (Tata et al., 2000a). Accumulating evidence suggests that ACh inhibits neurite outgrowth in different types of neurons (including DRG neurons), however, there are studies that show ACh increases neurite outgrowth (Abreu-Villaça et al., 2011; Lauder and Schambra, 1999; Owen and Bird, 1995). One example is the study by Zhong *et a*l. who showed ACh elongated neuronal growth cone filopodia through nAChRs in buccal ganglion of the pond snail *Helisoma trivolvis* (Zhong et al., 2013). ACh has a role in neurite extension in molluscan (the second-largest phylum of invertebrate animals) nervous systems (McCobb et al., 1988). Another example is a study on DRG neurons isolated from chick embryos at E12 that showed administration of carbachol and muscarine enhanced neurite outgrowth by mAChRs (Tata et al., 2003). Authors of the study explained that DRG neurons at E12 are post-mitotic, but their differentiation is in progress and they do not require neurotrophins for survival, thus they removed nerve growth factor (NGF) from the DRG culture in this study. It seems that differences in the methodologies and model systems underlie the inconsistencies between latter examples and previous studies. Studies in our lab on rat and mice DRG neurons showed that blocking mAChRs was associated with increased neurite outgrowth (and will be discussed in 1.4.7).

1.4.5 Acetylcholine at the motor endplate and muscarinic acetylcholine receptors

In the vertebrates, muscle fibers are innervated by motor neurons, whose cell bodies are located in the spinal cord/brainstem. Motor neurons have myelinated axons that project to muscles via a complex of peripheral nerves. Each axon enters the muscle and innervate many fibers through multiple branches. These branches are not myelinated and finally form the neuromuscular junction

(NMJ) (Hughes et al., 2006). The skeletal muscle fiber, the motor axon terminal, and perisynaptic Schwann cells (non-myelinating Schwann cells/terminal Schwann cells or PSCs) are the main components of the NMJ (Rudolf et al., 2019). The NMJ is considered a special type of synapse that transduces action potential pulses from neurons to muscles through ACh. NMJ structure and function differ between species. For example, while ACh is the chemical transmitter in the NMJ of vertebrates and *C. elegans*, glutamate is the main neurotransmitter in *Drosophila melanogaster* (Keshishian et al., 1996). Another example is that motor neurons of vertebrates make only one contact with a single muscle fiber, while neurons of insects make multiple contacts with a single muscle fiber (Slater, 2017).

There are several factors that contribute to the molecular organization of the NMJ structure and function. Among the nerve-derived components, agrin is known to activate the synthesis, aggregation and clustering of AChRs (mostly nAChRs) on the post synaptic site (Gautam et al., 1996; Nitkin et al., 1987; Tintignac et al., 2015) and Wnt signaling is known to induce and stabilize AChR clusters at post synaptic structures (Shen et al., 2018; Zhang et al., 2012). Also, muscle-derived components of the extracellular matrix (such as laminins and collagen) play an important role in the shaping of presynaptic structure during development. For example, laminin β2 directly binds to the presynaptic Ca^{2+} channels and deficiency of laminin β2 is associated with a significant decrease in neurotransmitter release due to shortage of synaptic vesicles (Nishimune et al., 2004; Rogers and Nishimune, 2017). Apart from stimulating nAChRs and mAChRs, ACh is one of the main regulators of the NMJ machinery as it often negatively regulates NMJ function by inducing dispersion and internalization of the post synaptic AChRs in a mechanism, which involves Cdk5 (Lin et al., 2005). To understand the importance of ACh in patterning and formation of mammalian NMJ, studies using ChAT $^{-/-}$ mice showed that these animals has no spontaneous and nerve-

induced postsynaptic potentials. Authors of this work showed that ChAT $^{-/-}$ embryos had an increase in the number of motor neurons, muscle hyperinnervation and AChR clusters (Brandon et al., 2003). Moreover, other studies used VAChT (vesicular acetylcholine transporter) knockout mice and showed significant aberrant changes in the neuromuscular structure and function similar to ChAT knockout mice (de Castro et al., 2009; Prado et al., 2006).

Emerging lines of research indicate the involvement of mAChRs in the development of NMJ. In early postnatal days, muscle fibers are multi-innervated by several motor axons in the NMJ, but following the competition between axons, each muscle fiber is innervated by a single axon. Evidence show that presynaptic mAChR, adenosine receptors and tropomyosin-related kinase B receptor (TrkB) orchestrate the communication between axons and regulate the competitive processes leading to synapse loss in the NMJ (Nadal et al., 2017; Nadal et al., 2016a; Nadal et al., 2016b). For example, a study by Garcia *et al*. showed that BDNF (Brain- derived neurotrophic factor) and NT-4 (neurotrophin 4) increased ACh release in the NMJ through the activation of TrkB receptor. Administration of atropine, PZ, and methoctramine blocked the effect of BDNF and NT-4 on ACh release. These results suggest that M_1R and M_2R auto-receptors are required for TrkB-associated ACh release (Garcia et al., 2010). Other studies suggest that presynaptic mAChRs (namely M_1R, M_2R, and M_4R) suppress ACh release by a mechanism dependent to PKC and voltage-gated Ca^{2+} channels in weak nerve endings, which consequently lead to their elimination at early stages of postnatal life (Santafe et al., 2009; Santafé et al., 2003, 2004; Wessler, 1989). Also, it has been reported that mAChRs are expressed in the muscle fibers and mediate the developmental effects of neurotransmitters (Furlan and Godinho, 2005; Reyes and Jaimovich, 1996). An interesting study by Wright *et al.* used several pharmacological and genetic manipulation approaches to determine the role of mAChR subtypes at mouse NMJs. They showed

that M_2R was the sole mAChR in motor neurons, and M_1R, M_3R and M_5R were expressed on PSCs/muscle fibers. Treating animals with atropine, non-selective mAChRs antagonist, resulted in terminal sprouting, terminal withdrawal, and muscle fiber atrophy suggesting that mAChRs were necessary for the NMJ formation and muscle function and structure. Treating animals with methoctramine, a selective M_2R/M_4R antagonist, resulted in terminal sprouting, terminal withdrawal, but not muscle fiber atrophy. In agreement with these results, $M_2R^{-/-}$ mice (and not other mAChRs knockout mice) showed spontaneous sprouting and loss of terminal nerve endings. Interestingly, authors found that $M_5R^{-/-}$ mice had small NMJs and muscle fibers and concluded that while M_2R was essential for the stability of axon terminals, muscle fiber growth depended on M_5R (Wright et al., 2009).

1.4.5.1 Schwann cells and motor endplate function

In the vertebrates, mature Schwann cells are divided into three main groups, which are myelinating Schwann cells, Remak Schwann cells, and perisynaptic Schwann cells (PSCs). Perisynaptic Schwann cells play a role similar to astrocytes in the CNS. In vertebrates, neural crest cells differentiate to glial lineage cells, and a subset of them gives rise to PSCs in the peripheral nervous system. PSCs are involved in the formation and development of NMJ and differ from myelinating Schwann cells, which are responsible for myelination of axons (Scherer, 1997; von Bernhardi et al., 2016). A study by Barik and colleagues showed that PSCs ablation at different stages of lifespan had devastating effects on the NMJ formation/function in mice. The authors of this work revealed that PSC ablation at E12.5 (a time nerves approach to muscle fibers) led to the formation of smaller and fewer nerve-induced AChR clusters, which suggest PSCs contribute to the maturation of AChR clusters. Further, they showed that PSC ablation at postnatal day (P) 30 (after NMJ maturation) was associated with terminal fragmentation and impaired neuromuscular

transmission (Barik et al., 2016). Previous studies demonstrate that axon-derived neuregulin 1 (NRG1) and its signaling pathway play a major role in Schwann cells development. Genetic studies on the ablation of NRG1 or ErbB receptor (NRG1 receptor) genes reveal that any defect in NRG1 signaling pathway result in the absence of Schwann cells (including PSCs), which are responsible for the formation and maintenance of the NMJ (Leu et al., 2003). In this regard, a number of studies used *ErbB3*$^{-/-}$, *α-MHC-ErbB2*$^{-/-}$, *Nrg1*$^{-/-}$ knockout mice and showed lack of PSCs was associated with retraction and degeneration of nerve terminals at NMJ (Jaworski and Burden, 2006; Lin et al., 2000; Riethmacher et al., 1997; Woldeyesus et al., 1999). Interestingly, ablation of *ErbB3* had no impact on the initial development of motor neurons and DRG sensory neurons, but at later stages, the majority of motor neurons and DRG sensory neurons died in *ErbB3*$^{-/-}$ embryos. These findings imply that Schwann cells not only contribute to NMJ development and function, but plays a pivotal role in motor and sensory neurons survival (Riethmacher et al., 1997). Liu *et al.* used *CRD-Nrg1*$^{-/-}$ mice that lack PSCs, muscle denervation and synaptic formation. Using these knockout mice, authors applied genetic manipulations at presynaptic regulators of NMJ formation by ablating particulars genes, such as *Snap25* (synaptosomal-associated 25 kDa protein) or *Chat* and found that the ablation of these genes prevented muscle denervation and synaptic loss in *CRD-Nrg1*$^{-/-}$ mice. These findings suggest that PSCs modulate ACh release and presynaptic vesicular activities, which are involved in synaptic formation and muscle innervation (Liu et al., 2019b). Collectively, these findings indicate PSCs play an indispensable role in the development of NMJ.

In the NMJ, PSCs express neurotransmitter receptors and synthesize a variety of neuromodulatory compounds such as ATP and ACh. The extracellular ATP is hydrolyzed to adenosine that activates purinergic receptors (such as P2Y1R) expressed by neurons, PSCs, and muscle fibers. Both ATP and ACh activate P2Y1R and mAChR (mainly $G\alpha_q$-coupled receptors),

respectively, and increase intracellular Ca^{2+} in PSCs. Increased intracellular Ca^{2+} is associated with increased ATP release by PSCs, and consequently increased adenosine formation that activate purinergic receptors on neurons (Darabid et al., 2013; Darabid et al., 2018). While high levels of adenosine activate A_1ARs A_2ARs on neurons and induces neuronal potentiation, low adenosine levels only activate A_1ARs leading to neuronal depression (Todd et al., 2010). Thus, PSCs regulate the patterns of neuronal activity and synaptic plasticity at the NMJ. Overall, cholinergic system plays an important role in the communication of NMJ components by regulating the development of NMJ , synaptic pruning, (Smith et al., 2013), and injury responses (Kang et al., 2014).

1.4.5.2 Schwann cells and myelination

Large axons are wrapped by the myelin sheaths, which improve rapid conduction of electrical messages in nerves and promote the efficiency of neuronal communications. Schwann cells and oligodendrocytes are responsible for the axon myelination in the peripheral nervous system and CNS, respectively. Accumulating evidence indicates that ACh is involved in the process of axon myelination (Fields et al., 2017). Previous studies revealed that myelin fractions obtained from the brain contain high level of muscarinic receptors, mainly M_1R and M_2R (Evans et al., 1985; Larocca et al., 1987; Watson et al., 1986). Previous studies showed that AChE activity was high during myelination process and AChE inhibitors improved myelin integration (Bartzokis, 2007; Fields et al., 2017; Kim et al., 1972). These findings suggest that cholinergic signaling is actively involved in the process of axon myelination. Also, a series of pharmacological studies showed the presence of muscarinic receptors on myelinating glia. In this regard, treating oligodendrocytes and oligodendroglioma cells with carbachol resulted in increased intracellular Ca^{2+} mobilization and inositol monophosphate accumulation, respectively (Post and Dawson, 1992; Takeda et al., 1995). Another study showed treating oligodendrocytes with carbachol

increased IP_3 and Ca^{2+} signaling and these effects were blocked by the administration of atropine and PZ indicating the role of M_1R in the oligodendrocyte development (Cohen and Almazan, 1994). Other studies also showed that muscarinic receptors were involved in the proliferation of oligodendrocyte progenitor cells (OPCs) by increasing ERK2 phosphorylation (Larocca and Almazan, 1997). A study by De Anglis and colleagues demonstrated that M_1R, M_3R, and M_4R were main mAChRs expressed in the OPCs and muscarine administration was associated with OPC proliferation *in vitro*. Authors of this work showed that treating OPCs with mAChRs antagonists, mainly atropine and PZ, suppressed OPCs proliferation and enhanced differentiation of OPCs (De Angelis et al., 2012). Similarly, Schwann cells also express muscarinic receptors and mice lacking M_2R and M_4R exhibit axon degeneration and abnormal myelin organization (Loreti et al., 2006; Uggenti et al., 2014). Stimulation of mAChRs induced the proliferation and survival of various types of glial cells (De Angelis et al., 2012; Murphy et al., 1986; Ragheb et al., 2001). Among mAChRs, M_2R is abundantly expressed in mature Schwann cells and activation of this receptor suppresses Schwann cell proliferation and enhances Schwann cells differentiation and upregulation of promyelinating factors (Loreti et al., 2007; Loreti et al., 2006; Uggenti et al., 2014). These findings indicate the involvement of cholinergic system in the development and function of myelinating glial cells.

Increasing lines of research during last decade indicate the therapeutic properties of anticholinergic drugs for myelinopathies, mainly multiple sclerosis (MS). Multiple sclerosis is a complex neurodegenerative disorder and is associated with various environmental factors, gender, and genetic background. This disorder is considered an inflammatory disorder that impairs axon myelination and results in axonal loss in the CNS (Buzzard et al., 2017). A research by Deshmukh and colleagues used an animal model of MS (cuprizone-induced demyelination) and experimental

autoimmune encephalomyelitis (EAE) model, and showed treating animals with benztropine induced remyelination (both *in vitro* and *in vivo*) through direct antagonism of M_1R and/or M_3R (Deshmukh et al., 2013). Using micropillar array screening, a recent study by Mei *et al.* identified a cluster of antimuscarinic compounds that effectively induced oligodendrocyte differentiation and membrane wrapping. These compounds (atropine, ipratropium, oxybutynin, trospium, tiotropium, quetiapine, benzatropine and clemastine) were suggested as potential drugs for the treatment of MS (Mei et al., 2024). Most of these drugs possess selective antagonistic activity against M_1R and M_3R more than other mAChRs. In relation to these findings, it has been reported that M_3R and M_1R are the most abundant mAChRs expressed on rat and human OPCs (Abiraman et al., 2015; De Angelis et al., 2012; Zhang et al., 2014b). In addition, Abiraman *et al.* used an animal model of hypomyelination and reported that solifenacin, an M_1R/M_3R selective antagonist, enhanced oligodendrocyte differentiation (Abiraman et al., 2015). Recently, the same research group showed that loss of M_3R in human OPCs led to improved cellular differentiation and reduced intracellular Ca^{2+} mobilization in response to oxotremorine-M. Further, authors of this research transplanted $M_3R^{-/-}$ OPCs to a mouse model of hypomyelination (*shiverer/rag2* mice) and observed a significant increase in remyelination *in vivo* (Welliver et al., 2018). Growing body of evidence indicates that FDA-approved drug clemastine, a muscarinic antagonist, possesses favorable effects on myelin formation. In this regard, clemastine was shown to enhance myelination in the prefrontal cortex of socially isolated rats by enhancing OPC differentiation (Liu et al., 2016). Using cuprizone mouse model of demyelination, Li *et al*., showed that clemastine treatment (10 mg/kg, 3 weeks, oral) enhanced myelination and increased the number of mature oligodendrocytes (Li et al., 2015b). Another study by Cree *et al*. induced neonatal hypoxia in wild-type and *Chrm1*-cKO (a mouse with conditional M_1R knockout in OPCs) mice. Clemastine treatment (10 mg/kg/day, P3-

P10, oral) significantly increased OPCs differentiation, myelination, and functional recovery in wild-type mice. However, same treatment failed to induce protective effects against hypoxia in *Chrm1*-cKO mice indicating the involvement of M_1R in mediating the effects of clemastine on myelination and OPCs maturation (Cree et al., 2018). Interestingly, a recent report by a randomized, controlled, double-blind, crossover trial showed that clemastine fumarate was a reliable remyelinating therapy for MS (Green et al., 2017). Overall, mAChRs antagonists possess potential remyelinating effects in different animal models of disease and are considered as novel therapeutic agents for the treatment of MS.

1.4.6 Non-neuronal cholinergic system in the epidermis

As mentioned above, NNCS plays an important role in the regulation of biological processes in living systems and NNCS dysregulation is observed in several human disorders (Beckmann and Lips, 2013; Wessler and Kirkpatrick, 2008). Indeed, cholinergic components (ACh, ChAT, AChE, mAChRs, and nAChRs) are present in many non-neuronal cells, including keratinocytes (KC), which are the main cellular component of epidermis. The epidermis envelops the skin and covers the oral and vaginal mucosa and consists of different layers (**Figure 7**). Keratinocytes synthesize, secrete, and degrade ACh and express both nAChRs and mAChRs. Ample evidence indicates that NNCS of human KCs (or ACh axis) plays a major role in the development and function of the skin (Grando, 2006; Grando et al., 2006). Acetylcholine exerts its modulatory effects on KCs through activation of both nAChRs and mAChRs. It is important to note that simultaneous activation of nAChRs and mAChRs occurs in a unique manner, through which the stimulation of a combination of nAChRs and mAChRs by ACh results in a balanced and synchronized biological response.

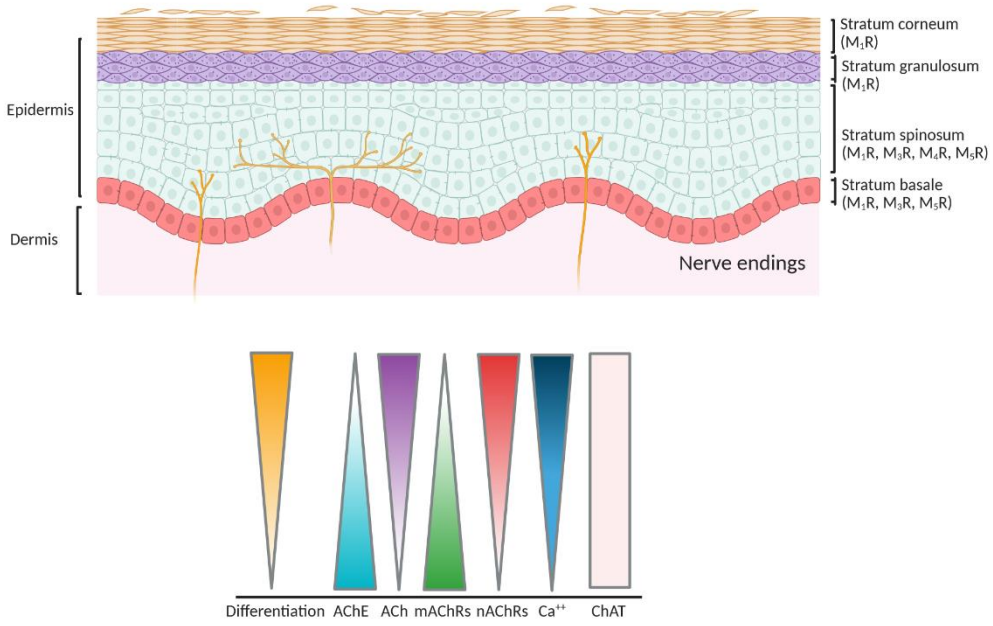


Figure 7. A simplified structure of epidermis and mAChRs expression. a) expression pattern of mAChR subtypes in different layers, and b) Alterations (increase/decrease) in cholinergic component during the differentiation of KC.

For example, increased Ca^{2+} influx following nAChRs stimulation is balanced by the activation of inhibitory mAChRs in the KCs (Grando, 1997). Immunohistochemical studies show that KCs express all types of mAChRs at the RNA and protein levels and the expression pattern of mAChRs varies during developmental stages of KCs. In this regard, M_3R (predominant subtype) and M_5R are expressed in the basal cells (Stratum basale layer), M_4R and M_5R are expressed in the spinous cells (Startum spinosum layer), and M_1R are the main subtype expressed by granular cells (Stratum granulosum layer) (Ndoye et al., 1998). Although there are controversial reports about the localization and expression of M_2R in the human epidermis (Elwary et al., 2004), but granular cells

of rat's epidermis express M_2R (Haberberger and Bodenbenner, 2000). Indeed, KCs express different combination of mAChRs at different stages of their development suggesting the distinct role of each mAChR subtype in the KCs maturation and function. Also, previous studies showed that ChAT was expressed equally throughout the epidermis, but AChE was predominantly expressed in Stratum basale (Nguyen et al., 2001). These findings suggest that ACh levels are higher in uppermost epidermal regions.

1.4.6.1 Muscarinic regulation of KC proliferation and outgrowth

The Stratum basale layer contains the KC progenitor cells, which give rise to transit amplifying cells. During the KC migration, these cells move upwards from Stratum basale layer and eventually experience terminal differentiation (Kirfel and Herzog, 2004). Previous studies on the role of cholinergic agonists and antagonists on the KCs development showed that both nAChRs and mAChRs were involved in the proliferation and outgrowth of KCs (Grando et al., 2006). In this regard, administration of propylbenzilylcholine mustard (PrBCM, irreversible muscarinic antagonist) to human KC culture caused a significant increase in cell number, while the administration of bromo-acetylcholine (BrACh, a specific ChAT inhibitor) suppressed KC proliferation and outgrowth suggesting that ACh is necessary for KC proliferation (Grando et al., 1993). On the other hand, administration of muscarinic agonists (such as muscarine and arecoline) to KCs (*in vitro*) was associated with mixed results with respect to cellular proliferation and outgrowth. Treatment with muscarine increased the KC proliferation and upregulation of factors associated with cellular proliferation and growth. These effects were abolished by the administration of atropine. In another study, administration of arecoline (a partial agonist for nAChRs and mAChRs) suppressed the KC proliferation (Arredondo et al., 2003a; Chang et al.,

2001; Thangjam and Kondaiah, 2009). These results suggest that mAChRs are involved in the KCs growth and proliferation. Moreover, nAChRs are also involved in the differentiation of KCs. In this context, previous studies showed that blocking the α7 nAChR signaling pathway resulted in the inhibition of terminal differentiation of KCs. Also, blocking of α7 nAChR pathway by α-bungarotoxin downregulated proapoptotic factors (such as Bad) suggesting that activation of α7 nAChR is associated with KCs apoptosis (Arredondo et al., 2002; Arredondo et al., 2003b) .

1.4.6.2 Muscarinic regulation of KC migration

The migration of KCs plays a major role in the wound healing process and any defect in KC migration is associated with clinical phenotype associated with non-healing wounds (Santoro and Gaudino, 2005). Within 24 hours after wounding, KCs in the basal layer undergo significant morphological and structural changes and start migrating laterally over the wounded area (Kirfel and Herzog, 2004; Ortonne et al., 1981). Crawling locomotion and migration of KCs depend to their ability to attach to the other cells and surrounding matrix. Interestingly, KCs are able to synthesize and express a wide range of extracellular matrix (ECM) materials, which play a major role in their cell-cell and cell-matrix interaction. The integrins are a family of proteins that link the cell cytoskeleton to the ECM and are involved in the KCs adhesion and migration process. Keratinocytes express several types of integrins (such as $\alpha_V\beta_5$, $\alpha_5\beta_1$, and $\alpha_V\beta_6$) that enable them to attach to ECM proteins such as fibronectin and vitronectin (Larjava et al., 1993). Since KCs express integrin receptors (recognize ECM of adjacent KCs), the lower cells facilitate the migration of upper KCs by providing an abutment for them (Kirfel and Herzog, 2004; O'Keefe et al., 1984). Acetylcholine is a highly positive charged molecule and has been reported as a chemoattractant, which determines the direction of migrating KCs (Chernyavsky et al., 2005).

M_1R and α7 nAChR are responsible for ACh chemotaxis and play the primary role in determining the direction of KCs migration. In relation to this, treating KCs with MT7, a negative allosteric modulator of M_1R) or silencing the M_1R gene resulted in a decrease in the number of migrating KCs toward the cathode in a direct current electric field (galvanotropism), a model system used for cellular migration studies. Further, antagonizing both M_1R and α7 nAChR resulted in the completed suppression of KCs migration. The stimulation of M_1R and α7 nAChR is associated with the activation of ERK and downregulation of sedentary integrins in KCs (Chernyavsky et al., 2005). To evaluate the effects of pharmacological agents on KCs migratory behaviors, researchers used agarose gel keratinocyte outgrowth system (AGKOS), which is a reliable test to investigate KC function during re-epithelization (Chernyavsky et al., 2004a; Grando et al., 1993). Using AGKOS, it has been shown that stimulation of M_3R and M_4R exhibit reciprocal effects on KCs migration distance. In this regard, Chernyavsky and colleagues showed that suppression of M_3R with siRNA resulted in the increased migration distance of KCs and treating the cells with MT3 (a M_4R antagonist) decreased the KCs migration. Conversely, suppressing M_4R with siRNA resulted in the decreased migration distance of KCs and treating the cells with 4-DAMP (a M_3R antagonist) had no effect on KCs migration. Authors of this work used $M_3R^{-/-}$ and $M_4R^{-/-}$ mice and showed KC migration increased in $M_3R^{-/-}$ mice, while there was a significant decrease in KC migration in $M_4R^{-/-}$ mice (Chernyavsky et al., 2004b). Interestingly, results of this work showed that M_3R inhibition was associated with the upregulation of migratory integrins, while M_4R inhibition resulted in the upregulation of sedentary integrins. Indeed, the inhibitory impact of M_3R stimulation on KC migration is mediated by calcium-dependent cGMP/protein kinase G signaling pathway and M_4R stimulation suppresses the effects of M_3R activation through adenylyl cyclase/cAMP/protein kinase pathway (Chernyavsky et al., 2004b). These results indicate that

stimulation of M_3R suppresses KC migration, while M_4R stimulation is associated with increased migration of KCs.

1.4.6.3 Muscarinic control of epidermal cohesion and cornification

Keratinocytes are strongly held together by adherens junctions (*Zonula* and *macula* adherens), which are necessary for the epithelium integrity and protection against environmental mechanobiological stressors. Evidence indicates that ACh signaling (both nAChRs and mAChRs) is highly involved in the regulation of KC adhesion (Grando, 2006). In this regard, previous research revealed that mAChRs antagonists induced acantholysis (loss of intercellular connections and KC adhesion) suggesting that stimulation of mAChRs is required for KC adhesion. For example, atropine has been reported to increase the permeability of KCs layers, and inducing acantholysis in an *in vitro* model of human epidermis (Kurzen et al., 2006; Nguyen et al., 2004b). Also, administration of atropine was shown to increase the phosphorylation of desmoglein 3, E-cadherins and catenins suggesting that phosphorylation of adherence proteins is involved in the atropine-induced acantholysis (Nguyen et al., 2003; Nguyen et al., 2004b) Interestingly, nAChRs antagonists are able to impair intercellular connections and KC adhesion indicating that both nAChRs and mAChRs are involved in the KCs adhesion (Grando et al., 1995; Nguyen et al., 2004b). Using knockout mice lacking M3, α3, and α9 cholinergic receptors, Nguyen and colleagues showed the abnormal expression of adherence molecules in KCs. Authors of this work also demonstrated that suppression of each of these three receptors induced acantholysis in human KCs, but simultaneous suppression of α3, α9, and M3 receptors by antisense oligonucleotides aggravated acantholysis (Nguyen et al., 2004b). The effects of cholinergic antagonists on KCs adhesion is reversible and previous studies showed that cholinergic agonists (such as carbachol

and methacholine) reversed the acantholysis mainly through the upregulation of adherence proteins like E-cadherin and desmoglein (Nguyen et al., 2003; Nguyen et al., 2004a). The anti-acantholytic effect of cholinergic agonists has been the focus of researchers for the treatment of pemphigus vulgaris (PV), a skin disease caused by the generation of IgG autoantibodies against adherence molecules that induces acantholysis (Fania et al., 2012; Grando, 2006; Nguyen et al., 2004a). It has been suggested that therapeutic effects of cholinergic agonists in PV are mediated by suppressing the IgG-induced phosphorylation of p120-catenin and γ-catenin (Chernyavsky et al., 2008; Nguyen et al., 2004a). Further research showed that stimulation of M_1R by pilocarpine increased serine/threonine and tyrosine phosphatase activities, reduced p120- and γ-catenin phosphorylation, and prevented KCs acantholysis *in vitro*. Treating cells with MT7 abolished pilocarpine-induced effects suggesting that M_1R is partly mediates the anti-acantholytic activity of cholinergic agonists. In addition, stimulation of α7 nicotinic receptor by AR-R17779 resulted in similar effects, but AR-R17779 treatment inhibited Src kinase as well. Treating cells with methyllycaconitine abolished AR-R17779 -induced effects suggesting that α7 nicotinic receptor is involved in the anti-acantholytic activity of cholinergic agonists (Chernyavsky et al., 2008).

Epidermal cornification is accompanied by a special form of KCs terminal differentiation and programmed cell death and results in the formation of the Stratum corneum (also hair and nails), which responsible for protecting the body from dehydration and environmental chemical injury (Eckhart et al., 2013). Terminally differentiated KCs synthesize different products such as lipids and humectants (such as filaggrin) and secrete them in the form of lamellar bodies (containing lipids and proteins) into the extracellular space. Concomitantly, granular cells lose their cellular organelles and nuclei and experience programmed cell death and consequently, become non-viable corneocytes in the stratum corneum. The humectants released by granular cells

are filaggrin degraded products, which serve as natural moisturizing factor (NMF) and are responsible for the plasticity of epidermis (Eckhart et al., 2013). Evidence indicates that ACh contributes to the epidermal cornification as a secretagogue that promotes the secretion of humectants. These effects of ACh are mediated by synergistic action of M1, α7, and α9 cholinergic receptors, which increase the intracellular levels of Ca^{2+} (Nguyen et al., 2001). Treating KCs with tropicamide (a M_4R antagonist) increased intracellular Ca^{2+} concentrations suggesting that M_4R stimulation may exert inhibitory effects on humectant secretion. As mentioned above, terminally differentiated KCs do not express M_4R, thus the discharge does not occur at early stages of KCs differentiation (Nguyen et al., 2001). Overall, ACh exerts its effects by orchestrating nAChRs and mAChRs during the different stages of KCs development.

As noted earlier, KCs are not only the building block of the skin and they release several neuroactivator and immunomodulator compounds, and express diverse receptors such as ligand- and voltage-gated ion channels as well as growth factor receptors. Accumulating evidence indicates that KCs are in close contact with sensory afferent nerves and several non-neuronal cells and are involved in the regulation of nociception and inflammation (Albanesi et al., 2005; Keppel Hesselink et al., 2017). A summary of the effects of cholinergic drugs on KC development was summarized in **Table 4**.

1.4.7 Role of cholinergic system in peripheral neuropathies

Peripheral neuropathy includes all disorders, which are associated with nerve injury in the peripheral nervous system. One of the common types of peripheral neuropathy is distal symmetric polyneuropathy (DSPN), which is observed in a variety of disorders such as diabetes, Friedreich ataxia, Charcot–Marie–Tooth disease type 2 and human immunodeficiency virus (HIV)-associated distal-symmetric neuropathy (Callaghan et al., 2015). Sensory and autonomic dysfunction are the

main manifestations of DSPN and are accompanied by positive symptoms (such as pain and dysesthesia) and negative symptoms (sensory abnormalities). In fact, distal dying-back process and axonal degeneration of small fibers are observed in DSPN (Ferrari et al., 2006; Klein, 2020; Morral et al., 2010).

As previously noted, both KCs and sensory neurons synthesize and release ACh that suppresses neurite outgrowth in different types of neurons, mainly through the stimulation of mAChRs. Studies in our lab examined the therapeutic potential of antimuscarinic drugs for preventing/reversing DSDP. We applied selective/specific antimuscarinic drugs to different animal models of peripheral neuropathy such as diabetic neuropathy, chemotherapy-induced peripheral neuropathy (CIPN) and HIV-associated neuropathy. Our *in vivo* and *in vitro* results showed that M_1R blockade by VU0255035, PZ, and MT7 stimulated AMPK and mitigated diabetes-induced mitochondrial dysfunction. In addition, these treatments attenuated the depletion of sensory nerve terminals, and improved nerve conduction and thermal hypoalgesia. Using $M_1R^{-/-}$ mice, we found that lack of M_1R enhanced neurite outgrowth of sensory neurons. and diabetic $M_1R^{-/-}$ mice did not exhibit physiological and structural indices of sensory neuropathy. Further, our results revealed that administration of PZ and MT7 protected animals against chemotherapy- or HIV-induced sensory neuropathy (Calcutt et al., 2017).

Previous studies from our lab and others highlighted the importance of mitochondrial dysfunction in the pathophysiology of peripheral neuropathy (Fernyhough, 2015; Leinninger et al., 2006; Roy Chowdhury et al., 2012). To investigate how M_1R blockade ameliorates the diabetes-induced mitochondrial dysfunction, Fernyhough lab administered MT7 to DRG sensory neurons of diabetic rats. Results showed that M_1R blockade increased AMPK phosphorylation and improved mitochondrial function in diabetic and CIPN animals through a mechanism

involving calcium/calmodulin-dependent protein kinase kinaseβ (CaMKKβ). Interestingly, topical delivery of MT7 reversed diabetes- or CIPN-induced decrease in corneal nerve density (Saleh et al., 2020). In another study, our lab examined the role of M_1R on mitochondrial trafficking and dynamics. Results showed that M_1R overexpression in DRG sensory neurons caused neurite outgrowth suppression via disruption of tubulin by $G\alpha_{13}$ protein and decreased mitochondrial trafficking in distal neurites. Treating transfected DRG sensory neurons with MT7 and PZ normalized the cytoskeletal alteration, neurite outgrowth and mitochondrial trafficking (Sabbir et al., 2018). Recent findings from our lab revealed that pharmacological blockade of M_1R induced neurite outgrowth in DRG sensory neurons in a mechanism associated with a biased β-arrestin dependent ERK-CREB pathway (Sabbir and Fernyhough, 2018). As mentioned above, KCs are in close communication with free nerve ending of C-fibers and other non-neuronal cells in the epidermis. Indeed, epidermal primary sensory afferents are responsible for the transition of environmental stimuli into sensations. KCs activate neurons innervating cutaneous and contribute to the initiation and maintenance of abnormal sensory transmission in pathological conditions (Baumbauer et al., 2015). According to these findings, KCs are appropriate therapeutic targets for the treatment of a variety of disorders including neuropathic pain (Keppel Hesselink et al., 2017; Knezevic et al., 2017). In this regard, we recently showed that topical delivery of muscarinic antagonists (such as PZ, atropine) to diabetic mice prevented and reversed peripheral neuropathy (Jolivalt et al., 2020). Also, our results demonstrated that topical delivery of oxybutynin (a muscarinic antagonist) improved structural and functional indices of small fiber function, and quality of life in persons with type 2 diabetes and exhibiting peripheral neuropathy (Casselini et al., 2024).

Table 4. A summary of the effects of cholinergic drugs on KC development.

Drug/genetic manipulation	Drug/genetic manipulation function	Effects on KC function	References
Proliferation and Differentiation			
PrBCM	mAChRs antagonist	Increased proliferation by blocking Gq-coupled mAChRs	(Grando et al., 1993)
bromoacetylcholine	ChAT inhibitor	Inhibits cell division	(Grando et al., 1993)
Arecoline	Partial nicotinic and muscarinic agonist	Inhibits KC proliferation by inducing cell cycle arrest	(Thangjam and Kondaiah, 2009)
Muscarine	mAChRs agonist	Increased proliferation and cell cycle proteins relevant to proliferation (reversed by atropine)	(Arredondo et al., 2003a)
Atropine, Mechamylamine, Strychnine	mAChRs and nAChRs antagonists	Blocked epidermal differentiation and proliferation	(Kurzen et al., 2006)
Orientation and Migration			
Carbachol	nAChRs and mAChRs agonist	Increased KC chemotaxis toward ACh through M_1R and α7 nAChR	(Chernyavsky et al., 2005)
anti-M_3R siRNA	Suppressing M_3R	Increased KC migration by down-regulation of "sedentary" integrins	(Chernyavsky et al., 2004b)

anti-M_4R siRNA	Suppressing M_4R	decreased KC migration by down-regulation of "migratory" integrins	(Chernyavsky et al., 2004b)
MT-3	M_4R antagonist	Reduced KC migration distance	(Chernyavsky et al., 2004b)
4-DAMP	M_3R antagonist	No effect on migration	(Chernyavsky et al., 2004b)
Epidermal cohesion and Cornification			
Atropine, tubocurarine, strychnine	mAChRs antagonist	Induced acantholysis by the phosphorylation of adherence proteins	(Nguyen et al., 2004b)
Atropine, Mechamylamine, Strychnine	mAChRs and nAChRs antagonists	Induced acantholysis	(Kurzen et al., 2006)
Pyridostigmine bromide, Carbachol	AChE inhibitor, nAChRs and mAChRs agonist	Anti-acantholytic activity through decreasing the phosphorylation of adherence molecules	(Nguyen et al., 2004a)
Pilocarpine	mAChRs agonist	Anti-acantholytic activity through decreasing the phosphorylation of adherence molecules by M_1R and the α7	(Chernyavsky et al., 2008)
A combination of mAChRs antagonists + nAChRs agonist (e.g. carbachol+Nicotine)	mAChRs antagonist and nAChRs agonists	Increased humectant release through M_1R, α7, and the α9 mediated intracellular Ca^{2+} elevation	(Nguyen et al., 2001)

1.5 Rationale, objectives, hypothesis, and aims of the study

1.5.1 Rationale, hypothesis, and objectives

Results from our lab and others showed that genetic/pharmacologic manipulations of M_1R in DRG sensory neurons revealed protective effects against peripheral neuropathies and axonopathies in animal models of disease, such as in diabetes (Calcutt et al., 2017; Casselini et al., 2024; Chauhan et al., 2025; Jolivalt et al., 2020; Sabbir et al., 2018; Saleh et al., 2020). These studies showed M_1R selective or specific antagonists PZ or MT7 enhanced neurite outgrowth in rodent DRG neurons, while antagonists at M2-M4 and nicotinic antagonists failed to induce neurite outgrowth. DRG neurons from M_1R knockout mice displayed augmented neurite outgrowth compared with neurons from wildtype mice, with improved mitochondrial function (Calcutt et al., 2017). M_1R overexpression in DRG sensory neurons caused neurite outgrowth suppression via disruption of tubulin mediated by $G\alpha_{13}$ protein and decreased mitochondrial trafficking in distal neurites. Treating transfected DRG sensory neurons with MT7 or PZ normalized the cytoskeletal alteration, neurite outgrowth and mitochondrial trafficking (Sabbir et al., 2018). M_1R blockade increased AMPK phosphorylation and improved mitochondrial function in diabetic and CIPN animals. Interestingly, topical delivery of MT7 reversed diabetes- or CIPN-induced decrease in corneal nerve density (Saleh et al., 2020). Despite these findings, there was no clear explanation about the molecular mechanism(s) through which PZ/MT7 exerted their effects via M_1R. Further studies from our lab revealed that pharmacological blockade of M_1R induces neurite outgrowth in DRG sensory neurons in a mechanism associated with a β-arrestin dependent ERK-CREB pathway (Sabbir and Fernyhough, 2018). As mentioned

above, the β-arrestin-ERK pathway is a well characterized pathway associated with β-arrestin biased signaling at GPCRs. With respect to M_1R, there are several examples of agonists displaying biased signaling (van der Westhuizen et al., 2020), while biased agonist activity of M_1R antagonists has not been observed (or studied).

Based on these findings, I **hypothesized** that selective/specific antagonists of M_1R exhibit biased activity at the M_1R via β-arrestin signaling. The **objective** was to determine molecular events occurring at the M_1R that underpin the mechanism of biased agonism mediated by these antimuscarinic drugs.

1.5.2 AIMS and proposed experiments

1.5.2.1 AIM 1: Effects of muscarinic antagonists on G protein and β-arrestin pathways

Regarding the protective effects of PZ/MT7 against peripheral neuropathies and axonopathies, investigating the biased agonist activity of these drugs may lead to better understanding of mechanisms underly their therapeutic effects. The first step for determining the biased agonism activity of a ligand is to examine the capability of a ligand to activate G protein or β-arrestin signaling pathways. Thus, for this aim, the activity of the G protein pathway should be tested by measuring 1) inositol monophosphate (IP1), an indicator of phosphoinositide flux via PLCβ ($G\alpha_{q/11}$ activity), and 2) cAMP in response to forskolin, for showing $G\alpha_{s/i}$ activity in HEK293 and/or DRG sensory neurons. For the evaluation of the β-arrestin-ERK pathway, it is necessary to examine the interaction between M_1R and β-arrestin2 in response to muscarinic drugs using a real-time protein-protein interaction technique such as NanoBRET (Bioluminescence Resonance Energy Transfer) in HEK293 cells and DRG sensory neurons. Further, the capability of

muscarinic drugs at inducing ERK activation in HEK293 cells and DRG sensory neurons should be tested.

1.5.2.2 AIM 2: Characterization of muscarinic antagonist-induced phosphorylation of M_1R

Binding of ligands to GPCRs is usually associated with phosphorylation of the receptor at intracellular domains, and β-arrestins subsequently bind to the phosphorylated receptors. Ligands control arrestin activity by modulating interactions of a GPCR with GRKs, which phosphorylate different sets of GPCR residues (Tobin, 2008). Thus, determining whether muscarinic drugs (e.g. PZ) are capable of phosphorylating M_1R is important. To achieve this, LC/MS/MS and phosphoproteomics studies at M_1R were pursued. After determining the serine/threonine phosphorylation status at the M_1R, it is necessary to discover the mechanistic role of phosphorylated residues within M_1R in mediating biased agonism activity. Using mutagenesis techniques and generation of M_1R mutants (lacking or with replaced phosphorylated residues) provide tools to test the effects of post-translational changes on β-arrestin-biased agonism activity of muscarinic drugs. These mechanistic studies are examined through their impact on protein-protein interaction (M_1R-β-arrestin2) and ERK activation as an indicator for β-arrestin-biased pathway.

1.5.2.3 AIM 3: Role of regulatory factors involved in M_1R biased signaling

There are several factors that contribute to ligand-induced GPCR signaling. In regard to this study, the role of GRKs, G proteins, CKs, receptor internalization and β-arrestins are determinant for ligand-induced signaling. Pharmacological and genetic tools are used to determine the role of these factors in regulation of M_1R signaling in response to antimuscarinic drugs. Using HEK293 cells lacking GRK/β-arrestin genes and inhibition of G protein/GRKs/CK activity are examples

of techniques used to show the importance of these regulatory elements in mediating biased agonism activity of antimuscarinic drugs.

Collectively, this study uses a wide range of molecular and cellular approaches to provide unprecedented insights into the molecular mechanisms of antimuscarinic drugs (PZ/MT7) at M_1R and highlights the importance of M_1R phosphorylation and biased agonism in mediating the physiological effects of these drugs on DRG sensory neurons.

Chapter 2

Antimuscarinic drugs exert β-arrestin-biased agonism at the muscarinic acetylcholine type 1 receptor

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One-sentence summary

Antimuscarinic drugs act as β-arrestin-biased agonists via casein kinase 2 activation to promote ERK1/2 phosphorylation and neurite outgrowth

Authors

Shayan Amiri[1, 2], Mohamad-Reza Aghanoori[1, 2], Darrell R. Smith[1], T. M. Zaved Waise[1], Ying Lao[3], Asuka Inoue[5,6], René P. Zahedi[3, 4], Henry A. Dunn[1,2] and Paul Fernyhough[1,2] *

[1] Division of Neurodegenerative Disorders, St. Boniface Hospital Albrechtsen Research Centre, Winnipeg, MB, Canada

[2] Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, MB, Canada

[3] Manitoba Centre for Proteomics and Systems Biology, University of Manitoba, Winnipeg, MB, Canada

[4] Department of Internal Medicine, University of Manitoba, Winnipeg, MB, Canada

[5] Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi, 980-8578, Japan

[6] Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan

*Corresponding author: Dr. Paul Fernyhough, Department of Pharmacology & Therapeutics, Rady Faculty of Health Sciences, University of Manitoba. E-mail: pfernyhough@sbrc.ca

Current address of Dr. Aghanoori: Department of Molecular Medicine, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

Current address of Dr. Waise: Department of Zoology, 3112 - 6270 University Blvd., University of British Columbia, Vancouver, BC, V6T 1Z4, Canada

2.1 Abstract

Previous studies indicate that both pirenzepine (PZ), a selective orthosteric muscarinic acetylcholine type 1 receptor (M_1R) antagonist, and muscarinic toxin 7 (MT7), a negative M_1R allosteric modulator (NAM), act via M_1R to promote neuritogenesis in cultured adult rodent primary dorsal root ganglia (DRG) sensory neurons, in part, through β-arrestin-dependent activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). Furthermore, these antagonists reverse nerve degeneration in a variety of rodent models of peripheral neuropathy through multiple complementary pathways. To understand the therapeutic effects and mechanism of M_1R antagonist-induced ERK1/2 phosphorylation, we tested the hypothesis that PZ and MT7 possess β–arrestin-biased agonism at M_1R to drive activation of ERK and enhance neurite outgrowth. Treatment for up to 30 min with PZ and MT7 dose-dependently recruited β-arrestin2 to M_1R (analyzed using nano-BRET) and increased ERK phosphorylation in both HEK293 cells and DRG neurons. DRG neurons of different sub-types express M_1R, and ERK activation by MT7 was only observed in M_1R-positive neurons. These novel pharmacological effects occurred in the absence of activation of G protein signaling or receptor internalization in HEK293 cells. PZ phosphorylated M_1R at six specific serine/threonine residues (Thr230, Ser251, Thr254, Ser321, Thr354, Ser356) of intracellular loop 3 (ICL3) and deletion mutation of these sites suppressed PZ and MT7 induction of β-arrestin binding to M_1R and inhibited ERK activation. β-arrestin-biased activity of PZ and MT7 involved the mobilization of casein kinase 2 (CK2) and this occurred in the absence of Gαq or G protein receptor kinase (GRK) activity. Pharmacological or siRNA-based inhibition of CK2 blocked PZ-induction of β-arrestin association, ERK activation and neurite outgrowth in DRG neurons. In conclusion, PZ/MT7 activated M_1R toward the β-arrestin signaling pathway in both HEK293 cells and DRG neurons to augment ERK activation and neurite

outgrowth via engagement of CK2. Results of this work provide novel findings with far reaching consequences on our understanding of the signaling mechanism of antimuscarinic drugs.

2.2 INTRODUCTION

G protein–coupled receptors (GPCRs) are dynamic transmembrane proteins that activate several signaling pathways via engagement of numerous intracellular molecules, including G proteins, G protein-coupled receptor kinases (GRKs), and β-arrestins (Magalhaes et al., 2012). Recruitment and activation of the different heterotrimeric G proteins and β-arrestins are ligand-dependent and cell-type-dependent processes (Wisler et al., 2014). Ligand binding to GPCRs triggers phosphorylation events at intracellular loops (ICLs) and the carboxyl terminus of the receptors that mobilizes binding of β-arrestins. These phosphorylation events are mostly controlled by kinases such as GRKs (Sulon and Benovic, 2021), protein kinase C (PKC) (Drube et al., 2022; Luo et al., 2017), and casein kinases (Jung et al., 2017; Torrecilla et al., 2007; Waugh et al., 1999). It is now clear that unlike balanced agonists and antagonists, biased ligands can specifically target a particular receptor-linked signaling pathway or subset of pathways (Smith et al., 2018; Wootten et al., 2018).

The muscarinic acetylcholine type 1 receptor (M_1R) is a class A GPCR and widely expressed in the central nervous system (CNS) (Levey, 1993; Levey et al., 1991) and the peripheral nervous system (PNS) (Bernardini et al., 1999; Harrington et al., 2007). In response to the endogenous agonist, acetylcholine, or a balanced synthetic agonist, such as carbachol, M_1R activates several signaling pathways via recruitment of $G\alpha_{q/11}$ to increase inositol (1,4,5) trisphosphate (IP_3) and intracellular calcium levels, and β-arrestins contributing to activation of extracellular signal-regulated protein kinases (ERKs) (Butcher et al., 2016; Sabbir et al., 2018; van der Westhuizen et al., 2020; Yeatman et al., 2014). It is important to note that M_1R is capable of binding to $G\alpha_s$, $G\alpha_i$, and $G\alpha_{13}$ (Avet et al., 2022; Sabbir et al., 2018). M_1R plays an important role in the regulation of neuronal function and its ablation causes absence of phosphoinositide

hydrolysis in cortical and hippocampal neurons in response to muscarinic agonists (Bymaster et al., 2003; Wess, 2004). M_1R is the major muscarinic receptor subtype that mediates ERK activation in neurons, and M_1R knockout (KO) mice show no ERK activation in hippocampal and cortical neurons after receiving carbachol and oxotremorine, respectively (Berkeley et al., 2001; Hamilton and Nathanson, 2001). M_1R selective agonists promote cellular excitability, synaptic transmission, and hippocampal long-term potentiation in mice, and this is suppressed in M_1R KO mice (Dennis et al., 2016). A study using chemogenetics and a phospho-deficient M_1R mouse model revealed that regulation of learning, memory, and anxiety-like behaviors depends on M_1R phosphorylation and relevant signaling pathways (such as β-arrestin recruitment and ERK activation), while $G\alpha_q$-related signaling was linked to M_1R-dependent adverse effects such as seizures (Bradley et al., 2020).

We previously reported that genetic or pharmacological blockade of M_1R exhibited protection against different types of peripheral neuropathy in rodents, including sensory and motor nerve dysfunction (Calcutt et al., 2017). Unlike M_2R, M_3R, and M_4R antagonists, treatment with pirenzepine (PZ), a selective orthosteric antagonist for M_1R, and muscarine toxin 7 (MT7), a negative allosteric modulator (NAM) for M_1R, enhanced mitochondrial function and neurite outgrowth in adult rodent dorsal root ganglia (DRG) sensory neurons (Calcutt et al., 2017; Sabbir et al., 2018). Further, we showed that ERK activation and β-arrestins contribute to the neuritogenic effect of PZ and MT7 (Sabbir and Fernyhough, 2018). However, the molecular mechanisms through which these prototypical antagonists activate M_1R-mediated ERK1/2 remain unknown. Interestingly, prototypical β-adrenergic receptor antagonists, such as the prominent β-blocker carvedilol, have also demonstrated β-arrestin-biased agonism leading to the induction of ERK activation (Nobles et al., 2011; Wisler et al., 2007). Similarly, in the case of angiotensin II type 1

receptor (AT_1R), antagonists toward $G\alpha_q$ can also show biased agonism toward β-arrestins (Violin et al., 2010). Studies with the β2-adrenergic receptor have assigned a barcode related to the distinct phosphorylation pattern controlled by GRKs that determines β-arrestin-dependent signaling (Wisler et al., 2007). Thus, in the current study, we tested the hypothesis that PZ and MT7 may possess β-arrestin-biased agonism at the M_1R.

To address this hypothesis, we used an orthosteric antagonist (PZ) and a NAM (MT7) to study signaling pathways and molecular mechanisms associated with M_1R, including G protein signaling, β-arrestin recruitment, ERK activation, receptor phosphorylation and internalization. We found that M_1R antagonists phosphorylated M_1R at specific residues at intracellular loop 3 (ICL3), recruited β-arrestin2, and activated ERK in both HEK293 and primary adult DRG sensory neurons without activating G protein signaling or receptor internalization. ERK activation depended on the phosphorylation of specific residues at M_1R. Unlike $G\alpha_q$ or GRKs, we found that β-arrestins and casein kinase 2 (CK2) mediate the effects of antimuscarinic drugs on ERK activation. Finally, inhibition of CK2 blocked PZ-induced ERK activation and neurite outgrowth in adult DRG sensory neurons.

2.3 RESULTS

2.3.1 Pirenzepine (PZ) and MT7 recruited β-arrestin2 to M_1R without activation of $G\alpha_q$ protein signaling

To explore whether MT7 and PZ mobilize β-arrestin signaling in the absence of G protein signaling we first showed the classical antagonism activity of PZ and MT7 at M_1R by measuring inositol monophosphate (IP1) production. As seen in Fig. 2.1A&B, PZ and MT7 blocked muscarine-induced IP1 production in a dose-dependent manner in HEK293 cells and cultured adult rat DRG sensory neurons (Fig. 2.3B&C). Next, we examined the induction of β-arrestin2 recruitment to M_1R using a Bioluminescence Resonance Energy Transfer (BRET) assay. The rationale for selecting β-arrestin2 over β-arrestin1 was that M_1R has more preference for β-arrestin2 (Davis et al., 2010; Song et al., 2009). BRET measurements showed that treatment with carbachol (1 mM) (Fig. 2S1) or muscarine (1 mM) rapidly increased β-arrestin2 recruitment to M_1R (Fig. 1C&D). Subsequent treatment over a 5 min period with the PZ (1 μM), or MT7 (100 nM) inhibited the muscarine-induced interaction between M_1R and β-arrestin2 (Fig. 2.1C&D); as expected and previously described (Yeatman et al., 2014). Further, we showed that PZ and MT7 treatments (but not muscarine) failed to increase IP1 in HEK293 cells (Fig. 2.2A). Interestingly, unlike muscarine, PZ and MT7 treatments did not change the cAMP response to forskolin in HEK293 cells (Fig. 2.2B). Since the maximum BRET response to M_1R agonists were observed after 5 min of incubation, a dose-response curve was performed at this time point. M_1R activation induced by carbachol over a 5 min period induced a dose-dependent recruitment of β-arrestin2 in HEK293 cells (Fig. 2.2C) and DRG sensory neurons (Fig. 2.3D). Unexpectedly, however, over a longer-term treatment period of 30 min PZ recruited β-arrestin2 to M_1R in a concentration-dependent manner in HEK293 cells (Fig. 2.2D) and DRG neurons (Fig. 2.3E). Similarly, over a

30 min period MT7 induced recruitment of β-arrestin2 to M_1R in a dose-dependent manner in HEK293 cells (Fig. 2.2E) and DRG sensory neurons (Fig. 2.3F).

2.3.2 PZ and MT7 increased ERK activity in HEK293 cells and DRG neurons

β-arrestins form signaling scaffolds with members of mitogen-activated protein kinases (MAPKs) including ERK (Luttrell et al., 2001; Song et al., 2009). We previously reported that treating DRG neurons with PZ (1 μM) or MT7 (100 nM) activated ERK via a β-arrestin-dependent pathway and increased neurite outgrowth (Sabbir and Fernyhough, 2018). Treating M_1R-transfected HEK293 cells (Fig. 2.2F&G) and DRG neurons (Fig. 2.3G&H) with PZ (1μM) and MT7 (100 nM) increased ERK phosphorylation in a time-dependent manner. To examine the role of M_1R in the PZ and MT7 stimulation of ERK phosphorylation in DRG neurons, we first stained intact DRG from WT (Fig. 2.4A, top panel) and M_1R KO mice (Fig. 2.4A, bottom panel) with β-tubulin III and MT7-ATTO590 (a functional fluorescently labelled MT7). Since MT7 possesses an extreme subtype specificity toward M_1R (Maeda et al., 2020), we used the MT7-ATTO590 as an M_1R marker in DRG neurons instead of unreliable commercial antibodies against M_1R. DRG neurons across a range of perikaryal sizes (and thus sensory sub-types) in WT mice expressed M_1R (Fig. 2.4A, top panel) while we confirmed M_1R KO mice expressed no M_1R (Fig. 2.4A, bottom panel). Treating DRG neurons with MT7-ATTO590 (90 min) significantly increased phospho-ERK expression in DRG neurons, but only in those expressing M_1R (Fig. 2.4B). The phospho-ERK signal was stronger in the nucleus than cytoplasm of DRG neurons (Fig. 2.4B).

2.3.3 M_1R phosphorylation by PZ is necessary for β-arrestin2 biased signaling activity

GPCR function is regulated by phosphorylation (Tobin, 2008), and β-arrestins bind to phosphorylated GPCRs with high affinity (Gurevich and Gurevich, 2004). To determine whether

PZ phosphorylated M_1R, and to establish the exact sites of phosphorylation, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results from 2 different proteomics centers (Fig. 2S2A&B, Table 2S1, University of Virginia Biomedical Research Facility for LC/MS/MS analysis) and (Fig. 2S3A-F, Table 2S2, Manitoba Centre for Proteomics and Systems Biology (MCPSB)) identified three serine (Ser^{251}, Ser^{321}, Ser^{356}) and three threonine (Thr^{230}, Thr^{254}, Thr^{354}) phosphorylation sites in the ICL3 of M_1R (Fig. 2.5A). For the MCPSB analysis accurate determination of the phosphorylation degree at individual sites required absolute quantification of both the phosphorylated and non-phosphorylated variants of the corresponding peptides, as previously described (Di Conza et al., 2017). In this study, we approximated phosphorylation levels by calculating, for each site, the ratio of high-confidence peptide-spectrum matches (PSMs) with site localization probabilities >90% to the total number of PSMs identifying peptides that included the respective amino acid residue. Across four control samples, Ser^{251} and Thr^{254} emerged as the most prominently phosphorylated residues, with 12.8% and 15.5% of PSMs phosphorylated, respectively, while all other sites ranged between 4.4% and 8.8%. Based on these LC-MS/MS results, 2 mutated M_1R plasmids were generated; 1) deletion of all six phosphorylation sites from ICL3 region of M_1R ($M_1R\Delta ICL3$), and 2) mutation at Ser^{251} and Thr^{254} (M_1R-S251A, T254A; alanine substitutions) in the ICL3 region. When compared to M_1R-WT, BRET results showed that $M_1R\Delta ICL3$ and M_1R-S251A, T254A mutations significantly decreased the efficacy (Emax response) of PZ to induce β-arrestin2 recruitment to M_1R (Fig. 2.5B). These mutations resulted in the failure of PZ to induce ERK phosphorylation in HEK293 cells (Fig. 2.6A&B). In comparison to M_1R-WT, $M_1R\Delta ICL3$ (but not M_1R-S251A, T254A) significantly decreased the efficacy of MT7 to induce M_1R-β-arrestin2 interaction (Fig. 2.5C) and ERK activation in HEK293 cells (Fig. 2.6C&D). Intriguingly, both mutations had no significant effect on the efficacy of

muscarine to recruit β-arrestin2 to M_1R (Fig. 5D). Surprisingly, these mutations significantly increased ERK phosphorylation in response to muscarine, when compared to the M_1R-WT response (Fig. 2.6E&F).

2.3.4 PZ and MT7 increased cell surface expression of M_1R at HEK293 cells and DRG neurons

It has been known for more than 20 years that β-arrestins bind phosphorylated GPCRs and contribute to the desensitization and internalization of GPCRs (Ferguson et al., 1996). To assess receptor internalization in response to muscarinic drugs, we used HEK293 cells and DRG sensory neurons transfected with N-terminally HiBiT-tagged M_1R. Muscarine, as an agonist, dose-dependently increased M_1R internalization in both HEK293 cells (Fig. 2.7A) and DRG sensory neurons (Fig. 2.7D) after 1 h, when compared to vehicle-treated counterparts. However, 1 h of treatment with different doses of PZ increased M_1R cell surface expression in a concentration-dependent manner in HEK293 cells, when compared to vehicle-treated groups (Fig. 2.7B). Similar treatment with different PZ concentrations did not significantly reduce/increase M_1R surface expression in cultured DRG sensory neurons (Fig. 2.7E). Treating HEK293 cells (Fig. 2.7C) and DRG sensory neurons (Fig. 2.7F) with MT7 for 1 h resulted in a dose-dependent increase in cell surface expression of M_1R. Also, treating HEK293 cells and DRG neurons with PZ (1 μM, Fig. 2S4A&C) and MT7 (100 nM, Fig. 2S4B&D) for up to 3 h did not reduce the surface expression of M_1R.

2.3.5 β-arrestins (but not GRKs) are necessary for ERK activation by PZ and MT7

We investigated the factors that might be involved in the β-arrestin-mediated signaling and ERK activation by PZ and MT7 in M_1R-transfected HEK293 cells. First, we examined the role of $G\alpha_q$

protein activity on the PZ/MT7-induced ERK activation. Results showed that inhibition of $G\alpha_q$ protein activity by YM-254890 had no effect on PZ/MT7-induced ERK activation in HEK293 (Fig. 2.8A&B). However, genetic ablation of β-arrestin1/2 abolished ERK activation induced by PZ/MT7 (Fig. 2.8C&D) and confirming our previous work (Sabbir and Fernyhough, 2018). Surprisingly, ablation of GRKs (2, 3, 5, and 6) failed to suppress ERK activation in response to PZ/MT7 (Fig. 2.8E&F). It is interesting that treating non-transfected HEK293 cells with CMPD101, a GRK2/3 inhibitor, decreased ERK activation (Fig. 2S5), while the same treatment increased ERK activation in M_1R-transfected HEK293 (Fig. 2.8G&H).

2.3.6 CK2 inhibition prevented PZ-induced ERK activation and neurite outgrowth in DRG neurons

Fig. 2.8E&F demonstrated that GRKs (2, 3, 5, and 6) had no role in ERK activation mediated by PZ/MT7. Hence, alternative kinases such as CK1 and CK2 might be responsible for M_1R phosphorylation, as previously reported (Drube et al., 2022; Jung et al., 2017; Tobin, 2008). We examined the role of CK2 based on the previously reported work indicating that CK2 inhibition blocks M_1R-mediated ERK activity in response to agonists (Jung et al., 2017). As seen in Fig. 2.9A, BRET results show that 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB; a well characterized CK2 inhibitor (Borgo et al., 2021), 1 µM, 10 min) pretreatment significantly reduced β-arrestin2 recruitment to M_1R by PZ (1 µM, 30 min). Similarly, TBB pretreatment blocked PZ-induced ERK activation in HEK293 cells (Fig. 2.9B) and DRG neurons (Fig. 2.9C). Further, knockdown of *Csnk2a1* and CK2 depletion (Fig. 2S6) significantly reduced PZ-induced ERK activation in HEK293 cells (Fig. 2.9D). PZ (1 µM, 42 h) significantly elevated total neurite outgrowth in DRG neurons, and co-administration of TBB (30 µM) attenuated the positive effect of PZ (Fig. 2.9E). This result revealed for the first time that PZ-dependent blockade of M_1R operated via CK2 to drive neurite outgrowth in primary adult neurons.

2.4 DISCUSSION

We provide evidence that PZ and MT7 act as β-arrestin-biased agonists at M_1R by recruiting β-arrestin2 and associated signaling pathways in preference over G protein activation in HEK293 cells and adult primary DRG sensory neurons. The data demonstrates that phosphorylation of M_1R at specific ICL3 residues is necessary for β-arrestin-mediated ERK activation. ERK activation by PZ/MT7 required β-arrestins and activation of CK2 (but not GRKs and $G\alpha_q$ protein). Lastly, CK2 inhibition blocked PZ-dependent enhancement of neurite outgrowth in DRG sensory neurons. The pharmacological profile of PZ and MT7 is consistent with previously reported β-arrestin biased agonists, such as carvedilol, including lack of G protein agonism, altered receptor phosphorylation and β-arrestin-dependent signaling (Carr III et al., 2016; Wisler et al., 2007).

Acute treatment (up to 5 min) with PZ and MT7, two structurally and functionally distinct M_1R ligands, exhibited antagonistic effects in response to muscarine/carbachol and blocked M_1R-arrestin interaction. However, longer treatment (e.g. 30 min) of PZ or MT7 in the absence of full agonist effectively recruited β-arrestin2 (Fig. 2.2D & E, Fig. 2.3E & F) and induced ERK activation (Fig. 2.2F&G, Fig. 2.3E&F). These pharmacological effects were markedly different from the orthosteric agonist carbachol/muscarine with much less ability to promptly recruit β-arrestin2 and initiate receptor internalization. PZ/MT7 time-dependently activated the β-arrestin-ERK pathway while inducing no G protein signaling and receptor internalization. This data suggests these ligands are capable of inducing specific receptor conformations and signaling as partial and biased agonists: acting as antagonists in the presence of a full unbiased agonist. In this regard, studies using transfected model systems showed PZ upregulated M_1R, induced stabilization of dimers/oligomers at cell surface (Ilien et al., 2009; Pediani et al., 2016), and resulted in slower diffusion of receptor, which might reduce the possibility of interactions between receptor and its

cognate G proteins (Zhou et al., 2024). Similarly, MT7 was reported to bind to the dimerized form of M_1R and stabilized this receptor state at the cell surface (Marquer et al., 2011; Marquer et al., 2010). Our results reveal that PZ/MT7 upregulated M_1R at the surface of HEK293 cells and DRG sensory neurons for up to 3 h (Fig. 2.7 and Fig. 2S4). Other studies reported that ERK activation by β-arrestin-biased signaling at muscarinic receptors did not necessarily internalize the receptor (Budd et al., 1999; Jung et al., 2017). Indeed, the unusual kinetics for the activation of β-arrestin2 recruitment might be associated with the dimerization/oligomerization of M_1R following PZ/MT7 treatment. Previous studies showed homodimerization (Liu et al., 2022) and heterodimerization (Chen et al., 2022) of GPCRs can activate biased signaling toward G protein, and β-arrestins, respectively.

In line with previous reports, we showed M_1R expression in different sub-types of DRG sensory neurons (Calcutt et al., 2017; Naznin et al., 2022), and only M_1R-positive neurons exhibited ERK activation in response to MT7. We recently demonstrated that M_1R overexpression markedly decreased neurite outgrowth and mitochondrial function in sensory neurons, and ERK activity played a major role in the neuritogenic effects of MT7 (Sabbir et al., 2018; Sabbir and Fernyhough, 2018).

Binding of β-arrestins to phosphorylated GPCRs regulates many aspects of cellular signaling. A significant body of evidence supports the "barcode hypothesis", which explains how different phosphorylation of residues at GPCRs results in diverse β-arrestin-associated signaling responses (Latorraca et al., 2020; Nobles et al., 2011; Tobin, 2008). A previous study by Butcher *et al*. revealed that acetylcholine treatment phosphorylated M_1R at 12 residues at ICL3 and 2 residues at the C-terminus (Butcher et al., 2016). In comparison to acetylcholine, our results showed that PZ phosphorylated hM_1R at 6 positions (similar to (Butcher et al., 2016)) at ICL3

(Fig. 2.5A), and subsequent mutation at these residues (deletion of all 6 positions or mutation at Ser^{251} and Thr^{254}) significantly reduced β-arrestin2 recruitment to M_1R and abolished ERK activation. In the case of MT7, deletion of all phosphorylated residues diminished β-arrestin2 recruitment and ERK activation, but mutation at Ser^{251} and Thr^{254} had no effect. This suggests that phosphorylation of a specific set of ICL3 residues is required for PZ- or MT7-induced β-arrestin biased activation of ERK. Surprisingly, ICL3 mutations had no effect on muscarine-induced β-arrestin2 recruitment, and even increased ERK activation. These results indicate that each ligand has a specific signature with respect to the phosphorylation pattern generated in the ICL3 of M_1R.

What factors might contribute to the β-arrestin biased activation of ERK by PZ/MT7? First, $G\alpha_q$ inhibition did not alter the PZ/MT7-induced ERK activity (Fig. 2.8A & B). Recent studies demonstrated that ERK activity requires active G proteins, including ERK activation by arrestins (Grundmann et al., 2018; Gurevich and Gurevich, 2024). Our data reveals that PZ/MT7 did not activate $G\alpha_q$ signaling, and $G\alpha_q$ inhibition did not modify the effects of PZ/MT7 on ERK activation. However, the effect of inhibition of alternative G proteins on putative ERK activity induced by PZ/MT7 was not tested. Furthermore, the data demonstrates that arrestins are necessary for PZ/MT7-induced ERK activity as lack of arrestins abolished the effect of these drugs. Other studies on β-arrestin biased ligands reported the same observation where arrestins play a major role in the biased effects of these ligands (Carr III et al., 2016; Rakesh et al., 2010; Wisler et al., 2007). Surprisingly, pharmacological inhibition of GRK2/3 with CMPD101 led to an increase in ERK activity mediated by PZ/MT7 (Fig. 2.8G & H), which indicates other kinases might be involved in M_1R phosphorylation. Recent work by Drube and colleagues showed that no specific GRK is assigned for M_1R recruitment of arrestins (Drube et al., 2022). Indeed, casein kinases phosphorylate muscarinic receptors (particularly M_1R and M_3R) (Jung et al., 2017; Tobin, 2002;

Torrecilla et al., 2007), and among them, CK2 contributes to ERK activation by M_1R (Jung et al., 2017). Our findings showed siRNA-based or pharmacological CK2 inhibition strikingly reduced ERK activation by PZ in HEK293 cells.

In DRG sensory neurons, the PZ-induced activation of ERK was dependent on CK2 (Fig. 2.9). Previous work by Jung *et al*., showed that CK2 inhibition abolished arrestin-mediated ERK activity by M_1R agonists (Jung et al., 2017). CK2 inhibition had no effect on basal ERK activity in DRG neurons, but significantly suppressed ERK activity in HEK293 cells, which explains drug-response differences between cell types. Indeed, CK2 is expressed in DRG sensory neurons, and plays a major role in neurite outgrowth where knockdown of CK2 suppressed neurite outgrowth (Ghosh et al., 2024; Sahoo et al., 2020). Intriguingly, our results showed that CK2 inhibition blocked PZ-induced neurite outgrowth in cultured DRG sensory neurons. These findings suggest that CK2 is necessary for arrestin-mediated ERK activity by PZ and suppressing ERK activity through CK2 inhibition blocks PZ-induced neurite outgrowth in sensory neurons.

Differences in signal transduction processes between PZ (as an orthosteric antagonist) and MT7 (as a NAM) were noted in this work. Unlike PZ, MT7 is a cell impermeable large molecule with a very slow dissociation rate, which determines its primary action is at the cell surface (Maeda et al., 2020; Morishima et al., 2013). Our findings showed that MT7 is more potent and rapid than PZ in inducing ERK activity and M_1R upregulation at the cell surface. Also, MT7 showed no sensitivity to Ser^{251}-Thr^{254} mutation in M_1R and its induction of β-arrestin2-ERK signaling was unaffected (Fig. 2.6). These discrepancies may be related to several factors such as differences in molecular size, binding location at M_1R, dissociation rate, and permeability of these two ligands.

Our study has some limitations. We used two model systems, HEK293 cells and adult DRG sensory neurons, and overexpressed modified proteins in these cells, which might not mimic real physiological conditions of sensory neurons. In addition, DRG sensory neurons are classified into several sub-types based on their soma size, expression of specific markers, and their conduction properties (Wang et al., 2023). Consequently, we showed M_1R expression (using specific MT7-ATTO590 marker) on DRG neurons with varying phenotypes, but did not delineate which sub-type of DRG neuron was responding to PZ/MT7 with β-arrestin biased activation of ERK.

Our future studies could focus on the cross-talk between M_1R and CK2 in different *in vivo* models of peripheral neuropathy, and uncover the molecular pathways associated with M_1R biased activity in different types of DRG neurons to find an appropriate treatment for peripheral neuropathy with minimal side effects. In summary, we showed that antimuscarinic drugs, PZ and MT7, activated M_1R towards the β-arrestin signaling pathway in both HEK293 and DRG sensory neurons. These drugs acted as β-arrestin-biased agonists and stimulated neurite outgrowth and could be potential treatment options for preventing/reversing nerve degeneration in different types of peripheral neuropathy.

2.5 MATERIALS AND METHODS

2.5.1 Chemicals

Muscarine, carbachol, atropine, and PZ were purchased from MilliporeSigma Canada (Oakville, ON, Canada). MT7, fluorescent dye ATTO Fluor 590-conjugated MT7 (MT7-ATTO590), and YM-254890 were purchased from Alomone Labs (Jerusalem, Israel) and Cayman Chemicals (Ann Arbor, MI, USA), respectively. CMPD101 and TBB were purchased from Tocris (Minneapolis, MN, USA).

2.5.2 Cell Culture and transfection

Cell lines

Wild-type (WT) Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin and streptomycin (MilliporeSigma Canada, Oakville, ON, Canada) at 37°C and 5% CO_2. The CRISPR/Cas9 mediated β-arrestin 1/2 knockout (KO) (Δβarr1/2) and GRK2, 3, 5, and 6 KO (ΔGRK2,3,5,6) HEK293 cell lines were designed and generated by Dr. Inoue laboratory (Kawakami et al., 2022; O'Hayre et al., 2017) . Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect the cells (details are provided in each experimental section). For siRNA transfection in HEK293 cells, 25 nM siRNA control (ON-TARGETplus Non-targeting siRNA, 5'UGGUUUACAUGUCGACUAA 3', Dharmacon, Cat#D-001810-01-05) and siRNA against CK2 (On-TARGETplus-SMART pool siRNA against *Csnk2a1*, Dharmacon, Cat#L-096197-02-0005) were used with Lipofectamine 3000 reagent and were incubated for 48 h.

Adult rat DRG sensory neuron culture

Adult male Sprague-Dawley rats, adult male wild-type (C57BL/6) and M_1R-KO (C57BL/6 background, line1784; Taconic Biosciences Inc., Hudson, NY, USA) were euthanized and DRG were dissected, dissociated, and cultured as previously described (Miyakawa et al., 2001). All animal procedures followed the guidelines of the University of Manitoba Animal Care Committee using the Canadian Committee on Animal Care (CCAC) rules. In brief, DRG neurons were cultured in Ham's F-12 media (Wisent Inc., Saint-Jean Baptiste, QC, Canada) supplemented with modified Bottenstein's N2 additives (0.1 mg/ml transferrin, 20 nM progesterone, 100 mM putrescine, 30 nM sodium selenite, 0.1 mg/ml BSA, all purchased from MilliporeSigma Canada, Oakville, ON, Canada). Media was also supplemented with 0.146 g/L L-glutamine (Thermo Fisher Scientific, Waltham, MA), a low dose cocktail of neurotrophic factors (0.1 ng/ml NGF, 1.0 ng/ml GDNF and 1 ng/ml NT-3, all from Thermo Fisher Scientific, Waltham, MA USA), 0.1 nM insulin and 1X antibiotic antimycotic solution (MilliporeSigma Canada, Oakville, ON, Canada). DRG neurons were transfected using NeuroMag (Oz Biosciences, San Diego, CA, USA) by magnetofection method (details are provided in each experimental section).

2.5.3 Plasmid construction

NLuc-M_1R, HaloTag-β-arrestin2, Halo-M_1R, and HiBiT-M_1R plasmids were purchased from GeneCopoeia Inc. (Rockville, MD, USA). All gene synthesis, cloning and mutagenesis for NLuc-M_1R was by GenScript (Piscataway, NJ, USA) and confirmed by DNA sequencing. Phospho-proteomics results were used to design M_1R mutant plasmids. Mutant M_1R plasmids used in this study were 1) NLuc-M_1R with Thr^{230}, Ser^{251}, Thr^{254}, Ser^{321}, Thr^{354}, and Ser^{356} deletion at ICL3

loop (M_1RΔICL3), and 2) NLuc-M_1R with Ser^{251} and Thr^{254} substitution with Alanine at ICL3 loop (M_1R-S251A, T254A). pcDNA3.1 and -22F cAMP pGloSensor.

2.5.4 β-arrestin recruitment

HEK293 cells

For BRET-based examination of β-arrestin2 recruitment to M_1R in HEK293 cells, 400000 cells were seeded onto 6-well plates per well. On the following day, cells were co-transfected using Lipofectamine 3000 with NLuc-hM_1R/mutant NLuc-hM_1R plasmids/pcDNA3.1 (20 ng) and HaloTag-β-arrestin2 (800 ng) per well (1:40 ratio), following the manufacturer's protocol. Calculation of the ratio and plasmid concentrations were obtained from saturation assay studies in the lab. After 24 h, cells were detached, centrifuged, and resuspended in OPTI-MEM media (no phenol red, with 4% FBS) plus HaloTag® NanoBRET™ 618 Ligand (Promega, Madison, WI, USA, final concentration 100 nM), and seeded (100 µl/well containing 25000 cells) into poly-D-L-ornithine hydrobromide (PDO)-coated 96-well white plates (OPTI-MEM from Thermo Fisher Scientific, Waltham, MA and PDO is from MilliporeSigma Canada, Oakville, ON, Canada). After 20 h, cell media was replaced with 90 µl HBSS (Thermo Fisher, Waltham, MA, USA) and 10 µl of 10X drugs. In the case of DRG neurons, 7500 cells were seeded into PDO and laminin-coated 96-well white plates and left unharmed for 36 h. DRG neurons were co-transfected with NLuc-hM_1R/pcDNA3.1 (50 ng) and HaloTag-β-arrestin2 (200 ng) per well (1:4 ratio) by magnetofection using NeuroMag (Oz Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Forty-eight hours after transfection, HaloTag® NanoBRET™ 618 Ligand (Promega, Madison, WI, USA, final concentration 100 nM) was added to wells, and then after 20 hours cell media was replaced with 90 µl of Ham's F-12 media (no phenol red) for 15 min, and then 10 µl of

10X drugs were added to wells. After incubation with drugs (5 min for carbachol/muscarine, and 30 min for PZ/MT7), 25 µl of Nano-Glo substrate (Promega, Madison, WI, USA, 1:400 final dilution) was added to wells, and a Synergy Neo2 plate reader (Biotek/Agilent Technologies, Santa Clara, CA, USA) was used to read luminescence and fluorescence to obtain the BRET ratio (610nm/460nm). β-arrestin2 recruitment was calculated as the change in BRET ratio (ΔBRET) following treatments and normalization to the baseline and test compound/vehicle response.

2.5.5 IP1 assay

As a measure of GPCR-associated $G\alpha_q$ signaling intracellular IP1 levels were measured using the IP-One assay (Cisbio Bioassays, Waltham, MA, USA). DRG sensory neurons were plated onto PDO and laminin-coated 384-well white microplates (5000 cells per well). HEK293 cells transiently transfected with plasmids containing the hM_1R-WT/pcDNA3.1, and were plated onto 384-well white microplates (20000 cells per well) and were treated with stimulation buffer with or without 1) different doses of M_1R agonist (muscarine), 2) different doses of M_1R antagonists (PZ and MT7) or 3) both M_1R antagonist (different doses of PZ/MT7) and agonist (muscarine, 1 mM) for different time points at 37 °C. Cells were lysed by addition of the supplied buffer containing d2-labeled IP1, followed by addition of terbium cryptate-labeled anti-IP1 antibody, according to the manufacturer's instructions. Plates were incubated for 1 h at room temperature and fluorescence signals were measured at 620 and 665 nm using the Biotek Synergy Neo2 plate reader. HTRF ratio 665 nm/620 nm of each well were extrapolated on the standard curve to obtain IP1 concentrations.

2.5.6 Glosensor cAMP assay

HEK293 cells (400000 cells/well, 6-well plate) transiently transfected with plasmids containing the hM_1R-WT (180 ng) and -22F cAMP pGloSensor (1080 ng) using Lipofectamine 3000. After 24 h, cells were detached, washed, centrifuged, resuspended in HBSS, and seeded (65µl/well containing 40000 cells) into 96-well white plates. Then, cells were incubated with Glosensor reagent (Promega, Madison, WI, USA, 10 µl/well) for two hours in the dark. Cells were pre-treated with 1) various concentrations (25 µl of 4X drug) of muscarine, PZ, and MT7, and 2) muscarine (1 mM) + various doses of PZ/MT7 and baseline luminescence was read on a Synergy Neo2 plate reader. After 5 min, cells were treated with 300 nM of forskolin (FSK, MilliporeSigma Canada, Oakville, ON, Canada)) and readings were continued for up to 20 min. Effects of drugs on M_1R-induced cAMP changes was calculated as the decrease/increase in FSK-induced luminescence amplitude.

2.5.7 Western blotting

HEK293 cells or DRG sensory neurons were lysed and homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA). DC protein assay (Bio-Rad, Hercules, CA, USA) was used for protein assay, and Western blot analysis was performed. Protein samples (20 µg total protein/lane for HEK293 cells and 5 µg total protein/lane for DRG neurons) were resolved and separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) and immunoblotted with specific antibodies against total ERK (T-ERK; 1:3000, Santa Cruz Biotechnology,Dallas, TX, USA, sc-514302), and phosphor-ERK (P-ERK, 1:3000, Cell Signaling Technology, Danvers, MA, USA, Cat# #9101).

HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used for secondary antibodies. The blots were incubated in Clarity™ Western ECL substrate (Bio-Rad, Hercules, CA, USA) or SignalFire™ ECL Reagent (Cell Signaling Technology, Danvers, MA, USA) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad, Hercules, CA, USA).

2.5.8 Mass spectrometry

Purification of hM_1R

For hM_1R purification, HaloTag® Mammalian Protein Purification System (Promega, Madison, WI, USA) was used. In brief, HEK293 cells (five 15-cm plates) were transfected with Halo-tagged hM_1R construct (20 µg/dish) using Lipofectamine 3000 for 24 h. Cells were washed twice with PBS, were incubated in serum-free DMEM for 6 h, and then media were removed and replaced with fresh serum-free DMEM containing 1 µM PZ for 30 min. Cells were scraped into 5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, (all from MillipoeSigma Canada, Oakville, ON, Canada) and protease inhibitor cocktail (Promega, Madison, WI, USA) and lysed by sonication on ice. Cellular debris was cleared by centrifugation, and 75 µl of HaloLink™ Resin (Promega, Madison, WI, USA), equilibrated with TBS containing 10XCMC or 0.087% n-Dodecyl-β-D Maltopyranoside (DDM) (Thermo Fisher Scientific, Waltham, MA, USA) was added. The samples were incubated overnight at 4 °C with rotation, and then supernatant was discarded and resin was washed (three times) with TBS+DDM10XCMC and TBS+DDM 3XCMC, respectively. The resin was treated with elution buffer (150 mM NaCl, 50 mM Tris-HCl (pH=7.5) + DTT (MilliporeSigma Canada, Oakville, ON, Canada, 1mM) + NaF (10mM) + DDM 3XCMC) containing HaloTEV Protease (Promega, Madison, WI, USA) overnight at 4 °C with rotation. Resin was cleared by centrifugation, and purified samples were collected and sent to the Manitoba

Centre for Proteomics and Systems Biology (MCPSB) and to the University of Virginia Biomedical Research Facility for LC/MS/MS analysis. The following LC-MS/MS analysis methodology is for the Manitoba Centre for Proteomics and Systems Biology (MCPSB). The methodology for the University of Virginia Biomedical Research Facility is in the supplementary materials.

LC-MS/MS analysis

Per sample, 10 µg of protein was separated by SDS-PAGE. The gel was fixed with 5% acetic acid in 50% methanol for 30 minutes and stained with GelCode Blue Safe Protein Stain (ThermoFisher Scientific, catalog # 24596) overnight, followed by destaining in water. The 50 kDa protein bands were excised and gel pieces were destained three times with 500 µL of acetonitrile and vortexed for 10 minutes (repeated twice). Gel pieces were dried in a speedvac, followed by incubation with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate (ABC) at 56 °C for 30 minutes to reduce disulfide bonds. Gel pieces were dehydrated in 500 µL acetonitrile for 10 minutes, followed by incubation with 55 mM iodoacetamide in 25 mM ABC for 30 min at RT in the dark, to alkylate free Cys residues. Gel pieces were dehydrated with 500 µL acetonitrile for 10 minutes, and excess iodoacetamide was quenched with 10 mM DTT in 25 mM ABC for 10 minutes at RT. Gel pieces were dehydrated in 500 µL acetonitrile for 10 minutes and rehydrated with digestion buffer containing 12.5 ng/µL trypsin/LysC (Promega) in 50 mM ABC. Proteins were digested at 37 °C overnight. Supernatants were collected and combined with four extracts collected after successively incubating the gel pieces once with H_2O and three times with 5% formic acid in 50% acetonitrile. Sample volumes were reduced in a speedvac, and generated peptides were desalted using C18 solid phase extraction tips (ThermoFisher Scientific, catalog #87782). Per sample, 60% of the peptide extract was analyzed by nano-LC-MS/MS on an Orbitrap Exploris 480 (Thermo

Fisher Scientific, Bremen, Germany) online-coupled to an Easy-nLC 1200 (Thermo Fisher Scientific). Mobile phase A was 0.1% (v/v) formic acid and mobile phase B was 0.1% (v/v) formic acid in 80% acetonitrile (LC-MS grade). Peptides were separated on a Luna C18(2) column (3 μm particle size; Phenomenex, Torrance, CA) packed in-house in a Pico-Frit (100 μm x 30 cm) capillary (New Objective, Woburn, MA) with the following gradient at a flow rate of 300 nL/min: 2-6% B in 3 minutes, 6-30% B in 36 min, 30-50% in 5 min, 50-90% in 1 min, remain at 90% for 5 min. The Orbitrap Exploris 480 was ran in data-dependent acquisition (DDA) mode. Survey scans were acquired from m/z 380-1500 at a resolution of 45,000 (at m/z 200), with a maximum ion injection time of 45 milliseconds, and a normalized automatic gain control (AGC) target of 200%. The top 12 precursor ions with charge states 2-5+ were isolated with a m/z 1.6 window, fragmented with a normalized collision energy of 30%, and MS/MS scans were acquired at a resolution of 30,000. AGC target value was set to 250%, with a maximum ion injection time set to auto.

Mass spectrometric data were searched using Proteome Discoverer v3.0 using SequestHT, MS Amanda 2.0, Percolator, and ptmRS nodes, against a human Swissprot protein database (downloaded from Uniprot.org in July 2022, 20399 target sequences) with the following search settings: trypsin as enzyme with a maximum of 2 missed cleavages, oxidation of Met (+15.995 Da), phosphorylation of Ser/Thr/Tyr (+79.966 Da) and deamidation of Asn/Gln (+0.984 Da) as variable and carbamidomethylation of Cys (+57.021 Da) as static modifications. MS and MS/MS tolerances were set to 10 ppm and 0.02 Da, respectively. The data was filtered to 1% false discovery rate on the protein, peptide, and peptide-spectrum-match levels, and only phosphopeptides with ptmRS site localization probabilities ≥99% were considered.

2.5.9 Internalization assay

Human M_1R internalization was measured by HiBiT-based receptor internalization assay (Promega). HEK293 cells were transiently transfected with hM_1R-HiBiT plasmid (100 ng) and pcDNA3.1 (9900 ng) in suspension (OPTI-MEM no phenol red) using Lipofectamine ® 3000. Then, cells were seeded (95 µl/25000 cells) into PDO-coated white 96-well plates. After 20 h, cells were treated with 10 µl of (10X) different doses of muscarine, PZ, and MT7 for 1 h, and were incubated at 37^0C + 5% CO_2 for 1 h. Then 100 µl of 2× substrate buffer consisting of 1:100 of a LgBiT stock solution and 1:50 dilution of Nano-Glo® HiBiT Extracellular Substrate in the OPTI-MEM (no phenol red) was added to wells, and a Synergy Neo2 plate reader was used to read luminescence. Luminescence signals were averaged and normalized to the vehicle stimulation count.

For the DRG sensory neurons, 7500 cells were seeded into PDO and laminin-coated 96-well white plates and left unharmed for 36 h. DRG neurons were transfected with hM_1R-HiBiT plasmid (25 ng) per well by magnetofection using NeuroMag according to the manufacturer's protocol. Forty-eight hours after transfection, media was removed and replaced with 90 µl of Ham's F-12 media (no phenol red) supplemented with N2 additives, and cells were incubated at 37^0C + 5% CO_2 for 15 min. Then, 10 µl of (10X) different doses of muscarine, PZ, and MT7 for different time-points. Finally, 100 µl of 2× substrate buffer consisting of 1:100 of a LgBiT stock solution (Promega) and 1:50 dilution of Nano-Glo® HiBiT Extracellular Substrate in the Ham's F-12 media (no phenol red) was added to wells, and a Synergy Neo2 plate reader was used to read luminescence. Luminescence signals were averaged and normalized to the vehicle stimulation count.

2.5.10 Immunohistochemistry (IHC) and immunocytochemistry (ICC)

For the IHC, mouse DRG tissues derived from C57BL/6 mice (Miyakawa et al., 2001) were exposed to MT7-ATTO590 (100 nM, overnight) at 37 °C CO_2 incubator, and then were fixed in PFA 2% (Thermo Fisher Scientic, Waltham, MA, USA), cryoprotected in 20% sucrose, and finally embedded in Tissue-Tek O.C.T. (Sakura Finetek USA, Torrence, CA, USA) to prepare 7 µm tissue sections. All sections were incubated with β-tubulin III antibody (1:500; T8578, MilliporeSigma Canada, Oakville, ON, Canada) overnight at 4 °C, and then stained for 1 h with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. For the ICC mouse DRG neurons were cultured on glass coverslips, incubated with MT7-ATTO590 (100 nM, 90 minutes) at 37° C in a CO_2 incubator, and then were fixed with PFA 2%, and permeabilized with 0.3% Triton X-100 (MilliporeSigma Canada, Oakville, ON, Canada). Neurons were blocked by 5% BSA for 1 h, and then incubated with phospho-ERK antibody (P-ERK, 1:500, Cell Signaling Technology, Danvers, MA, USA Cat# #9101) overnight. Cells were washed with PBS (3X), and then stained for 1 h with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. Coverslips were mounted on slides using VECTASHIELD antifade mounting medium with DAPI (Vectorlabs, Inc., Newark, CA, USA). All images were taken by using a Carl Zeiss LSM510 confocal or Axioscope-2 fluorescence microscope.

2.5.11 Neurite outgrowth in adult DRG sensory neurons

DRG sensory neurons were cultured on glass coverslips and were treated with 1) vehicle 2) PZ (1 µM), 3) TBB (30 µM), or 4) combination of PZ (1 µM) + TBB (3, 10, and 30 µM). Appropriate doses for TBB were obtained from pilot studies in the lab. After 24 h, cells were fixed with 2% paraformaldehyde (pH 7.4, 15 min), and permeabilized with 0.3% Triton X-100 in PBS for 5 min.

Cells were blocked by 10% blocking reagent (Roche Diagnostics, Laval, QC, Canada) and 20% FBS in PBS for 1 h, and then incubated with Peripherin antibody (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-377093) overnight. Cells were washed with PBS (3X), and were incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. Coverslips were mounted on slides using PERMAFLUOR mounting medium (Thermo Fisher Scientific, Waltham, MA, USA) and imaged using a Carl Zeiss Axioscope-2 fluorescence microscope equipped with equipped with an AxioCam camera. Neurite outgrowth was determined by collecting fluorescent signals as total pixel area for neurites and were analyzed by the high throughput NeurphologyJ plugin in ImageJ software after image enhancement. Total pixel area was normalized to number of cell bodies to calculate total neurite outgrowth per neuron.

2.5.12 Statistical analysis

Excel (Microsoft, Redmond, WA, USA) and graphed in Prism 9.0 (GraphPad, San Diego, CA, USA) were used to analyze the data. Statistical tests were performed using a *t*-test, one or two-way ANOVA followed by multiple comparison's tests. Details of statistical analysis and replicates are included in the figure legends. Lines represent the mean, and error bars signify the SEM, unless otherwise noted.

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2.7 Figures

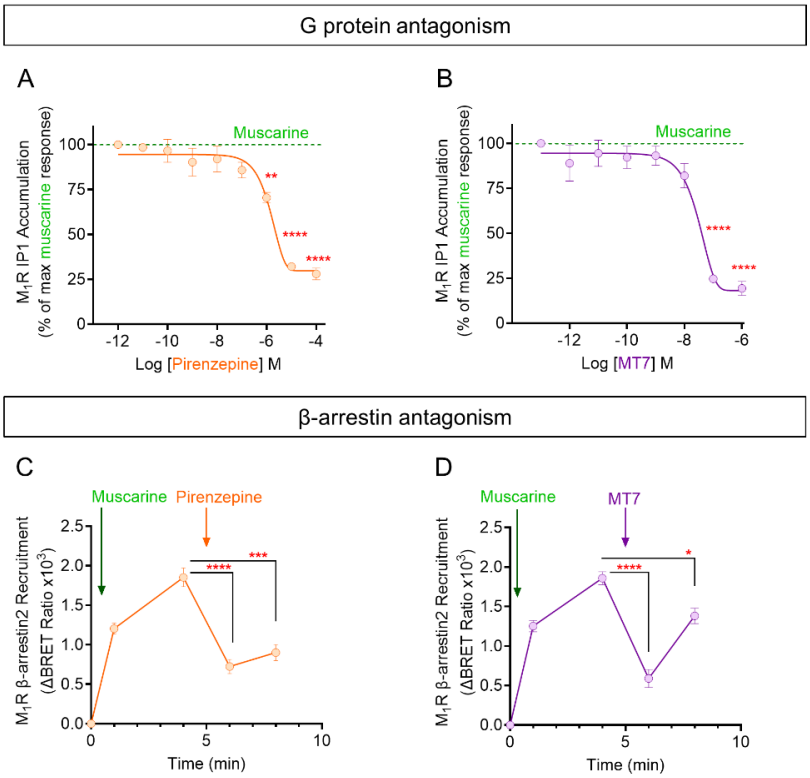

Fig. 2.1 Antagonism activity of pirenzepine and MT7 against muscarine-induced signaling at M_1R. **(A)** Inhibitory effect of different doses of pirenzepine and **(B)** MT7 on muscarine (1 mM)-induced IP1 production in transiently hM_1R-transfected HEK293 cells. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Data normalized to baseline with multiple comparisons to the lowest concentration of drug; ****P<0.0001, **P<0.01, n=3. **(C&D)** Ligands were added at the times indicated. Muscarine (1 mM) induced βArr2-Halo recruitment to hM_1R-Nluc, and acute treatment with **(C)** pirenzepine (1 μM) and **(D)** MT7 (100 nM) blocked the effects of muscarine in transiently co-transfected HEK293 cells. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Antagonist-treated groups were compared to muscarine-treated groups at minute 4; ****P<0.0001, ***P<0.001, and *P<0.05, n=3.

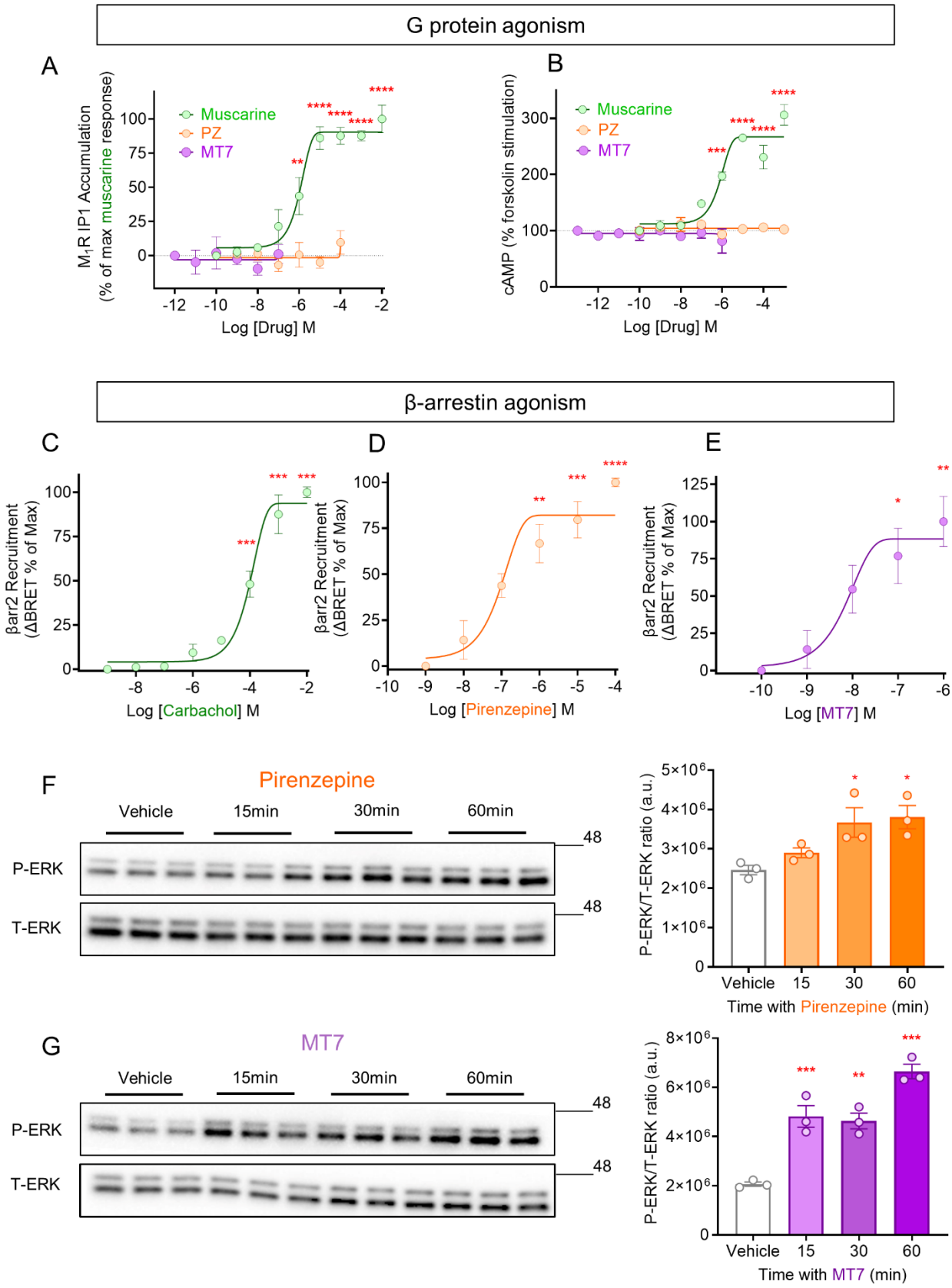
G protein agonism
A
M1R IP1 Accumulation (% of max muscarine response)
Muscarine
PZ
MT7
Log [Drug] M
B
cAMP (% forskolin stimulation)
Muscarine
PZ
MT7
Log [Drug] M
β-arrestin agonism
C
βarr2 Recruitment (ΔBRET % of Max)
Log [Carbachol] M
D
βarr2 Recruitment (ΔBRET % of Max)
Log [Pirenzepine] M
E
βarr2 Recruitment (ΔBRET % of Max)
Log [MT7] M
F
Pirenzepine
Vehicle
15min
30min
60min
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
Vehicle
15
30
60
Time with Pirenzepine (min)
G
MT7
Vehicle
15min
30min
60min
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
Vehicle
15
30
60
Time with MT7 (min)

Fig. 2.2 Pirenzepine and MT7 activated β-arrestin (but not G protein) pathway in HEK293 cells. (A) shows a dose-response curve for treatment with muscarine, pirenzepine (PZ), and MT7 and levels of IP1 production in hM_1R-transfected HEK293 cells (at 45 min of treatment). **(B)** shows a dose-response curve for the effects of ligands (muscarine (5 min), PZ (30 min), and MT7 (30 min)) on forskolin (300 nM)-induced cAMP production in hM_1R-transfected HEK293 cells. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Data normalized to baseline with multiple comparisons to the lowest concentration of drug; ****P<0.001, ***P<0.001, and **P<0.01, n=4. **(C-E)** Recruitment of HaloTag-β-arrestin2 to hM_1R in transiently co-transfected HEK293 cells following treatments with **(C)** carbachol (5 min) , **(D)** pirenzepine (30 min) and **(E)** MT7 (30 min). Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Data normalized to baseline with multiple comparisons to the lowest concentration of drug; ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05, n=3. **(F&G)** Immunoblotting for T-ERK and P-ERK proteins was conducted on hM_1R-transfected HEK293 cells, which were treated with **(F)** pirenzepine (PZ, 1 μM) or **(G)** MT7 (100 nM) at different time-points. Data represent P-ERK/T-ERK ratio (a.u.). Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to vehicle-treated group; ***P<0.001, **P<0.01, and *P<0.05, n=3.

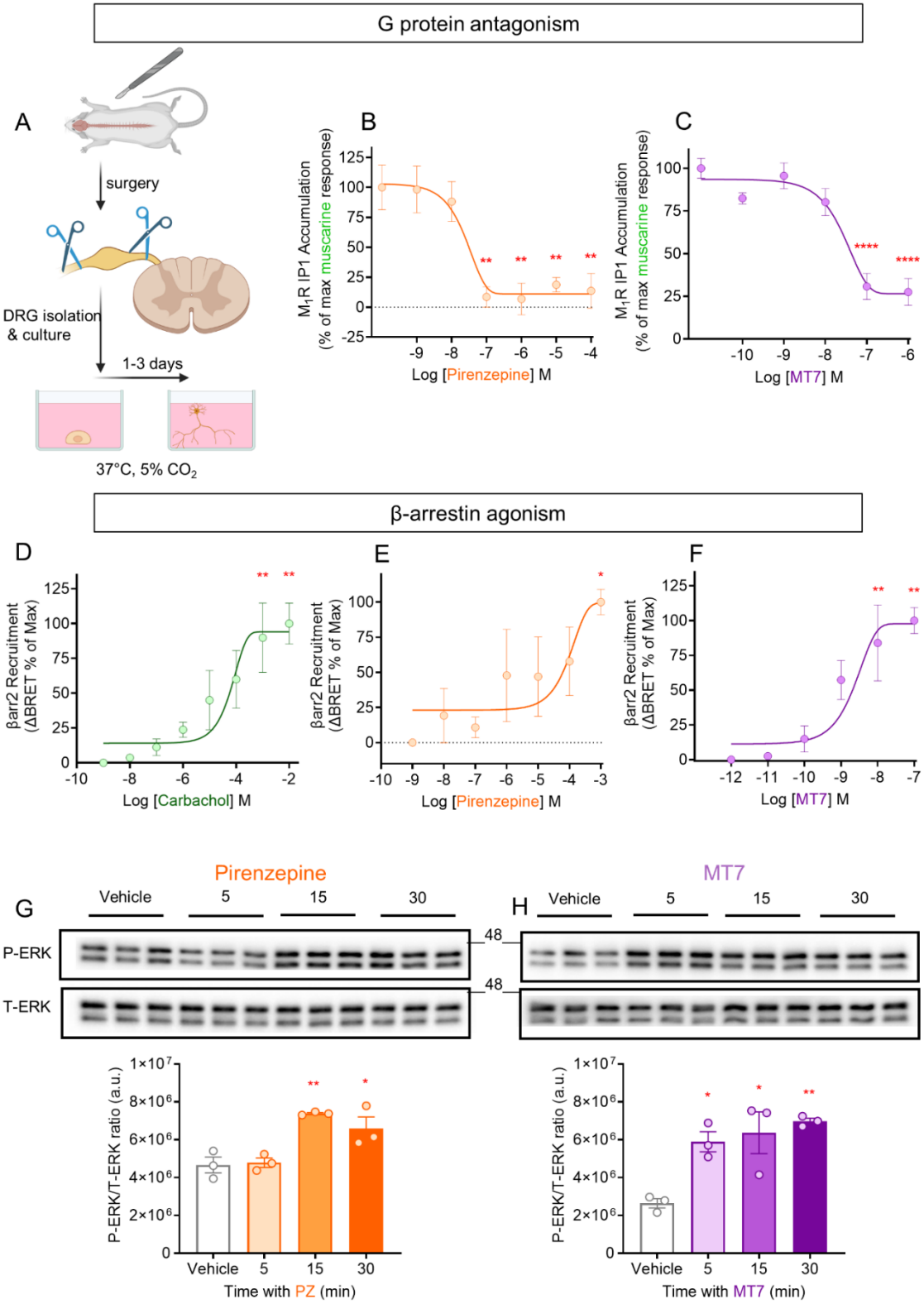
G protein antagonism
A
surgery
DRG isolation
& culture
1-3 days
37°C, 5% CO2
B
M1R IP1 Accumulation
(% of max muscarine response)
Log [Pirenzepine] M
C
M1R IP1 Accumulation
(% of max muscarine response)
Log [MT7] M
β-arrestin agonism
D
βarr2 Recruitment
(ΔBRET % of Max)
Log [Carbachol] M
E
βarr2 Recruitment
(ΔBRET % of Max)
Log [Pirenzepine] M
F
βarr2 Recruitment
(ΔBRET % of Max)
Log [MT7] M
Pirenzepine
MT7
G
H
Vehicle
5
15
30
P-ERK
T-ERK
48
P-ERK/T-ERK ratio (a.u.)
Time with PZ (min)
Time with MT7 (min)

Fig. 2.3 Pirenzepine and MT7 activated β-arrestin (but not G protein) pathway in DRG sensory neurons. (A) shows a schematic procedure for adult rat dorsal root ganglion (DRG) neuron isolation and culture. Inhibitory effect of **(B)** different concentrations of pirenzepine and **(C)** MT7 on muscarine (1 mM)-induced IP1 production in DRG sensory neurons. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Data normalized to baseline with multiple comparisons to the lowest concentration of drug; ****P<0.0001, **P<0.01, n=3. **(D-F)** Recruitment of HaloTag-β-arrestin2 to hM_1R in transiently co-transfected DRG sensory neurons following treatments with **(D)** carbachol (5 min) , **(E)** pirenzepine (30 min) and **(F)** MT7 (30 min). Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Data normalized to baseline with multiple comparisons to the lowest concentration of drug; **P<0.01, and *P<0.05, n=3. **(G&H)** Immunoblotting for T-ERK and P-ERK proteins was conducted on DRG sensory neurons, which were treated with **(G)** pirenzepine (PZ, 1 μM) or **(H)** MT7 (100 nM) at different time-points. Data represent P-ERK/T-ERK ratio (a.u.). Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to vehicle-treated group **P<0.01, and *P<0.05, n=3.

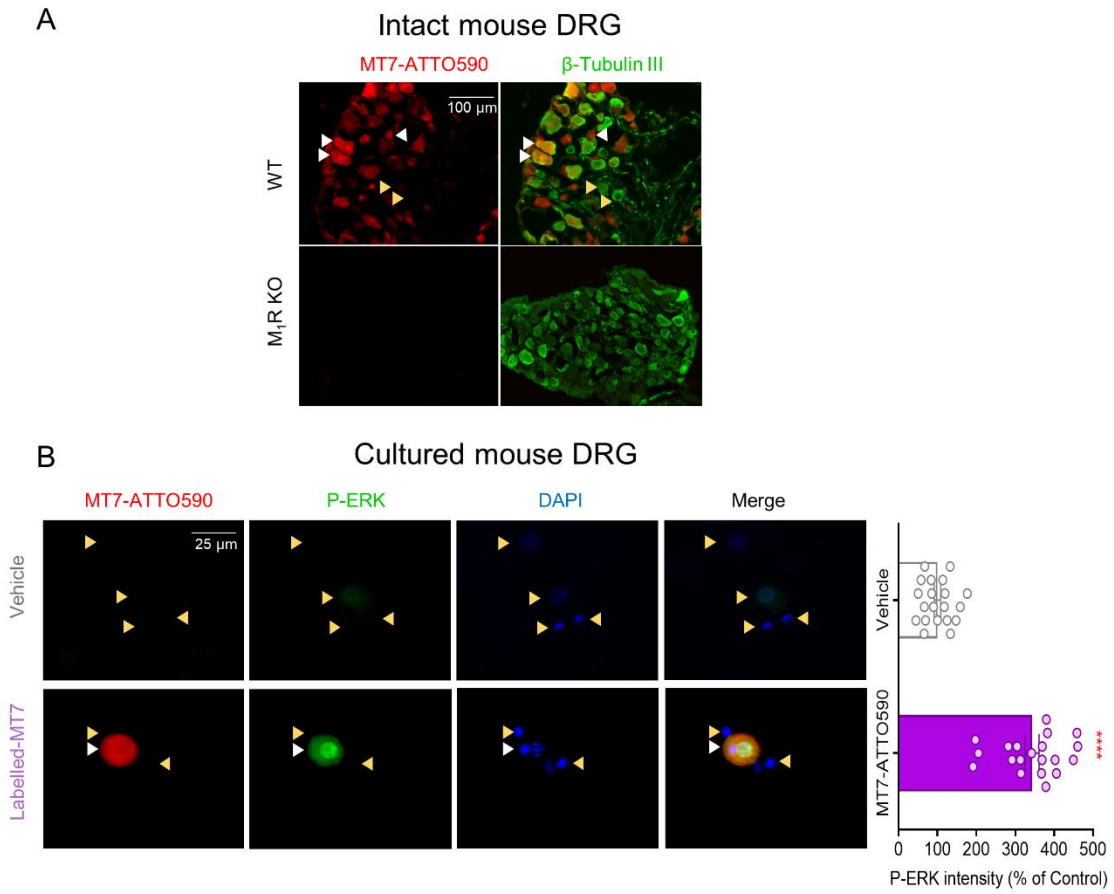

Fig. 2.4 M_1R expression and single cell analysis of ERK activation in mouse DRG sensory neurons. **(A)** Immunocytochemistry images of adult mouse DRG tissue sections for wild-type (WT) and M_1R KO mice treated with fluorescently tagged MT7-ATTO590 (100 nM), and labelled with antibody to neuron-specific ß-tubulin III. White arrows indicate M_1R positive and yellow arrows indicate M_1R negative neurons. **(B)** Immunocytochemistry images of cultured adult mouse DRG sensory neurons with vehicle/ MT7-ATTO590 treatment (100 nM, 90 min). Neurons were labelled with MT7-ATTO590, phospho-ERK, and DAPI. Values for fluorescence intensity of P-ERK are expressed as the mean ± SEM from 20 neurons and analyzed using *t*-test, ****P<0.0001.

A

```
1           11          21          31          41          51          61          71
MNTSAPPAVS  PNITVLAPGK  GPWQVAFIGI TTGLLSLATV TGNLLVLISF KVNTELKTVN NYFLLSLACA  DLIIGTFSMN
81          91          101         111         121         131         141         151
LYTTYLLMGH  WALGTLACDL WLALDYVASN ASVMNLLLIS FDRYFSVTRP  LSYRAKRTPR RAALMIGLAW LVSFVLWAPA
161         171         181         191         201         211         221         231
ILFWQYLVGE  RTVLAGQCYI  QFLSQPIITF  GTAMAAFYLP VTVMCTLYWR IYRETENRAR  ELAALQGSET  PGKGGGSSSS
241         251         261         271         281         291         301         311
SERSQPGAEG SPETPPGRCC RCCRAPRLLQ AYSWKEEEEE DEGSMESLTS SEGEEPGSEV VIKMPMVDPE  AQAPTKQPPR
321         331         341         351         361         371         381         391
SSPNTVKRPT  KKGRDRAGKG QKPRGKEQLA KRKTFSLVKE KKAARTLSAI LLAFILTWTP YNIMVLVSTF CKDCVPETLW
401         411         421         431         441         451
ELGYWLCYVN STINPMCYAL  CNKAFRDTFR LLLLCRWDKR RWRKIPKRPG SVHRTPSRQC
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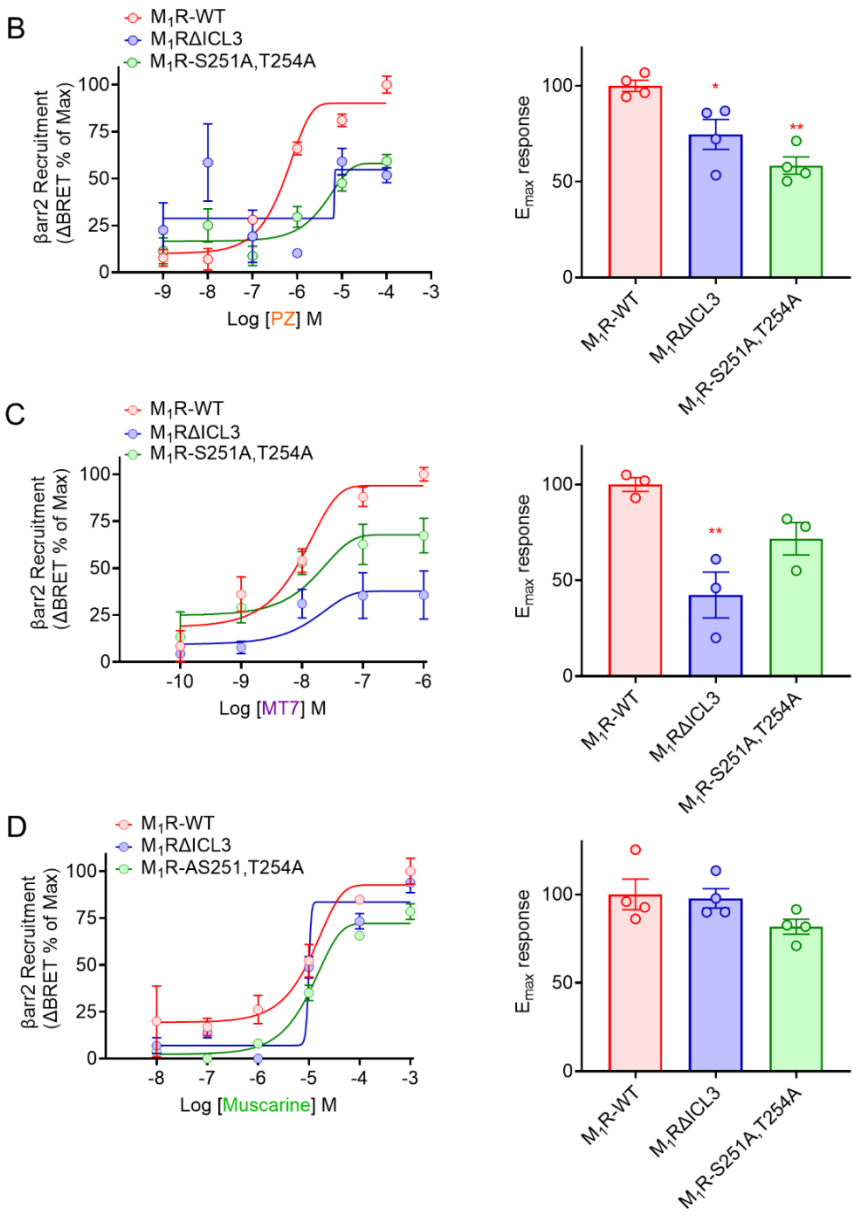


Figure 2.5 Mass spectrometry for Ser/Thr phosphorylation at hM$_1$R and effects of mutagenesis studies on Emax response to pirenzepine. (A) M$_1$R serine and threonine phosphorylation sites identified by nano-LC-MS/MS with high confidence and with site localization probabilities >0.99. Phosphorylated residues are in the red color within ICL3 region of M$_1$R in gray color. Concentration-response results for **(B)** pirenzepine (PZ, 30 min), **(C)** MT7 (30 min) and **(D)** muscarine (5 min) for β-arrestin2 recruitment to M$_1$R-WT, M$_1$RΔICL3 (mutation of all 6 Phos-sites at M$_1$R ICL3 region), and M$_1$R-S251A, T254A (mutations at Ser^{251} and Thr^{254} sites at M$_1$R ICL3 region) in HEK293 cells. Emax response was calculated for each drug and data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparisons to M$_1$R-WT group; **P<0.01 and *P<0.05, n=4.

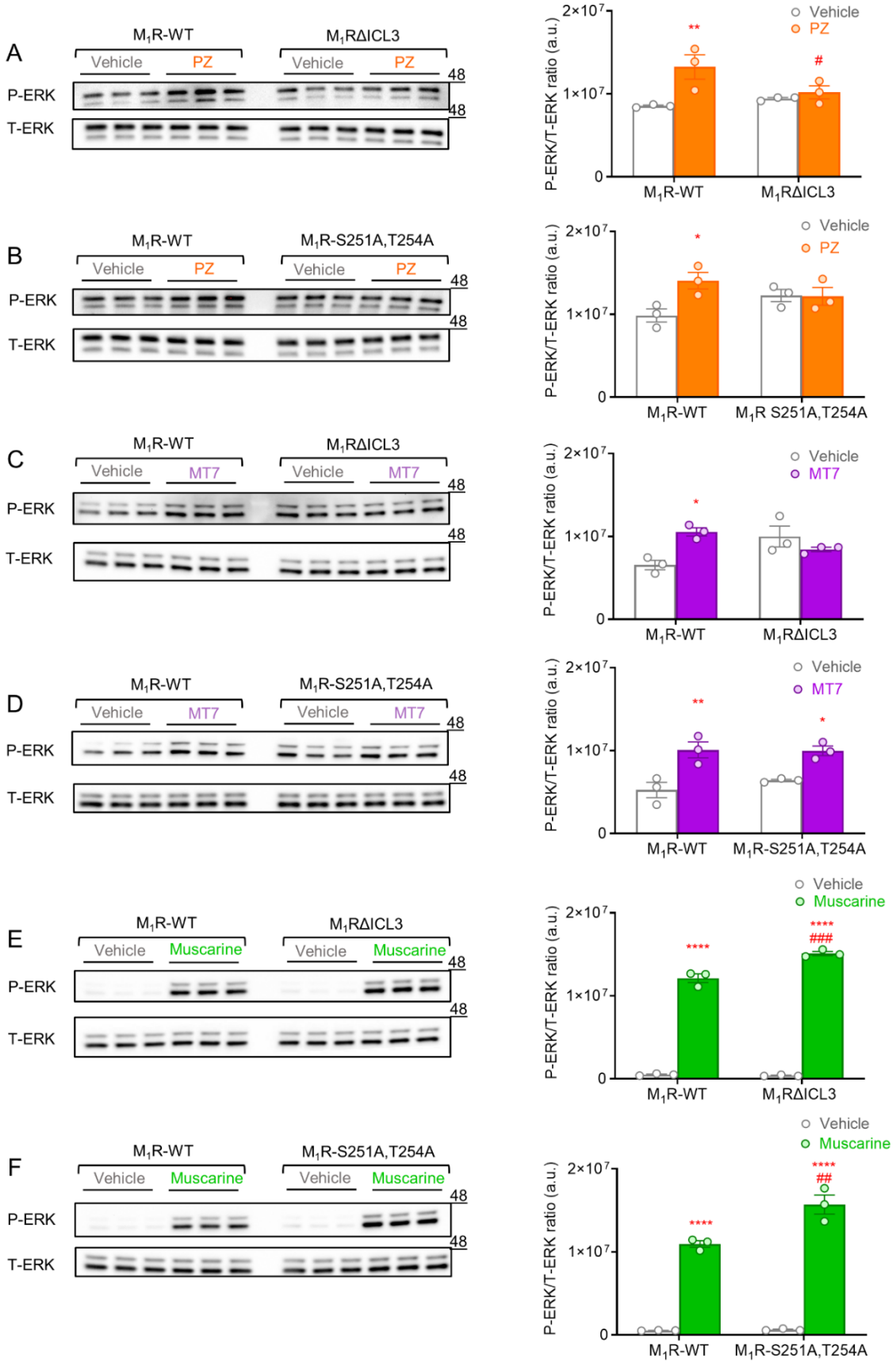

A
M1R-WT
M1RΔICL3
Vehicle
PZ
Vehicle
PZ
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
2×10^7
1×10^7
0
**
#
Vehicle
PZ
M1R-WT
M1RΔICL3
B
M1R-WT
M1R-S251A,T254A
Vehicle
PZ
Vehicle
PZ
P-ERK
T-ERK
48
48
*
Vehicle
PZ
M1R-WT
M1R S251A,T254A
C
M1R-WT
M1RΔICL3
Vehicle
MT7
Vehicle
MT7
P-ERK
T-ERK
48
48
*
Vehicle
MT7
M1R-WT
M1RΔICL3
D
M1R-WT
M1R-S251A,T254A
Vehicle
MT7
Vehicle
MT7
P-ERK
T-ERK
48
48
**
*
Vehicle
MT7
M1R-WT
M1R-S251A,T254A
E
M1R-WT
M1RΔICL3
Vehicle
Muscarine
Vehicle
Muscarine
P-ERK
T-ERK
48
48
****
****
###
Vehicle
Muscarine
M1R-WT
M1RΔICL3
F
M1R-WT
M1R-S251A,T254A
Vehicle
Muscarine
Vehicle
Muscarine
P-ERK
T-ERK
48
48
****
****
##
Vehicle
Muscarine
M1R-WT
M1R-S251A,T254A

Fig. 2.6 M_1R mutations altered ERK phosphorylation in response to muscarinic drugs in HEK293 cells. (A, C, and E) HEK293 cells were transfected with M_1R-WT and $M_1R\Delta$ICL3 (mutation of all 6 Phos-sites at M_1R ICL3 region) and received **(A)** pirenzepine (PZ, 1 μM, 60 min), **(C)** MT7 (100 nM, 60 min), and **E**) muscarine (100 μM, 5 min). **(B, D, and F)** HEK293 cells were transfected with M_1R-WT and M_1R-S251A, T254A (mutations at Ser^{251} and Thr^{254}) and received **(B)** pirenzepine (PZ, 1 μM, 60 min), **(D)** MT7 (100 nM, 60 min), and **(F)** muscarine (100 μM, 10 min). Immunoblotting for T-ERK and P-ERK proteins was conducted. Data represent P-ERK/T-ERK ratio (a.u.). Data show mean ± SEM (2-way ANOVA with Tukey's post-hoc test). Comparison between vehicle-treated groups and drug-treated groups for WT/mutation conditions; ****P<0.0001, **P<0.01, and *P<0.05. Comparison between PZ-treated groups; #P<0.05, comparison between muscarine-treated groups; ###P<0.001 and ##P<0.01, n=3.

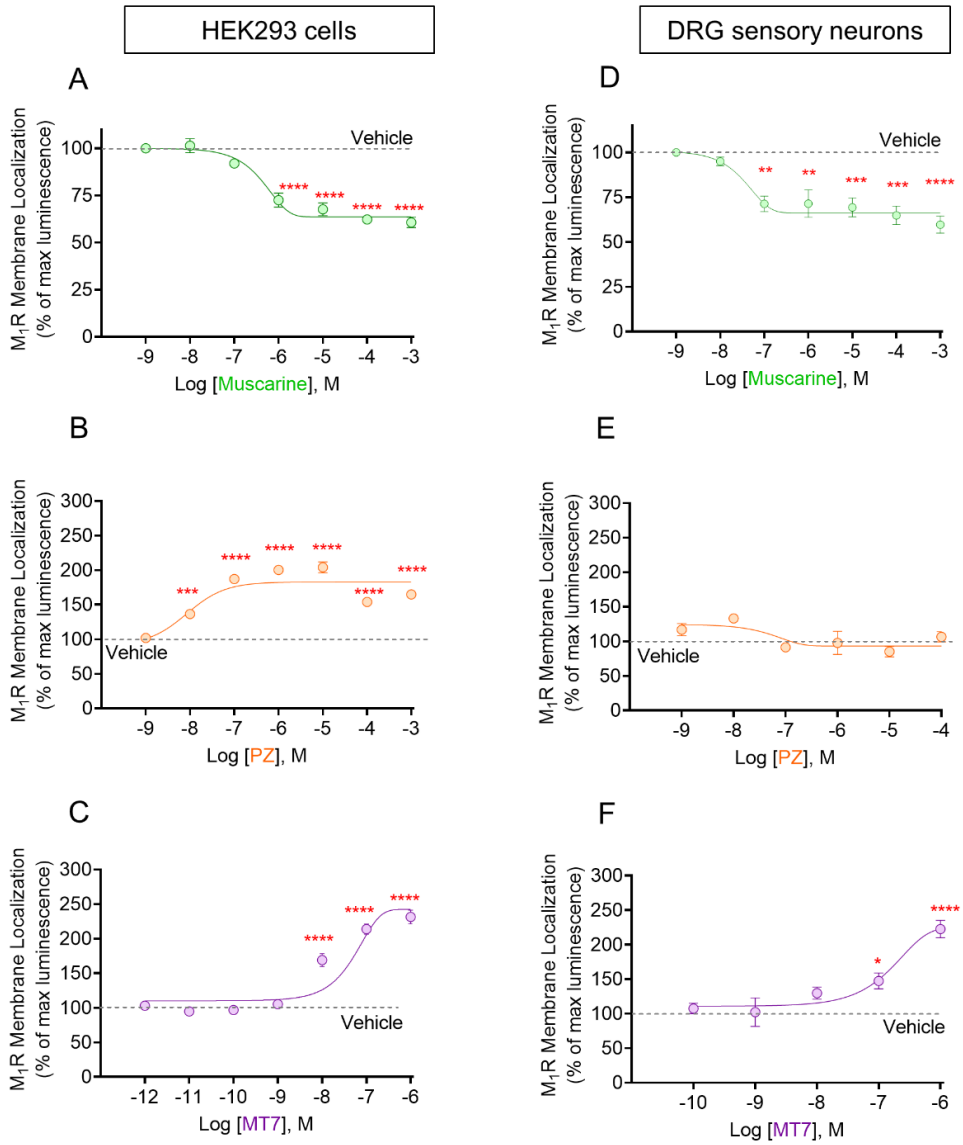


Fig. 2.7 Pirenzepine and MT7 enhance accumulation of M_1R at the surface of HEK293 cells DRG neurons. Dose response curve for receptor internalization performed for muscarine (**A&D**, 60 min), pirenzepine (**B&E**, 60 min), and MT7 (**C&F**, 60 min) in both HEK293 cells and DRG sensory neurons. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to vehicle-treated group; ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05, n=4.

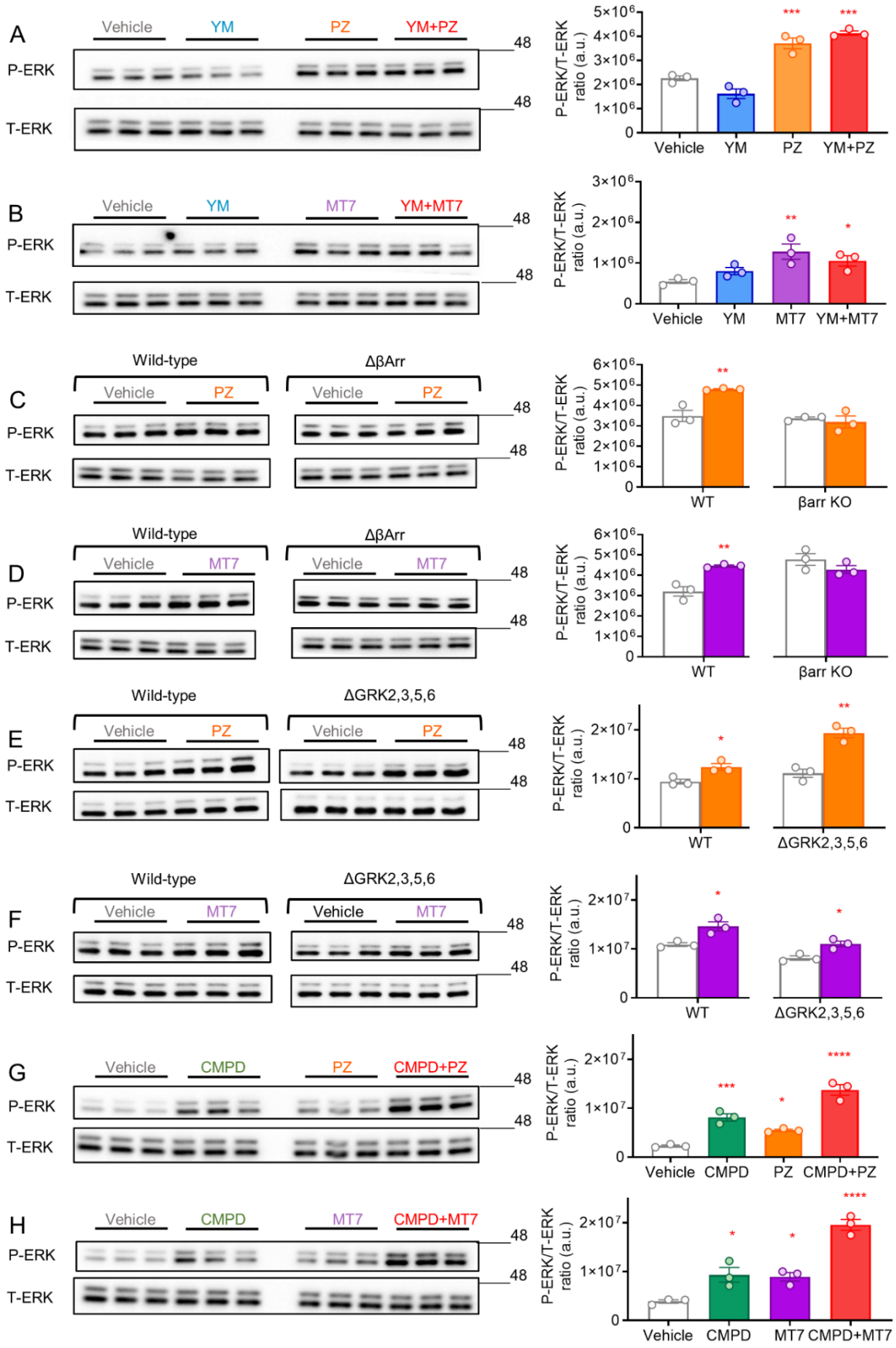

A
Vehicle
YM
PZ
YM+PZ
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
***
***
Vehicle
YM
PZ
YM+PZ
B
Vehicle
YM
MT7
YM+MT7
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
3×10^6
2×10^6
1×10^6
0
**
*
Vehicle
YM
MT7
YM+MT7
C
Wild-type
ΔβArr
Vehicle
PZ
Vehicle
PZ
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
6×10^6
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
**
WT
βarr KO
D
Wild-type
ΔβArr
Vehicle
MT7
Vehicle
MT7
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
6×10^6
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
**
WT
βarr KO
E
Wild-type
ΔGRK2,3,5,6
Vehicle
PZ
Vehicle
PZ
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
2×10^7
1×10^7
0
*
**
WT
ΔGRK2,3,5,6
F
Wild-type
ΔGRK2,3,5,6
Vehicle
MT7
Vehicle
MT7
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
2×10^7
1×10^7
0
*
*
WT
ΔGRK2,3,5,6
G
Vehicle
CMPD
PZ
CMPD+PZ
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
2×10^7
1×10^7
0
***
*
****
Vehicle
CMPD
PZ
CMPD+PZ
H
Vehicle
CMPD
MT7
CMPD+MT7
P-ERK
T-ERK
48
48
P-ERK/T-ERK ratio (a.u.)
2×10^7
1×10^7
0
*
*
****
Vehicle
CMPD
MT7
CMPD+MT7

Fig. 2.8 β-arrestins (but not GRKs and Gq-protein) are necessary for ERK phosphorylation following M_1R antagonist treatment. (A&B) M_1R-transfected HEK293 cells were pre-treated with vehicle or YM-254890 (1 μM, 60 min) before treatment with **(A)** pirenzepine (PZ, 1 μM, 60 min) or **(B)** MT7 (100 nM, 60 min). Immunoblotting for T-ERK and P-ERK proteins was conducted. Data represent P-ERK/T-ERK ratio (a.u.). Data are reported as the mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to vehicle-treated group; ***P<0.001, **P<0.01, and *P<0.05, n=3. **(C-F)** hM_1R transfected HEK293 cells (WT, ΔβArrestin1/2 or ΔGRK2,3,5,6) were treated with **(C&E)** pirenzepine (1 μM, 60 min) or **(D&F)** MT7 (100 nM, 60 min). Data are reported as the mean ± SEM. *t*-test was used for comparing vehicle-treated and drug-treated groups; **P<0.01 and *P<0.05, n=3. **(G&H)** hM_1R transfected HEK293 cells were pretreated with vehicle/CMPD101 (30 μM, 10 min), and then received **(G)** PZ (1 μM, 60 min), and **(H)** MT7 (100 nM, 60 min). Data are reported as the mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to vehicle-treated group; ****P<0.0001, ***P<0.001, and *P<0.05, n=3.

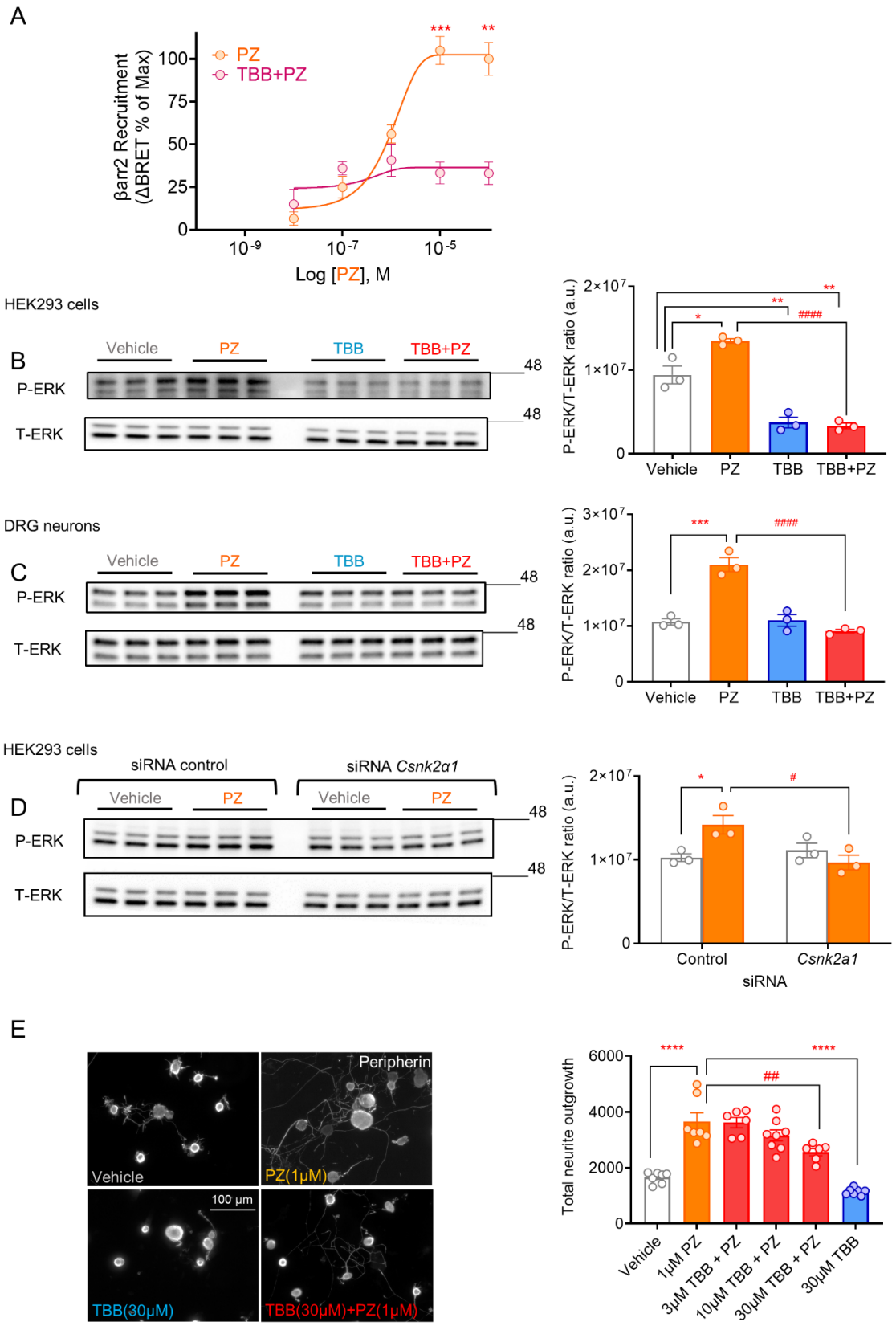

A
βarr2 Recruitment (ΔBRET % of Max)
PZ
TBB+PZ
Log [PZ], M
HEK293 cells
B
Vehicle
PZ
TBB
TBB+PZ
P-ERK
T-ERK
48
P-ERK/T-ERK ratio (a.u.)
DRG neurons
C
HEK293 cells
siRNA control
siRNA Csnk2α1
D
Control
Csnk2a1
siRNA
E
Peripherin
Vehicle
PZ(1μM)
100 μm
TBB(30μM)
TBB(30μM)+PZ(1μM)
Total neurite outgrowth
1μM PZ
3μM TBB + PZ
10μM TBB + PZ
30μM TBB + PZ
30μM TBB

Fig. 2.9 CK2 inhibition attenuated β-arrestin recruitment, ERK activation and neurite outgrowth by pirenzepine in DRG sensory neurons. (A) TBB (1 μM, 10 min) pretreatment blocked pirenzepine-induced β-arrestin2 recruitment to hM_1R in HEK293 cells. Values are expressed as the mean ± SEM, and were analyzed using *t*-test. Comparison between PZ (100 μM) and TBB + PZ (100 μM); ***P<0.001 and comparison between PZ (10 μM) and TBB + PZ (10 μM); **P<0.01, n=4. **(B)** M_1R-transfected HEK293 cells and **(C)** DRG neurons were pretreated with TBB (1 μM or 10 μM, 10 min, respectively), and then received pirenzepine (PZ, 1 μM, 60 min). Data are reported as the mean ± SEM (1-way ANOVA with Tukey's post-hoc test). Comparison to vehicle; ***P<0.001, **P<0.01 and *P<0.05. Comparison to PZ-treated group; ####P<0.0001, n=3. **(D)** HEK293 cells were co-transfected with 25 nM of control or *Csnk2a1* siRNA and hM_1R, and then received vehicle or pirenzepine (PZ, 1 μM, 60 min). Values are expressed as the mean ± SEM (2-way ANOVA with Tukey's post-hoc test). Comparison between vehicle and PZ-treated cells in siRNA control group; *P<0.05, and comparison between PZ-treated cells in siRNA control and *Csnk2a1* groups; #P<0.05, n=3. **(E)** DRG sensory neurons were treated with pirenzepine (PZ ,1 μM), and different doses of TBB in combination with (PZ, 1 μM) for 42 h. Cells were fixed and immune-stained for peripherin, and total neurite outgrowth was assessed. Values are means ± SEM (1-way ANOVA with Tukey's post hoc test**)**. Comparison between vehicle/TBB 30 μM-treated cells with PZ (1 μM)-treated group; ****P<0.0001, and comparison between PZ (1 μM)-treated cells and PZ (1 μM) + TBB (30 μM) combination group; ##P<0.01, n = 6-8 replicate cultures.

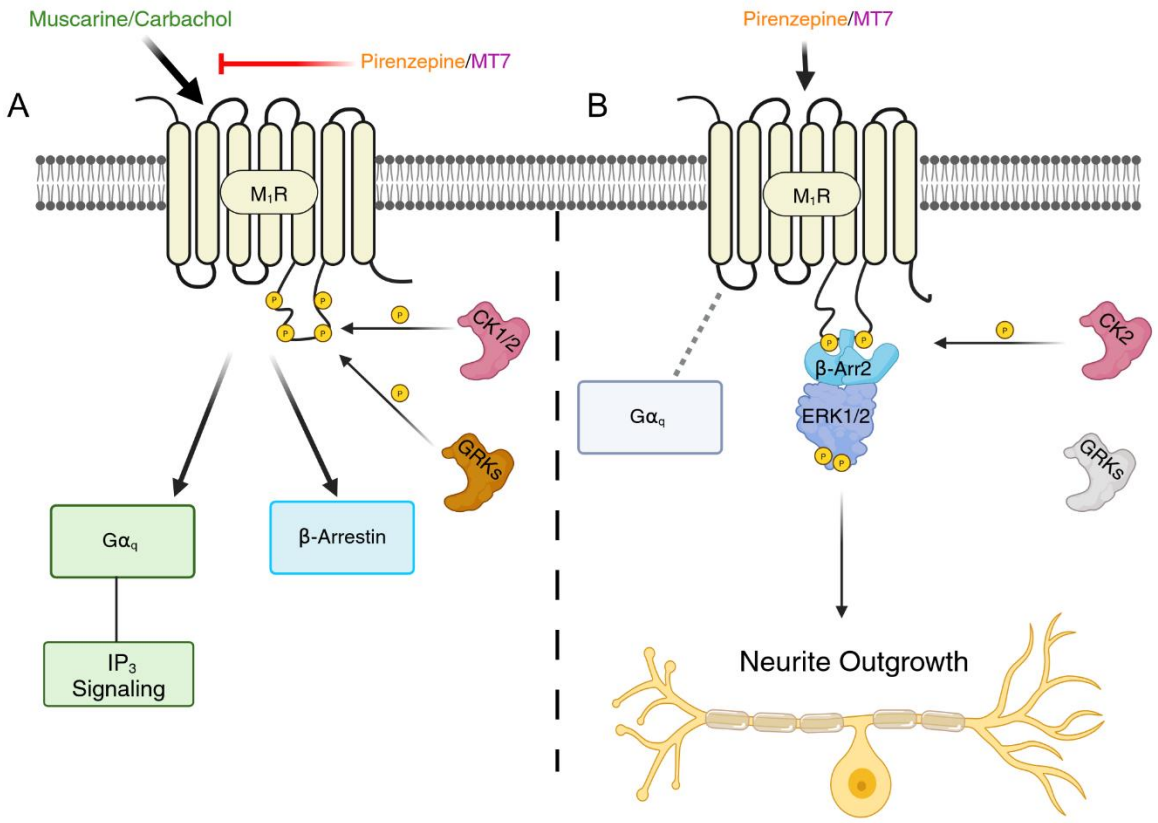


Fig 2.10 Graphical abstract. Schematic presentation of the effect of muscarinic ligands at M_1R associated signaling pathway. (A) Muscarine/carbachol acts as a balanced ligand by engaging both $G\alpha_q$ and β-arrestin signaling pathways and treatment with pirenzepine/MT7 blocks these effects. **(B)** Pirenzepine/MT7 acts as a β-arrestin biased ligand by 1) phosphorylating of ICL3 region of M_1R via CK2 (but not GRKs), 2) no activation of G protein signaling, 3) recruitment of β-arrestin 2 and 4) ERK1/2 activation leading to neurite outgrowth in DRG sensory neurons. This figure was generated by BioRender under license number EK285MUOPQ.

2.8 ACKNOWLEDGMENTS

We thank Dr. Nicholas Sherman at the University of Virginia Biomedical Research Facility for supervising the initial LC-MS/MS analysis and bioinformatic work on phosphorylation sites of M_1R in response to pirenzepine. **We also** thank Dr. Jürgen Wess, NIDDK, for the kind gift of the M_1R KO mouse line. Graphical figures were created with BioRender.

2.9 FUNDING

Canadian Institutes of Health Research (CIHR) grant # PJT-162172 to PF. Graduate Enhancement of Tri-Council Stipends (GETS) to SA. University of Manitoba and Shared Health to RPZ. A.I. was funded by KAKENHI JP21H04791 and JP24K21281 from the Japan Society for the Promotion of Science (JSPS); JP22ama121038 and JP22zf0127007 from the Japan Agency for Medical Research and Development (AMED); JPMJFR215T and JPMJMS2023 from the Japan Science and Technology Agency (JST); The Uehara Memorial Foundation.

2.10 AUTHOR CONTRIBUTIONS

SA performed and designed all experiments (except where specified in the following paragraph). In addition, SA carried out data analysis and construction of figures. MA constructed and tested the M_1R and β-arrestin2 plasmids for the BRET analysis. DRS performed the neurite outgrowth studies with cultured DRG sensory neurons. TMZW set up the use of fluorescently tagged MT7 and performed the related labelling studies in vitro and in sections of DRG. YL processed samples and performed mass spectroscopy for proteomic analysis. RPZ supervised the proteomic work and performed bioinformatic analysis. AI designed and generated the β-arrestin and GRK knockout

HEK293 cells lines using CRISPR-Cas9 technology. HAD contributed to supervision of some methodology, data analysis, and design/construction of figures. SA wrote the 1st draft of the manuscript which was then edited and refined by PF. All authors reviewed and edited the manuscript prior to submission. PF helped to design experiments, supervised all the work and obtained supporting funding.

2.11 COMPETING INTEREST

The corresponding author, PF, declares he is a co-founder, shareholder and member of the scientific advisory board of WinSanTor Inc., which has licensed intellectual property from the University of Manitoba in the area of antimuscarinic drugs.

2.12 DATA AND MATERIALS AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. All plasmids generated in this study will be distributed upon reasonable request and completion of a materials transfer agreement with University of Manitoba. All data reported in this paper will be shared by the lead contact upon request.

2.13 Supplementary materials

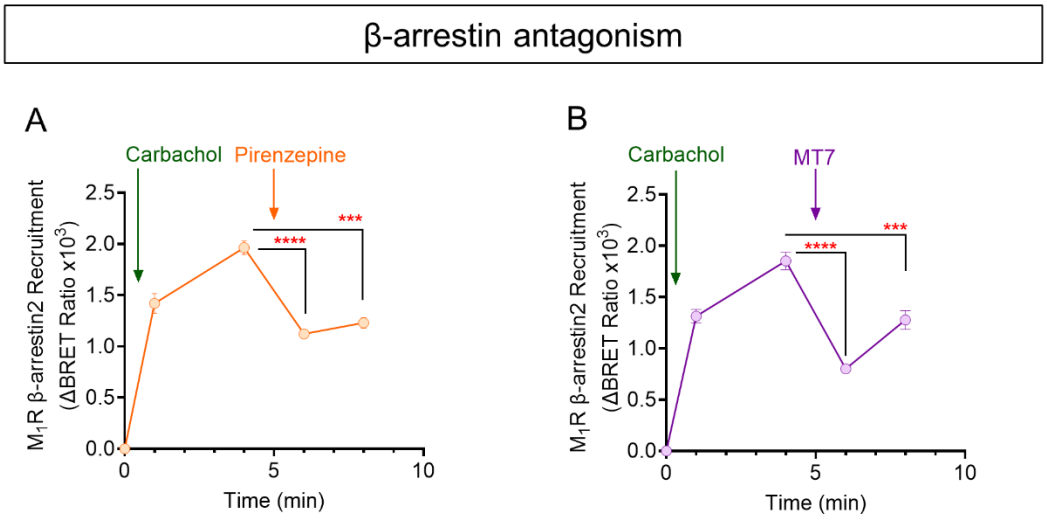
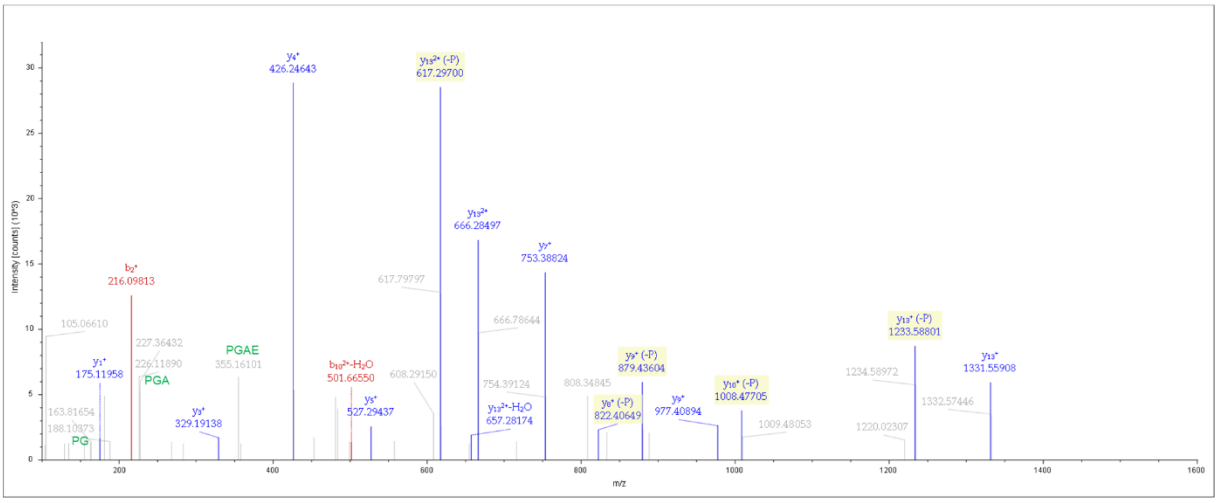


Fig. 2 S1. Antagonism of the carbachol-induced recruitment of HaloTag-β-arrestin2 to hM$_1$R. Ligands were added at the times indicated. Carbachol (1 mM)-induced βArr2-Halo recruitment to hM$_1$R-Nluc was blocked by **(A)** pirenzepine (1 μM) and **(B)** MT7 (100 nM) in transiently co-transfected HEK293 cells. Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). ****P<0.0001, and ***P<0.001, n=3. Antagonist-treated groups were compared to carbachol-treated groups at minute 5.

A

SQPGAEGpS(251)PETPPGR



B

SQPGAEGSPEpT(254)PPGR

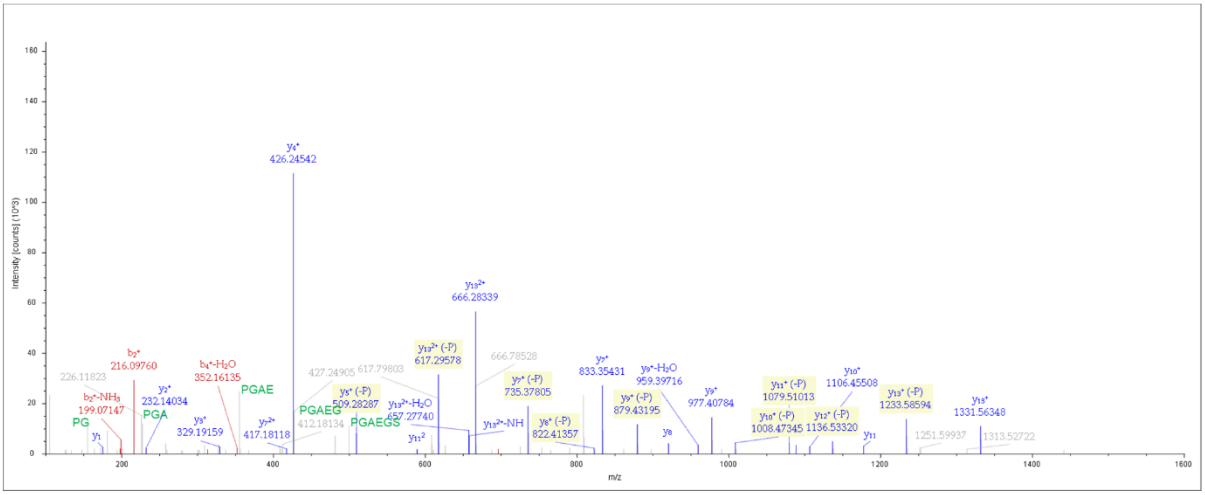


Fig. 2 S2. LC-MS/MS analysis identifies sites of hM$_1$R phosphorylation in HEK293 cells following pirenzepine treatment. LC-MS/MS analysis performed by the Biomolecular Analysis Facility at University of Virginia Health System. **(A)** MS2 spectra of identified hM$_1$R phosphorylation at Ser^{251} in ICL3. **(B)** MS2 spectra of identified hM$_1$R phosphorylation at Thr^{254} in ICL3.

Methodology for Fig. S2. The gel pieces from the band were transferred to a siliconized tube and washed in 200 µL 50% methanol. The gel pieces were dehydrated in acetonitrile, rehydrated in

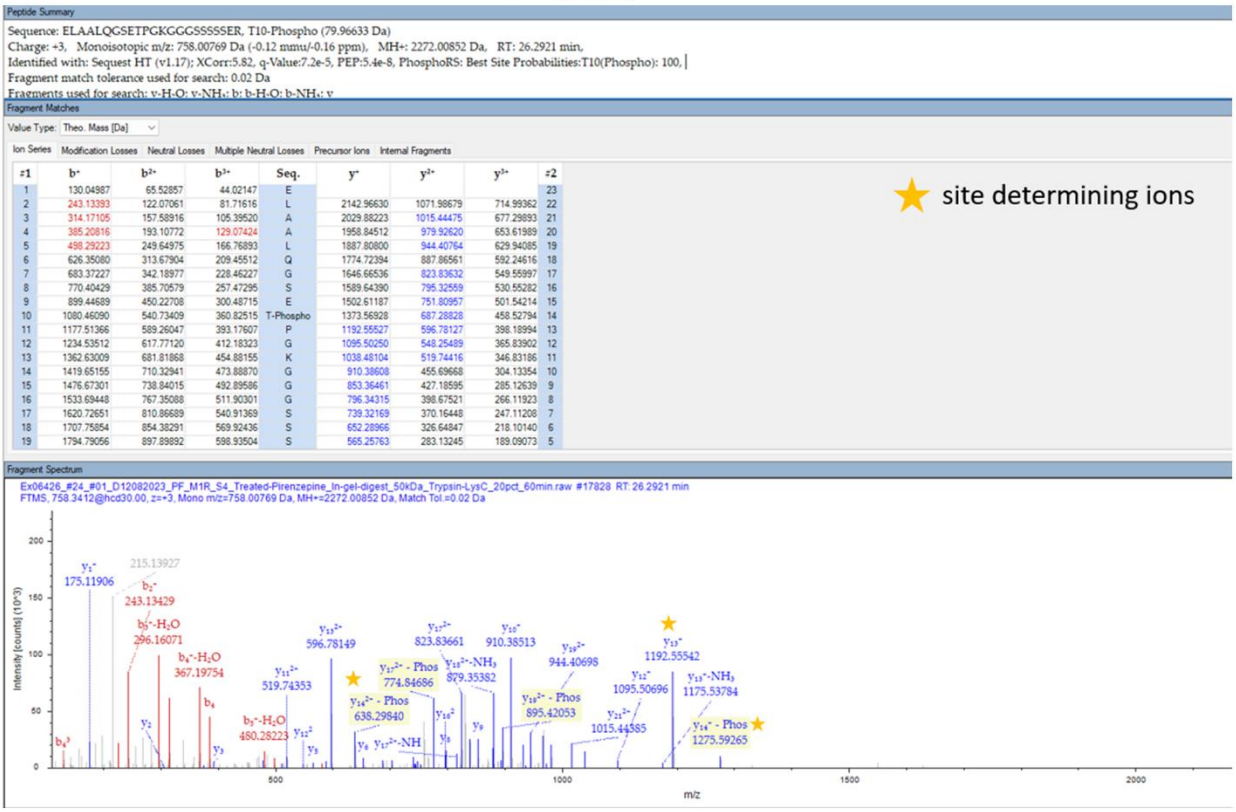
30 µL of 10 mM dithiolthreitol in 0.1 M ammonium bicarbonate and reduced at room temperature for 0.5 h. The DTT solution was removed and the sample alkylated in 30 µL 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The reagent was removed and the gel pieces dehydrated in 100 µL acetonitrile. The acetonitrile was removed and the gel pieces rehydrated in 100 µL 0.1 M ammonium bicarbonate. The pieces were dehydrated in 100 µL acetonitrile, the acetonitrile removed and the pieces completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/µL (Trypsin, Chymotrypsin, and Thermolysin separately) in 50 mM ammonium bicarbonate on ice for 30 min. Any excess enzyme solution was removed and 20 µL 50 mM ammonium bicarbonate added. The sample was digested overnight at 37 ^{o}C and the peptides formed extracted from the polyacrylamide in a 100 µL aliquot of 50% acetonitrile/5% formic acid. This extract was evaporated to 15 µL for MS analysis.

The LC-MS system consisted of a ThermoFisher Orbitrap Exploris 480 mass spectrometer system with an Easy Spray ion source connected to a Thermo 75 µm x 15 cm C18 Easy Spray column (through pre-column). 5 µL of the extract was injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.3 µL/min over 1.0 hours. The nanospray ion source was operated at 1.9 kV. The digest was analyzed using the rapid switching capability of the instrument acquiring a full scan mass spectrum to determine peptide molecular weights followed by product ion spectra (Top10 - 10 HCD) to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 25000 MS/MS spectra of ions ranging in abundance over several orders of magnitude. Not all MS/MS spectra

are derived from peptides. The data were analyzed by database searching using the Sequest search algorithm against investigator's provided sequence.

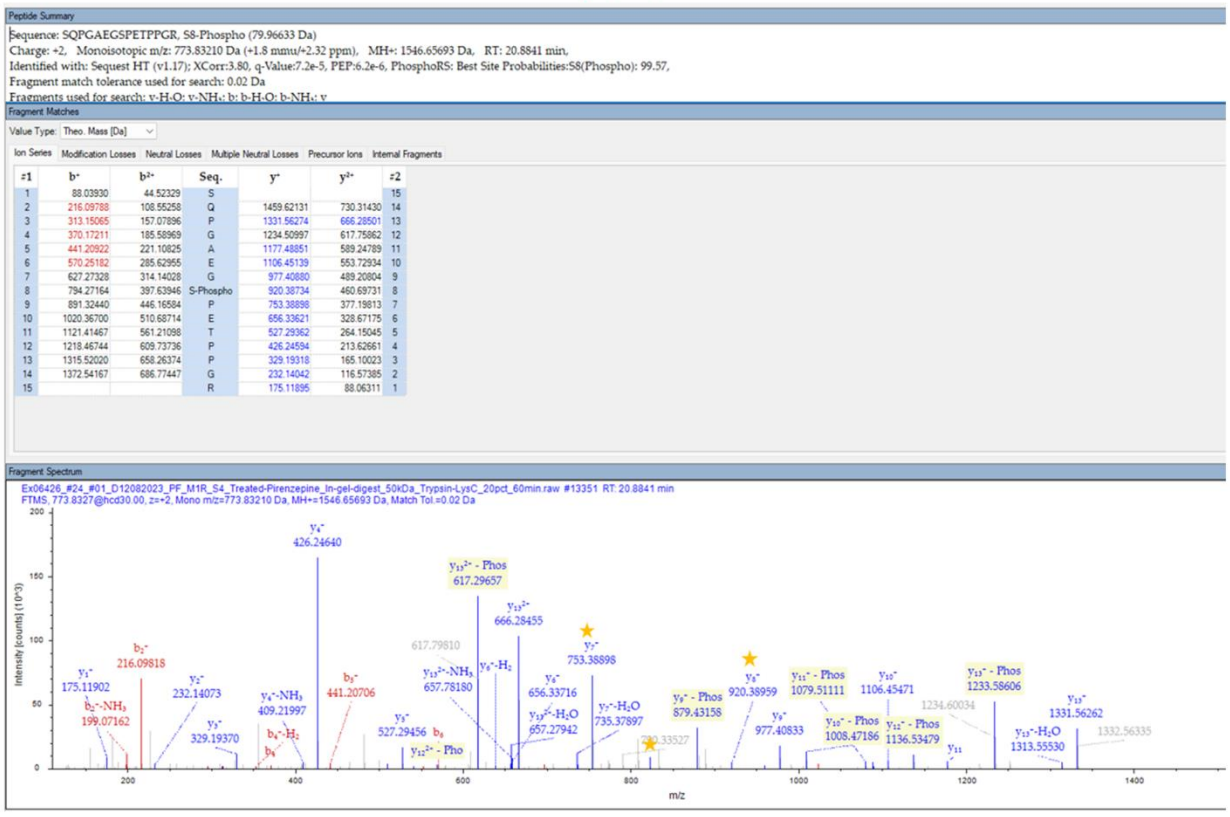
A

ELAALQGSEpT(230)PGKGGGSSSSSER



B

SQPGAEGpS(251)PETPPGR



C

SQPGAEGSPEpT(254)PPGR

Peptide Summary

Sequence: SQPGAEGSPETPPGR, T11-Phospho (79.96633 Da)
Charge: +2, Monoisotopic m/z: 773.83000 Da (-0.31 mmu/-0.4 ppm), MH+: 1546.65272 Da, RT: 21.4967 min,
Identified with: Sequest HT (v1.17); XCorr:4.23, q-Value:7.2e-5, PEP:2.8e-6, PhosphoRS: Best Site Probabilities:T11(Phospho): 100,
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: y-H$_2$O; y-NH$_3$; b; b-H$_2$O; b-NH$_3$; y

Fragment Matches

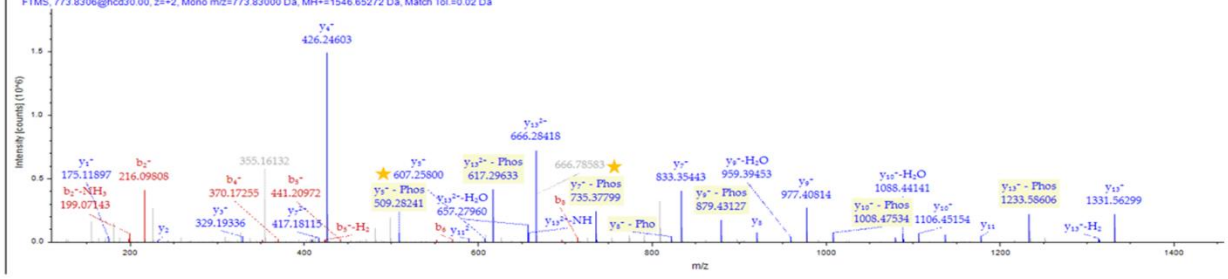
Value Type: Theo. Mass [Da]

Ion Series | Modification Losses | Neutral Losses | Multiple Neutral Losses | Precursor Ions | Internal Fragments

#1	b+	b2+	Seq.	y+	y2+	#2
1	88.03930	44.52329	S			15
2	216.09788	108.55258	Q	1459.62131	730.31430	14
3	313.15065	157.07896	P	1331.56274	666.28501	13
4	370.17211	185.58969	G	1234.50997	617.75862	12
5	441.20922	221.10825	A	1177.48851	589.24789	11
6	570.25182	285.62955	E	1106.45139	553.72934	10
7	627.27328	314.14028	G	977.40880	489.20804	9
8	714.30531	357.65629	S	920.38734	460.69731	8
9	811.35807	406.18267	P	833.35531	417.18129	7
10	940.40067	470.70397	E	736.30255	368.65491	6
11	1121.41467	561.21098	T-Phospho	607.25995	304.13361	5
12	1218.46744	609.73736	P	426.24594	213.62661	4
13	1315.52020	658.26374	P	329.19318	165.10023	3
14	1372.54167	686.77447	G	232.14042	116.57385	2
15			R	175.11895	88.06311	1

Fragment Spectrum

Ex06426_#24_#01_D12082023_PF_M1R_S4_Treated-Pirenzepine_In-gel-digest_50kDa_Trypsin-LysC_20pct_60min.raw #13791 RT: 21.4967 min
FTMS, 773.8306@hcd30.00, z=+2, Mono m/z=773.83000 Da, MH+=1546.65272 Da, Match Tol.=0.02 Da



D

MPMVDPEAQAPTKQPPRpS(321)SPNTVK

Peptide Summary

Sequence: MPMVDPEAQAPTKQPPRSSPNTVK, S18-Phospho (79.96633 Da)
Charge: +4, Monoisotopic m/z: 672.32661 Da (+2.95 mmu/+4.38 ppm), MH+: 2686.28462 Da, RT: 28.6018 min,
Identified with: Sequest HT (v1.17); XCorr:4.24, q-Value:4.5e-5, PEP:2.1e-5, PhosphoRS: Best Site Probabilities:S18(Phospho): 98.2,
Fragment match tolerance used for search: 0.02 Da
Fragments used for search: y-H$_2$O; y-NH$_3$; b; b-H$_2$O; b-NH$_3$; y

Fragment Matches

Value Type: Theo. Mass [Da]

Ion Series | Modification Losses | Neutral Losses | Multiple Neutral Losses | Precursor Ions | Internal Fragments

#1	b+	b2+	b3+	Immonium	Seq.	y+	y2+	y3+	#2
1	132.04776	66.52752	44.68744	104.05285	M				24
2	229.10053	115.05390	77.03836	70.06513	P	2555.23236	1278.11982	852.41564	23
3	360.14101	180.57414	120.71852	104.05285	M	2458.17960	1229.59344	820.06472	22
4	459.20942	230.10835	153.74133	72.08078	V	2327.13912	1164.07320	776.38456	21
5	574.23637	287.62182	192.08364	88.03930	D	2228.07070	1114.53899	743.36175	20
6	671.28913	336.14820	224.43456	70.06513	P	2113.04376	1057.02552	705.01944	19
7	800.33172	400.66950	267.44876	102.05496	E	2015.99099	1008.49914	672.66852	18
8	871.36884	436.18806	291.12780	44.04948	A	1886.94840	943.97784	629.65432	17
9	999.42741	500.21735	333.81399	101.07094	Q	1815.91129	908.45928	605.97528	16
10	1070.46453	535.73590	357.49303	44.04948	A	1687.85271	844.42999	563.28909	15
11	1167.51729	584.26228	389.84395	70.06513	P	1616.81560	808.91144	539.61005	14
12	1268.56497	634.78612	423.52651	74.06004	T	1519.76283	760.38505	507.25913	13
13	1396.65993	698.83360	466.22483	101.10732	K	1418.71515	709.86122	473.57657	12
14	1524.71851	762.86289	508.91102	101.07094	Q	1290.62019	645.81373	430.87825	11
15	1621.77127	811.38928	541.26194	70.06513	P	1162.56161	581.78445	388.19206	10
16	1718.82404	859.91566	573.61286	70.06513	P	1065.50885	533.25806	355.84113	9
17	1874.92515	937.96621	625.64657	129.11347	R	968.45609	484.73168	323.49021	8
18	2041.92351	1021.46539	681.31269	140.01072	S-Phospho	812.35498	406.68113	271.45651	7
19	2128.95554	1064.98141	710.32336	60.04439	S	645.35662	323.18195	215.79039	6
20	2226.00830	1113.50779	742.67428	70.06513	P	558.32459	279.66593	186.77971	5
21	2340.05123	1170.52925	780.68859	87.05529	N	461.27182	231.13955	154.42879	4
22	2441.09891	1221.05309	814.37115	74.06004	T	347.22890	174.11809	116.41448	3
23	2540.16732	1270.58730	847.39396	72.08078	V	246.18122	123.59425	82.73192	2
24				101.10732	K	147.11280	74.06004	49.70912	1

Fragment Spectrum

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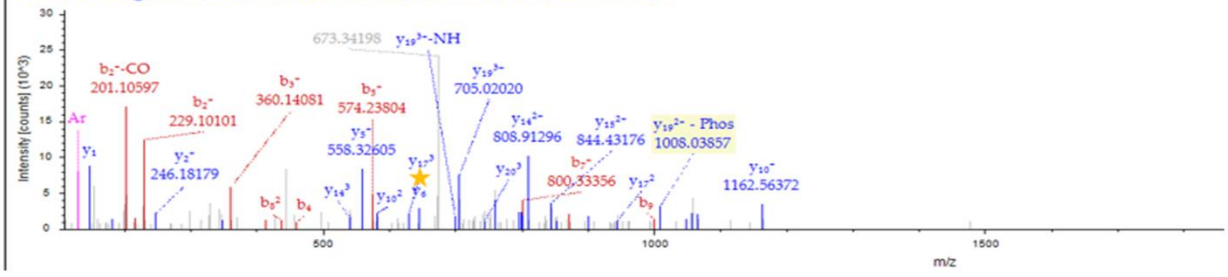
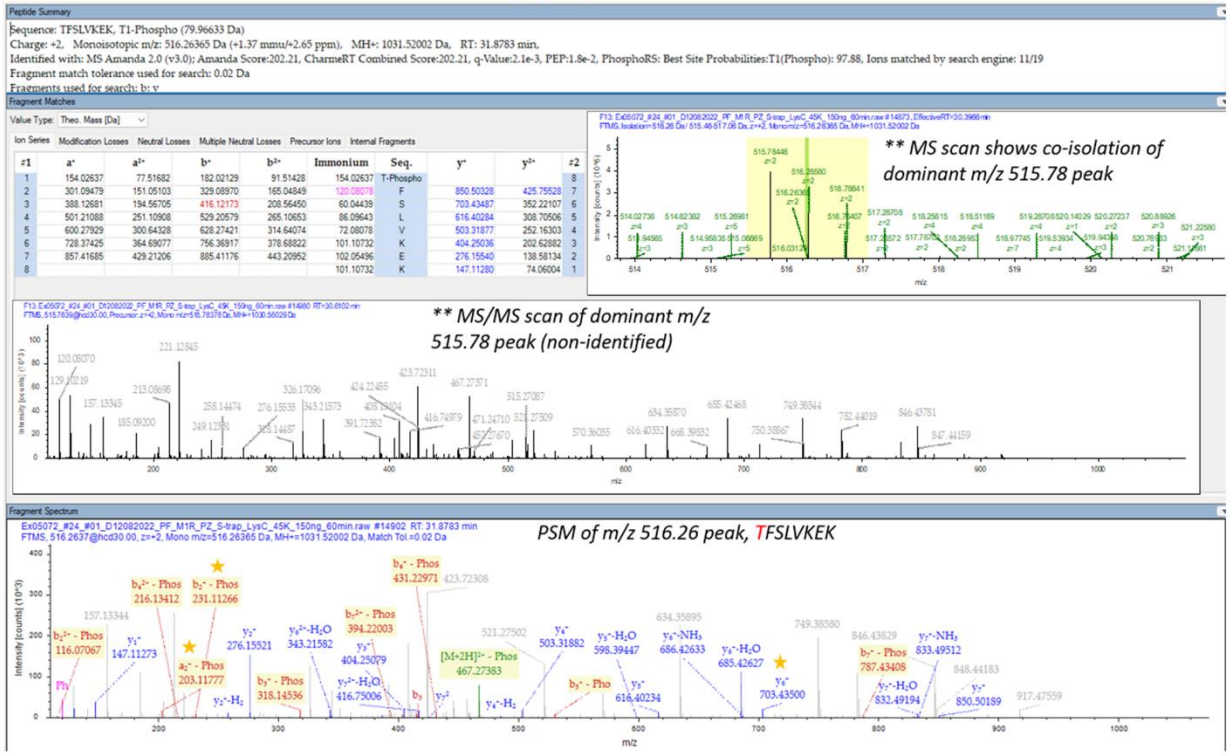
FTMS, 672.5775@hcd30.00, z=+4, Mono m/z=672.32661 Da, MH+=2686.28462 Da, Match Tol.=0.02 Da



E

pT(354)FSLVKEK



F

TFpS(356)LVK

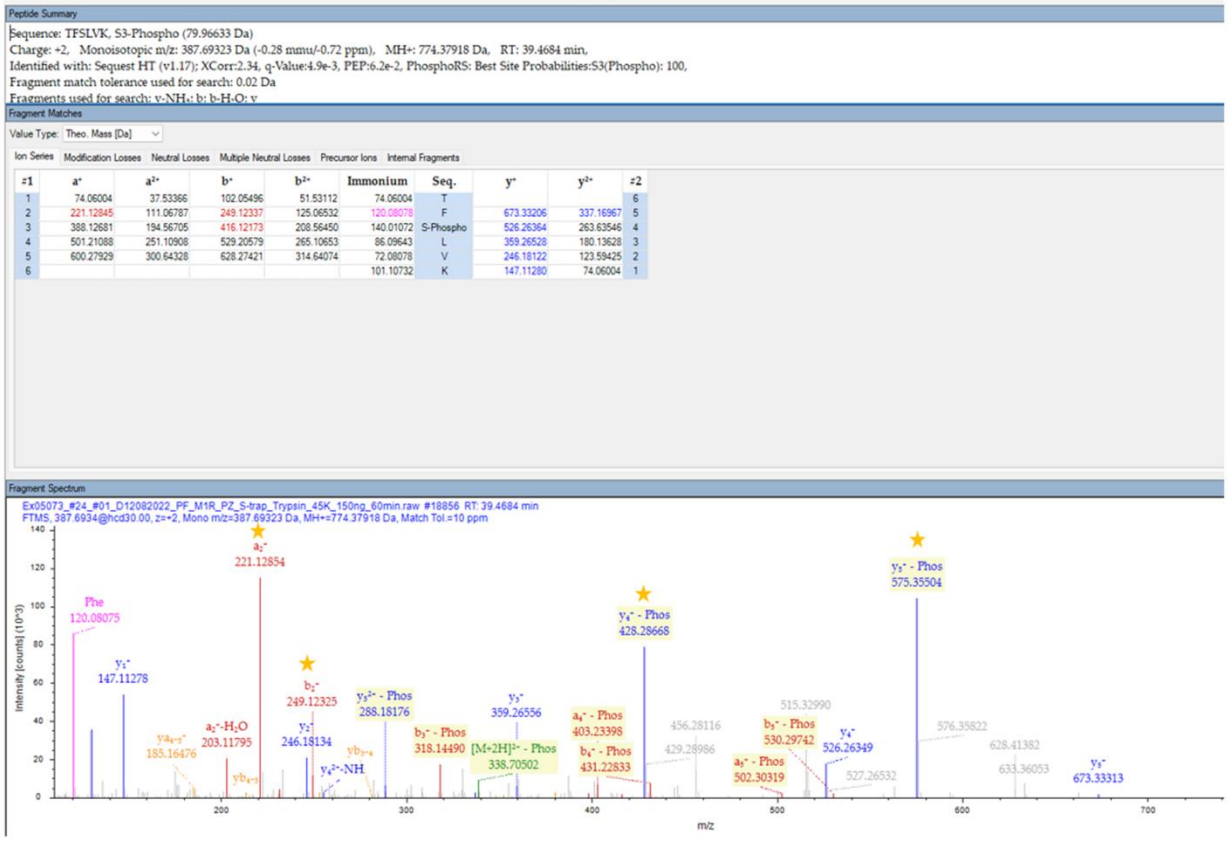


Fig. 2 S3. LC-MS/MS analysis identifies sites of hM_1R phosphorylation in HEK293 cells following pirenzepine treatment. LC-MS/MS analysis performed by the Manitoba Centre for Proteomics and Systems Biology (MCPSB) **(A**) MS2 spectra of identified hM_1R phosphorylation at Thr^{230}, **(B)** at Ser^{251}, **(C)** at Thr^{254}, **(D)** at Ser^{321}, **(E)** at Thr^{354}, and **(F)** at Ser^{356} in ICL3.

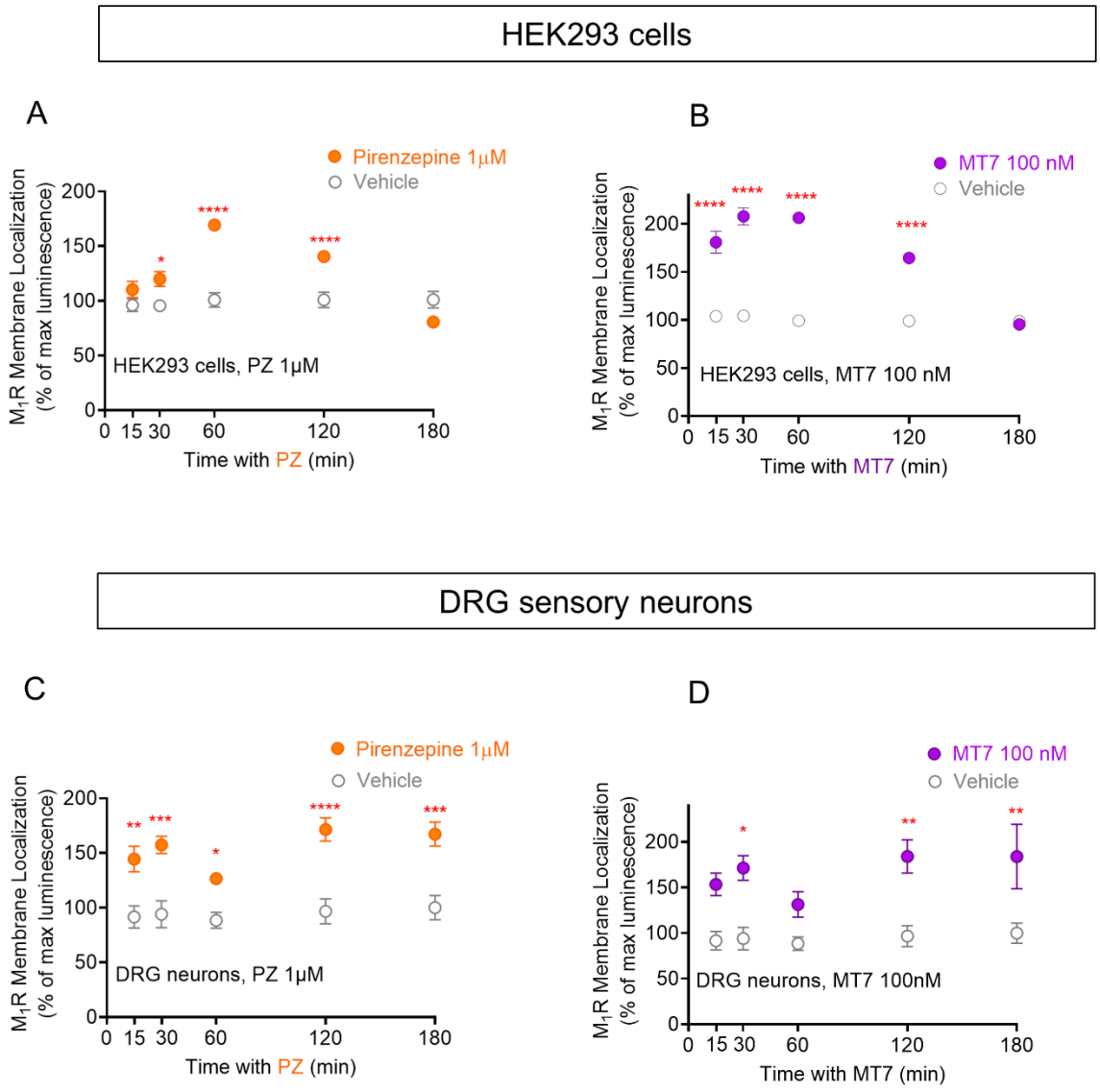


Fig. 2 S4. Time course of PZ and MT7 induced accumulation of M_1R at the cell surface. Time-response curve for receptor internalization performed for **(A)** pirenzepine (PZ, 1 μM, HEK293 cells), **(B)** MT7 (100 nM, HEK293 cells), **(C)** pirenzepine (PZ, 1 μM, DRG sensory neurons), and **(D)** MT7 (100 nM, DRG sensory neurons). *t*-test was used to compare the values of drug-treated groups with their vehicle-treated groups at each time-point. Data show mean ± SEM; ****P<0.0001, ***P<0.001, **P<0.01 and *P<0.05, n=4.

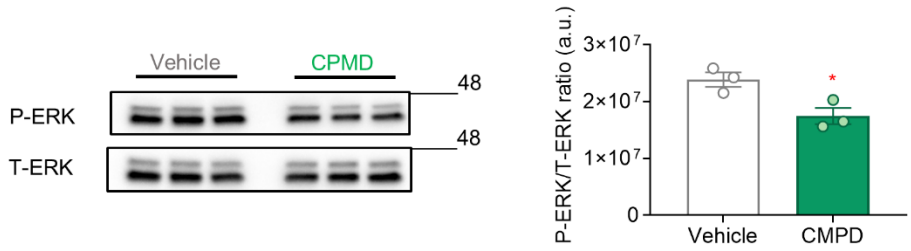


Fig. 2 S5. CPMD101 treatment in non-transfected HEK293 cells. HEK293 cells received vehicle/CMPD101 (30 µM, 10 min) and immunoblotting for T-ERK and P-ERK proteins was conducted. Data represent P-ERK/T-ERK ratio (a.u.). Data show mean ± SEM; *t*-test, *P<0.05, n=3.

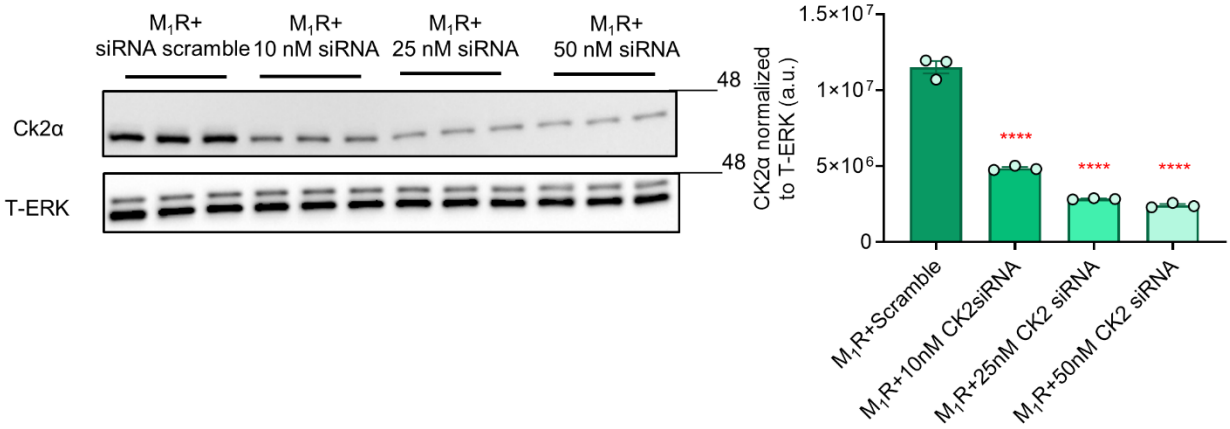


Fig. 2 S6. CK2α knock-down in HEK293 cells. HEK293 cells received scramble siRNA (50 nM) or target pool siRNA for CK2α at different concentrations (10, 25, and 50 nM) for 24h, and then were transfected with hM_1R plasmid (100 ng/well) for another 24h in 6-well plates. Immunoblotting for CK2α and T-ERK proteins was conducted. Data represent CK2α values normalized to T-ERK (a.u.). Data show mean ± SEM (1-way ANOVA with Dunnett's post-hoc test). Comparison to siRNA scramble; ****P<0.0001, n=3.

2.14 Supplementary tables

Annotated Sequence	m/z [Da]	MH+ [Da]	Site
SQPGAEGSPEtPPGR	773.8308	1546.6542	Thr^{254}
SQPGAEGsPETPPGR	773.8309	1546.6545	Ser^{251}

Table. 2 S1. Table of identified phosphorylated peptides on hM_1R. Phosphorylated residues were determined by label-free LC-MS/MS on trypsin-digested and phosphorylation-enriched in HEK293 cells following overexpression of hM_1R (24h) and treatment with pirenzepine (1 μM, 30 min). Results from Biomolecular Analysis Facility at University of Virginia Health System.

Annotated Sequence	m/z [Da]	MH+ [Da]	Site
ELAALQGSEtPGKGGGSSSSSER	758.0076	2272.0085	Thr^{230}
SQPGAEGsPETPPGR	773.8321	1546.6569	Ser^{251}
SQPGAEGSPEtPPGR	773.8300	1546.6527	Thr^{254}
MPMVDPEAQAPTKQPPRsSPNTVK	672.3266	2686.2846	Ser^{321}
tFSLVKEK	516.2636	1031.520	Thr^{354}
TFsLVK	387.6932	774.3791	Ser^{356}

Table. 2 S2. Table of identified phosphorylated peptides on hM_1R. Phosphorylated residues were determined by label-free LC-MS/MS on trypsin-digested and phosphorylation-enriched in HEK293 cells following overexpression of hM_1R (24h) and treatment with pirenzepine (1 µM, 30 min). Results from Manitoba Centre for Proteomics and Systems Biology (MCPSB).

Chapter 3

General discussion (all citation of figures refers to BioRxiv paper)

A large body of evidence indicated the importance of M_1R in the regulation of learning and memory (Conn et al., 2009b; Wess et al., 2007). As a result, the M_1R was studied to discover new therapeutic drugs for a range of neurological and psychiatric disorders. Studies from our lab and others also highlighted the fact that antagonists of M_1R could be therapeutic agents for other neurological disorders, particularly those which involve the PNS, such as neuropathies (Calcutt et al., 2017; Casselini et al., 2024; Chen et al., 2025; Poon et al., 2024). However, the mechanism(s) of action through which M_1R antagonists exert their therapeutic effects are not well understood.

In this work, I provide evidence that PZ and MT7 acted as β-arrestin-biased agonists at M_1R by recruiting β-arrestin2 and associated signaling pathways in preference over G protein activation in HEK293 cells and adult primary DRG sensory neurons. The data demonstrated that phosphorylation of M_1R at specific ICL3 residues was necessary for β-arrestin-mediated ERK activation. Further, ERK activation by PZ/MT7 required β-arrestins and activation of CK2 (but not GRKs and $G\alpha_q$ protein). Lastly, CK2 inhibition blocked PZ-dependent enhancement of neurite outgrowth in DRG sensory neurons. The pharmacological profile of PZ and MT7 was consistent with previously reported β-arrestin biased agonists, such as carvedilol, including lack of G protein agonism, altered receptor phosphorylation and β-arrestin-dependent signaling (Carr et al., 2016; Wisler et al., 2007). In recent years, great attempts have been made to discover biased ligands for their unique therapeutic effects. Functional selectivity of biased ligands enables them to activate either G proteins or engage β-arrestins to activate a particular downstream signaling pathway. For example, agonists for the D_1R (Urs et al., 2015), D_2R (Allen et al., 2011), AT_1R (Violin et al.,

2010), δ-opioid receptor (DOR) (Charfi et al., 2015), and MOR (Soergel et al., 2014) were reported in the literature.

In this work, acute treatment (up to 5 min) with PZ and MT7, two structurally and functionally distinct M_1R ligands, exhibited antagonistic effects in response to muscarine/carbachol and blocked the M_1R-arrestin interaction (Fig. 2.1C&D, Fig. 2S1). Similarly, Yeatman *et al*., showed that atropine, a non-specific orthosteric muscarinic antagonist, effectively blocked M_1R-arrestin interaction in Chinese hamster ovary (CHO) cells overexpressing M_1R (Yeatman et al., 2014). However, longer treatment (e.g. 30 min) effectively recruited β-arrestin2 (in HEK293 cells Fig. 2.2D&E, in DRG neurons Fig. 2.3E&F) and induced ERK activation (in HEK293 cells Fig. 2.2F&G, in DRG neurons Fig. 2.3G&H). These pharmacological effects of PZ and MT7 were markedly different from the orthosteric agonist carbachol/muscarine with much less ability to promptly recruit β-arrestin2 and initiate receptor internalization. PZ/MT7 time-dependently activated the β-arrestin-ERK pathway while inducing no G protein signaling (Fig. 2.1A&B, Fig. 2.2A&B, Fig. 2.3B&C) and receptor internalization (Fig. 2.7). This data suggests these ligands are capable of inducing specific receptor conformations and signaling. Indeed, in the case of PZ and MT7, these ligands showed β-arrestin biased activity at M_1R by recruiting β-arrestin2 and activation of ERK1/2 without triggering $G_{\alpha q}$ protein signaling. One key question is how PZ and MT7 could activate biased signaling at M_1R? In this regard, studies using transfected model systems showed PZ upregulated M_1R, induced stabilization of dimers/oligomers at the cell surface (Ilien et al., 2009; Pediani et al., 2016), and resulted in slower diffusion of receptor. These molecular events may reduce the possibility of interactions between receptor and its cognate G proteins (Zhou et al., 2024). Similarly, MT7 was reported to bind to the dimerized form of M_1R and stabilized this receptor state at the cell surface (Marquer et al., 2011; Marquer et al., 2010).

Indeed, my results revealed that PZ/MT7 upregulated M_1R at the surface of HEK293 cells and DRG sensory neurons for up to 3 hours (Fig. 2.7 and Fig. 2S4). There is evidence in the literature indicating chaperone activity of some chemicals on GPCRs. Many wild-type GPCRs are not folded optimally, causing genetic diseases due to GPCR mutations. Both general and receptor-specific molecular chaperones facilitate the folding of GPCRs. Chemical chaperones are able to correct the misfolding in mutant GPCRs and increasing the cell surface expression of wild-type GPCRs (Tao and Conn, 2014). An example of these compounds is tolvaptan, an inverse agonist of V_2R, which is capable of partially restoring the cAMP generating function of the mutant receptor in response to vasopressin stimulation (Szalai et al., 2022). In the case of M_1R, I found no evidence of pharmaco-chaperone activity of PZ/MT7, and increased cell surface expression of M_1R is not related to chaperone activity of drugs.

Other studies reported that ERK activation by β-arrestin-biased signaling at muscarinic receptors did not necessarily internalize the receptor (Budd et al., 1999; Jung et al., 2017). Interestingly, a report by Paradis *et al*., showed that homodimerization of CXCR4 was associated with altered signaling toward biased agonism with less β-arrestin2-ERK1/2 engagement in response to the agonist (Paradis et al., 2022). Another study on GPCR heterodimerization revealed that DOR-MOR dimerization altered signaling to DAMGO toward β-arrestin biased signaling (Chen et al., 2022). Thus, it seems that ligand-induced dimerization/oligomerization of GPCRs may be associated with altered GPCR signaling including biased signaling. Similar to results of my work, an interesting study by Rosethorne and Charlton reported that an antagonist of histamine H_4 (H_4R), JNJ7777120, exhibited β-arrestin biased agonism activity without affecting the G protein pathway. This compound increased ERK phosphorylation for a long period of time (60 min), similar to PZ and MT7 (Rosethorne and Charlton, 2011).

In line with previous reports, results from my study showed M_1R expression in different sub-types of DRG sensory neurons (Calcutt et al., 2017; Naznin et al., 2022), and only M_1R-positive neurons showed ERK activation (mostly in the nucleus) in response to MT7 (Fig. 2.4). Our lab recently showed that M_1R overexpression markedly decreased neurite outgrowth and mitochondrial function in sensory neurons, and ERK activity played a major role in the neuritogenic effects of MT7 (Sabbir et al., 2018; Sabbir and Fernyhough, 2018). These results showed that DRG sensory neurons (approximately 60%) expressed M_1R and the ERK response depended on the existence of expression of M_1R. Also, these results revealed that PZ/MT7 induced-ERK activation did not require over-expression of M_1R, and DRG sensory neurons were capable of responding to ligands in their native form. In GPCR studies, it is important to consider the fact that different cell types possess their own unique transcriptome and proteome. They express different kinases (in regard to GRKs and CKs), arrestins, and other proteins associated to GPCR regulation (Tobin et al., 2008). Thus, I tested both HEK293 cells and DRG neurons (for most of the experiments) in this work to have a better picture of the mechanism of action of these drugs.

Binding of β-arrestins to phosphorylated GPCRs regulates many aspects of cellular signaling. A significant body of evidence supports the "barcode hypothesis", which explains how different phosphorylation of residues at GPCRs results in diverse β-arrestin-associated signaling responses (Latorraca et al., 2020; Nobles et al., 2011; Tobin, 2008). A previous study by Butcher *at al*. showed that acetylcholine treatment phosphorylated M_1R at 12 residues at ICL3 and 2 residues at the C-terminus (Butcher et al., 2016). In comparison to acetylcholine, results of my study showed that PZ phosphorylated hM_1R at 6 positions (similar to Butcher *et al*) at ICL3 (Fig. 2.5A), and subsequent mutation at these residues (deletion of all or mutation at Ser^{251} and Thr^{254})

significantly reduced β-arrestin2 recruitment to M_1R (Fig. 2.5) and abolished ERK activation (Fig. 2.6). Based on the proteomics results, two different mutations were used for evaluation of muscarinic drugs' responses because I received two sets of phospho-proteomics results from two different proteomics centers. Results from the Manitoba Centre for Proteomics and Systems Biology (MCPSB) found 6 phosphorylated residues (Thr^{230}, Ser^{251}, Thr^{254}, Ser^{321}, Thr^{354}, and Ser^{356}) at ICL3 region of the M_1R (Fig. 2S3), while results from the University of Virginia Biomedical Research Facility also identified Ser^{251} and Thr^{254} (Fig. 2S2).

In this study, we approximated phosphorylation levels by calculating, for each site, the ratio of high-confidence peptide-spectrum matches (PSMs) with site localization probabilities >90% to the total number of PSMs identifying peptides that included the respective amino acid residue. Across four control samples, Ser^{251} and Thr^{254} emerged as the most prominently phosphorylated residues, with 12.8% and 15.5% of PSMs phosphorylated, respectively, while all other sites ranged between 4.4% and 8.8%. Thus, based on these results, I used two different mutant M_1Rs in my work; one plasmid had a deletion of all six phosphorylated residues ($M_1R\Delta ICL3$), and the other had mutations at Ser^{251} and Thr^{254} (M_1R-S251A, T254A). Although these results were from PZ-treated cells, I wanted to see the effects of these mutations on MT7 and muscarine treatments as well. In the case of MT7, deletion of all phosphorylated residues diminished β-arrestin2 recruitment (Fig. 2.5C) and ERK activation, but mutation at Ser^{251} and Thr^{254} had no effect (Fig. 2.6C&D). This result suggested that phosphorylation of a specific set of ICL3 residues was required for PZ- or MT7-induced β-arrestin biased activation of ERK. Surprisingly, ICL3 mutations had no effect on muscarine-induced β-arrestin2 recruitment (Fig. 2.5D), and even increased ERK activation (Fig. 2.6E&F). These results indicated that each ligand had a specific signature with respect to the phosphorylation pattern generated in the ICL3 of M_1R.

What factors might contribute to the β-arrestin biased activation of ERK by PZ/MT7?

In regard to my work, recent studies demonstrated that ERK activity was not possible in the absence of active G proteins, and G proteins were required for ERK activation by arrestins (Grundmann et al., 2018; Gurevich and Gurevich, 2024). Using a specific $G\alpha_{q/11}$ blocker (YM-254890), $G\alpha_q$ inhibition did not alter the PZ/MT7-induced ERK activity (Fig. 2.8A&B). Similarly, a recent study on the biased signaling activity at histamine H_1 receptor (H_1R) showed that YM-254890 blocked G protein biased signaling without affecting β-arrestin-ERK1/2 pathway. However, blocking β-arrestin2 (by siRNA), and GRK2/3 (by CMPD101) inhibited ERK phosphorylation (Michinaga et al., 2023). My data also revealed that PZ/MT7 did not activate $G\alpha_q$ / $G\alpha_i$ /$G\alpha_s$ signaling, and $G\alpha_q$ inhibition did not modify the effects of PZ/MT7 on ERK activation. However, the effect of inhibition of alternative G proteins on putative ERK activity induced by PZ/MT7 was not tested. In this regard, a previous study by our lab showed that PZ and MT7 also inhibited $G\alpha_{13}$ activity without affecting ERK activation (Sabbir et al., 2018). Furthermore, the data demonstrated that arrestins were necessary for PZ/MT7-induced ERK activity as lack of arrestins abolished the effect of these drugs (Fig. 2.8C&D). Other studies on β-arrestin biased ligands reported the same observation where arrestins played a major role in the biased effects of these ligands (Carr et al., 2016; Rakesh et al., 2010; Wisler et al., 2007). Surprisingly, genetic or pharmacological inhibition of GRKs did not block the activation of ERK mediated by PZ/MT7 (Fig. 2.8E-H), which indicates other kinases might be involved in M_1R phosphorylation. Pretreatment of M_1R-transfected HEK293 with CMPD101, a specific GRK2/3 inhibitor, did not reduce PZ/MT7-induced ERK phosphorylation, and surprisingly increased the ERK response (Fig. 2.8G&H). Interestingly, CMPD101 treatment reduced ERK phosphorylation in non-transfected HEK293 (Fig. 2S5), while the same treatment increased ERK activity in M_1R-treansfected

HEK293 cells. These results indicated the importance of M_1R in mediating the effects of GRK2/3 in HEK293 cells. In line with these results, Jung and colleagues showed that CMPD101 failed to block ERK activity in cells overexpressing M_1R and treated with oxotremorine (Jung et al., 2017). Interestingly, recent work by Drube and colleagues showed that no specific GRK was assigned for M_1R recruitment of arrestins (Drube et al., 2022).

Casein kinases were reported to phosphorylate muscarinic receptors (particularly M_1R and M_3R) (Jung et al., 2017; Tobin, 2002; Torrecilla et al., 2007), and among them, CK2 contributed to ERK activation by M_1R (Jung et al., 2017). Other studies reported the involvement of CK2 in the regulation of GPCR-related responses. For example, CK2 was reported to phosphorylate M_3R (Torrecilla et al., 2007), and also regulate insulin release via M_3R *in vitro* and *in vivo* (Rossi et al., 2015). CK2 also was reported to regulate internalization of thyrotropin-releasing hormone receptor (TRHR) and gonadotropin-releasing hormone receptors (GRHR) via arrestins (Hanyaloglu et al., 2001). My findings showed genetic/pharmacological CK2 inhibition strikingly reduced ERK activation by PZ in HEK293 cells (Fig. 2.9, Fig. 2S6). In DRG sensory neurons, the PZ-induced activation of ERK was dependent on CK2 (Fig. 2.9). Jung *et al*., showed that CK2 inhibition abolished arrestin-mediated ERK activity by M_1R agonists (Jung et al., 2017). Further, CK2 inhibition had no effect on basal ERK activity in DRG neurons, but significantly suppressed ERK activity in HEK293 cells, which explains drug-response differences between cell types.

CK2 is expressed in DRG sensory neurons, and plays a major role in neurite outgrowth where knockdown of CK2 suppressed neurite outgrowth (Ghosh et al., 2024; Sahoo et al., 2020). Intriguingly, my results showed that CK2 inhibition blocked PZ-induced neurite outgrowth in cultured DRG sensory neurons (Fig. 2.9). These findings suggest that CK2 is necessary for arrestin-mediated ERK activity by PZ, and suppressing ERK activity through CK2 inhibition

blocks PZ-induced neurite outgrowth in sensory neurons. Indeed, there are multiple factors that might be involved in the biased agonist activity of ligands (Kenakin and Christopoulos, 2013). First, this process is ligand dependent, and it refers to the ability of a ligand to induce a unique conformational change in the GPCR, leading to activation of a G protein/β-arrestin biased signaling (Michel and Charlton, 2018). Second, it is system dependent, and it refers to the stochiometric ratio of β-arrestins, G proteins, and other signaling proteins associated to that particular GPCR. For example, high expression of a particular signaling partner might lead to the preferential activity of that pathway regardless of GPCR affinity (Michel and Charlton, 2018; Onfroy et al., 2017). Third, it is dynamic biased, and it refers to the patho(physiological) factors that may change the expression of a particular G protein or GRK (Michel et al., 2014).

HEK293 cells (a cell line, female, from human origin) and adult post-mitotic DRG sensory neurons (primary neurons, from male rats) are completely different cells, and the drug responses in these two cell types differ in some experiments. For example, DRG sensory neurons express GRK2 and GRK6, but HEK293 cells express higher levels of GRK2 and GRK6 in comparison to DRG sensory neurons (von Banchet et al., 2011; Wang et al., 2011; Zhou et al., 2016). Also, DRG sensory neurons express β-arrestin2 (Muchhala et al., 2021; Rowan et al., 2014; Walwyn et al., 2007), but one report has shown that small size DRG neurons express more β-arrestin1 than β-arrestin2 (Komori et al., 1999). DRG sensory neurons also express M_2R (De Angelis et al., 2014), M_3R (Zhang et al., 2011a), and M_4R (Cao et al., 2011) while expression of muscarinic receptors in HEK293 cells are negligible. DRG sensory neurons in my work were derived from healthy adult rats/mice. Results from our lab showed the efficacy of PZ to increase neurite outgrowth, ERK phosphorylation, and improve mitochondrial function in a variety of disease models including diabetic neuropathy in animals (Calcutt et al., 2017). However, there is no data how a pathological

condition such as diabetic neuropathy, or chemotherapy-induced neuropathy may alter the expression of M_1R, arrestins, GRKs, and CKs in DRG neurons. These possible alterations may affect the biased signaling activity of PZ/MT7.

Differences in signal transduction processes between PZ (as an orthosteric antagonist) and MT7 (as a NAM) were noted in this work. Unlike PZ, MT7 is a cell impermeable large molecule with a very slow dissociation rate, which determines its primary action is at the cell surface (Maeda et al., 2020; Morishima et al., 2013). My findings showed that MT7 was more potent and rapid than PZ in inducing ERK activity and M_1R upregulation at the cell surface. Also, MT7 showed no sensitivity to Ser^{251}-Thr^{254} mutation in M_1R and its induction of β-arrestin2-ERK signaling was unaffected. These discrepancies may be related to several factors such as differences in molecular size, binding location at M_1R, dissociation rate, and permeability of these two ligands.

It is important to note that my study has some limitations. I used two model systems, HEK293 cells and adult DRG sensory neurons, and overexpressed modified proteins in these cells, which might not mimic real physiological conditions of sensory neurons. In addition, DRG sensory neurons are classified into several types based on their soma size, expression of specific markers, and their conduction properties (Wang et al., 2023). Consequently, we showed M_1R expression (using specific MT7-ATTO590 marker) on DRG neurons with varying phenotypes, but did not delineate which sub-type of DRG neuron was responding to PZ/MT7 with β-arrestin biased activation of ERK. Another limitation of this study is that I did not investigate the role of CK1 on the PZ/MT7-induced signaling. As mentioned earlier, CK1 is also involved in phosphorylation of GPCRs including M_1R (Tobin, 2002). Despite the crucial role of CK2 in the pathogenesis of a variety of diseases (Borgo et al., 2021; Guerra and Issinger, 2008), CK1 is also associated with several disorders (Li et al., 2021), and many inhibitors were developed for both CK1 and CK2

with some of them under clinical trials (Chen et al., 2023; Li et al., 2021). Among these CKs, CK2 was reported to play a role in hypersensitivity induced by nerve injury and inflammation (Deng et al., 2019; Li et al., 2005).

Another limitation of this study was that I did not test the possible biased agonism activity of other muscarinic drugs. These drugs could be non-selective muscarinic agonists (such as atropine), selective M_1R-M_4R agonist (xanomeline), selective M_1R positive allosteric modulators (PAMs)(BQCA), and other M_1R antagonists (oxybutynin, telenzepine, PIPE-307). Performing experiments on these drugs could give us a better picture about how muscarinic drugs regulate neurite outgrowth in DRG neurons, and whether biased agonism is a major player in their therapeutic effects. Another limitation of this work is that I used healthy DRG sensory neurons in this study. As mentioned above, system bias is a factor that should be considered when concluding the results of a study. Thus, DRG neurons under pathological conditions may respond differently to PZ/MT7 due to the alterations in GPCR-related regulatory elements such as alterations in M_1R, GRKs, CKs, and arrestins expression and distribution in DRG. Another limitation of this work is lack of evidence for biased agonism activity of PZ/MT7 *in vivo*. Recent studies by the Dr. Tobin team in UK used genetically manipulated mice, which expressed G protein-biased M_1R, and showed no β-arrestin pathway activity due to lack of phosphorylation sites at ICL3. These studies showed that M_1R phosphorylation and the β-arrestin pathway played a protective role against adverse effects of G protein-related signaling such as seizure activity, anxiety-like behaviors, hyper locomotion, and prion-associated neurodegeneration (Bradley et al., 2020; Scarpa et al., 2021). Using such mutant mice in my study could reveal how agonism/antagonism at M_1R may affect DRG neurons behaviors in the context of pathological conditions such as peripheral neuropathies. Least but not last, I did not determine whether biased agonism activity of PZ/MT7

is sex-specific. Although HEK293 cells were female and DRG sensory neurons were male in this study, the effects of hormones and system biology on PZ/MT7 effects should be investigated in a sex-dependent manner *in vivo*. Another limitation of this study is that we did not conduct rescue studies for the β-arrestin 1 and 2 (with respect to the studies using the β-arrestin knockout cell line). Using rescuing studies could be useful to determine the role of individual β-arrestins (1 or 2) in mediating the PZ/MT7-induced signaling pathways.

In conclusion, results of this work showed that antimuscarinic drugs, PZ and MT7, activated M_1R towards the β-arrestin signaling pathway in both HEK293 and DRG sensory neurons. These drugs acted as β-arrestin-biased agonists and stimulated neurite outgrowth and could be potential treatment options for preventing/reversing nerve degeneration in different types of peripheral neuropathy. Another interesting result of this work was the role of CK2 in regulation of PZ-induced effects. CK2 is known as a kinase with more than 300 substrates, which involves in many signaling pathways such as DNA damage response, PI3K/Akt pathway, and NFκB pathway. Given that CK2 has a diverse range of substrates, it is involved in the pathophysiology of several diseases such as cancer, neurodegenerative disorders, infections (viral and bacterial), ophthalmic disorders, psychiatric disorders, and diabetes and obesity (Borgo et al., 2021). This led to the development of several CK2 inhibitors for therapeutic purposes, and some of them are at clinical trial stage (Al-Qadhi et al., 2024). Despite many reports about the roles of CK2 in diabetes and its associated comorbidities, there is no evidence about the role of CK2 in diabetic neuropathy. My future studies could focus on the cross-talk between M_1R and CK2 in different *in vivo* models of peripheral neuropathy, and uncover the molecular pathways associated with M_1R biased activity in different types of DRG neurons to find an appropriate treatment for peripheral neuropathy with minimal side effects. Recent studies highlighted the negative role of CK2 in diabetes and its

associated comorbidities (Ampofo et al., 2019), and it could be possible to investigate the role of CK2 on the pathobiology of diabetic peripheral neuropathy by using conditional knockout mice lacking CK2 in DRG sensory neurons. Another interesting result of my work was discovering PZ-induced phosphorylated residues at M_1R, which regulated the effects of drug. It would be interesting to investigate the role of these specific residues by developing mutant mice lacking 6 amino acids at the ICL3 region of M_1R. This study will shed more light on the importance of GPCR phosphorylation, barcode hypothesis, and ligand selectivity on the therapeutic impact of a drug under pathological conditions and also in communication with other cells. This study would reveal the importance of these phosphorylation sites in regulation of a variety of behavioral, cellular, physiological and metabolic processes governed by M_1R in the context of pathological conditions such as peripheral neuropathies.

Another interesting idea for the future of my work is investigating the role of other GPCRs in the pathobiology of disorders associated with DRG sensory neurons. This was the main subject of a very recent review paper entitled "Targeting sensory neuron GPCRs for peripheral neuropathic pain". Application of omics studies, specially GPCRome to animal models/human samples associated with DRG sensory neurons pathology (e.g. peripheral neuropathies) would open new avenues to discover new druggable GPCRs involved in the pathophysiology of peripheral neuropathies (Hsiao et al., 2021; Lorente et al., 2025). Indeed, results of my study and other lab members indicated that PZ, a medication under ongoing clinical trial studies, antagonized $G\alpha_q$-coupled GPCR, blocked G protein signaling, but activated β-arrestin-related signaling and more importantly PIP_2 related signaling (Chauhan et al., 2025) in DRG sensory neurons. These new findings would pave the way to target other $G\alpha_q$-coupled GPCRs to develop new therapeutics for peripheral neuropathies. For example, a study on a $G\alpha_q$-coupled GPCR, CXCR5 (a chemokine

receptor), showed that this receptor was expressed in non-peptidergic unmyelinated DRG neuron (IB4+), peptidergic DRG neuron (CGRP+) and myelinated DRG neurons. Antagonizing this receptor by depletion of its ligand (Cxcl13) in DRG neurons via siRNA inhibited mechanical and heat hypersensitivity in mice (Ma et al., 2021). Another example of a $G\alpha_q$-coupled GPCR is calcitonin gene-related peptide (CGRP) receptor, which is expressed in DRG sensory neurons and its activation increases IP_3 and intracellular calcium. A study on CGRP receptors showed that olcegepant, an antagonist for this receptor, inhibited temporomandibular disorder associated pain (Suttle et al., 2023). More interestingly, angiotensin II type 1 and 2 receptors (AT_1Rs) are expressed in DRG neurons and are well-known $G\alpha_q$-coupled GPCRs. Antagonizing these receptors by systemic administration of losartan (antagonist) inhibited cytokine release in DRG neurons and mechanical hypersensitivity in a CIPN animal model (Kim et al., 2019). According to these findings, it would be interesting to investigate ligands of these receptors for β-arrestin biased agonism in order to discover novel and safe therapies for peripheral neuropathies and DRG sensory neurons associated pathologies.

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