FUNCTIONAL ANALYSIS OF THE DIMERIZATION POTENTIAL OF THE P2Y12 RECEPTOR AND TWO NATURALLY-OCCURING MUTANT VARIANTS

by

Fawziya Barnawi

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Fall 2017

FUNCTIONAL ANALYSIS OF THE DIMERIZATION POTENTIAL OF THE P2Y12 RECEPTOR AND TWO NATURALLY-OCCURING MUTANT VARIANTS

by

Fawziya Barnawi

Approved: _____
Donna S. Woulfe, Ph.D.
Professor in charge of Thesis

Approved: _____
E. Fidelma Boyd, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of Arts and Science

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I am sincerely thankful to my advisor Dr. Donna Woulfe for her constant support and encouragement. Dr. Woulfe was more than an advisor, who empathized and encouraged during my difficult times, motivated me to be persistent and unyielding and led to my successful completion of this graduate program.

The door to Dr. Woulfe office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this project to be my own work, but steered me in the right the direction whenever he thought I needed it.

I am thankful to my committee members Dr. Jeffrey Caplan and Dr. Erica Selva for their valuable suggestions. I am thankful to Aasma Khan for helping with the initial stages of my project. I would like to thank all previous and current Woulfe lab members – Conroy Field, Cara Giordano, Martin Ciskanik for their support.

I would like to thank Michael Moore and Deborah Powell from Delaware Biotechnology Institute for helping me with confocal microscopy and Dr. Jeffrey Caplan for helping with super resolution microscopy. Also, I extend my sincere thanks to Dr. Robert Sikes for allowing me to use cell culture equipment's. A special thanks to Dr. Melinda K Duncan and Cowgill Betty for helping throughout my residency. I thank all my colleagues and friends Ramachandran Balasubramanian, Salma Alsaai and Suhair Najm for their inputs and encouragement.

Finally, I must express my very profound gratitude to my parents, my brother and my husband for providing me with unfailing support and continuous

encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

An expanding body of evidence on GPCR oligomerization has challenged the classical concept that GPCRs are non-interacting or monomeric membrane receptors (Bouvier, 2001; Ferre et al., 2014; Milligan, 2008; Pin et al., 2007; Salahpour et al.,2000). Furthermore, numerous number of evidence that state that G-Protein-Coupled-Receptor dimers are required for effective G protein coupling have challenged the belief that one GPCR interacts to one G protein. Therefore, the monomeric one GPCR: one G protein pattern cannot be generalized for all the receptors (Maurice et al., 2011).

For the Class A family of GPCRs, there is some evidence that some family members form dimers; however, it has been more challenging to combine the observed oligomerization status with whether direct homomeric association of GPCRs is required for function, given that the interactions are sometimes transient or dynamic in nature and other times constitutive. It has been observed that these interactions sometimes correlate with function and sometimes not. The suggested use of dopamine D2 and adenosine A2A receptor heteromer-specific tools as an approach to treat some diseases (Armentero et al., 2011) possibly best represents the pharmacological advantages of further understanding GPCR dimerization and their functional relevance. Thus, we set out to investigate GPCR dimerization and functional relevance for the platelet GPCR, P2Y12, in this thesis.

ADP receptors P2Y1 and P2Y12 belong to class A GPCRS and are essential for normal platelet function, hemostasis and thrombosis (Gachet, 2008; Lin et al.,

2013; Nakata et al., 2010). However, the role of P2Y12-P2Y12 dimer or oligomer status in receptor function has never been elucidated. This work assesses the ability of the WT P2Y12 receptors to form homo-dimers and demonstrates the ability of P2Y12 mutants reported to occur concurrent with bleeding diatheses to exhibit a notably changed dimerization ratio compared to the WT receptor.

P2Y12 is a crucial target of anti-platelet drugs, but the role of its dimer/oligomerization in receptor function has not been clarified, although its in vitro oligomeric status has been reported. In this project, I investigated the ability of P2Y12 protomers to homodimerize using Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histogram (PCH) analyses of confocal microscopy. Previous studies from our lab demonstrated that P2Y12 receptors could specifically interact in a saturable manner in a saturation Bifluorescence complementation (BiFC) assay. To determine the importance of oligomerization to receptor physiological relevance, we chose to evaluate the dimerization potential of two mutant variants of P2Y12, P2Y12-R256Q and -R265W, which were detected in a patient with a mild bleeding disorder and was found to have compound heterozygous expression of the mutations (Cattaneo et al., 2003). The platelets from the patient expressing these mutant variants had compromised Gi function, although the platelets displayed normal ADP binding and membrane expression (Mao et al., 2010). It is unknown how Arg256 and Arg265 affect structural integrity of the receptor and how they may influence Gi function of the receptor. The existence of an asymmetric model in which one G-protein may couple to two GPCRs has been hypothesized and there is evidence for this stoichiometry for various class A GPCRs.

Taking these models into account, we hypothesized that the mutant variants (R256Q and R265W) might weaken the ability of P2Y12 receptors to dimerize which in turn could affect their ability to couple to Gi, leading to the observed functional defect. WT P2Y12 and mutant variants R256Q and R265W did not show altered expression or altered trafficking when observed by live cell imaging (Khan,2015). However, in contrast to my hypothesis, both R256Q and R265W mutants displayed significantly high molecular brightness, indicating enhanced dimerization abilities compared to WT P2Y12.

Such a modified oligomerization profile of these receptors due to the R265W / R256Q mutations might alter the abilities or proportion with which Gi might associate with receptors, thus affecting the net Gi function. We have shown that the R256Q mutant variant of P2Y12 in fact fails to activate Gi optimally. The change in oligomerization status of this variant receptor may affect Gi activation either by disturbing the surface interface for receptor association to G protein or by altering the conformation which allows G protein to exchange GDP for GTP. These possibilities will be discriminated in future work. In addition, altered internalization, recycling or membrane stability of receptors, could be impacted by enhanced oligomerization, hence reducing the net Gi function of the mutant variants of P2Y12. These future lines of investigation will help us further understand the potential role of P2Y12 oligomerization in receptor structure/function and dynamics.

Chapter 1

INTRODUCTION

1.1 G- Protein Coupled Receptors (GPCRs)

G-Protein-Coupled Receptors are the most diverse group of membrane receptors in eukaryotic cells. GPCR families bind to a vast and varied array of ligands which include photons, lipids, inorganic ions, nucleotides, neurotransmitters, peptides, proteases, hormones and pheromones(Rozenfeld and Devi 2011b). GPCRs exist at the interface of a cell's external and internal environments and play a crucial role in human physiology, perception, homeostasis and when dysregulated, disease. GPCRs are the biggest family of receptors, consisting of about 800 receptors in the human genome. Approximately 30-40% of current medical drugs target GPCRs (Zhang K. et al. 2014b), thus signifying their indispensable role in human physiology, health and disease. Many GPCRs act as basic receptors for sensory functions like sight and smell and hence are not prime therapeutic targets; however, about 300 GPCRs are potential candidates for drug discovery initiatives. About 200 of these have known natural ligands. Among the diverse cellular processes regulated by GPCRs are cell differentiation and proliferation, leukocyte migration in response to inflammation, neurotransmission (muscarinic acetylcholine, dopamine, and adrenergic receptors), vision (rhodopsins), and hormonal response (thyroid-stimulating hormone receptor; (Zhang K. et al. 2014b).

All GPCRs have a common motif of seven-transmembrane domains comprised of 7 alpha helices. Each of the 7 helices is made up primarily of hydrophobic amino

acids (around 25-30) that cross the cell membrane and are joined by six loops (three intracellular and extracellular). Therefore, GPCRs may also be referred to as Seven transmembrane (TM) receptors. Given that GPCRs bind a vast array of ligands and exist at the cell membrane, they serve as an important interface between the extracellular and intracellular environments (Ferre et al. 2014b). Thus, these receptors deliver information sent from other cells or environmental stimuli.

Many drugs, including some antibody-based drugs, function by binding to specific GPCRs and inhibiting or initiating their intracellular actions-therefore GPCRs have been and remain an important subject of basic research and therapeutic intervention. Pharmaceutical companies desire to develop new drugs that can more specifically and potently bind to these receptors. Understanding binding of receptors to ligands (agonists and antagonists), the arrangement of the receptors in the membrane, and their regulatory control after agonist binding are crucial properties for the development, optimization, and indications for new medicines (Waldhoer et al. 2005). To completely understand the arrangement and regulatory control of GPCRs, fluorescent ligands are used to study the regulation and role of receptors in live cell systems.

GPCRs were originally classified based on sequence homology into six categories (Waldhoer et al. 2005). These categories were as follows: Class A that are rhodopsin-like proteins, Class B (or secretin receptors), Class C or glutamate receptors, Class D (fungal mating receptors), Class E (cAMP receptors) and Class F (frizzled/smoothened). Classes D and E of these aren't found in vertebrates. An alternative classification, "GRAFS", divides vertebrate GPCRs into five categories, overlapping with the A-F nomenclature: Glutamate family, Rhodopsin family (class

A), Adhesion family, Frizzled family and Secretin family (Sommer, Hofmann, and Heck 2012).

Agonist-activated GPCRs can induce distinctive signaling pathways to modify cellular functions because of their ability to specifically interact with functionally diverse heterotrimeric guanine nucleotide-binding proteins (G proteins). The versatility and rapid activation kinetics of the G protein-mediated signaling system might explain why it is the principal mediator of early-stage platelet activation during thrombosis and hemostasis, which requires the coordinated and rapid action of diffusible mediators to activate platelets and to recruit them to the growing thrombus (Waldhoer et al. 2005).

1.2 G Proteins: Types and Mechanism of Function

G-Proteins are specialized proteins that bind the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP). Some G Proteins, such as the signaling proteins Ras, are small proteins with a single subunit. GPCRs couple to heterotrimeric G proteins, made up α, β, and ϒ subunits, on the intracellular membrane through their intracellular loops 3 and 2 (Dupré, Hébert, and Jockers 2012). After binding with ligands, the G protein is activated by the GPCR as a result of exchanging Guanosine diphosphate for Guano triphosphate on the alpha subunit. This leads to the dissociation of alpha subunit from both the receptor as well as from the βϒ subunits; these in turn trigger downstream effector signaling (Figure 1). Depend on the classification of the Gα subunit; there are four main kinds of G proteins, Gs, Gi, Gq, and G12/13.

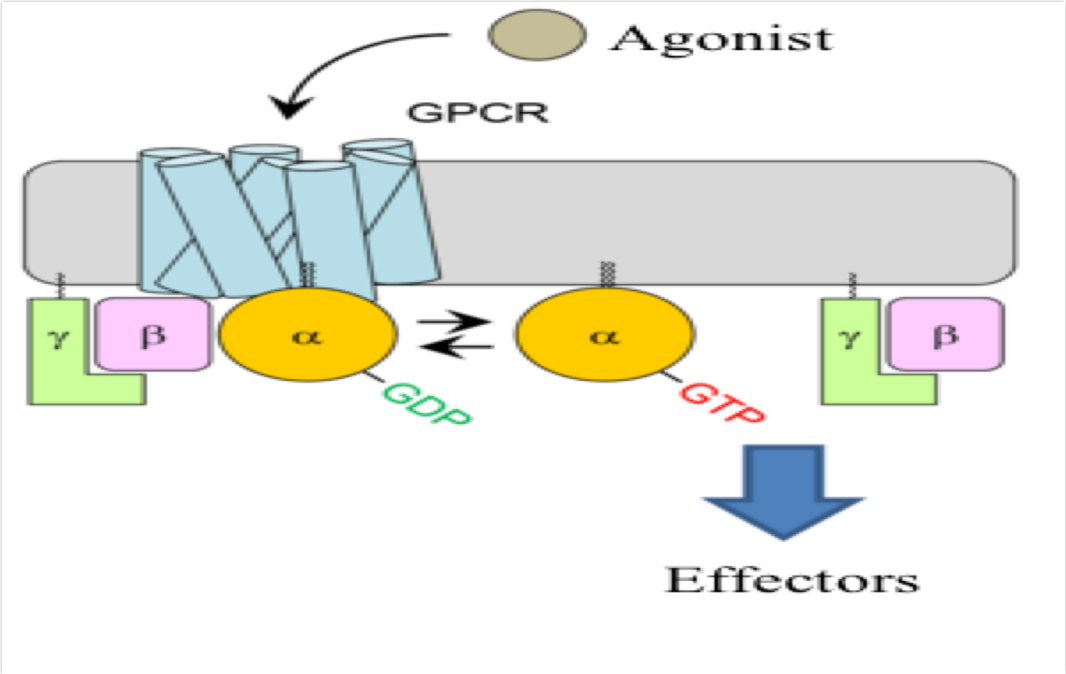

Figure 1: Mechanism of GPCRs signaling. When GPCRs bind to their agonists, G-proteins are activated. The Gα subunit is released from the βϒ subunits to activate different downstream pathways through a variety of second messengers (Masuho, n.d.) Retrieved from http://ikuo-masuho.squarespace.com/projects.

Gαs proteins stimulate adenylyl cyclase, which produces cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Gαi hinders the function of Gs by inactivating adenylyl cyclase. Gq activates phospholipase C, which cleaves the phospholipid Phosphatidyl inositol bisphosphate (PIP2) to produce Di-Acyl Glycerol (DAG) and Inositol 1, 4, -5 triphosphate (IP3). Subsequently, IP3 binds to its receptors on the ER, which allows opening of calcium channels and release of stored calcium into the cytoplasm. DAG, along with high intracellular calcium concentrations, activates protein kinase C, with many consequences for cellular function. The final class of G protein is the Gα12/13 family, which activate Rho

kinases to contribute to cytoskeletal rearrangements and frequently cell motility processes.

GPCR signals are terminated by receptor desensitization and internalization (Dupré, Hébert, and Jockers 2012). Once activated, the C-terminal cytoplasmic tail is subject to GRK-mediated phosphorylation that allows the recruitment of arrestins to GPCRs (Figure 2). Mammals express two types of arrestins, which include the visual (arrestin 1 and 4) and non-visual arrestins (arrestin-2 and 3). In all mammalian cells, at least one form of non- visual arrestin is expressed (Nisar et al. 2012).

Recent studies have shown that arrestins can act as scaffolding molecules for GPCRs by recruiting other mediators to the receptor-arrestin complexes. Some of these proteins include clathrin, mdm2, adaptin, and of particular relevance to the present study, SRC family kinases (SFK), which may then elicit further G-protein-independent signaling events(Li et al. 2010b). GRK phosphorylation undoubtedly prepares the triggered receptor for Arrestin recruitment. Thus, arrestins prevent additional G Protein-mediated signaling, target receptors for internalization and transmit additional signals to potential G-protein-independent pathways.

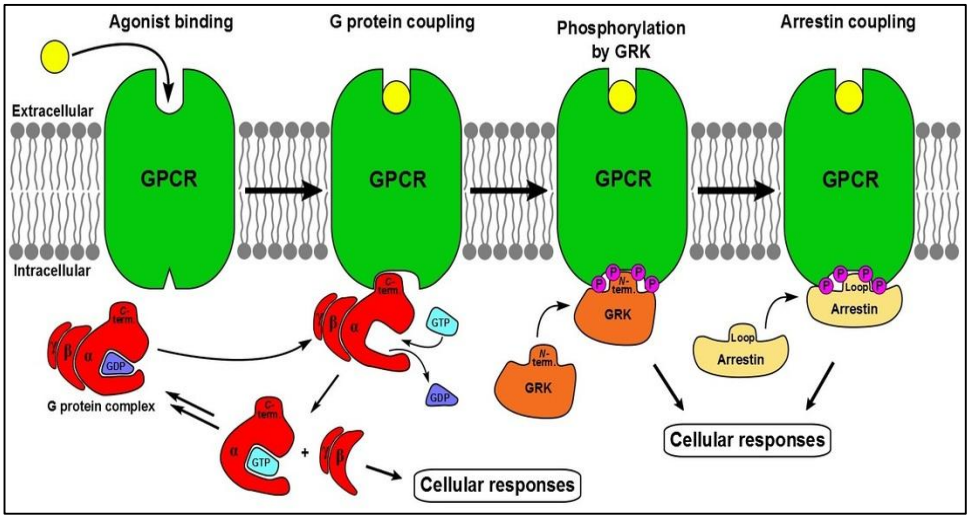

Figure 2: GPCRs mediate signals via G proteins and Arrestins. Upon ligand binding GPCRs trigger G proteins, that releases G-alpha and βϒ from the receptor and from each other to trigger downstream ancillary mediators. Following G protein activation, C-terminus of GPCRs is phosphorylated by GPCR Kinases. Then, β- Arrestins are added to the phosphorylated C-terminal tail of GPCRs. Illustration obtained from (GPCR Signalling Project - The Sejer Research Group).

1.3 Platelets in Hemostasis and Thrombosis

Platelets are anuclear blood cells created by the release of proplatelets into the circulation. Their major function is to prevent blood loss (haemostasis) during an event of vascular injury (G. 1962). Thrombosis is the pathophysiological result of dysregulated platelet activation, and it takes place at sites of arterial wall disease, local inflammation, or atherosclerotic plaque buildup (Schulze and Italiano 2016). This causes the formation of platelet plugs (thrombi) that may block blood vessels, causing heart attacks and strokes.

Platelet activation is a multistep process reached through associated and/or constant stimulation of a group of receptors held on the platelet membranes (Offermanns 2006). A blood vessel damage exposes the extracellular matrix (ECM)

proteins like Collagen and Von Willebrand Factor (VWF) located below the endothelial cell layer (Colman, p. 43). These proteins are recognized or associated with their receptors on platelet membranes, GP1b/V/IX, GPVI and integrin α2β1, respectively. Platelets bind to vWF and collagen via cascades result in platelet spreading and secretion of dense granules. Platelet dense granules hold small molecules like ATP, ADP, and 5HT that activate platelet GPCRs and help the circulating platelets to recruit at the site of injury. Platelet GPCRs activation leads to platelet shape change and activation of integrin. Activated integrin αIIbβ3 binds divalent fibrin molecules thus enabling platelets to create an aggregate or platelet plug at the site of injury.

1.4 The Role of GPCRs in Platelet Activation

Creation of a stable platelet fibrin clot and maximal platelet activation in homeostasis requires the stimulation of platelet GPCRs. These include purinergic (ADP) receptors like P2Y1 and P2Y12, Thromboxane A2 (TxA2 receptor TPα and TPβ), and thrombin receptors. Although these were historically thought to exist as separate monomeric molecules, current studies suggest that each one of these receptors may form dimeric or multimeric complexes in the platelet membrane. In this study, I will define the known function of ADP receptors and I will describe our rationale for concentrating on understanding their homodimeric interactions.

Platelets flow in the blood and attach to sites of vascular damage to the vessel wall resulting in the growth of platelet plugs that are required for crucial hemostasis. Platelets become activated is by their communication with soluble molecules like collagens and von Willebrand factors at the sub-endothelial surface, leading to the recruitment of other platelets through diffusible mediators. These adhesive molecules

involve Serotonin, Thromboxane A2, and adenosine diphosphate (ADP) that are released from the triggered platelets together with thrombin formed on the surface of stimulated platelets (Schulze, 2016). The platelet inducements are usually transduced through GPCRs. In comparison to ADP, Thrombin, and Serotonin, Prostaglandin I2 hinders platelet activation.

Concomitant activation of Gα-13 by protease activated receptors like PAR1 and PAR4 activates small GTPases like RhoA. ROCK kinases, on the other hand, phosphorylate LIMK1 and LIMK2, that then phosphorylate Cofilin, then restructuring of the actin cytoskeleton takes place. Cytoskeleton rearrangement leads to an alteration in platelet structure that is essential for appropriate platelet aggregate formation (Arachiche et al.,2013).

Gαq is activated by adenosine diphosphate (ADP) through P2Y1, Serotonin through HTR2A, Thromboxane A2 via TBXA2R, and Thrombin through PAR1 and PAR4. After the activation of PLC-beta2 and PLC-beta3 by G-protein alpha –q, 1,2, Diacylglycerol and IP3 are produced (Schulze, 2016). The release of calcium ions and stimulation of PKC –alpha which is a protein kinase C follows. PKC-alpha plays a major role in regulating platelet aggregation. This is accomplished by facilitating the subsequent secretion of soluble agonists which include Serotonin and ADP among others from the dense granules, thus triggering platelets stimulation and activation of integrins.

Alpha-IIb/beta-3 integrin is a protein complex made up of ITGA2B and ITGB3 subunits and is known to be the major thrombocyte integrin that is essential for binding and accumulation of platelets, therefore, allows for the interconnecting of

platelets with proteins like fibrinogen; hence it is essential in the blood clotting process.

1.5 The Purinergic Receptors of Platelets

Currently, there are 8 known members in the P2Y group of metabotropic purinergic receptors, these include the P2Y1, 2, 4, 6, 11, 12, 13, and 14. On the basis of evolutionary and structural relationships and functional information about the class of G proteins to which they bind, they are classified into two major subgroups. P2Y1, 2, 4, 6 and 11 mainly join to the Gq family of G proteins, as a consequence activating the PLCβ/IP3 pathways, this leads to an increase in the concentration of cytosolic calcium. P2Y12, 13, and 14 on the other hand, couple to Gi/o G proteins, which in turn hinder adenylyl cyclase. Nucleotides such as ADP, ATP, UDP, UTP and UDP-glucose stimulate these receptors. Platelets of humans express two groups of the P2Y family: P2Y1 and P2Y12. They are essential for maximum integrin activation and for stable platelet aggregates (Jin and Kunapuli, 1998; Dorsam and Kunapuli 2004; Kahner et al. 2006).

P2Y1 and P2Y12 are activated by ADP, which is secreted from platelet dense granules at the sites of injury and from the nearby broken endothelial cells in vivo. P2Y12 couples to Gi family members, the alpha subunit of which inhibits adenylyl cyclase to control production of cAMP, consequently counter-balancing the influence of natural platelet suppressive agonists, such as Prostacyclin, which help in the production of cAMP in inactive platelets. The main pathways leading to permanent platelet aggregation are started by the βϒ subunits of the Gi family members of G proteins. The βϒ subunits activate PI3K/AKT pathways, hence promoting stable αIIbβ3 integrin stimulation. On the other hand, P2Y1 binds to Gq and stimulates the

IP3/DAG signaling pathway to mobilize calcium from the dense tubular system calcium stocks of platelets. The increased concentrations of calcium, together with intensification of signaling due to DAG- and PKC-dependent condensed granule secretion, activates αIIbβ3 integrin through CalDAGGEF-1, mediating alterable platelet accumulation (Seifert, p. 141). Fascinatingly, magnification of signaling through P2Y1 activation is vital for optimum thrombin and collagen-mediated reactions in mouse. Both P2Y1 and P2Y12 are essential for maximum integrin initiation and formation of stable permanent platelet aggregates, as evaluation of bleeding times in knock-out mouse models have shown that P2Y12 knockout mice have a severe persistent bleeding time when compared to the wild type mice (Cattaneo, 2015; Foster et al., 2001; Leon et al., 1999). On the other hand, P2Y1 mice have a slighter bleeding syndrome. This happens because, in contrast to the P2Y1, P2YI2 performs two roles: an initiating role via βϒ, and an inhibiting function of Gi on the production of cAMP, thus contrasting the tonic inhibitory role of the endogenously antiplatelet mediators, like prostacyclin. Furthermore, P2Y12 is found in large numbers on platelets, having around 400 sites of binding per platelet (Ohlmann et al., 2013) in comparison to the P2Y1 which has around 150 binding sites per platelet (Ohlmann et al., 2010;Kauffenstein et al. 2001). Therefore, P2Y12 is a chief target for production of anti-platelet drugs (Schulze et al., 2016).

P2Y12 is highly expressed in platelets, frontal cortex, fetal brain, cerebral cortex, spinal cord, and retina. It also plays a role in the activation and migration of microglial cells, activation of dendritic cells and in nociception (Erb and Weisman 2012). P2Y12 is an essential target for drugs for instance, Thienopyridine molecules have been widely used in the prevention of thrombosis in human since the 1980's;

nevertheless, the molecular target of these molecules was only recognized as the ADP receptor P2Y12 in 2001(Tapon-Bretaudiere 2003). The critical role of P2Y12 in thrombosis was established by the identification of P2Y12 as the receptor for known anti-thrombotic compounds, along with knock-out of the receptor in mice (Hollopeter et al. 2001). Currently, the combination of Aspirin and Clopidogrel, an irreversible antagonist, is the most widely prescribed antiplatelet drug therapy.

One-third of the population treated with Clopidogrel is resistant to the effects of the drug although it is the drug of choice for most cases of bleeding disorders. Around 0.1% of patients treated with Ticagrelor, which is an orally-active P2Y12 antagonist, experienced intracranial-bleeding in the PLATO trial (Wallentin et al., 2009). Therefore, development of numerous allosteric modulators and reversible antagonists continues, aiming to reduce the bleeding risk of antiplatelet therapies while limiting thrombotic events. Knowing the homo/heterophilic interactions of P2Y12, their regulation of receptor function and platelet activation will help to identify novel potential drug targets that could help in minimizing thrombotic events while maintaining the haemostatic risk associated with it.

In this work, I have characterized the interactions of P2Y12 and analyzed the oligomeric potential of P2Y12 mutants to acquire an insight into the function of P2Y12 oligomerization in receptor function.

1.6 GPCR Oligomerization: Current View on GPCR Status and Stoichiometries

Traditionally, the functional units of GPCRs were believed to be monomeric receptors coupled to single G proteins. Nevertheless, evidence from as early as 1985 using radio-ligand joining and cross-linking experimentations have shown that GPCRs

may exist and carry out their functions as larger molecules rather than single entities (Salahpour, Angers, and Bouvier 2000). Over the last two decades, the belief that GPCRs are monomeric non-interacting classes has slowly changed. The use of biochemical and biophysical methods has confirmed that GPCRs can occur and play their role as constitutive dimers or create more active oligomers or homo- or heteromeric dimers to create new pharmacologically manageable diverse receptor pairs (Qanbar and Bouvier 2003; Ferre et al. 2014a). The best and most studied obligate GPCR dimers are the class C GPCRs. For instance, the mGluR1 dimers and Glutamate receptor mGluR5 dimers are joined by covalent bonds. In B receptors for instance the ϒ-aminobutyric acid (GABA) are connected by coiled-coiled interfaces of c-end polypeptides of one protomer, are transferred to the cell membrane and are necessary for the functioning of the receptor (Bonde, Sheikh, and Hansen 2006). The oligomeric state of the receptors members of class A of the GPCR family has not been identified so far because the GPCR interactions for this family has been observed to change quickly. Applying different methods such as Atomic force microscopy of mouse optic discs, Rhodopsin has been revealed to be in a dimeric configuration(Fotiadis et al. 2003; Jastrzebska et al. 2013b). Biophysical techniques with single molecule sensitivity like Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histograms (PCH) can determine the oligomer size of freely diffusing fluorescently tagged GPCRs on the cell membrane. Some members from the class A GPCRs family, for instance, biogenic amine, muscarinic (M1 and M2) and Dopamine (D1), adrenergic (β2-AR, α1b-AR) receptors have been revealed to exist as homogeneous populations of constitutive homodimers applying such techniques (Giraldo, 2013). Oligomer organization of probed muscarinic M-2 and M-3

acetylcholine receptor has been shown to form homodimers and exist in a dynamic equilibrium of homodimers by multiple studies utilizing Total Internal Reflection Fluorescence Microscopy (TIRFM) and FRET spectrometry (Fotiadis et al. 2003; Jastrzebska et al. 2013b).

1.7 GPCR Oligomerization Boundaries

The transmembrane helices (TM), of GPCRs have been proven to make up the dimerization interfaces of homo and heteromeric GPCR interactions using several methods, such as interpretations made from high resolution crystallized structures, computational molecular models in lipid and water environments and cysteine cross-linking. The trans-membrane helix 6 was suggested to participate in the homodimerization of leukotriene receptor BLT1, as well as β2-adrenoreceptor using interfering synthetic TM mimetic peptides for TM6 (Hebert et al. 1996; Pellissier et al. 2011). TM5 is also involved in homodimerization of Serotonin, 5-HT2C dopamine D2 and muscarinic M3 receptor. (Ferre et al., 2014). Clarifications made from crystallized dimeric configurations of CXCR4 and μ-opioid receptors suggested that the dimer interface of these receptors is made by TM5 and TM6 (Manglik et al. 2012).

1.8 Controversy on P2Y12 Oligomerization

P2Y12 is reported to exist as oligomers in platelet lipid rafts, characterized predominantly by covalently bounds, slowly migrating bands of higher molecular weight than that predicted for monomeric forms on immunoblots, although this has never been detected in intact cells. The structure of P2Y12 receptors displays TM5 to be at the interface of two parallel P2Y12 molecules from adjacent units, potentially implicating its role in P2Y12 homomeric interactions (P. Zhang et al. 2016). To

generally determine the existence of GPCRs in cell membranes as dimers and whether their interactions are stable, transient, or change upon agonist stimulation, techniques such as Fluorescence energy transfer or single molecule imaging methods are now available. Currently, P2Y12 is a critical drug target of the antiplatelet therapies. Particular mutations of P2Y12 are related with slight to severe blood loss phenotypes due to a single amino acid mutations that have impacts on the functioning of the receptor or loss of P2Y12 expression, the purpose of my thesis research was to evaluate the oligomerization properties of P2Y12 using FCS and PCH techniques. In addition, there are several P2Y12 mutant modifications that have been described to cause slight bleeding illnesses in human beings, yet the fundamental molecular deficiencies in these variant forms of P2Y12 is not well understood. In this study, therefore, I seek to understand whether two of the described variants might have altered oligomerization features, may explain the deficiencies in signaling and platelet accumulation to some extent. I have considered and compared their oligomerization potential to that of wild type P2Y12.

1.9 GPCR G Protein Association and Stoichiometry

Questions regarding the stoichiometry of G protein pairing to a GPCR have arisen since GPCRs have been proposed to dimerize. To reconcile the prior model of one G protein binding to one GPCR, it has now been proposed that two G proteins bind per GPCR dimer. Nevertheless, several research studies propose the asymmetrical stoichiometry of 2:1 between GPCR and G proteins. One example of this is shown for the Class C GPCRS that exist as obligate dimers. In addition, Rhodopsin, a prototypical class A GPCR, has been thoroughly investigated for its stoichiometry of binding with G protein transducin (Gt). Rhodopsin's crystal

structures suggest an arrangement of the receptor dimer associated with one transducin molecule (Giraldo, 2013). Additional computational docking and molecular modeling analyses of Rhodopsin and its G protein transducin (Gt) suggest that the G protein surface is much larger than the cytoplasmic face of the receptor, thus providing a requirement of Rhodopsin dimerization to accommodate one transducing molecule (Seifert and Wieland 2005).

1.10 Applications to Discover GPCRs Dimerization

To understand and evaluate the regulatory interactions between hormones, pharmacological ligands, and other mediators of GPCR signaling and kinetics, it is important to consider whether the dimerization and oligomerization of GPCRs could affect their activity, trans-activation, localization or down-regulation. Different structural, biochemical and biophysical methods may be employed to obtain information about the dimerization and oligomerization of the receptors and their significance. Despite years of diverse approaches to understand these questions, there remains deep controversy over the extent and functional relevance of receptor oligomerization. Among the techniques employed to resolve these arguments biochemical methods which include co-immunoprecipitation, photo bleaching FRET, time-resolved FRET, BRET and additional structural studies (Giraldo et al., 2013).

Structural studies of the rhodopsin GPCRs using atomic force microscopy show evidence that many receptors are made up dimers. The studies were carried out on the membranes of murine rod outer segments. The results showed a complex arrangement of rhodopsin molecules. These results have been confirmed in different organisms; for instance, in mice, it has also been shown that rhodopsin can arrange itself in dimeric configurations (Jastrzebska et al. 2013a). Use of bimolecular

fluorescence complementation is another method useful to demonstrate direct interactions between proteins of living cells of organisms (Giraldo and Ciruela 2013). The method is founded on the reconstruction of a fluorescent protein molecule after the re- association its non-fluorescent fragments. For instance, if YFP is fragmented into C-terminal and N-terminal fragments, neither of the two fragments demonstrates fluorescence on its own, but fluorescence is achieved if the fragments are brought close enough to reassemble. Some advantages of this method are their high sensitivity and that specialized imaging apparati are not required, because the interactions can be easily sensed using a simple fluorescence microscope.

BRET, is also a technique that is widely used to detect dimerization and oligomerization in GPCRs, in which a luminescent substrate is used to excite an acceptor, (eYFP) to emit at a unique wavelength when they are within sufficient proximity. BRET has several advantages, one of which is that from the use of luminescence to excite the fluorophore causes less auto-bleaching than some other methods (Devi, p. 168). The main limitation of this method is as a result of the low amount of fluorescent light that is emitted, which can be insufficient to detect some GPCR associations.

FRET is another method that can also be used to detect oligomerization in GPCRs when ligands are conjugated to fluorophores. This method can also be used together with the fluorescence lifetime imaging (FLIM) method which measures the average time taken by an excited molecule to return to its ground state. Co-immunoprecipitation, is a biochemical method that is commonly used to discover oligomerization in GPCRs. The method is usually completed using dissimilar molecules tagged with epitopes articulated in collective systems. In this method, cells

transfected with both tagged receptors are lysed and a receptor is precipitated using one antibody and detected via immunoblotting with antibody to the second, candidate associated receptor. Despite being a common method used in the detection of GPCR oligomers, it has some limitations which are mainly associated with the lysis and solubilization processes. Firstly, the method is not appropriate for the study of cellular interactions in live cells, because it requires solubilization. Secondly, because of the lipophilic nature of all the TM domains of GPCRs, artifactual oligomers are potentially formed. It can be challenging to select an appropriate concentration of detergents, which prevents unnatural aggregation that happens during the solubilization procedure, but also is not in excess, such that naturally occurring interactions may be disrupted. In addition, receptors are frequently not naturally expressed at high enough levels to detect these interactions using this method (Herrick-Davis and Mazurkiewicz 2013).

.

1.11 Fluorescence Correlation Spectroscopy and Photon Counting Histograms

Fluorescence correlation spectroscopy (FCS) / photon counting histogram (PCH) analysis performed using confocal microscopes are techniques with single molecule sensitivity and are valued for their use in live cells. FCS can be used for studying biophysical characteristics of protein complexes, for instance, determining the oligomeric size and diffusion coefficients (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). FCS measures the fluctuations fluorescent molecules as they move into and out of the laser-illuminated observation volume. A PCH then uses the recorded FCS data to determine the average molecular brightness of the sample (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).

FCS was introduced over 40 years ago by Magde, Elson, and Webb (1972) when they wanted to investigate chemical reaction kinetics of the association of ethidium bromide with DNA in solution (Briddon and Hill 2007; Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). After that, studies discovered that not only could FCS measure diffusion coefficients but also aggregation, rotational dynamics, and chemical rate constants (reviewed by Hess et al. 2002). In the 1990s, FCS was paired with confocal microscopy to afford a sensitive method to monitor protein dynamics in living cells (Briddon and Hill 2007; Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). FCS has some crucial advantages compared to other techniques used to study these interactions. It provides real-time information and requires very low amounts of proteins to obtain both temporal and spatial resolution of protein properties at physiological expression levels (Briddon and Hill 2007; Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).

FCS experiments are conducted using a small detection volume formed by focusing a laser beam to a diffraction-limited spot (~0.3μm) utilizing an objective lens with a high numerical aperture (Figure 3). A detection volume is created by positioning a pinhole in the confocal plane (reviewed by Briddon and Hill 2007; Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013).

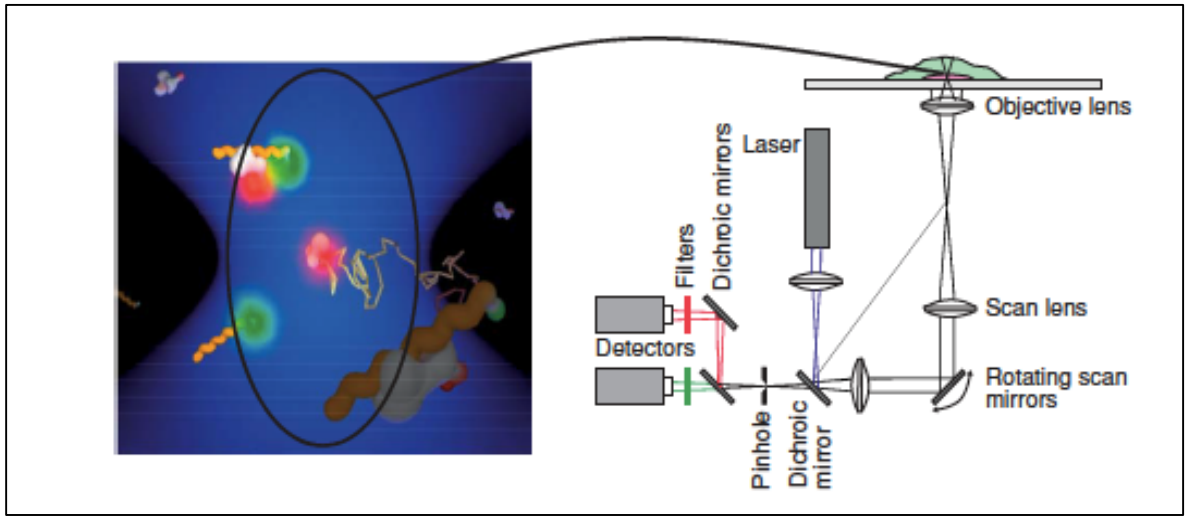

Figure 3: Schematic principle of the FCS method: A high numerical aperture objective and the laser are utilized to create a small detection volume. Fluorescent molecules are excited by the laser as they pass through the detection volume. The objective lens captures the emitted fluorescence which is focused through a pinhole onto a photon detector passes through dichroic mirrors and selected emission filters. The detector records the fluctuations in fluorescence over time. The spontaneous fluctuations in fluorescence emission of the molecules in thermodynamic equilibrium can be used to detect whether molecules are specifically interacting (reviewed by Haustein and Schwille 2007). So, when fluorescent molecules pass through this volume they are excited, and the emitted photons are recorded in real time by a photon counting detector.

Autocorrelation analysis of the fluorescence signal describes the fluctuations as a function of diffusion time and particle number (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). It resembles the fluctuation size (δI) from the average intensity ($\langle I \rangle$) at time (t) with a following fluctuation at a time $t + \tau$ (reviewed by Hess et al. 2002; reviewed by Briddon and Hill 2007; reviewed by Haustein and Schwille 2007; Herrick-Davis and Mazurkiewicz 2013). The autocorrelation function, $G(\tau)$, results from using a range of values for τ. It can yield information on the average

dwell time (τD) and the number of molecules (N) of the fluorescent particles in the volume during measurements (Figure 4)(Hess et al. 2002; Briddon and Hill 2007; Haustein and Schwille 2007; K. Herrick-Davis et al. 2013). Moreover, the diffusion coefficient of the species can be calculated with information of the size of the detection volume; however, that was not completed as part of this project.

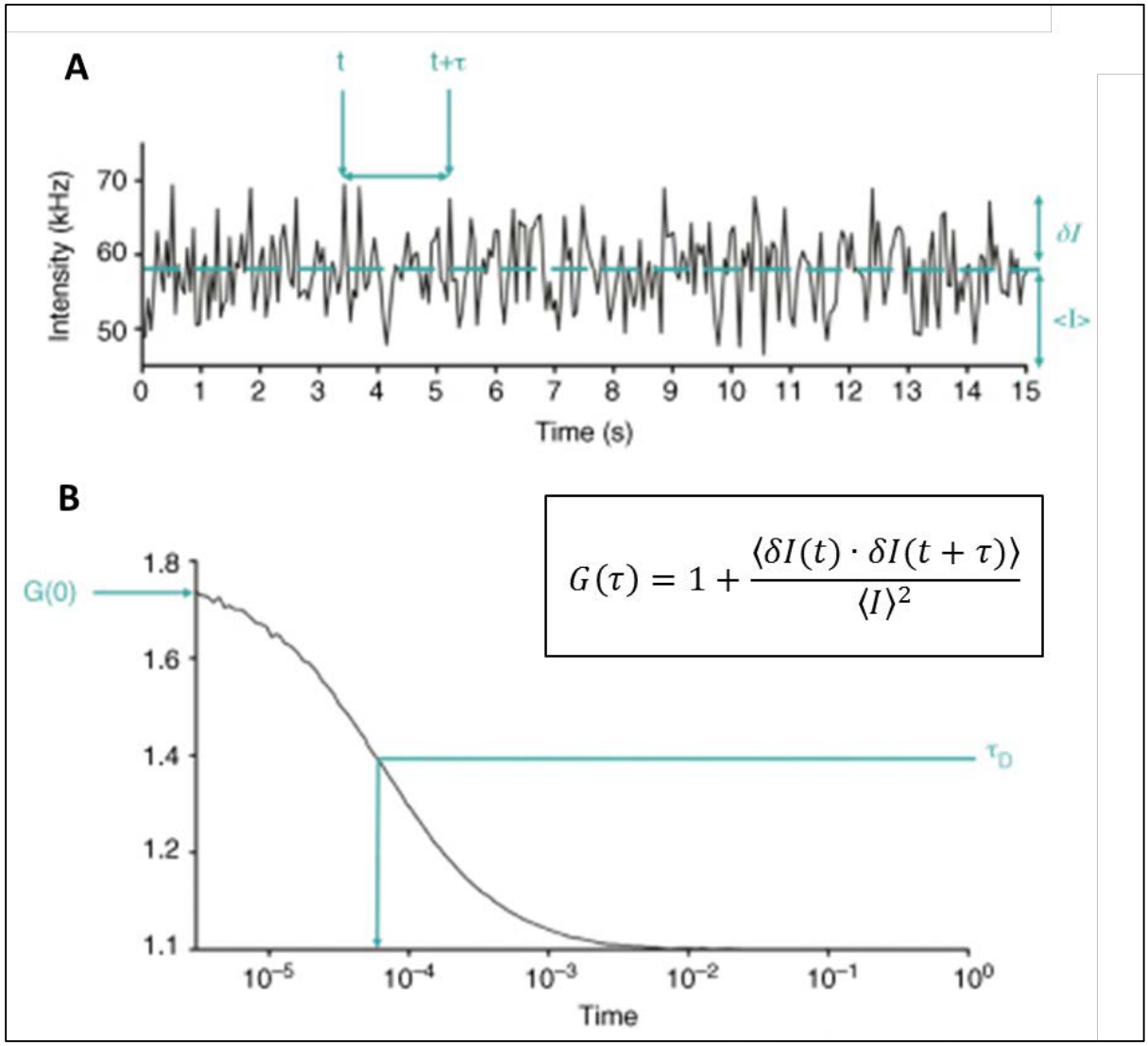

Figure 4: An example of an autocorrelation curve for a single FCS experiment. (A) The variations in fluorescent intensities are shown as they are recorded in real time by the photon detector. Frequently, five to ten consecutive 10-second observation intervals are taken through each FCS recording. Usually, some photobleaching can occur in the first 10-second interval and hence, may not be included in the data. (B) Autocorrelation analysis of the fluorescence intensity trace. The dwell time (τD) can be calculated from the midpoint of an autocorrelation curve, as drawn on the graph. The inverse relationship between the autocorrelation function at time zero [G(0)] and the number of diffusing particles (N) is not shown here. The image adopted from Briddon and Hill, 2007.

Analysis of the amplitude of the fluctuations in fluorescence intensity can be utilized to create a photon counting histogram (PCH) and estimate the molecular brightness of a fluorescent species (Herrick-Davis et al. 2012). Molecular brightness is a term used to describe the number of photon counts per molecule (Herrick-Davis et al. 2012; Herrick-Davis and Mazurkiewicz 2013). The molecular brightness of a protein oligomer can be used to determine the oligomeric size of that complex because it is directly proportional to the number of fluorescent molecules present in the complex (Herrick-Davis et al. 2012; Katharine Herrick-Davis and Mazurkiewicz 2013). Hence, its application to the study of potential oligomers formed by the GPCRs in vivo provided us a novel method for us to detect the forms of P2Y12 occurring in whole cells in real time.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture and Transfection

2.1.1 Plasmid Constructs and Cell Culture

Our lab performed site-directed mutagenesis on P2Y12mEGFP to generate R256Q and R265W mutant variants fused to mEGFP. For R256Q mutants of P2Y12: the cds of P2Y12 was mutagenized from CGA at 766-768 to CAA. For R265W mutants of P2Y12: the cds of P2Y12 was altered at base pairs 793-795 from CGG to TGG.

2.2 Controls and Reference Standards for Molecular Brightness:

CD86 coding-plasmids were tagged with either mEGFP or tandem mEGFP-mEGFP (Herrick-Davis et al. 2013) were gifted from (Prof. Graeme Milligan - Unversity of Glasgow). CD86-mEGFP was used as a monomeric receptor control and CD86-mEGFP- mEGFP was used as a dimeric control to determine the increase in molecular brightness. All plasmids described in this part were verified by Sanger sequencing (Genewiz). To eliminate self-aggregation of GFP (Zacharias et al. 2002), all GFP constructs contained an A206K mutation.

2.3 Fluorescent Correlation Spectroscopy (FCS)

2.3.1 Cells Preparation for FCS:

Dulbecco's Modified Eagle Medium (Cellgro 10-013 CV) (containing 10% fetal calf serum and 1% penicillin/streptomycin (Hy Clone SV300010) at 37ºC and 5% CO2) was used to maintain Human Embryonic kidney (HEK)293T cells. Cells were then grown in a cell media of DMEM (Cellgro 10-013 CV)) that is made up of 5% carbon dioxide, 10% fetal bovine serum (FBS – Gemini) and 1% Penicillin-Streptomycin ((Hy Clone SV300010 at 37ºC and 5% CO2). Laminin(25µg/ml) coated 4well Nunc chambers (1.5mm cover glass bottom) were used to plate 15000 cells per well at 37c overnight. The following day, cells were transfected with 50ng of each plasmid constructs (CD86-EmGFP, CD86-mEGFP-mEGFP, P2Y12-mEGFP, P2Y12R265W-mEGFP, and P2Y12R256Q-mEGFP), TransIT293 transfection reagent (Mirus BioLLC) in a 1:3 ratio of DNA: transfection reagent. pcDNA 3.0 empty vector was used to keep the total DNA amount in the transfection mixture to 50ng. The transfection complexes were prepared in 250ul OptiMEM and incubated for 30 minutes. 50ul of this transfection complex was added per well. Before imaging or loading the wells onto the microscope, the cell media (DMEM/10 %FBS) was replaced with 1x live cell imaging media (Life Technologies).

2.3.2 Imaging Settings

A Zeiss LSM-880 Confocal microscope equipped with a gallium arsenide phosphide photon detector at the Delaware Biotechnology Institute Bio-imaging Centre (Newark, DE) was used to take FCS measurements. The system was switched on and then warmed up for at least an hour before imaging. To observe the extent of excitation of the mEGFP-tagged proteins, the argon laser line was used as one photon

excitation source, using a 40X 1.2NA C- Apochromat water immersion objective with collar adjustment at 0.17, the main beam splitter of 458/514, laser intensity of 0.2%; Pinhole of 1 airy unit was used. A correlator bin time of 0.20us was used with a data acquisition time of 10s for five consecutive replications per cell. With an intensity of 0.2%, the 488-nm laser excited the mEGFP-tagged proteins as they moved through the observation volume. The emitted fluorescence was then captured by the objective lenses, passed through a beam splitter and it was focused onto the photon detector utilizing a pinhole of 1 Airy unit (32 μm). Analysis of the recordings was made using the Zeiss Zen 2.1 (black) software.

2.3.3 System Alignment

To align the system, a calibration dye rhodamine 123 (1.6 nM dilution) was prepared and positioned in LabTekII Chambered 1.5 German Coverglass System wells. With a microscope fixated in the 1.6 nM rhodamine solution, the X and Y planes changes were made by adjusting the microscope's pinhole. Rotational FCS recording on almost five of each cell that were transfected with the control plasmids (containing clones CD86-mEGFP and CD86-mEGFP-mEGFP) and those transfected with the investigational plasmids (P2Y12-R265W and P2Y12-R256Q) fused with GFP, was made after the alignment and calibration of the instruments. Selection of cells with an average plasma membrane photon count rate ranging from 50 to 200 kHz was made. Regions on the membranes having filopodia were not considered. Some photobleaching was observed in most of the first of five 10-second intervals even with a low laser intensity of 0.2%. It is crucial to use the lowest laser power possible, while still maintaining a good signal to noise ratio since higher laser powers can result in photobleaching of the fluorescent probe.

2.3.4 Data Representation and Statistics

Raw data were imported in Graph pad prism 6 for graphical representation. Bar graphs are represented as mean ± SEM and were analyzed by unpaired t-tests and Welch's correction to account for unequal standard deviations. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$).

2.4 GloSensor™ cAMP Assay

To measure the levels of cAMP, I used GloSensor cAMP assay from Promega that gives a straightforward strategy to track GPCR action through a shift in the intracellular cAMP concentration. It is a dynamic assay that is made up of luciferase merged with a cAMP binding domain; the system allows instant detection of an agonist effect that is forskolin-free. This assay employs a mutant form of Photinus pyralis luciferase into which a cAMP-binding protein moiety was inserted (Figure 5) Upon binding of cAMP, a conformational change is triggered resulting in increased light output that allows assessing the activity of ligands at the receptor under study. After pre-equilibration using a substrate, cells stably expressing a biosensor variant can be used to assess GPCR function that enables easy kinetic measurements of cAMP accumulation or turnover in living cells.

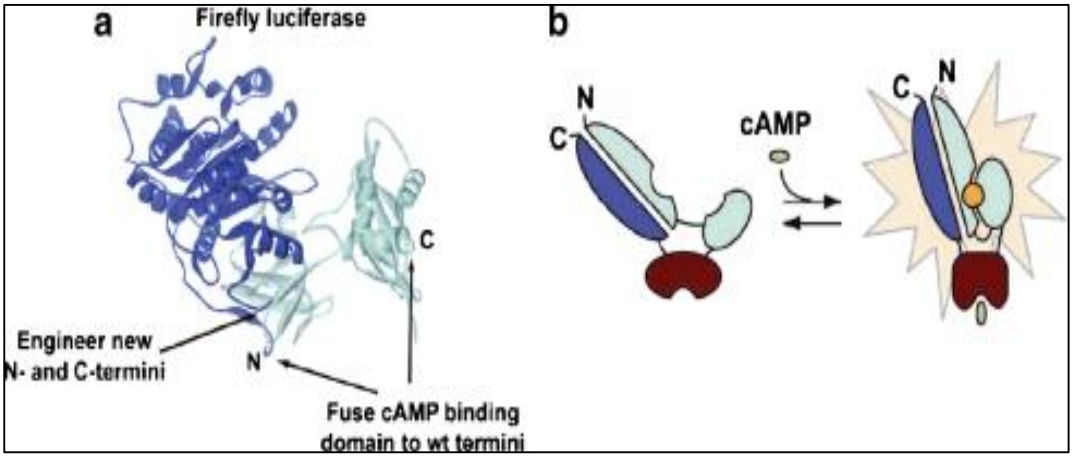

Figure 5: Summary of the GloSensor cAMPassay. A- a representation of the biosensor used in the GloSensor cAMP assay. B- Conformational change upon binding to cAMP that generates an increase of light output. Adapted from Promega (Kumar et al. 2017).

2.4.1 Cell Culture Preparation

HEK293 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and transferred to tissue culture-treated, flat white, 96-well assay plates Immediately after transfection, described below.

2.4.2 Transiet Transfection and Cell Culture

pGloSensor-20F cAMP and/or pGloSensor-22F cAMP plasmids (Promega), TranIT transfection reagent (Mirus BioLLC), Opti-MEM® I reduced-serum medium (Invitrogen).

2.4.3 Pre-equilibration Requirements

GloSensor cAMP Reagent (Promega), GloSensor cAMP Reagent Stock Solution: suspend GloSensor cAMP Reagent in 10 mm HEPES, pH 7.5 (25 mg/817 ml; 250 mg/8.17 ml.

2.4.4 Equilibration Medium

Medium independent of CO2 (Invitrogen) added with 10% FBS and comprising 2% v/v GloSensor cAMP Reagent Stock Solution.

2.4.5 Compound Addition and Luminescent Measurements

Forskolin: 10 μM with ADP 10 μM and Plate reader GloMax plate reader). Human embryonic kidney (HEK293) T cells were grown adherently in DMEM media and incubated at 37°C in an atmosphere of 5% CO_2 overnight. Cells were harvested in CO2-independent medium and were counted to the desired number. Then were incubated in equilibration medium containing a 2% v/v GloSensor cAMP reagent stock solution, 10% FBS and 88% CO2 independent medium. The cells were dispensed in wells of 96-well plate after 2 hours of incubation. When a steady-state basal signal was obtained, agonist (ADP) 10 μM was added in the presence of adenylyl cyclase activator Forskolin (FSK) 10 μM. Luminescence measurements were made at indicated incubation times.

Chapter 3

RESULTS

3.1 HEK293T Cells Expressions in Heterologous Systems

To study the stoichiometry of the P2Y12 receptor, we performed Fluorescence Correlation Spectroscopy (FCS) and Photon Counting Histograms (PCH). FCS measures the fluctuations in the fluorescence intensities of the molecules moving through the observation volume (Confocal volume) on the cell membrane (Elson 2013). P2Y12 receptor-encoding plasmids tagged to mEGFP were expressed in HEK293T cells. Fluorescence was observed in cells 36 hrs. post-transfection. For FCS measurements, fluorescence intensity fluctuations of the receptor molecules freely diffusing in the confocal volume were made over a period of 10 sec per measurement and repeated five times per confocal volume. Photon counting histograms (PCH) created from the FCS data recorded determine the oligomeric state of the diffusing molecules in the confocal volume. PCH measures the molecular brightness of a given fluorescent species or a heterogeneous mixture of fluorescent species from the probability distribution of photon counts which is directly proportional to the oligomer size of the fluorescent species (K. Chen et al. 1999).

Autocorrelation curves were obtained using the Zeiss Aim 4.2 software, depicting the autocorrelation function G (τ) on the Y axis and diffusion time on the X axis. It represents the decay of fluorescence signal of the molecules over time.

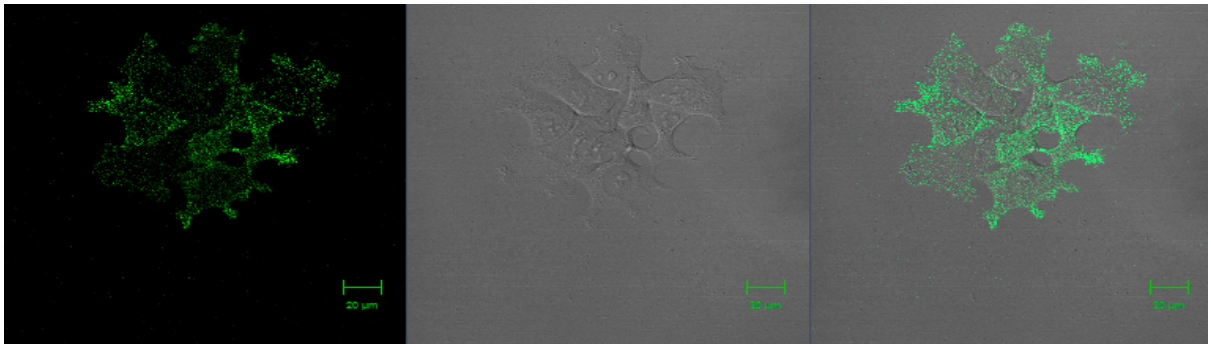

Figure 6: Expressions of P2Y12 and variant receptors in HEK293T cells. HEK293T cells transfected with P2Y12-encoding plasmids to achieve low receptor expression were selected. All images were taken at approximately 36 hours' post-transfection using a Zeiss Axio Observer D1 microscope and a 40x objective. FCS is optimal at low levels of protein expression to mimic the physiological range of receptor expression levels (Bleicken, Otsuki, and J. Garcia-Saez 2011).

3.2 The Molecular Brightness of the Dimer Mimic Control is Significantly Different than the Monomeric Control

Transfected HEK293T cells with the specified plasmids and proteins were given 36 hours to express before carrying out FCS and PCH experiments. The mean molecular illumination of 30 cells conveying the monomeric CD86-mEGFP construct was established to be 15,234 CPSM whereas 36 cells expressing the dimer mimic had a mean illumination of 25758.45 CPSM. Although the molecular brightness of the dimer is expected to be twice that of the monomer, the difference between the two is statistically significant (Figure 7). The fact that the brightness is not exactly double that of the monomeric control may be caused by misfolding of the second mEGFP or its photobleaching (Monillas et al. 2015).

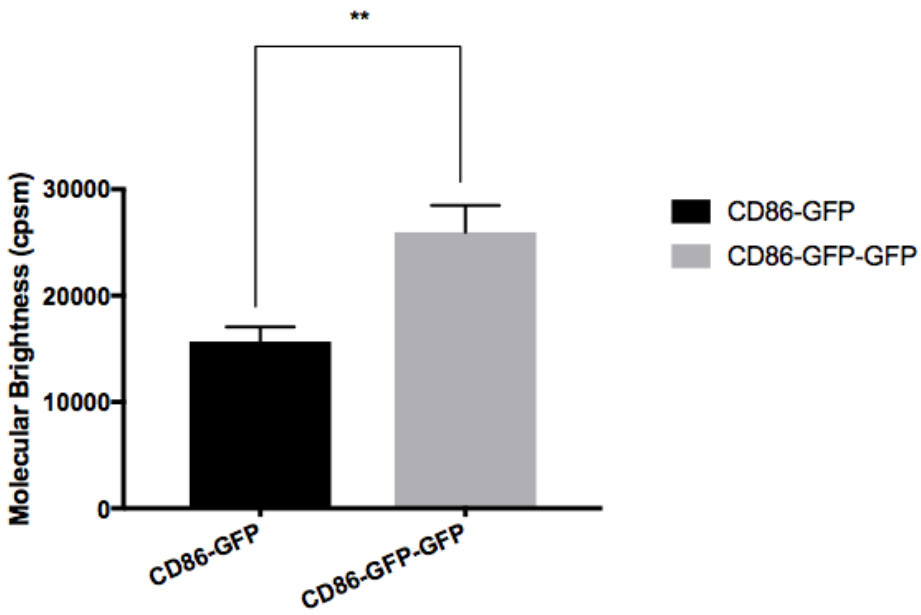

Figure 7: Molecular brightness of CD86-mEGFP and CD86-mEGFP-mEGFP controls. Error bars represent the standard deviation and the asterisk means statistically significant difference between molecular brightness of monomer and dimer (student's t-test, $p < 0.001$)

3.3 P2Y12 Receptors Exist Predominantly as Monomers

We applied FCS in cells transfected with P2Y12-mEGFP, as well as two mutant variant receptors, to determine the oligomeric state of the receptors in the cell membrane. The mean brightness of the molecules of (25 cells) that have P2Y12-mEGFP was 16234.76 CPSM. The intensity of P2Y12-mEGFP mixture at the cell membrane was not statistically different from that of the monomer control and was statistically distinct from that of cells expressing a receptor tagged with two GFP molecules to mimic the fluorescence expected of a dimer (dimeric control, CD86-GFP-GFP) (Figure 8). Thus, it appears that the predominant species of P2Y12 detected in P2Y12-expressing cells is monomeric.

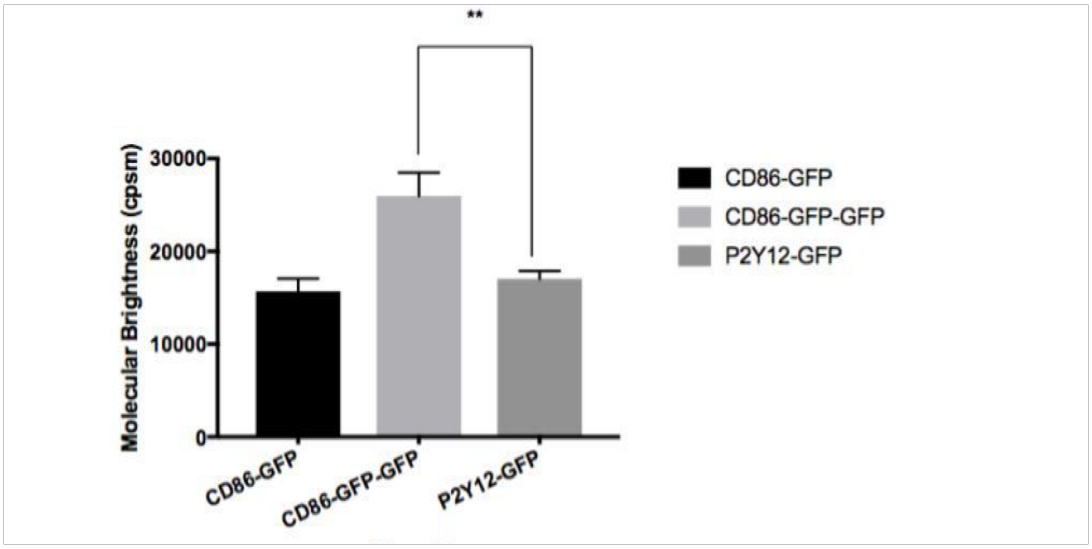

Figure 8: Molecular brightness of CD86-mEGFP and -mEGFP-mEGFP controls compared to the WT P2Y12mEGFP. Error bars present standard deviations and the asterisks designate the statistically significant difference between the brightness of the CD86-mEGFP-mEGFP dimer mimic and P2Y12-mEGFP (student's t-test, $p < 0.001$). There was no significant difference between CD86-mEGFP brightness and P2Y12-mEGFP brightness (student's t-test, $p = 0.699$).

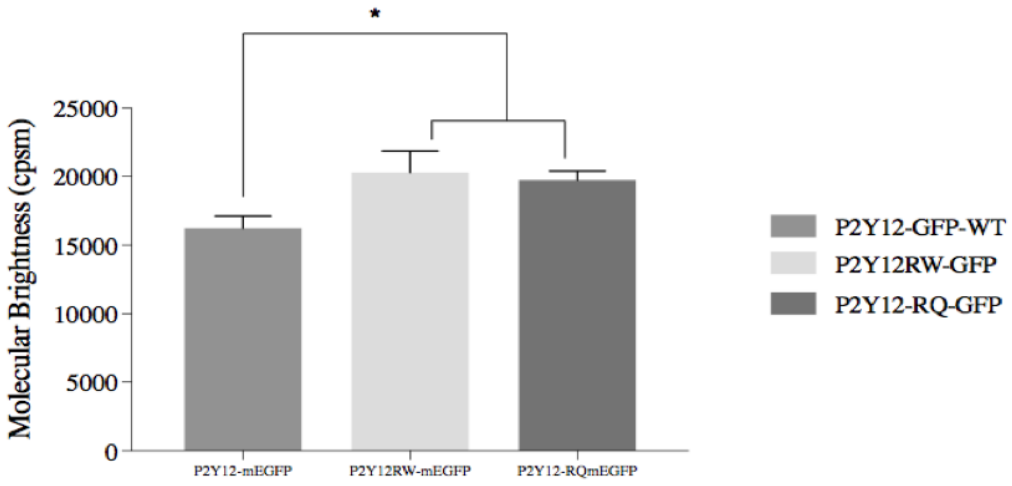

Figure 9: The molecular brightness of P2Y12WT compared to its mutants R256Q and R265W. Both mutants form a higher proportion of dimers than WT P2Y12 (student's t-test, $p < 0.01$).

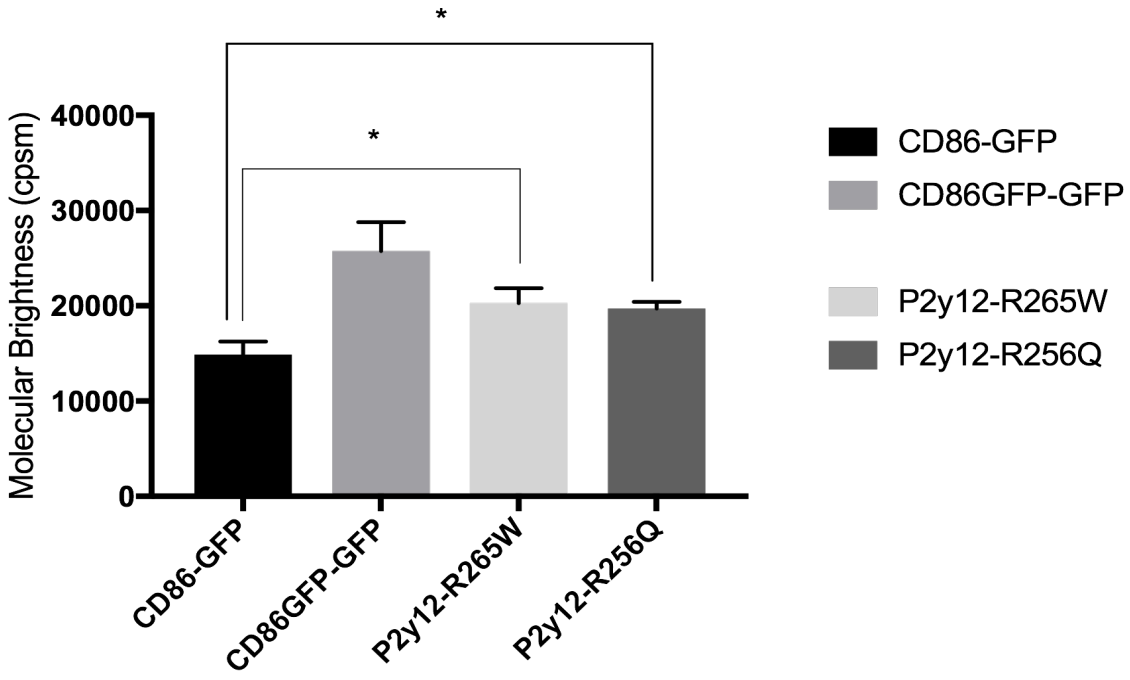

Figure10: Molecular brightness of mEGFP controls and P2Y12-GFP mutant variants. Consistent with our lab's previous studies, the two P2Y12 mutant variants, P2Y12R256Q and P2Y12R265W have enhanced potential to homo-oligomerize and interestingly, the P2Y12-R256W mutant variant exhibits a greater potential to form dimers than either P2Y12-R256Q or WT receptors (student's t-test, $p < 0.01$).

3.4 R256W Mutant of P2Y12 has Increased Ability to Oligomerize than P2Y12

We next used FCS to evaluate the species of a mutant variant of P2Y12 that had been detected and isolated from a patient with a defined bleeding disorder. This patient was a compound heterozygote, expressing two different P2Y12 mutations. Thus, we evaluated the dimerization capacity of each of the mutant variants in isolation. For the first, the molecular brightness of R265W-mEGFP 20293.07 CPSM was significantly greater than P2Y12mEGFP 16234.76 CPSM. This indicates that mutation R265W in P2Y12 enhances the ability of the receptor protomers to self-

associate into dimers (forming more dimers per cell). This residue, Arg256, is at the boundary of transmembrane domain 6 (TM6) and extracellular loop 3 (EL3). We next evaluated the dimerization of P2Y12 containing a mutation in the second residue, detected as the second mutant variant of P2Y12 in the patient with compound heterozygous expression of P2Y12 variants. This mutation, R256Q-mEGFP showed a statistically higher molecular brightness than that of P2Y12, but was not significantly different from R256Q-mEGFP (Figure 10). This suggests that the second mutant variant found in the patient also exists as a more dimeric species than the native P2Y12. Arg265 is a part of EL3.

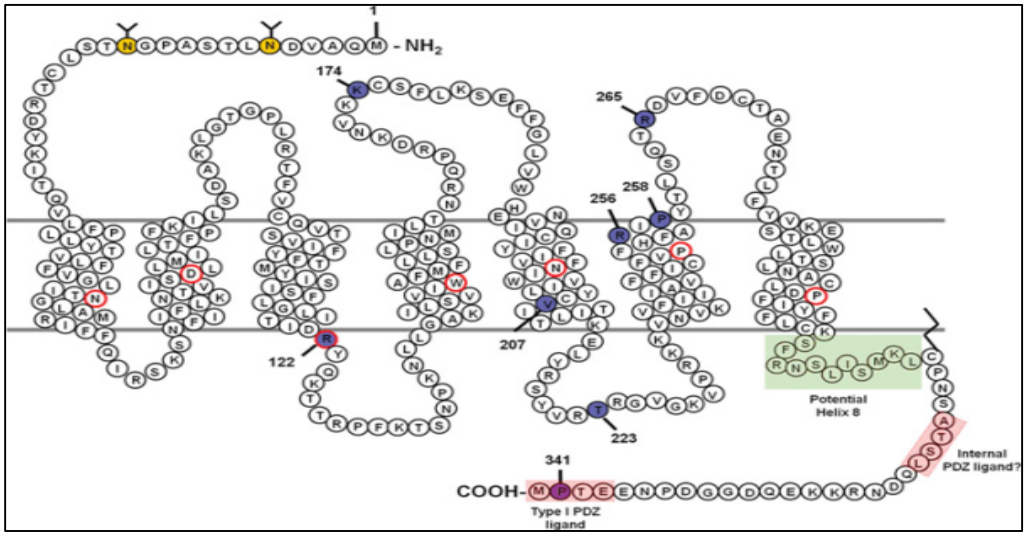

Figure11: The sites of amino acid substitution in patients with dysfunctional P2Y12 R265W and R256Q mutant variants are shown at EL3 and TM6 (Margaret et al, 2013).

Previous studies of agonist and antagonist-binding to these variant-expressed receptors done by others have revealed that both R256Q and R265W mutants were more sensitive to P2Y12 antagonists than the wild type receptor, with R265W showing a stronger sensitivity than R256Q (Mao et al. 2010). This suggests that mutation of R265W alters the conformational state of the receptor, increasing the affinity of the receptor for the antagonist. Our work indicates that this change in the conformational state of the receptor might alter P2Y12 self-association affinities as well.

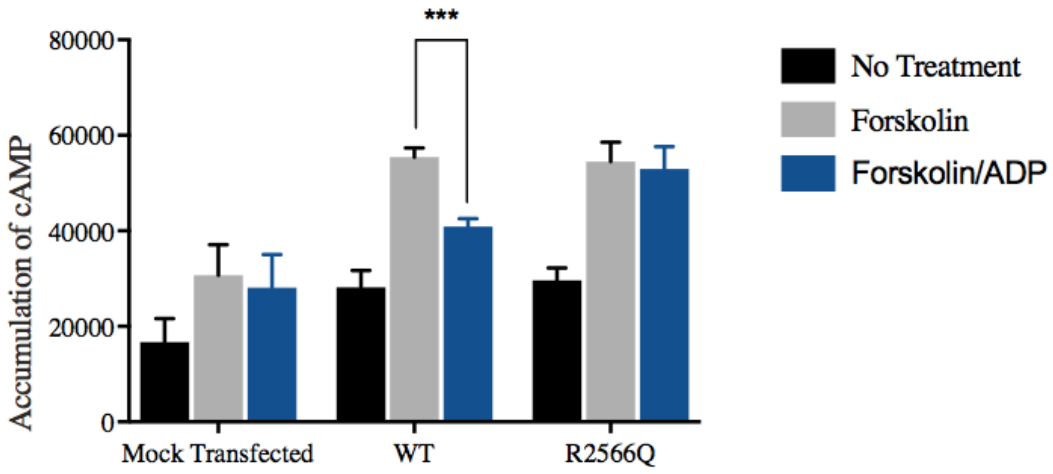

Figure 12: R256Q failed to activate Gαi proteins. HEK293T cells transfected with WT P2Y12(WT) or P2Y12(R256Q). The results represent the mean +/- SDs of three experiments ($P<0.0001$) ANOVA. In addition, ADP inhibited the Forskolin-induced increase of cAMP in cells transfected with WT P2Y12; in contrast, it had a minimal or no effect in cells transfected with P2Y12(R256Q) when used at concentrations between 1 and 5 µM, and caused only marginal inhibition at 10 µM.

3.5 R256Q Failed to Activate Gi Proteins

In order to understand whether oligomerization of P2Y12 might affect receptor function, we next evaluated the ability of these two mutant variant receptors to activate G protein. Both of these mutant variants, P2Y12R256Q and P2Y12R265W exhibit higher potential to form dimeric forms, as discussed earlier. This demonstrates that the structural integrity of P2Y12 at these sites might affect receptor conformational states, which influence both oligomerization potential as well as Gi function of the receptors. To evaluate Gi activity, a cAMP glosensor assay was used to quantify cAMP levels in P2Y12-WT- expressing cells and in P2Y12-R256Q compared to un-transfected control cells. When compared to P2Y12-expressing cells, the mutant variant P2Y12R256Q failed to elicit an ADP-dependent reduction in forskolin-stimulated adenylyl cyclase activity (Figure 11). The cyclase-activator forskolin increased cAMP in both P2Y12 and P2Y12-R256Q-expressing cells. However, the reduction in cAMP following ADP stimulation was significantly blunted in P2Y12-R256Q-expressing cells compared to P2Y12-expressing cells. This suggests that the mutant receptor is not coupling efficiently to Gi (interesting for our purposes). Future investigations of G protein coupling using different techniques will further test these hypotheses. These results are consistent with previous studies that show that platelets from the compound heterozygote fail to show efficient ADP-induced cAMP suppression as well. Interestingly, the Gi defective function in the mutants is present although the sites of mutations, R265W and R256Q, are far from where Gi-binding takes place. Therefore, it is possible that oligomerization status of the receptors influences the efficiency of Gi coupling.

Chapter 4

CONCLUSIONS AND DISCUSSION

The question of GPCRs oligomerization and their stoichiometry in vivo is one that remains unanswered at present. It is known that most GPCRs can form homo- and/or heterodimers in heterologous expression systems (Hébert and Bouvier 1998; Prinster et al. 2005; Bulenger et al. 2005; Milligan 2009). However, the jury remains undecided concerning the functional roles of such dimers in vivo. GPCR crystal-structure studies have confirmed that some GPCRs can crystalize as dimers (Manglik et al. 2012; Wu et al. 2012). In contrast, it is additionally evident from studies using reconstitution of G-protein coupled receptors (GPCRs) into proteoliposomes, that they can signal as monomeric proteins (Whorton et al. 2007, 2008).

Over the last decade, platelet GPCRs have been shown to exist in both homo- and hetero-dimeric forms (Arachiche et al. 2013; María de la Fuente et al. 2012; Frey et al. 2013a; Leger 2006; Savi et al. 2006); however, their influence on physiologically relevant platelet signaling pathways and pathophysiological manifestations is still poorly understood. In this study, we seek to determine whether the homodimerization of P2Y12 influences the already-established signaling or binding events essential for platelet function. Our laboratory has previously shown that P2Y12 can form heterodimers with the GPCR PAR4 on membrane surfaces. However, the ability of P2Y12 to form functional homo-dimeric complexes is controversial. In our own laboratory, we have previously demonstrated using bi-molecular fluorescence complementation (biFC), a very sensitive technique involving reconstitution of two

portions of a single fluorescent molecule tagged to two different P2Y12 protomers, that P2Y12 can self-associate. However, this technique is unable to yield any insight into the amount or ratio of monomeric, dimeric, or other multimeric forms of the receptor. Therefore, we sought to understand whether the functional form of P2Y12 exists as monomers or dimers in live cells. In this work, using FCS and PCH analysis, I demonstrate that P2Y12 exists predominantly as a monomer on the membrane and that two specific P2Y12 mutant variants, P2Y12R256Q and P2Y12R265W, known to cause a mild bleeding disorder in a compound heterozygote human subject, have enhanced potential to homo-dimerize. Interestingly, the P2Y12-R256W mutant variant exhibits a higher potential to form a dimer than either P2Y12-R256Q or WT receptors.

4.1 P2Y12 Oligomerization and Platelet Activation

P2Y12 has previously been stated to be detected as an oligomeric form confined to platelet lipid rafts when analyzed exclusively by SDS-PAGE (Savi et al. 2006). In this study, the P2Y12 receptors isolated from human platelets were characterized predominantly by covalently bound, slowly migrating bands of higher molecular weight than that predicted for monomeric forms on immunoblots, although this technique precluded direct evaluation of the oligomerization in intact cells. Interestingly, the dimeric species was a minor species in SDS-PAGE; however, more recently, the crystal structure of P2Y12 was solved and shown to consist of a dimeric form, with TM5 at the interface of two parallel P2Y12 molecules from adjacent units, potentially implicating a role for this transmembrane domain in P2Y12 homomeric interactions (K. Zhang et al. 2014a). Unfortunately, neither of these techniques allowed evaluation in live cells or direct correlation of the forms to the active or regulated state of the receptor. Thus, our study sought to determine whether P2Y12

exists predominantly as monomer, dimer or other species in intact, live cell membranes. This is now possible with the recent application of techniques such as Fluorescence energy transfer or single-molecule imaging methods.

The homo-oligomeric forms of P2Y12 receptors detected in heterologous expression systems (HEK293 and CHO cells) and cell lysates of human platelets by Savi et al. could be disrupted by incubation with a clinically applicable antagonist, with Clopidogrel (Savi et al. 2006). Mutation of Cys97 residue rendered P2Y12 insensitive to the disruption of the oligomers by clopidogrel detected on SDS-PAGE. Therefore, it was proposed that the antagonistic properties of clopidogrel might be associated with the disruption of P2Y12 oligomers into monomers. This concept was refuted, since it has been observed that the antagonistic effect of clopidogrel on P2Y12 function can be detected before the detection of disruption of the oligomers in SDS-acrylamide gels (Ding et al., 2009). It is likely that the action of clopidogrel is more direct than in disruption of dimeric structure, and therefore the role of P2Y12 dimerization on its function remains to be further explored.

To better understand this aspect of P2Y12, I asked the following questions 1) Do P2Y12 monomers self-associate in live cells? What is the oligomer status of P2Y12 receptors freely diffusing on cell membranes? Moreover, is it required for receptor function? By employing FCS and PCH on HEK293T cell membranes and measuring the level of cAMP in them, my primary data show that P2Y12 receptor is predominantly a monomer on cell membranes. Although previous experiments done in our lab, using systems with high sensitivity to detect dimers i.e. BifC and BRET, have revealed that P2Y12 can be detected in a dimeric form, these techniques were not able to assess the ratio of monomer to dimeric form, nor able to assess the correlation

of these forms with activity. Thus, we evaluated not only the ability of P2Y12 to exist as a monomer or dimer in live cells, but also evaluated the monomer/dimer species of two mutant variants that have been reported to have reduced activity in vivo in human subjects. In FCS/PCH experiments, we analyzed heterologously-expressed fluorescently-tagged version of the P2Y12 receptor. Our results demonstrate that the predominant species of P2Y12 in live cell membranes is monomeric were unexpected. It is likely that a small number of dimers may exist, but the ratio of naturally-occuring receptor in the membrane is likely monomeric.

In addition, endogenous P2Y12 expression may influence the percentage of each species detected by FCS/PCH experiments, because untagged endogenous receptor may compete/interfere with the GFP-tagged detection of dimer. However, this may explain results that are intermediate between controls for monomeric and dimeric species, as we have shown that untagged receptor species can compete for those that are tagged (data not shown). Furthermore, it is conceivable that a Fluorescent protein can influence the diffusion rates and the ability of receptor monomers to interact with each other due to the conformational constraints they can confer on the proteins. This will be resolved in future experiments by testing P2Y12 tagged to different FPs like citrine.

A survey of reported studies detecting multimeric interactions of GPCRs reveals a diverse array of potential interaction structures. For example, a tetrameric organization of GPCRs was revealed with the combination of RET and PCA for A2-adenosine receptor homo-oligomers and hetero-oligomers of cannabinoid/D2 dopamine receptors CXCR4 multimers were detected using a similar combination of techniques (Hamatake et al., 2009). FRET spectrometry, which is a relatively new

approach, has demonstrated that muscarinic receptors M3 are present in a dimer-tetramer equilibrium (Patowary et al. 2013), supporting an earlier finding by quantitative FRET that predicted quaternary structures of M3 muscarinic receptors (Pisterzi et al. 2010). Contrary to these results, muscarinic receptors M1 and M2 receptors were reported to be dimeric in FCS and PCH analyses without any detection of trimers or tetramers (Herrick-Davis et al. 2013). Our current studies demonstrate that P2Y12 exists predominantly as a monomer when expressed in heterologous systems. The R256W mutant variant is probably different from the WT receptor in its oligomerization behavior. In our PCH studies, this mutant variant of P2Y12 displays enhanced dimerization potential, suggesting that a conformational change initiated at this site pushes the receptor towards forming higher- order clusters. For future FCS experiments, it may be useful to add an additional control, such as evaluation of naturally-occuring dimers, as has been reported for CD28, with additional comparison of this to our dimer-mimetic control of membrane receptor CD86-GFP-GFP used in the above experiments. FCS can also be used to determine not only the oligomerization state of P2Y12 or other plasmids but also to resolve their diffusion coefficient. In addition, since the homomeric interactions of P2Y12 have not been probed with other biophysical approaches that are currently employed extensively to determine receptor interactions, techniques such as TIRF or FRET might provide additional insights into the regulation of the receptor by agonist application in real time.

To determine whether oligomerization may play a role in regulating receptor function, we assessed both the oligomerization properties and began to explore the activation potential of two P2Y12 mutant variants (R256Q and R265W) detected in a

patient with a defined bleeding disorder. Both R265W and R256Q mutant forms of the receptor displayed enhanced potential to dimerize compared to WT control. R265W especially exhibited a propensity to form a higher proportion of receptor oligomers than the WT receptor. This demonstrates the influence of structural integrity on receptor oligomerization dynamics and in turn, alludes to a possible mechanism by which oligomerization could influence G protein receptor interaction/function and vice versa.

Reported molecular and functional analyses of inherited P2Y12 mutants documented in patient cohorts presenting with a range of bleeding disorders has shed light on a structure-function relationship for P2Y12. Of specific interest to this project: ECL3 has been shown to be critical in maintaining the integrity of receptor signaling (Cunningham, Nisar, and Mundell 2013). Mutations R256Q and R265W in P2Y12 are at the periphery of the TM6-ECL3 interface and a part of ECL3, respectively. They do not impair receptor expression or ligand binding, but some studies reported that they may have altered the receptor function of platelets, (Cattaneo, 2015; Cattaneo et al., 2003). Yet the functional analysis of each individual mutant when expressed in isolation remained to be explored. G proteins are generally reported to bind to ICL2 and ICL3 of GPCRS: the most well-studied G protein interaction sites within receptors are reported for β2 adrenergic receptor-Gs and Rhodopsin-Gt (Gahbauer and Böckmann 2016). It is, therefore, unclear why mutations R265Q and R265W in P2Y12 ECL3 (far from the putative G protein-interaction sites on the intracellular surfaces of receptors) may have a defective Gi function. Some GPCR dimers like Rhodopsin, Serotonin receptors, and Neuropeptide Y receptor are increasingly being

reported to be essential for efficient G-protein function (Maria de la Fuente et al. 2012; K. Herrick-Davis et al. 2013a; Jastrzebska et al. 2013a; Parker 1972).

Similarly, Thromboxane A2 receptor DRY motif mutants fail to homodimerize in saturation BRET assays(Frey et al. 2013b). Loss of receptor interactions of the mutants is reported to influence G protein function in both these cases. To comprehend how enhanced homodimerization abilities of P2Y12 mutants R256Q and R265W may regulate the receptor function, we examined whether the ability of these mutants to dimerize differentially versus the WT also correlated with a change in the individual functional coupling.

To address the hypothesis that mutations R256Q and R265W might alter oligomerization properties and in turn, lead to an inefficient binding of Gi to the receptor, we began to analyze Gi function of each mutant variant. Moreover, this approach would allow us to link the physiological relevance of P2Y12 oligomerization to platelet function and hemostasis since R256Q, and R265W mutations in P2Y12 are associated with a mild bleeding disorder. Contrary to our original hypothesis R265W/R256Q had higher molecular brightness compared to the WT. This suggests that these mutants have enhanced abilities to self-associate or an increase in the affinities for homodimer formation. This is uncommon, as most prior investigations of mutant receptors have reported a reduction in their dimerization potential. To comprehend how enhanced homodimerization abilities of P2Y12 mutants R256Q and R265W might regulate receptor function, it would be beneficial to understand the oligomer organization of P2Y12, not only with itself, but with other receptors in platelet membranes.

4.2 Biological and Functional Impact of GPCR Oligomerization

Homo- and heteromeric receptor structures produce an extra layer of pharmacological diversity, as they moderate the binding of ligands as well as the ability of the individual receptor to initiate signaling cascades that are different from what they would as monomers. The heterodimers of GPCR can elicit signals different from those that are detected because of expression of either alone. For instance, heterodimers of α2c adrenergic receptors, δ-κ opioid receptors, and α2a- and δ-μ opioid receptors produce arrestin-dependent signaling responses as heterodimers that would possibly not be observed after expression the monomers only (Rozenfeld and Devi , 2007,2010). Homodimers and heterodimers formed by the platelet GPCRs are necessary for the functioning of receptors when expressed in a heterologous expression system; although, their physiological influence to platelet activation pathways/thrombosis is unclear (Tapon-Bretaudiere 2003a). For instance, homodimerization of Thromboxane A2 receptor has been shown to be essential for the functioning of Gq, shown by the ability of TP receptor homodimerization mutant variants to produce IP3 after stimulation in both HEK293 cells and megakaryocytic cell lines (Frey et al. 2013a). Of particular relevance to P2Y12 interactions, work from our own laboratory has demonstrated that PAR4 and P2Y12 heterodimerize upon PAR4 activation and regulate arrestin-mediated Akt activation (Khan et al. 2014b) which has been shown to influence fibrinogen binding in platelets (Li et al. 2010a), signifying its physiological relevance.

4.3 CONCLUSION

In summary, these studies provide evidence that P2Y12 are monomers on cell membranes. PCH analyses reveal monomers of P2Y12 on HEK293T cell membranes.

Mutations R256Q and R265W of P2Y12 vary in their oligomerization properties from the WT receptor. Both R256Q and R265W mutants show significantly enhanced homodimerization as compared to P2Y12-WT. One and two component PCH analyses reveal that R265W and R256Q mutant receptor complexes have a significantly higher average molecular brightness than P2Y12, in turn suggesting that the composition of the receptor clusters of R265W and R256Q mutants are different from that of P2Y12. Further, we demonstrate that the R256Q mutant variant form of P2Y12 has significantly impaired Gi function compared to P2Y12-WT. It is unclear how enhanced oligomerization might impair Gi function of the R256Q and R265W mutant variants of P2Y12 or in fact, whether the altered dimerization is rather a consequence of deficient signaling. It is also possible that these two alterations are independent of one another. Nevertheless, enhanced oligomerization of the receptors on the cell membrane could alter the proportion of Gi associated with the P2Y12 receptor, as this has been implied in studies with other GPCRs. Increased receptor oligomer formation could also affect receptor life cycle (membrane stability, internalization, and recycling)(Khan , 2015). These leads will be pursued in future investigations to fully understand the role of oligomerization in P2Y12 receptor function.

Chapter 5

FUTURE DIRECTIONS

5.1 To Analyze Gi Coupling of R265W

Mutation of P2Y12 at residue 265 has been shown to exist in a patient with a bleeding disorder and is thus likely to cause dysfuction in the receptor (Cattaneo 2011, 2005). Since our experiments showed that the other mutation associated with altered dimerization properties (P2Y12-R256Q) failed to activate Gi in the presence of even high concentration of ADP, it will be of equal significance interest to determine whether R265W mutation also alters coupling to Gi proteins Interestingly, R265W has a higher potential of oligomerization than R256Q mutant variants.

5.2 To Test the Ability of P2Y12 Mutants to Associate with Gi: GTPγS Binding Assay

Although we have shown that R256Q and R265W have a functional defect in Gi activation, it has not been investigated whether these mutants cannot adequately bind Gi or whether they are simply unable to activate them. Thus, we propose to conduct both GTPϒS binding assays and G protein- P2Y12 co- immunoprecipitation studies. Regarding the stoichiometry of G protein-GPCR coupling, Asymmetric (2:1 ratio of GPCR: G-protein) and symmetric (1:1 ratio) coupling of GPCRs to G protein is still a debated area, although there is strong evidence for both (Maurice, Kamal, and Jockers 2011). We expect that we will need to co-transfect cells with both Gi as well

as P2Y12 forms to adequately immunoprecipitate and detect G protein-GPCR associations.

5.3 To Examine the Membrane Stability and Recycling Dynamics of P2Y12 Mutant Variants

No gross differences in trafficking and expression pattern of the WT P2Y12 or its mutants R256Q and R265W were observed. However, the enhanced oligomerization of R256Q and R265W mutants of P2Y12 might alter their internalization, recycling, and membrane stability. P2Y12 is known to get internalized in an arrestin-dependent manner and is recycled with the help of Rab GTPases to the membrane to undergo another round of activation, thus amplifying the platelet activation responses (Mundell et al. 2006). Reduced stability of the membrane or impaired recycling can also result in the apparently reduced Gi function. These inteeractions may be evaluated using live cell immunoflourescent confocal microscopy and by co-staining for known markers of intracellular vesicular compartments.

5.4 To Test the Ability of R265W and R256Q Mutant Variants to Associate with Arrestin

Bioluminescence resonance energy transfer (BRET) has been utilized to observe arrestin recruitment to GPCRs. BRET is universally used to examine protein-protein interactions in intact cells. One protein is fused with a donor protein that is Renilla luciferase, and the other protein is fused to a green fluorescent protein(GFP) or the acceptor protein to investigate proteins interactions in living cells using this technique. When the donor and acceptor proteins are within ~10 nm of each other, the donor transfers energy to the acceptor and exits it. This will result in emission from

the acceptor protein. To evaluate the ability of P2Y12 mutant variants to recruit arrestin, R265W or R256Q mutant variants will be genetically fused with the acceptor protein and arrestin tagged with GFP their relative BRET association measured relative to that of arrestin with the WT P2Y12 (Donthamsetti et al. 2015).

REFERENCES

1. Arachiche, Amal, Michele M. Mumaw, María de la Fuente, and Marvin T. Nieman. 2013. “Protease-Activated Receptor 1 (PAR1) and PAR4 Heterodimers Are Required for PAR1-Enhanced Cleavage of PAR4 by $\upalpha$-Thrombin.” *Journal of Biological Chemistry* 288 (45):32553–32562. https://doi.org/10.1074/jbc.m113.472373.

2. Bleicken, Stephanie, Miki Otsuki, and Ana J. Garcia-Saez. 2011. “Quantification of Protein-Protein Interactions within Membranes by Fluorescence Correlation Spectroscopy.” *Current Protein & Peptide Science* 12 (8):691–98. https://doi.org/10.2174/138920311798841744.

3. Bonde, Marie, Soren Sheikh, and Jakob Hansen. 2006. “Family C 7TM Receptor Dimerization and Activation.” *Endocrine, Metabolic & Immune Disorders - Drug Targets* 6 (1):7–16. https://doi.org/10.2174/187153006776056594.

4. Briddon, Stephen J., and Stephen J. Hill. 2007. “Pharmacology under the Microscope: The Use of Fluorescence Correlation Spectroscopy to Determine the Properties of Ligand{\textendash} receptor Complexes.” *Trends in Pharmacological Sciences* 28 (12):637–45. https://doi.org/10.1016/j.tips.2007.09.008.

5. Cattaneo, Marco. "The P2 Receptors and Congenital Platelet Function Defects." *Seminars in Thrombosis and Hemostasis* 31, no. 02 (2005): 168-73. doi:10.1055/s-2005-869522.

6. Chen, Kuo-ching, Keng-tso Chuang, Han-hsueh Liu, and Ching-ya Chen. 2013. “Two-Speed Automatic Transmissions of Electric Scooters.” {SAE} International. https://doi.org/10.4271%2F2013-32-9087.

7. Chen, M.-H. 2004. “Palmitoylation Is Required for the Production of a Soluble Multimeric Hedgehog Protein Complex and Long-Range Signaling in Vertebrates.” *Genes & Development* 18 (6):641–59. https://doi.org/10.1101/gad.1185804.

8. Cunningham, Margaret R., Shaista P. Nisar, and Stuart J. Mundell. 2013. "Molecular Mechanisms of Platelet P2Y12 Receptor Regulation." *Biochemical Society Transactions* 41 (1):225–30. https://doi.org/10.1042/BST20120295.

9. Donthamsetti, Prashant, Jose Quejada, Jonathan A. Javitch, Vsevolod V. Gurevich, and Nevin A. Lambert. 2015. "Using Bioluminescence Resonance Energy Transfer (BRET) to Characterize Agonist Induced Arrestin Recruitment to Modified and Unmodified G Protein Coupled Receptors." *Current Protocols in Pharmacology*, 2.14. 1--2.14. 14. https://doi.org/10.1002/0471141755.ph0214s70.

10. Dorsam, Robert T., and Satya P. Kunapuli. 2004. "Central Role of the P2Y12 Receptor in Platelet Activation." *Journal of Clinical Investigation* 113 (3):340–45. https://doi.org/10.1172/JCI20986.

11. Dupré, Denis J, Terence E Hébert, and Ralf Jockers, eds. 2012. *{GPCR} Signalling Complexes {\textendash} Synthesis, Assembly, Trafficking and Specificity*. Springer Netherlands. https://doi.org/10.1007/978-94-007-4765-4.

12. Elson, Elliot L. 2013. "40 Years of FCS." *Methods in Enzymology*, 1–10. https://doi.org/10.1016/b978-0-12-388422-0.00001-7.

13. Erb, Laurie, and Gary A. Weisman. 2012. "Coupling of P2Y Receptors to G Proteins and Other Signaling Pathways." *Wiley Interdisciplinary Reviews: Membrane Transport and Signaling* 1 (6):789–803. https://doi.org/10.1002/wmts.62.

14. Ferre, S., V. Casado, L. A. Devi, M. Filizola, R. Jockers, M. J. Lohse, G. Milligan, J.-P. Pin, and X. Guitart. 2014a. "G Protein-Coupled Receptor Oligomerization Revisited: Functional and Pharmacological Perspectives." *Pharmacological Reviews* 66 (2):413–34. https://doi.org/10.1124/pr.113.008052.

15. Ferre, S, V Casado, L A Devi, M Filizola, R Jockers, M J Lohse, G Milligan, J.-P. Pin, and X Guitart. 2014b. "G Protein-Coupled Receptor Oligomerization Revisited: Functional and Pharmacological Perspectives." *Pharmacological Reviews* 66 (2). American Society for Pharmacology & Experimental Therapeutics ({ASPET}):413–34. https://doi.org/10.1124/pr.113.008052.

16. Fotiadis, Dimitrios, Yan Liang, Slawomir Filipek, David A. Saperstein, Andreas Engel, and Krzysztof Palczewski. 2003. “Atomic-Force Microscopy: Rhodopsin Dimers in Native Disc Membranes.” *Nature* 421 (6919):127–128. https://doi.org/10.1038/421127a.

17. Frey, Alexander J., Salam Ibrahim, Scott Gleim, John Hwa, and Emer M. Smyth. 2013a. “Biased Suppression of {TP} Homodimerization and Signaling through Disruption of a {TM} {GxxxGxxxL} Helical Interaction Motif.” *Journal of Lipid Research* 54 (6):1678–90. https://doi.org/10.1194/jlr.m036673.

18. Frey, Alexander J, Salam Ibrahim, Scott Gleim, John Hwa, and Emer M Smyth. 2013b. “Biased Suppression of {TP} Homodimerization and Signaling through Disruption of a {TM} {GxxxGxxxL} Helical Interaction Motif.” *Journal of Lipid Research* 54 (6). American Society for Biochemistry & Molecular Biology ({ASBMB}):1678–90. https://doi.org/10.1194/jlr.m036673.

19. Fuente, Mar\’\ia de la, Daniel N. Noble, Sheetal Verma, and Marvin T. Nieman. 2012. “Mapping Human Protease-Activated Receptor 4 ({PAR}4) Homodimer Interface to Transmembrane Helix 4.” *Journal of Biological Chemistry* 287 (13):10414–23. https://doi.org/10.1074/jbc.m112.341438.

20. Fuente, María de la, Daniel N. Noble, Sheetal Verma, and Marvin T. Nieman. 2012. “Mapping Human Protease-Activated Receptor 4 (PAR4) Homodimer Interface to Transmembrane Helix 4.” *Journal of Biological Chemistry* 287 (13):10414–10423. https://doi.org/10.1074/jbc.m112.341438.

21. G., VRBO. 1962. “Aggregation of Blood Platelets by Adenosine Diphosphate and Its Reversal.” *Nature* 194 (4832):927–29. https://doi.org/10.1038/194927b0.

22. Gahbauer, Stefan, and Rainer A. Böckmann. 2016. “Membrane-Mediated Oligomerization of G Protein Coupled Receptors and Its Implications for GPCR Function.” *Frontiers in Physiology* 7:494. https://doi.org/10.3389/fphys.2016.00494.

23. Giraldo, Jesús, and Francisco Ciruela. 2013. “Preface.” In *Progress in Molecular Biology and Translational Science*, xix--xxii. Elsevier. https://doi.org/10.1016%2Fb978-0-12-386931-9.10000-5.

24. Haustein, Elke, and Petra Schwille. 2007. “Fluorescence Correlation Spectroscopy: Novel Variations of an Established Technique.” *Annual Review of Biophysics and Biomolecular Structure* 36 (1):151–69. https://doi.org/10.1146/annurev.biophys.36.040306.132612.

25. Hebert, Terence E., Serge Moffett, Jean-Pierre Morello, Thomas P. Loisel, Daniel G. Bichet, Cécile Barret, and Michel Bouvier. 1996. “A Peptide Derived from a $\upbeta$2-Adrenergic Receptor Transmembrane Domain Inhibits Both Receptor Dimerization and Activation.” *Journal of Biological Chemistry* 271 (27):16384–16392. https://doi.org/10.1074/jbc.271.27.16384.

26. Herrick-Davis, K., E. Grinde, A. Cowan, and J. E. Mazurkiewicz. 2013a. “Fluorescence Correlation Spectroscopy Analysis of Serotonin, Adrenergic, Muscarinic, and Dopamine Receptor Dimerization: The Oligomer Number Puzzle.” *Molecular Pharmacology* 84 (4):630–42. https://doi.org/10.1124/mol.113.087072.

27. Herrick-Davis, Katharine, and Joseph E. Mazurkiewicz. 2013. “Fluorescence Correlation Spectroscopy and Photon-Counting Histogram Analysis of Receptor–Receptor Interactions.” In *Methods in Cell Biology*, 181–196. Elsevier. https://doi.org/10.1016/b978-0-12-408143-7.00010-4.

28. Hess, Samuel T., Shaohui Huang, Ahmed A. Heikal, and Watt W. Webb. 2002. “Biological and Chemical Applications of Fluorescence Correlation Spectroscopy: A Review†.” *Biochemistry* 41 (3):697–705. https://doi.org/10.1021/bi0118512.

29. Hollopeter, Gunther, Hans-Michael Jantzen, Diana Vincent, Georgia Li, Laura England, Vanitha Ramakrishnan, Ruey-Bing Yang, et al. 2001. *Nature* 409 (6817):202–7. https://doi.org/10.1038/35051599.

30. Jastrzebska, B., T. Orban, M. Golczak, A. Engel, and K. Palczewski. 2013a. “Asymmetry of the Rhodopsin Dimer in Complex with Transducin.” *The {FASEB} Journal* 27 (4):1572–84. https://doi.org/10.1096/fj.12-225383.

31. Kahner, B. N., H. SHANKAR, S. MURUGAPPAN, G. L. PRASAD, and S. P. KUNAPULI. 2006. “Nucleotide Receptor Signaling in Platelets.” *Journal of Thrombosis and Haemostasis* 4 (11):2317–26. https://doi.org/10.1111/j.1538-7836.2006.02192.x.

32. Kauffenstein, G., W. Bergmeier, A. Eckly, P. Ohlmann, C. Léon, J. P. Cazenave, B. Nieswandt, and C. Gachet. 2001. “The P2Y12 Receptor Induces Platelet Aggregation through Weak Activation of the AIIbβ3 Integrin – a Phosphoinositide 3-kinase-dependent Mechanism.” *FEBS Letters* 505 (2):281–90. https://doi.org/10.1016/S0014-5793(01)02824-1.

33. Khan, A., D. Li, S. Ibrahim, E. Smyth, and D. S. Woulfe. 2014a. “The Physical Association of the P2Y12 Receptor with {PAR}4 Regulates Arrestin-Mediated Akt Activation.” *Molecular Pharmacology* 86 (1):1–11. https://doi.org/10.1124/mol.114.091595.

34. Kumar, Boda Arun, Poonam Kumari, Chandan Sona, and Prem N. Yadav. 2017. “GloSensor Assay for Discovery of GPCR-Selective Ligands.” *Methods in Cell Biology*, 27–50. https://doi.org/10.1016/bs.mcb.2017.07.012.

35. Leger, A. J. 2006. “Blocking the Protease-Activated Receptor 1-4 Heterodimer in Platelet-Mediated Thrombosis.” *Circulation* 113 (9):1244–54. https://doi.org/10.1161/circulationaha.105.587758.

36. Li, Dongjun, Lauren D\textquotesingleAngelo, Massiel Chavez, and Donna S. Woulfe. 2010a. “Arrestin-2 Differentially Regulates {PAR}4 and {ADP} Receptor Signaling in Platelets.” *Journal of Biological Chemistry* 286 (5):3805–14. https://doi.org/10.1074/jbc.m110.118018.

37. Manglik, A., A.C. Kruse, T.S. Kobilka, F.S. Thian, J.M. Mathiesen, R.K. Sunahara, L. Pardo, W.I. Weis, B.K. Kobilka, and S. Granier. 2012. *Crystal Structure of the Mu-Opioid Receptor Bound to a Morphinan Antagonist*. https://doi.org/10.2210/pdb4dkl/pdb.

38. Mao, Yingying, Lili Zhang, Jianguo Jin, Barrie Ashby, and Satya P. Kunapuli. 2010. “Mutational Analysis of Residues Important for Ligand Interaction with the Human P2Y12 Receptor.” *European Journal of Pharmacology* 644 (1–3):10–16. https://doi.org/10.1016/j.ejphar.2010.06.023.

39. Maurice, Pascal, Maud Kamal, and Ralf Jockers. 2011. “Asymmetry of GPCR Oligomers Supports Their Functional Relevance.” *Trends in Pharmacological Sciences* 32 (9):514–20. https://doi.org/10.1016/j.tips.2011.05.006.

40. Monillas, Elizabeth S., Jeffrey L. Caplan, Anastasia F. Thévenin, and Brian J. Bahnson. 2015. “Oligomeric State Regulated Trafficking of Human Platelet-Activating Factor Acetylhydrolase Type-{II}.” *Biochimica et Biophysica Acta ({BBA}) - Proteins and Proteomics* 1854 (5):469–75. https://doi.org/10.1016/j.bbapap.2015.02.007.

41. Müller, J. D., Y. Chen, and E. Gratton. 2000. “Resolving Heterogeneity on the Single Molecular Level with the Photon-Counting Histogram.”

42. Mundell, Stuart J., Jiansong Luo, Jeffrey L. Benovic, Pamela B. Conley, and Alastair W. Poole. 2006. “Distinct Clathrin-Coated Pits Sort Different G Protein-Coupled Receptor Cargo.” *Traffic* 7 (10):1420–31. https://doi.org/10.1111/j.1600-0854.2006.00469.x.

43. Masuho, Ikuo. "Deconvolution of complex GPCR signaling pathways."Accessed December 17, 2017. http://ikuo-masuho.squarespace.com/projects/.

44. Nisar, Shaista P., Margaret Cunningham, Kunal Saxena, Robert J. Pope, Eamonn Kelly, and Stuart J. Mundell. 2012. “Arrestin Scaffolds NHERF1 to the P2Y12 Receptor to Regulate Receptor Internalization.” *Journal of Biological Chemistry* 287 (29):24505–15. https://doi.org/10.1074/jbc.M112.347104.

45. Offermanns, Stefan. 2006. “Activation of Platelet Function Through G Protein–Coupled Receptors.” *Circulation Research* 99 (12):1293–1304. https://doi.org/10.1161/01.res.0000251742.71301.16.

46. Parker, Leonard. 1972. “Particle Creation in Isotropic Cosmologies.” *Physical Review Letters* 28 (11):705–8. https://doi.org/10.1103/physrevlett.28.705.

47. Patowary, Suparna, Elisa Alvarez-Curto, Tian-Rui Xu, Jessica~D Holz, Julie~A Oliver, Graeme Milligan, and Valeric\ua Raicu. 2013. “The Muscarinic M3acetylcholine Receptor Exists as Two Differently Sized Complexes at the Plasma Membrane.” *Biochemical Journal* 452 (2):303–12. https://doi.org/10.1042/bj20121902.

48. Pellissier, Lucie P., Gaël Barthet, Florence Gaven, Elisabeth Cassier, Eric Trinquet, Jean-Philippe Pin, Philippe Marin, et al. 2011. “G Protein Activation by Serotonin Type 4 Receptor Dimers.” *Journal of Biological Chemistry* 286 (12):9985–97. https://doi.org/10.1074/jbc.m110.201939.

49. Pisterzi, Luca F., David B. Jansma, John Georgiou, Michael J. Woodside, Judy Tai-Chieh Chou, Stéphane Angers, Valerica Raicu, and James W. Wells. 2010. “Oligomeric Size of the M2 Muscarinic Receptor in Live Cells as Determined by Quantitative Fluorescence Resonance Energy Transfer.” *The Journal of Biological Chemistry* 285 (22):16723–38. https://doi.org/10.1074/jbc.M109.069443.

50. Qanbar, Riad, and Michel Bouvier. 2003. “Role of Palmitoylation/Depalmitoylation Reactions in G-Protein-Coupled Receptor Function.” *Pharmacology & Therapeutics* 97 (1):1–33. https://doi.org/10.1016/s0163-7258(02)00300-5.

51. Rozenfeld, Raphael, and Lakshmi~A Devi. 2011a. “Exploring a Role for Heteromerization in {GPCR} Signalling Specificity.” *Biochemical Journal* 433 (1):11–18. https://doi.org/10.1042/bj20100458.

52. Rozenfeld, Raphael, and Lakshmi~A. Devi. 2011b. “Exploring a Role for Heteromerization in {GPCR} Signalling Specificity.” *Biochemical Journal* 433 (1). Portland Press Ltd.:11–18. https://doi.org/10.1042/bj20100458.

53. Salahpour, Ali, Stéphane Angers, and Michel Bouvier. 2000. “Functional Significance of Oligomerization of G-Protein-Coupled Receptors.” *Trends in Endocrinology & Metabolism* 11 (5):163–68. https://doi.org/10.1016/s1043-2760(00)00260-5.

54. Savi, P., J.-L. Zachayus, N. Delesque-Touchard, C. Labouret, C. Herve, M.-F. Uzabiaga, J.-M. Pereillo, et al. 2006. “The Active Metabolite of Clopidogrel Disrupts P2Y12 Receptor Oligomers and Partitions Them out of Lipid Rafts.” *Proceedings of the National Academy of Sciences* 103 (29):11069–74. https://doi.org/10.1073/pnas.0510446103.

55. Schulze, Harald, and Joseph Italiano, eds. 2016. *Molecular and Cellular Biology of Platelet Formation*. Springer International Publishing. https://doi.org/10.1007/978-3-319-39562-3.

56. Seifert, Roland, and Thomas Wieland, eds. 2005. *G Protein-Coupled Receptors as Drug Targets*. Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/352760734x.

57. Sommer, Martha E, Klaus Peter Hofmann, and Martin Heck. 2012. “Distinct Loops in Arrestin Differentially Regulate Ligand Binding within the {GPCR} Opsin.” *Nature Communications* 3 (August). Springer Nature:995. https://doi.org/10.1038/ncomms2000.

58. Tapon-Bretaudiere, J. 2003. “Hemostasis and Thrombosis. Basic Principles and Clinical Practice, Fourth Edition. Robert W. Colman, Jack Hirsh, Victor J. Marder, Alexander W. Clowes, and James N. George, Eds. Philadelphia, {PA}: Lippincott Williams & Wilkins, 2001, 1578 Pp., {\textdoll.” *Clinical Chemistry* 49 (2):345–46. https://doi.org/10.1373/49.2.345/-a.

59. Waldhoer, M, J Fong, R M Jones, M M Lunzer, S K Sharma, E Kostenis, P S Portoghese, and J L Whistler. 2005. “A Heterodimer-Selective Agonist Shows In vivo Relevance of G Protein-Coupled Receptor Dimers.” *Proceedings of the National Academy of Sciences* 102 (25). Proceedings of the National Academy of Sciences:9050–55. https://doi.org/10.1073/pnas.0501112102.

60. Zacharias, David A., Jonathan D. Violin, Alexandra C. Newton, and Roger Y. Tsien. 2002. “Partitioning of Lipid-Modified Monomeric GFPs into Membrane Microdomains of Live Cells.” *Science (New York, N.Y.)* 296 (5569):913–16. https://doi.org/10.1126/science.1068539.

61. Zhang, Kaihua, Jin Zhang, Zhan-Guo Gao, Dandan Zhang, Lan Zhu, Gye Won Han, Steven M. Moss, et al. 2014a. “Structure of the Human P2Y12 Receptor in Complex with an Antithrombotic Drug.” *Nature* 509 (7498):115–18. https://doi.org/10.1038/nature13083.

62. Zhang, Kaihua, Jin Zhang, Zhan-Guo Gao, Dandan Zhang, Lan Zhu, Gye Won Han, Steven M Moss, et al. 2014b. “Structure of the Human P2Y12 Receptor in Complex with an Antithrombotic Drug.” *Nature* 509 (7498). Springer Nature:115–18. https://doi.org/10.1038/nature13083.

63. Zhang, Peng, Lujun Zhou, Chunli Pei, Xinhua Lin, and Zengqiang Yuan. 2016. “Dysfunction of Wntless Triggers the Retrograde Golgi-to-{ER} Transport of Wingless and Induces {ER} Stress.” *Scientific Reports* 6 (1). https://doi.org/10.1038/srep19418.