Novel implications of lost serotonin transporter function on platelet biology

By

Kendra Helen Oliver

Dissertation
Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in

Pharmacology
December, 2016
Nashville, Tennessee

Approved: Date:

_____ _____
Jonathan Schoenecker, M.D., Ph.D. (Chair)

_____ _____
Heidi E. Hamm, Ph.D.

_____ _____
Ana M.D. Carneiro, Ph.D.

_____ _____
Digna R. Velez Edwards Ph.D., M.S.

_____ _____
Vsevolod Gurevich, Ph.D.

DEDICATION

To my parents, Michael Cherry and Kimberlee Oliver,
to my brilliant sister, Briana Oliver,
and to my partner in crime, Dr. Max Joffe.

ACKNOWLEDGEMENTS

There are so many wonderful people that have I would like to thank for their support and guidance during this challenging and daunting process. Most importantly, I would like to thank my mentors, Dr. Heidi E Hamm and Dr. Ana M.D. Carneiro. Dr. Carneiro has pushed me beyond my limits and has provided my scholarly foundation. Dr. Hamm has also been an outstanding mentor and role model as a scientist. I would also like to thank my committee members for their guidance during my scientific scholarship. The direction and administrative support received from Dr. Christine Konrodi and Karen Geig, and Dr. Joey Barrett, has been extraordinary, and for that I am very thankful.

Additionally, I would like to thank and acknowledge my lab members. In particular, Dr. Matthew Duvernay has helped me navigate the world of platelet biology and Dr. Ali Kali for his support and encouragement. I would also like to thank Dr. Matthew Mazalouskas and Dr. Michael Dohn. Dr. Mazalouskas has not only been there to discuss the grueling hardships of graduate studies, but he is also a role model for deep scientific rigor and inquisitiveness. I would also like to acknowledge the rest of the Hamm Lab. Additionally, there have been a number of undergraduates who have contributed to this work, notably including Cecillia Li and Cynthia Mairink. I would also like to thank Jae Maeng for assisting with animal husbandry and experiments.

I would like to acknowledge the wonderful guidance and inspiration of Dr. John Wikswo, whose mentorship has meant a great deal towards my progression as a scientist. Dr. Wikswo has provided me numerous opportunity to expand my intellectual phase space, for which I am tremendously appreciative. He also enabled me with the opportunity to work with The Systems Biology and Bioengineering Undergraduate Research Experience (SyBBURE) and Dr. Christina Marasco. I would like to thank Dr. Wikswo and Dr. Marasco for allowing me to mentor a number of undergraduates and fellows through the SyBBRE program. In particular, Babtunde Bello and Attiyah Houston have been instrumental in the design and creation of the serotonin homeostasis model.

Finally, I would like to thank others at Vanderbilt who have given me extraordinary opportunities to follow my interests in teaching and learning including Dr. Cynthia Brame, Danielle Picard, and Dr. Derek Bruff at the Center for Teaching.

This work would not have been possible without the financial support of the American Heart Association, and the T32 Training Program in Mechanisms of Vascular Disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The cardiovascular system, hemostasis and the role of platelets

The cardiovascular system (CVS) is the body's highway that can be modulated by external factors lead to abnormalities in either cardiac, vascular, and/or blood function imperative to CVS maintenance (Potočnjak et al. 2016). Consequently, the CVS can be considered one of the most vital aspects of a living system that connects all the individual "parts" into a "whole." Hemostasis maintains normal CVS function (Zarbock, Polanowska-Grabowska, and Ley 2007; I. Cornelissen et al. 2010; W. Bergmeier and Hynes 2012). If rupture of the CVS occurs, i.e. bleeding, blood flow through the vasculature needs to be quickly retained inside the damaged vessel. This process requires the contraction of the vasculature, binding of cells and platelets to the injury site, and coagulation (Versteeg et al. 2013) (Figure 1). Pathologies arise following imbalances in hemostatic factors leading to either insufficient hemostasis, (bleeding or hypocoagulability pathologies) or improper initiation of repair mechanisms (thrombosis or hypercoagulability pathologies) (Stutz et al. 2013). The vasculature is a network of arteries, veins, and capillaries, which houses the blood component. Platelets are small, anucleated, cellular fragments that maintain the vascular integrity by binding sites of injury and forming a platelet plug. Upon vessel damage, platelets adhere to the damaged site and aggregate through interactions of platelet receptors with extracellular ligands and soluble proteins. The plasma contains factors important in clot formation that act to stabilize platelet plug formation. These three components have overlapping functions but also play distinct roles in hemostasis. Vascular damage and exposure of subendothelial tissue factors generate thrombin with multiple

effects on other coagulation factors and platelets. Platelets not only contribute to the hemostatic plug formation, but also act to accelerate the coagulation system and therefore play a vital role in hemostasis (Versteeg et al. 2013) .

Formation of a clot, or thrombs, results in the sustained or temporary loss of blood flow. If blood flow is reduced within the peripheral arteries it may lead to limping due to pain in the leg. Occlusion of peripheral blood flow can also lead to critical limb ischemia which is the lack of oxygen to the limb or leg when it is at rest. In all of these cases, it is a blockage or limited blood flow through the vasculature that leads to pathologic conditions. Within the central nervous system, reduced vascular function can lead to stroke or a transient ischemic attack (Kahn et al. 1998; Bynagari-Settipalli et al. 2014).

One pathology that can initiate obstruction of vascular blood flow is atherosclerosis. Atherosclerosis, which is the buildup of fatty plaques leading to vessel stiffening, is one of the most common vascular diseases in western culture (Goldstein et al. 2015a). While it remains unclear how atherosclerosis pathology is initiated, the disease can advance rapidly leading to life threatening conditions (Wozniak et al. 2011). Obstruction of vascular blood flow occurs when fatty plaques lining the inner walls of the vasculature thicken or rupture leading to platelet accumulation. In some cases, this leads to decreased blood flow and to the complete occlusion of local circulation. There are various types of fatty lesions that can develop: non-atherosclerotic intimal lesions, progressive atherosclerotic lesions, lesions with acute thrombi and lesions with healed thrombi (Virmani et al. 2000).

In addition to vascular occlusions, several other vascular diseases can occur. For example, an aortic aneurysm results from bulging of a weakened area in the wall of a blood

vessel, which creates turbulence in blood flow and increases the likelihood of platelet activation. Other pathologies related to the vasculature include thoracic vascular disease, abdominal vascular disease, peripheral venous disease, lymphatic vascular disease, and vascular disease of the lungs including hypertension (Virmani et al. 2000; Angiolillo, Capodanno, and Goto 2010; Wozniak et al. 2011). These conditions lead to an abnormal environment for circulating platelets, and can alter baseline function and responsiveness. Premature or inappropriate platelet activation can further exacerbate these conditions by increasing the likelihood of vessel occlusion.

From both a clinical and biological prospective, platelet function is dependent on the function of the other hemostatic components including the vasculature and plasma factors. The relationship of these components serve to limit blood loss, preserve tissue perfusion, and stimulate local repair processes. Each component will be discussed in greater detail below. In this chapter, I will particularly focus on the function of platelets, their activation mechanisms, and describe the fundamental functional importance of these small cellular fragments within hemostasis.

Role of the vasculature in hemostasis

The vasculature system is a series of blood vessels that carry the liquid and cellular components of the blood throughout the body. However, it is more than just a system of conduits or tubes that contain the blood. Composed of multiple cell types including endothelial cells, smooth muscles, and fibroblasts, the tone of the vasculature is regulated by local and distal factors (Koba et al. 1999; Paul M Vanhoutte et al. 1986; Ware 2004). It

is the dynamics of vessel structure and function that makes the vasculature a key player in hemostasis.

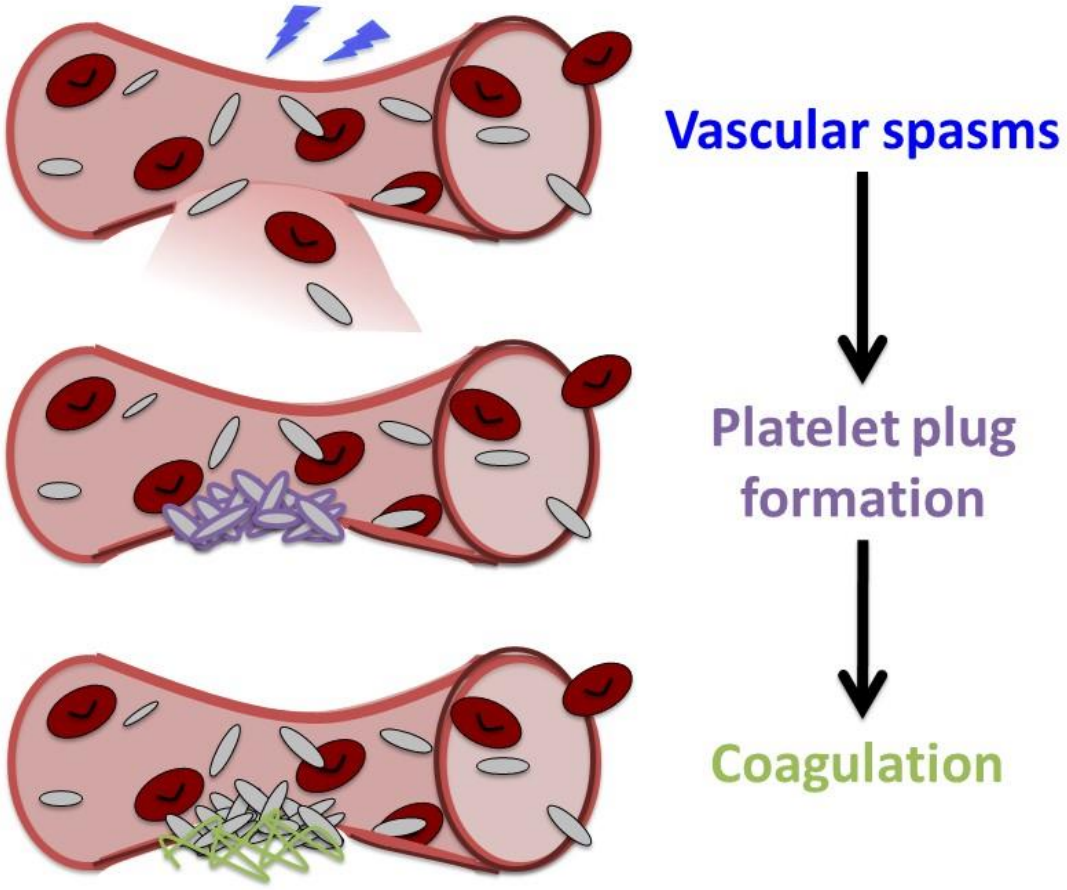


Figure 1. ***Hemostasis.*** *Hemostasis is the maintenance of the body's circulation system. The processes can be divided into three distinct categories once injury occurs to the blood vessel: 1) vascular contraction or spasms 2) platelets binding to injured endothelium and form a platelet plug and 3) coagulation that stabilizes the clot.* (Stalker et al. 2013)

The vasculature can be characterized by the size and complexity of its regions including arteries, veins, and capillaries. The arteries carry oxygenated blood to the tissues, while veins carry blood back from the tissues to the heart and lungs for re-oxygenation

(Bing and Hammond 1949). Capillaries, located between the arteries and the veins, are the site of distribution for oxygen-rich blood and absorption of waste to be carried away by the veins (Morel 1950). Veins are considered a capacitance vessel and have a blunted pressure-volume relationship. Veins serve as the volume reservoir to retain a relatively constant volume of the blood.

In hemostasis, the vessel's role depends on its structural dynamics (Figure 1). The primary vasoconstriction substance released during injury is thromboxane A2 (Gryglewski, Dembínska-Kieć, and Korbut 1978). Thromboxane A2 is released by activated platelets and is responsible for most of the vascular contraction. However, other factors like ADP can also lead to smooth muscle contraction (Wihlborg et al. 2004). These factors along with other platelet agonists will be discussed in the platelet section.

Role of coagulation in hemostasis

While primary hemostasis requires vessel constriction and binding of platelets to the injury site, it is the coagulation cascade, or secondary hemostasis, that regulates the stabilization of thrombus growth (L Shen 1983; Champy 1952). Coagulation is the process by which the blood changes from a liquid to a solid, gel-like state(Lister 1891). Fibrinogen and other coagulation factors found in the plasma secure the platelet plug through the deposition and maturation of fibrin that solidifies platelet-platelet contacts (Palta, Saroa, and Palta 2014). This process requires coagulation factors or clotting factors that respond in a complex cascade to form fibrin strands that strengthen the integrity platelet plug to prevent dissociation for the injury site (Palta, Saroa, and Palta 2014). Two distinct pathways are part of the coagulation cascade and converge onto one common path

(Mackman 2009). The extrinsic pathway has a cell-associated tissue factor component while the intrinsic pathway is initiated by collagen exposure following vascular damage. The common pathway consists of the plasma components that lead to thrombin activation from prothrombin. Cross-linked fibrin is the final product of coagulation regardless of which pathway is initiated. Fibrin is produced when activated thrombin cleaves fibrinogen, and crosslinking occurs through the activation of Factor XIII (Nahrendorf et al. 2006; Ádány, Ph, and Sc 1996; Komanasin et al. 2005; Lee et al. 2000).

In order to determine where thrombin generation occurs during *in vivo* thrombi formation, studies using antibodies that target either fibrinogen or fibrin have been used to localize the final product of the coagulation cascade (Stalker et al. 2013). These studies suggest that fibrin formation only occurs within the interior region of a growing thrombus but is required for initial thrombus formation (Stalker et al. 2013). Moreover, these studies suggest that thrombin is localized within the core region of a growing thrombus. Therefore, it is likely that coagulation is occurring directly at the site of injury and not necessarily throughout the entirety of the thrombus (Figure 3). These observations suggest the spatially and temporally regulated exposure of platelet to varying agonist within the thrombus structure.

Role of platelets in hemostasis

Platelets, also called thrombocytes, stop bleeding by binding at sites of vascular injury (Figure 1). Platelets are small, anucleated, cellular fragments that are derived from megakaryocytes. The megakaryocyte produces pro-platelets while lodged in the lung capillaries and shed cellular fragments that become platelets (Machlus and Italiano 2013).

Platelets are produced in a blebbing process, that resembles soap bubbles blown on a windy day (Machlus and Italiano 2013). Therefore, platelets are not technically cells but rather small collections of cellular components, or cellular dust, approximately 1-3μm in diameter (Machlus and Italiano 2013). Platelets reside in circulation for about 7-10 days in a human and between 4-6 days in a mouse (Tsakiris et al. 1999; Ware 2004). To be cleared from circulation, platelets are degraded following recognition by macrophages found in the liver and spleen (R. Li, Hoffmeister, and Falet 2016). Platelets are found only in mammals, whereas other animals such as birds and amphibians have circulating mononuclear cells (Levin 1997).

Studying platelets allows for a unique perspective into cell biology because they do not have a typical nucleus. However, platelets do contain transcripts that might relate to other physiological or pathological conditions (Harrison and Goodall 2008; McManus and Freedman 2015). An entire field has emerged from the discovery that platelets contain precursor mRNA and have the ability to translate mature mRNA into protein (Schubert, Weyrich, and Rowley 2014; Rowley, Schwertz, and Weyrich 2012; Stritt et al. 2015). These studies elucidate the complex and dynamic interaction that platelets within their environment.

In an attempt to simplify the role of platelets in hemostasis, platelet function can be divided into three key components: adhesion, activation, and aggregation (Baker-Groberg et al. 2016; Hom et al. 2016). The binding and activation of platelet to the injury site is the initial step to maintain CVS integrity. These functions are dependent on one protein in particular, the αIIbβ3 integrin, which is described in detail later in this chapter. Additionally, later stages of platelet activation lead to aggregation due to platelet-platelet

fibrinogen bridge formation mediated through this key integrin (Bodary, Napier, and McLean 1989; Versteeg et al. 2013; Bonnefoy et al. 2001).

In a recent paper published by Stalker et al. (Stalker et al. 2013), a combination of approaches was used to explore the dynamics of the platelet signaling network. They demonstrated a tiered organization of *in vivo* clot structure consisting of an inner core of fully-activated, P-selectin expressing, platelets overlaid with an unstable shell of non-fully-activated platelets retained on the surface of the thrombus. It is critical that experiments examining platelet activation do so in the context of the platelet activation *in vivo* in order to increase the physiological implication of platelet research. Below, I will discuss four key aspects of platelets: platelet agonists and signaling, shape change, granule exocytosis, and finally integrin activation. Most importantly, all of these elements will be positioned within the hierarchical structure of *in vivo* clot formation (Figure 3).

G-proteins, GPCRs, and platelets

Most of the platelet receptors that have be discussed are G-protein coupled receptors. G-protein coupled receptors (GPCRs) are the largest family of transmembrane receptors, which transduce extracellular signals into intracellular responses. All GPCRs share basic structural motifs, the best-known of these being seven hydrophobic transmembrane domains that adopt an alpha-helical conformation, giving the receptors the alternative name "7-transmembrane receptors". GPCRs bind to their extracellular ligand and change conformations to activate G-proteins (Figure 2).

GPCRs are guanine nucleotide exchange factors (GEFs). After the agonist binds to the GPCR, a complex consisting of agonist, receptor, and heterotrimeric G-protein is

formed. When all three of these components are bound, nucleotide exchange occurs. This has been demonstrated using the crystal structures of β2 adrenergic receptor as a model for GPCR signaling (Rasmussen et al. 2011). The heterotrimeric G-proteins consist of an α subunit that contains a GTP-binding domain and a βγ subunit. When it is in the inactive confirmation the Gα subunit is bound to GDP. When activated by a GPCR, GDP is exchanged for GTP and the G-protein acquires and activate conformation (Yeagle and Albert 2003; Siekhaus and Drubin 2003). The other half of the heterotrimeric G-protein consists of the bound β and γ subunits which has distinct signaling capacities including the activation of PI_3K and PLD (Preininger et al. 2006). A structure of the G-protein β γ dimer was published using multiwavelength anomalous diffraction data and computational approaches (Sondek et al. 1996). This structure demonstrated that the β subunit contains a seven-blade β-propeller that is surrounded by the γ subunit. The βγ subunit can regulate a diverse array of effects including ion channels and enzymes(Ford et al. 1998). The organization of the β subunit account for different regions that interact with specific effectors (Ford et al. 1998). These regions overlap with α subunit interaction site. Therefore dissociation is crucial for signal transmission through the βγ subunits. There is a substantial effort in the lab of Dr. Hamm to elucidate alternative roles of the βγ subunit including its interactions with the SNARE protein for the secretion of granules (Blackmer et al. 2005; Zurawski et al. 2016; Yim, Betke, and Hamm 2015).

The type of signaling initiated by the G-protein is based on the α subunit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ as illustrated in Figure 2. $G\alpha_s$ activates adenylate cyclase, leading to cAMP production and the activation of PKA, Ras, and Rap small GTPases. $G\alpha_{i/o}$ signaling inhibits adenylate cyclase and reduces cAMP levels, while also activating Rap1 and c-Src. $G\alpha_q$

activates PLC-β which causes the production of phosphatidylinositol and DAG production. $G\alpha_{12/13}$ can activate Rho leading to activation of the MAPK pathway and initiating platelet shape change. Additionally, phosphatidylinositol activates IP_3 receptors on the endoplasmic reticulum leading to increases in intracellular Ca^{2+} levels. Finally, the Gβγ subunit can also lead to activation of PI_3K and the downstream activation of Rac1 and CD42 (Touhara et al. 1994; Sondek et al. 1996; Woulfe et al. 2002).

Platelet agonists and signaling

Platelet agonist can be characterized based on the strength of the subsequent platelet activation (Figure 2). One of the strongest platelet agonists *in vivo* is thrombin after it is activated following cleavage in the final step of the coagulation cascade (Figure 1) (Palta, Saroa, and Palta 2014). A fundamental functional role of thrombin is activation of the protease-activated receptor (PAR) family found on platelets (Kahn et al. 1998; Covic, Gresser, and Kuliopulos 2000; M. Duvernay et al. 2013). This family includes PAR1 and PAR4 in humans and PAR3 and PAR4 in mice (Weiss et al. 2002). The mechanism by which PARs are activated is unique, in that an extracellular cleavage event on the exterior portion of the receptor is transduced into an intercellular signaling cascade. In this sense, thrombin does not act like a typical ligand, and instead cleaves the receptor's amino-terminal exodomain at the Arg41/Ser42 peptide binding site within the sequences LDPR41/S42FLLRN (J. Chen et al. 1994). It has also been suggested that intramolecular tethered ligand-ing between receptors can occur (J. Chen et al. 1994; Vu et al. 1991).

Thrombin can activate platelets at a concentration as low as 0.1nM. Within seconds of thrombin addition, the cytosolic calcium (Ca^{2+}) concentration in the platelet increase tenfold (Obydennyy et al. 2016). While these receptors are similary activated by peptide

products following thrombin cleavage, PAR1 and PAR4 have very different functional roles mediating platelet activation (M. Duvernay et al. 2013). In order to examine the functions of individual PARs, specific peptides have been developed to mimic the cleaved product (Bernatowicz et al. 1996). However, the synthetic peptides display considerably reduced efficacy as compared to thrombin, suggesting possible alternative mechanisms underlying the high potency of platelet activation seen with thrombin. Both PAR1 and PAR4 bind to $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$ differences in signaling exist (Holinstat et al. 2006; M. Duvernay et al. 2013; Muehlschlegel et al. 2012; Hirano et al. 2007). Increases in platelet cytosolic Ca^{2+} lead to activation of PLA_2 (Yoda et al. 2014). Phospholipase A2α (cPLA2α) may play a significant role in platelet function by producing a novel eicosanoid in response to platelet activation that represents a large component of PAR4- and GPVI-mediated responses (M. T. Duvernay et al. 2015). Thrombin also activates Rho, leading to actin cytoskeleton rearrangement and shape change that are linked to $G\alpha_{13}$ (Huang et al. 2007). Finally, thrombin also either directly or indirectly causes inhibition of adenylyl cyclase activity. Furthermore, several interesting relationships between PARs and other platelet receptors exist. For instance, although purinergic receptor P2Y G protein-coupled 12 ($P2Y_{12}$), is thought to act downstream of PARs to amplify platelet activation, $P2Y_{12}$ has been shown to have a greater impact than PAR signaling on hemostasis in models of arterial thrombosis (I. Cornelissen et al. 2010).

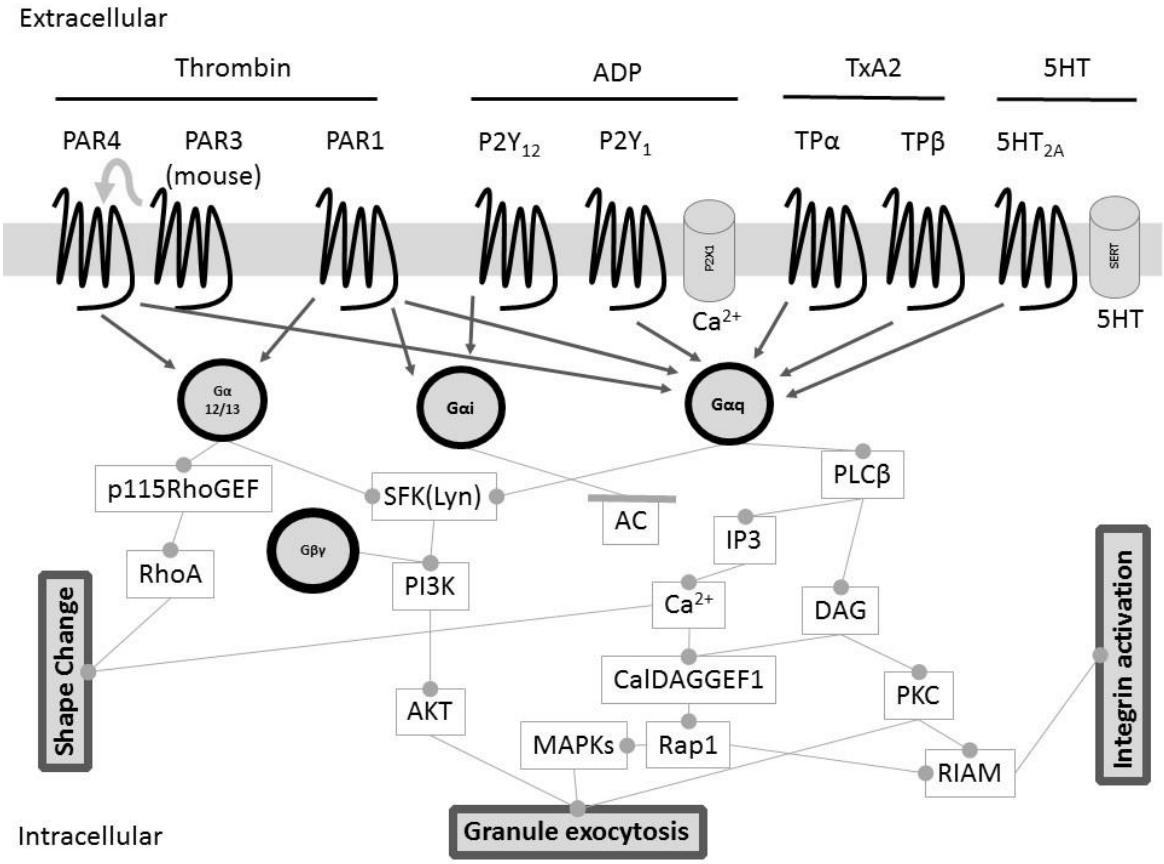


Figure 2. ***GPCRs involved in platelet activation.*** *Shown are a number of G-protein coupled receptors (GPCRs) that play a critical role in platelet activation. These proteins are activators of heterotrimeric G-proteins that can signal to downstream effectors as shown. Activation of these downstream effectors leads to cellular responses including shape change, granule exocytosis, and integrin activation.* (Offermanns 2006)

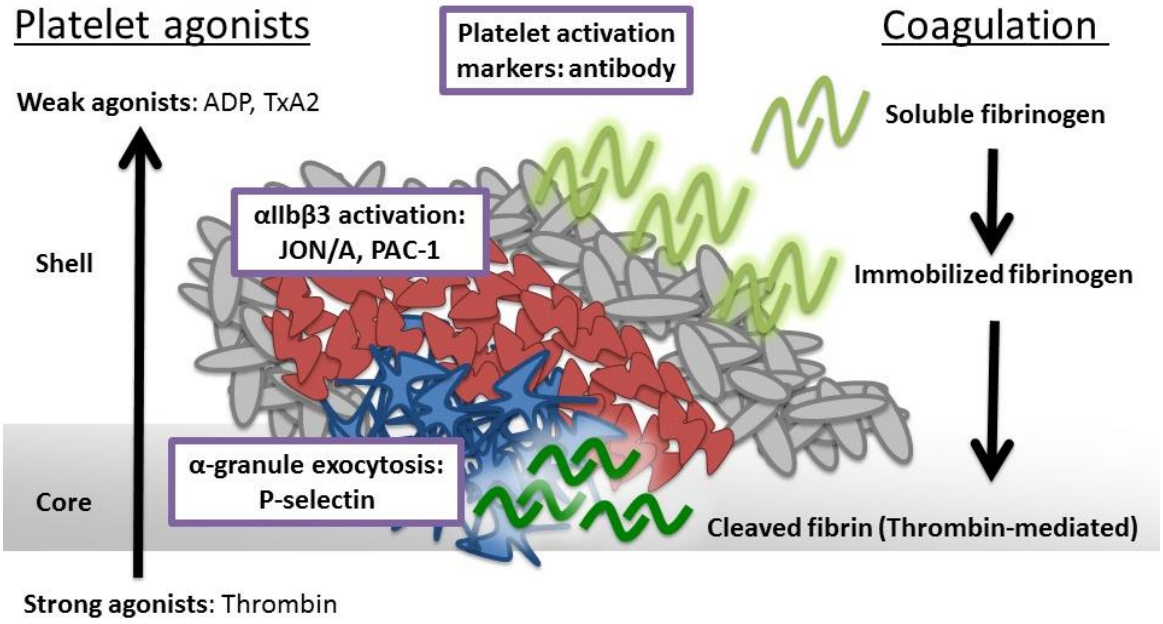


Figure 3. ***Clot structure and hierarchy.*** *The platelet plug (clot, thrombus) can be separated into regions based on component gradients. Regarding platelet agonists, strong agonists such as thrombin are important in forming the inner region or core of the clot. Weaker agonists like ADP and thromboxane A2 are important in mediating the size of the outer shell region of the platelet plug. Extracellular proteins like fibrinogen also can be divided into distinct regions within the clot structure. Fibrinogen is found in the outer area of the platelet plug but is only converted into fibrin in the final stages of the coagulation cascade by thrombin within the core region. The clear distinctions between different sections can be mirrored based on platelet activation markers: αIIbβ3 activation occurs through the clot while α-granule exocytosis is primarily found within the inner core region.* (Stalker et al. 2013)

It has been well established that PAR signaling plays a significant role in thrombosis (Weiss et al. 2002). However, a very complicated relationship exists between receptor interactions and their resulting physiological consequences. PAR1 is a high-affinity thrombin receptor on platelets and the target of the antiplatelet drug vorapaxar (Stalker et al. 2013; Friedman et al. 2016). While PAR3 is typically considered as only a cofactor of PAR4 activation in mice, knockout of PAR3 demonstrated disruptions in hemostasis *in vivo* (Weiss et al. 2002). PAR inhibition is an active area of research being

explored for the prevention or treatment of thrombosis in humans (Holinstat et al. 2006; M. Duvernay et al. 2013; Friedman et al. 2016; M. T. Duvernay et al. 2015).

Regarding the hierarchical organization of a thrombus or clot, thrombin is only produced within the core or interior region of a clot, close to the site of injury (Stalker et al. 2013). Thrombin is the primary effector protein at the end of the coagulation cascade. Studies suggest that thrombin is crucial for initiating thrombus formation and determining the core region of a mature thrombus, whereas weaker agonists ADP and thromboxane are important for mediating the size of the outer shell (Stalker et al. 2013; Woulfe et al. 2004; Sakurai et al. 2015; Versteeg et al. 2013).

The full dynamics of thrombin platelet activation remain complex and unresolved. Platelet activation by thrombin, eliminated by knocking out PAR4 in mouse platelets, has been shown unnecessary for the initial accumulation of platelets at or near the vessel wall (Jackson 2007; Davì et al. 1994; Kulkarni and Jackson 2004; Vandendries et al. 2007). Additionally, limited accumulation of platelets and platelet-independent mechanism(s) of thrombin generation are sufficient for normal fibrin deposition (Vandendries et al. 2007). Further work is necessary to unmask the complex and intricate role of the *in vivo* dynamics of thrombin activation in platelets.

The ADP receptors expressed on platelets are $P2Y_1$ and $P2Y_{12}$ as well as the ion channel $P2X_1$. $P2Y_1$ is a $G\alpha_q$-coupled receptor while $P2Y_{12}$ is a $G\alpha_i$-coupled receptor. Together, these two receptors can also signal through $G\alpha_{12/13}$. $P2X_1$ is a ligand-gated Ca^{2+}-channel that leads to an increase in intracellular Ca^{2+} once activated. Although ADP activates both $P2Y_1$ and $P2Y_{12}$ receptors independetly, dual activation is required for full ADP-mediated effects (Malyszko et al. 1994). While ADP is able to inhibit cAMP

formation through $P2Y_{12}$ alone, its ability to cause an increase in cytosolic Ca^{2+}, shape change, and aggregation is substantially impaired when $P2Y_1$ is blocked. $P2Y_1$ receptors have been shown to activate the Rac effector, P21-activated kinase (PAK), but $P2Y_1$ is not coupled to Gαi family members (Soulet et al. 2005; Woulfe et al. 2002; Xiang et al. 2012). When $P2Y_{12}$ activation is blocked with antagonists, ADP still elicits shape change and PLC activation but is unable to inhibit cAMP formation. However, loss of $P2Y_{12}$ activation impairs normal aggregation in response to ADP (Wihlborg et al. 2004; Tseng et al. 2013; Soulet et al. 2005; Woulfe et al. 2002; Ohlmann et al. 2013).

ADP is stored in platelet dense granules and is released upon platelet activation (Packham and Rand 2011). Platelet activation via ADP receptors reaches approximately half-maximal activation around 1μM ADP as assessed by shape change, aggregation, TxA_2 production, and release of granule contents (Z. Li et al. 2010). 250,000 platelet /μL contains approximately 3-4μM for ADP based on the measurement of serum following platelet activation (Packham and Rand 2011). However, the plasma concentration of ADP is very low before platelet activation (Malyszko et al. 1994).

One of the weakest platelet agonists is serotonin (5HT; 5-hydroxytryptamine). Serotonin activates the $5HT_{2A}$ receptor on platelets as well as being sequestered and stored primarily in platelet dense granules once it reaches the circulation via the serotonin transporter (SERT) (Zeinali et al. 2013; Namkung, Kim, and Park 2015). How serotonin fits into the platelet activation hierarchy is not well understood. However, it is clear that both ADP and serotonin each independently contribute to myosin light chain phosphorylation and platelet shape change (Bismuth-Evenzal et al. 2012a; Joseph E. Aslan et al. 2012; Paul, Daniel, and Kunapuli 1999; Tseng et al. 2013; Steiner, Carneiro, and

Blakely 2008). As both $5HT_{2A}R$ and $P2Y_1$ are bound to Gαq, one could reasonably predict that the Gαq pathway is crucial of platelet shape change. However, it has been demonstrated that platelets lacking the α-subunit of $G\alpha_q$ are still able to undergo shape change (Woulfe et al. 2004). This activation pathway results in the phosphorylation of MLCK in platelet in a non-$G\alpha_q$-dependent manner. These findings suggest that alternative signaling pathways downstream of Rho/Rho kinase are sufficient to mediate platelet shape change (Klages et al. 1999; Offermanns and Offermanns 2013; Glatt et al. 2004). Alternatively, novel signaling pathways downstream of $5HT_{2A}R$ activation could play an important component in platelet activation.

In hemostasis, modulating the growth of the outer shell region determines the balance between proper platelet recruitment to a growing thrombus versus over recruitment of circulating platelets resulting in vascular occlusion (Stalker et al. 2013; Woulfe et al. 2004; Woulfe et al. 2002). Because of this, targeting weaker agonists allows for a more subtle modulation of platelet activation as compared to thrombin. The growth of the shell region is known to be mediated by weak agonists like ADP and TxA2, as shown using *in vivo* imaging methods (Stalker et al. 2013). Therefore, one would predict that serotonin is also important for determining the shell size, however this has not be determined. Additionally, while serotonin may mediate platelet shape, from filopodia and lamellipodia extensions that increase platelet surface area and contact points, it is not clear how this process translates to *in vivo* platelet accumulation (Joseph E. Aslan et al. 2012). Further research is required to determine how serotonin modulates platelet activation within the context of the platelet agonist hierarchical organization.

Platelet shape change

While they do not have a nucleus, platelets contain other cell organelles and microtubules which hold the platelet in a crisp, discoid shape (Agbani et al. 2015; Antkowiak et al. 2016). Following platelet activation the shape of the platelet changes drastically (Soulet et al. 2005; Kuwahara et al. 2002; Paul, Daniel, and Kunapuli 1999; Huang et al. 2007; Z. Li et al. 2010). Platelets have internal membrane invaginations, referred to as the open canalicular system (White and Conard 1973). These invaginations provide expanded signaling surface that can be used during platelet shape change and spreading (Golebiewska and Poole 2014).

The stages of platelet shape change can be divided into three steps (Figure 4). First, platelet rolling on a surface is followed by formation of a firm platelet adhesion on the exterior of a growing clot (Kuwahara et al. 2002). Platelet rolling is mediated by the glycoprotein IB-IX (GPIb-IX) when it binds immobilized von Willebrand Factor (vWF) on the thrombus (Z. Li et al. 2010). Stabilization via αIIbβ3 activation mediates platelet-platelet adhesions and stabilizes platelet focal adhesions through clustering (Dormond et al. 2004; Delaney et al. 2012; J E Aslan and McCarty 2013). Shape change following platelet clustering and integrin outside-in signaling occurs through both Ca^{2+}-dependent and Ca^{2+}-independent pathways (Paul, Daniel, and Kunapuli 1999). Ca^{2+} and calmodulin-stimulate myosin light chain kinase (MLCK) and p160 Rho-associated coiled-coil-containing protein kinase- kinase and cofilin (Randriamboavonjy et al. 2012; Irina Pleines et al. 2012).

Agonists such as ADP, TxA_2, and serotonin play important roles in enhancing the platelet activation and spreading in both a paracrine and autocrine fashion (Z. Li et al.

2010). $G\alpha_q$ and $G\alpha_{13}$ pathways are imperative to alterations in platelet function involving the actin cytoskeleton (Flaumenhaft et al. 2005; Tadokoro et al. 2011; N. T. Thompson, Scrutton, and Wallis 1986). Ca^{2+}-dependent activation of myosin light chain kinase downstream of $G\alpha_q$ family members and activation of Rho family members downstream of $G\alpha_{13}$ have a significant implication on platelet shape change and spreading (J E Aslan and McCarty 2013; Soulet et al. 2005). Gα-containing domains and GEF domains can link $G\alpha_{12}$ family members to Rho family members, including p115RhoGEF. $G\alpha_{12/13}$ activation regulates platelet shape change through linking receptor mediated activation to Rho/Rho-kinases and myosin light chain phosphorylation (Klages et al. 1999). It has been shown that $G\alpha_{13}$-dependent Rho activation leads to shape change via pathways that include the Rho-activated kinases (p160ROCK) and LIM-kinases (Gohla, Harhammer, and Schultz 1998). Through these pathways, platelet shape change and spreading occurs and may contribute to the retention of platelets at the thrombus surface.

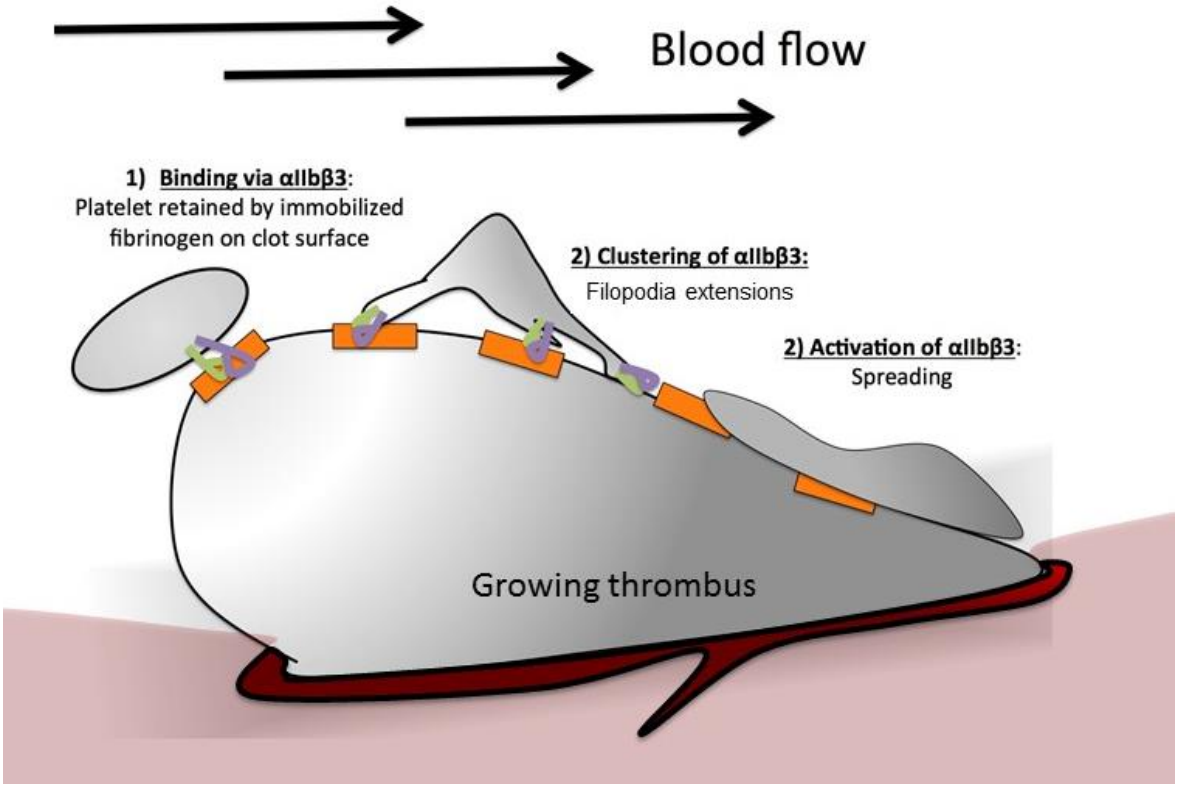


Figure 4. ***Stages of platelet recruitment to a platelet plug****. Platelet attachment and recruitment to a platelet plug can be divided into three distinct phases. Initial binding to the platelet is mediated by binding of integrins including αIIbβ3 and GPIb. The accumulation of platelets leads to an increase in clot stability that occurs after clustering of integrins creating a signaling complex. Finally, the platelet begins to spreading, increasing its surface area and point of contact to the growing clot surface.*

Granule exocytosis

One of the main properties of platelet activation is the exocytosis of dense granules, α granules (Figure 5), and lysosomes (Flaumenhaft 2012b; Flaumenhaft et al. 2005; Rendu and Brohard-Bohn 2001). In total there are over 300 different components that are released following platelet activation (Golebiewska and Poole 2014). Within the α-granules, P-selectin, fibrinogen, fibronectin, Factor V, Factor VIII, Platelet Factor IV, Platelet-Derived Growth Factor (PDGF), and tumor growth factor- α are all found (Palta, Saroa, and Palta

2014; Heemskerk, Bevers, and Lindhout 2002). In contrast, the dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), Ca^{2+}, serotonin, histamine, and epinephrine (Palta, Saroa, and Palta 2014; Heemskerk, Bevers, and Lindhout 2002). The release of these components promotes platelet activation in both an autocrine and paracrine fashion. The release of platelet granular contents is both spatially and temporally regulated to ensure appropriate platelet responses within the thrombus microenvironment (Kuwahara et al. 2002; Stalker et al. 2013; Jackson 2007).

α-granules

The content of the α-granules are sorted by the megakaryocyte after the proteins are synthesized in the endoplasmic reticulum, exported to the Golgi for maturation and subsequently, and stored in the trans-Golgi network (Cramer et al. 1989). This content includes both membrane bound proteins that become expressed on the platelet surface and soluble proteins that are released into the extracellular space. Some of the membrane-bound proteins include integrins as well as immunoglobulin family receptors like GPVI, Fc receptors, and PECAM (Maynard et al. 2007; Niiya et al. 1987; P. Nurden et al. 2004). Additionally, tetraspanins and other receptors like CD36 ad Glut-3 are found in platelet α-granules (Maynard et al. 2007; G. Berger et al. 1993; Suzuki et al. 2003). Some proteins are only expressed on the surface following α-granule exocytosis, like P-selectin (Maynard et al. 2007). In addition to the vast array of membrane proteins, there are also hundreds of soluble proteins released by platelets, including vWf (Cramer et al. 1988), coagulation factors such as Factor V, XI, XIII, and antithrombin localized with the α-granule (Rendu and Brohard-Bohn 2001; Hayward et al. 1995; Kiesselbach and Wagner 1972).

α-granule exocytosis is believed to be an irreversible step in platelet activation, and these granules are the most abundant within the platelet (Golebiewska and Poole 2014; Wolfgang Bergmeier et al. 2002). In order to measure platelet activation, we can examine surface binding of the α-granule component P-selectin using an anti-P-selectin antibody that is tethered to a fluorescent probe (Wolfgang Bergmeier et al. 2002). P-selectin is a surface protein that helps to recruit immune cells to a growing clot and is only expressed on the surface of the platelet following α-granule exocytosis.

Disorders that have reduced or absent α-granules include ARC (arthrogryposis-Renal Dysfunction- Cholestasis Syndrome) or GPS (Grey Platelet Syndrome) leads to significant clotting deficiencies (A. Nurden and Nurden 2011). Both of these granules are produced from multivesicular bodies or late endosomes following budding from the megakaryocytes (Ambrosio, Boyle, and Di Pietro 2012; Machlus and Italiano 2013).

Dense granules

Serotonin is found in platelet dense granules along with ADP, histamine, and other small molecule platelet activators (Meyers, Holmsen, and Seachord 1982). Experimentally, dense granule exocytosis has typically been measured by pre-incubating the platelets with radiolabeled serotonin and measuring the release of the radiolabeled serotonin during platelet activation (F. Cerrito et al. 1993a; Giuliana Gobbi et al. 2003). ATP release, which is also found in dense granules, can also be measured using a fluorescent probe in an aggregometer. Very little is known about the context of dense granule release during *in vivo* thrombus formation. Future work is required to localize and clarify the release of dense

granule content during different stages of platelet activation. Disorders that have reduced or absent dense granules include Hermansky, Pudlak, and Chediak-Higashi Syndromes.

The localization of the granular factors within the thrombus hierarchical organization suggest distinct release triggers of α- and dense granules (Stalker et al. 2013; Versteeg et al. 2013; Sakurai et al. 2015). The heirarchical structure occurs based on the exposure of the injury site, the diffusion of platelet agonists and the activated enzymes produced via the coagulation cascade as well as a flow based mechanical stress that is exerted on the clot (Figure 3). For weaker platelet agonists, ADP and TxA_2 activation modulate the growth of the outer shell region, while stronger platelet agonists like thrombin are essential for mediating the initiation and size of the inner core (Stalker et al. 2013). These findings could suggest that dense granules are initially released during platelet rolling and adhesion or weak agonist activation as compared to α-granule exocytosis which occurs only within the inner core. This hypothesis is supported by the differential regulation of α- and dense- granule secretion (Flaumenhaft 2012a; Flaumenhaft 2012b; Flaumenhaft et al. 2005). However, further work is needed to understand the spatial and temporal release of α- versus dense platelet granules.

Integrin function and activation: Aggregation

The properties of platelets to bind and form a thrombus was initially described over 100 years ago (Zahn 1874) . Platelets are recruited and retained to the growing thrombus via a process called aggregation. Traditionally, platelet aggregation was considered a straightforward process involving the noncovalent bridging of integrin αIIbβ3 receptors on the platelet surface with dimeric fibrinogen binding sites (O'Brien 1964; Malyszko et al. 1994; N. T. Thompson, Scrutton, and Wallis 1986; Jackson 2007). However, this process

also involves a number of other factors exposed using microfluidic flow-based studies (Kuwahara et al. 2002). These studies have demonstrated novel mechanisms in platelet adhesion (mediated by VWF and collagen) as well as platelet aggregation (fibrinogen) (Figure 4).

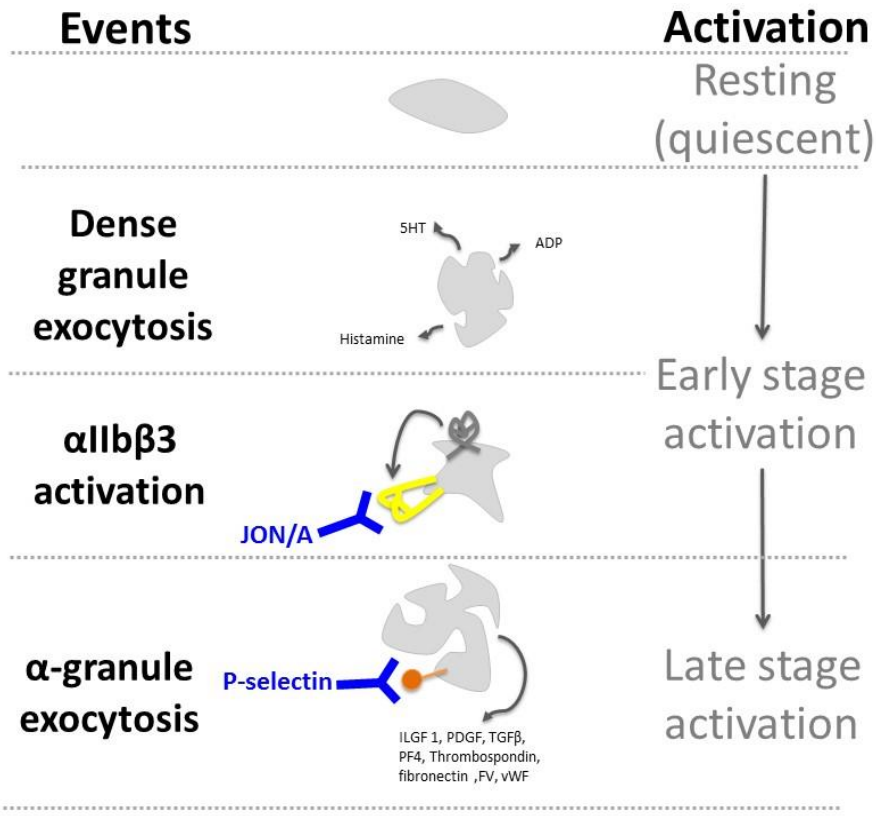


Figure 5. ***Platelet activation.*** *Platelet activation by release of dense granules can be separated into three distinct events and recognized by two standard platelet activation markers: JON/A (PAC-1 in humans) that recognize the activate confirmation of the αIIbβ3 integrin and P-selectin. First, dense granules are released which occurs during early stages of platelet activation. Later, α-granule exocytosis occurs leading to the exposure of P-selectin onto the platelet surface.*

Integrins are a unique family of noncovalently associated α and β transmembrane heterodimers that bridge extracellular ligands to intracellular signaling pathways (Bennett, Berger, and Billings 2009; Tadokoro et al. 2011; Gibbins 2004; Oliver et al. 2014; Jackson 2007). Platelets contain five different types of integrins (Table 1). There is a total of three

β1 integrins that mediate platelet adhesion to the matrix protein collagen, fibronectin, and laminin including α1β1, α2β1, and α7β1 (Clemetson and Clemetson 2001). Additionally, there are two β3 integrins, αVβ3, and αIIbβ3 (Bennett, Berger, and Billings 2009). The nomenclature for platelets is described in Table 1. Integrins bind to extracellular matrix proteins such as collagen, laminin, fibronectin, and fibrinogen and are essential for platelet aggregation.

Common name	**Platelet protein composition**	**Compos.**	**Ligands**	**Number of surface receptors per platelet (assuming a platelet surface expression of 22.2μm^2)**
Collagen receptor	Ia/IIa (VLA-2)	α 2 β1	Collagen	~1,000
Fibrinogen receptor	Ic/IIa (VLA-5)	α 5 β 1	Fibronectin	~1,000
Laminin receptor	Ic/IIa (VLA-6)	α 5 β 1	Laminin	~1,000
Vitronectin receptor	αv/IIIa	α v β 3	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin	~100
Fibrinogen receptor	GPIIb/IIIa	α IIb β 3	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin	~50,000

Table 1. ***Integrins found on platelets.*** *Listed are all the integrin subtypes found in platelets along with their platelet protein composition name, their ligands. Based on the number of receptors, there is a vast amount of αIIbβ3 integrin as compared to other subtypes and are vital in initiating and propagating platelet activation. Adapted from* (Coller 1992).

Integrins undergo two distinct signaling processes: inside-out activation and outside-in activation (Tadokoro et al. 2011; Oliver et al. 2014; Z. Li et al. 2010). Inside-

out signaling refers to the binding of proteins to the intracellular side of the leading to phosphorylation of the β-tail region. αIIbβ3 undergoes a conformational switch that "opens" the ligand binding pocket. Inside-out signaling requires that intracellular proteins bind to the intracellular side of the integrin and initiate a structural rearrangement on the extracellular surface. Once the integrin is in the active conformation, it binds the ligand and changes conformation to initiate "outside-in" signaling, or signaling that transduced following integrin ligand binding. Additionally, force exerted on the integrin based on substrate stiffness modulates the outside-in signaling processes (Qiu et al. 2014). While all integrins expressed on platelets play a role in platelet function, the αIIbβ3 integrin is the highest expressed integrin and is crucial for platelet function (Schaff et al. 2012; Bennett, Berger, and Billings 2009; Gibbins 2004).

Inside-out activation of the integrin is primarily mediated through talin binding to the inactive state of the αIIbβ3 integrin on the cytosolic side of the plasma membrane (Figure 6). Platelets attach or adhere to exposed extracellular matrix proteins outside the injured vasculature including the basement membrane. The adhesion of platelets is an essential function in response to vascular injury that is required for proper plug formation. Talin activation of αIIbβ3 is mediated through calpain, Rap1, PIP2 and RIAM binding and leads to a separation of the tail regions of the integrin inducing a conformational change on the extracellular side of the integrin (Oliver et al. 2014; Legate and Fässler 2009). This seperation of the tail regions allows the integrin to bind its ligand, fibrinogen, and initiate outside-in signaling that propagates platelet activation (Z. Li et al. 2010; Oliver et al. 2014). The platelets integrin ligation mediates thrombus formation and outside in signaling, which requires $G_{\alpha 13}$ (B. Shen et al. 2013). This activation event allows for the formation of

platelet-platelet connection by bridging fibrinogen between two αIIbβ3 binding sites (aggregation) and greatly expands the thrombi.

There are a number of platelet signaling pathways that are essential for inside-out αIIbβ3 activation. Initiation of $G\alpha_q$ activation leads to phosphoinositide hydrolysis, cytosolic Ca^{2+} increases, and integrin activation (Z. Li et al. 2010). The major defects seen in $G\alpha_q^{-/-}$ platelets demonstrate the importance of Gαq signaling in integrin activation (Simon et al. 1997; Woulfe et al. 2004). However, additional experiments are needed to clarify this signaling pathway. Once initiated, the raising Ca^{2+} concentration in activated platelets can trigger integrin activation via the CalDAG-GEF/RAP1/RIAM pathway (Woulfe et al. 2002). αIIbβ3 can also be activated via PKC without increasing cytosolic Ca^{2+} (Xiang et al. 2012; Z. Li et al. 2010; Delaney et al. 2012). However, to form the strongest and irreversible platelet aggregation require combined activation of all three-$G\alpha_q$, and $G\alpha_i$, and $G\alpha_{12/13}$ G protein families (Simon et al. 1997; Klages et al. 1999).

To measure platelet inside-out activation on an intact platelet, there are antibodies that can be used that recognize the active conformation of αIIbβ3. In human platelets, PAC-1 can be used to measure αIIbβ3 activation on platelet in suspension (JON/A antibody for mouse platelets) (Tadokoro et al. 2011; Wolfgang Bergmeier et al. 2002). The αIIbβ3 integrin binds to the Arg-Gly-Asp (RGD) sequence of the fibrinogen A α chain. PAC1 was designed to recognize the platelet αIIbβ3 in a region that is only exposed following inside-out activation. Therefore, PAC-1 only measures αIIbβ3 integrin that are in their active conformation (Taub et al. 1989). CD41 and CD61 antibodies are also available and recognize the αIIb and β3 integrins, respectively. Because αIIbβ3 activation is a critical

step in aggregation, measuring the active conformation of the integrin, in addition to platelet spreading studies, allows for investigation into platelet aggregation *ex vivo*.

Outside-in activation refers to the sequence of protein-protein interactions that occur following ligand binding. Proteins that are capable of binding αIIbβ3 and initiating signaling following ligand binding include β3-endonexin, CIB1, talin, kindling, myosin, Shc, Src (Xiang et al. 2012), Fyn, and SyK (Z. Li et al. 2010; Delaney et al. 2012; Ana Marin D Carneiro et al. 2008; Oliver et al. 2014; Legate and Fässler 2009; Bennett, Berger, and Billings 2009; Woulfe et al. 2002). Both the inside-out and outside-in activation of the integrin is important in mediating full platelet spreading, irreversible aggregation and clot retraction (Ono et al. 2008; I. Pleines et al. 2012).

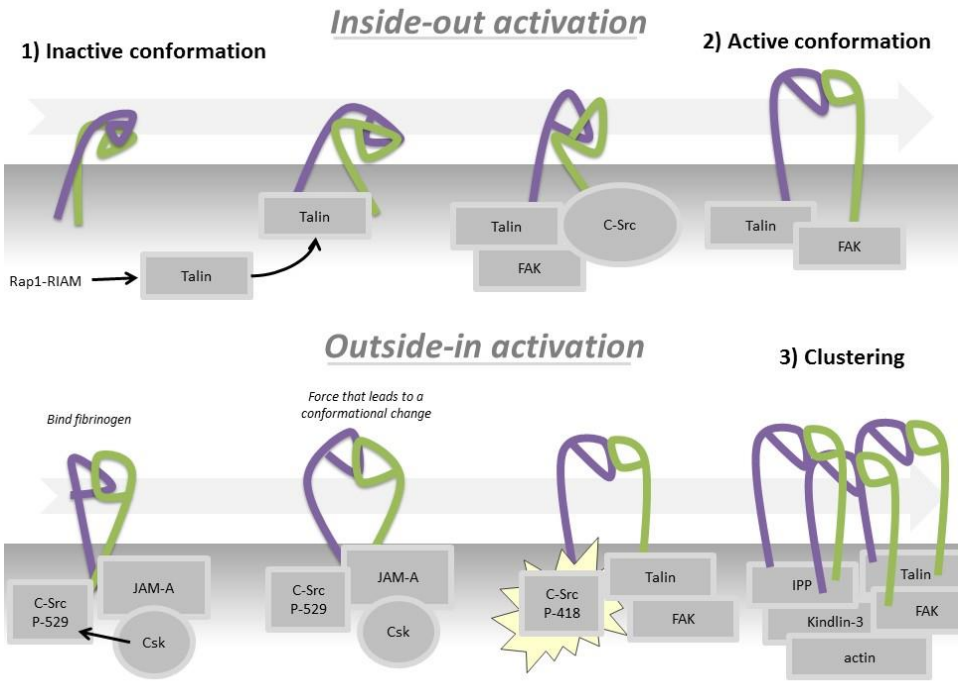


Figure 6. ***Integrin activation****. There are two distinct part to platelet activation. Initially, the integrin undergoes inside-out activation that is caused by the separation of the tail regions by binding of talin. The binding of talin and the separation of the integrin legs allows for activation of FAK and c-Src which can then be released to exert its action on other signaling pathways. Additionally, this intracellular action change the extracellular side of the integrin allowing for ligand binding. This process is distinct from outside-in activation where the integrin binds to a ligand and initiated a separate set of intracellular signaling events. In particular, the accumulation of activate integrins leads to integrin clustering and cytoskeletal rearrangement.*

Extracellular matrix proteins

There are multiple extracellular matrix proteins involved in platelet activation. Some of these include collagen, laminin, vitronectin, fibronectin, and fibrinogen (Table 2). Collagen in one of the first extracellular matrix components that the platelet will come in contact with following platelet injury (Clemetson and Clemetson 2001; Watson 2009). It is considered a relatively strong platelet agonist similar to PAR4-mediated thrombin activation (Bynagari-Settipalli et al. 2014). When PAR4 activation is lost, it uncovered large effects of GPVI deficiency (Bynagari-Settipalli et al. 2014). This observation implies that PAR4 and GPVI modulate platelet function independently, but have partially redundant contributions to occlusive thrombus formation in the carotid and hemostatic clot formation (Bynagari-Settipalli et al. 2014). At face value, these results suggest that thrombin- and collagen-induced platelet activation can play partially redundant roles, despite substantial and vast differences in how these agonists are made available to platelets.

Collagen is exposed at the injury site and binds to integrins that are present on the platelet surface. Binding of integrin to collagen leads to platelet recruitment to the wound site (Alberio et al. 2000; Yoshinaga et al. 1994). Von Willebrand factor (vWF) is also exposed with collagen at the injury site, leading to the recruitment and retainment of platelets initially following injury (Kuwahara et al. 2002; Delaney et al. 2012; A. Nurden and Nurden 2011). vWF is a glycoprotein that is found in the blood plasma. It is produced by the endothelium, megakaryocytes, and connective tissue. vWF binds to proteins including FVIII, collagen, and the platelet receptor GPIb in order to enhance platelet recruitment (Cramer et al. 1989; Weiss et al. 2009). Loss of vWF leads to bleeding

disorders and dysregulation of hemostasis (Rayes et al. 2010). Fibronectin may also play a significant role in platelet aggregation (Thurlow, Kenneally, and Connellan 1990). However, it is thought that fibrinogen is the dominant ligand supporting platelet aggregation, principally attributed to its high concentration in plasma relative to other αIIbβ3 ligands (Heemskerk, Bevers, and Lindhout 2002).

Fibrinogen is the major protein that the coagulation cascade cleaves following the activation of thrombin. Activation of thrombin leads to the cleavage of fibrinogen to form fibrin. The coagulation cascade also activates FXIII, a transglutaminase, that crosslinks fibrin (Hummerich et al. 2012; Bennett, Berger, and Billings 2009). The polymerized fibrin form a fibrin mesh that stabilizes and strengthens a platelet plug and prevents detachment. However, fibrinogen can be found in its free form circulating throughout the plasma (Sentí et al. 1998; Cramer et al. 1989). During thrombus formation, fibrinogen becomes entangled with the growing shell region of the clot. This is distinct from the inner region where thrombin is being produced and cleaves fibrinogen to form fibrin (Figure 3). Therefore fibrin is only found within the inner core region of a thrombus, but fibrinogen on the outer clot surface can recruit circulating platelets (Stalker et al. 2013). This suggests a hierarchical organization within the clot not only applies to agonists but also to extracellular matrix proteins. Platelet recruitment to a growing thrombus is based on the organization of both extracellular matrix proteins and agonists. Therefore investigating platelet function is physiological relevant contexts is crucial.

Extracellular matrix proteins	Localization
Fibrinogen	Platelets, plasma
Fibronectin	Platelets, plasma, endothelial basement membrane (BM), adventitum, smooth muscle cells, interstitium
Thrombospondin	Platelets
Vitronectin	Plasma
vWF	Platelets, plasma, endothelial BM
Laminins	Endothelial BM
Type IV collagen	Endothelial BM
Fibrillar collagens	Adventitium, interstitium
Other collagens	Adventitium, smooth muscles, interstitium

Table 2. ***Extracellular matrix proteins in the circulation system***. (W. Bergmeier and Hynes 2012). *There are many extracellular matrix proteins that play an important role in platelet function. The extracellular ligands and there localization in the hemostasis process is noted.*

Serotonergic signaling in platelets

Serotonin and the cardiovascular system

Serotonin was first discovered in the periphery for its ability to increase vascular tone ("ser (serum) tone (tone)") (Haddy, Gordon, and Emanuel 1959). However, since that time much research as concentrated on its role as a neurochemical transmitter that modulates neuronal processes including mood, sleep, and appetite (Best, Nijhout, and Reed 2010). In the periphery, serotonin is secreted from the enterochromaffin (EC) cells in the gastrointestinal tract (Dalglesh anf Dutton 1957). Once released into the plasma, serotonin is quickly taken up by platelets via the serotonin transporter (SERT) (Beikmann et al. 2013). When platelets become activated, they release serotonin and other factors (Bismuth-Evenzal et al. 2012a; Hara et al. 2011; Przyklenk et al. 2010a). The release of serotonin can alter the vascular tone, potentiate the blood clot growth through platelet amplification, and may play a role in coagulation (F. Cerrito et al. 1993a; Cloutier et al. 2012; Watts, Priestley, and Thompson 2009a; Hummerich and Schloss 2010). Below, the role of serotonin in the cardiovascular system will be explored.

Production and modulation of peripheral serotonin

EC cells are an enteroendocrine and neuroendocrine cell that lines both the lumen and the respiratory tract (Zeinali et al. 2013). Within the gastrointestinal tract, the EC cells are found in the stomach, small bowel and colon. These cells function is to produce serotonin by the rate-limiting enzyme tryptophan hydroxylase-1 (TPH-1) (Figure 7) (Dees et al. 2011; Dürk et al. 2013). There are two different TPH isoforms found in the body. One isoform is centrally located (TPH-2) while one is peripherally located (TPH-1)

(Amireault, Sibon, and Côté 2013). Serotonin is released in response to chemical, mechanical or pathological stimuli within the lumen of the gut (Rios-Avila et al. 2013). The release of serotonin leads to peristaltic reflexes and activate vagal afferents that are downstream of $5HT_3$ receptors signal to the brain leads to nausea via enteric neurons impulses (S. M. Stahl et al. 2013; M. Berger, Gray, and Roth 2009; Sherwin et al. 1986).

The serotonin produced by EC cells requires dietary tryptophan to be taken up by the amine transporters in the gut lumen (Figure 8) (Rios-Avila et al. 2013; Keszthelyi, Troost, and Masclee 2009). Tryptophan is then metabolized by the action of TPH -1 leading to the production of 5-hydroxy-L-tryptophan (5HTP) (Racke et al. 1996). The second step catalyzed by L-amino acid decarboxylase, which is also present in EC cells, produces serotonin. In EC cells, serotonin is packaged into granules via the vesicular monoamine transporter 1 (VMAT-1) and is then released on both the apical and basal end of the EC cell(Höltje, Winter, Walther, et al. 2003; Zeinali et al. 2013; Racke et al. 1996).

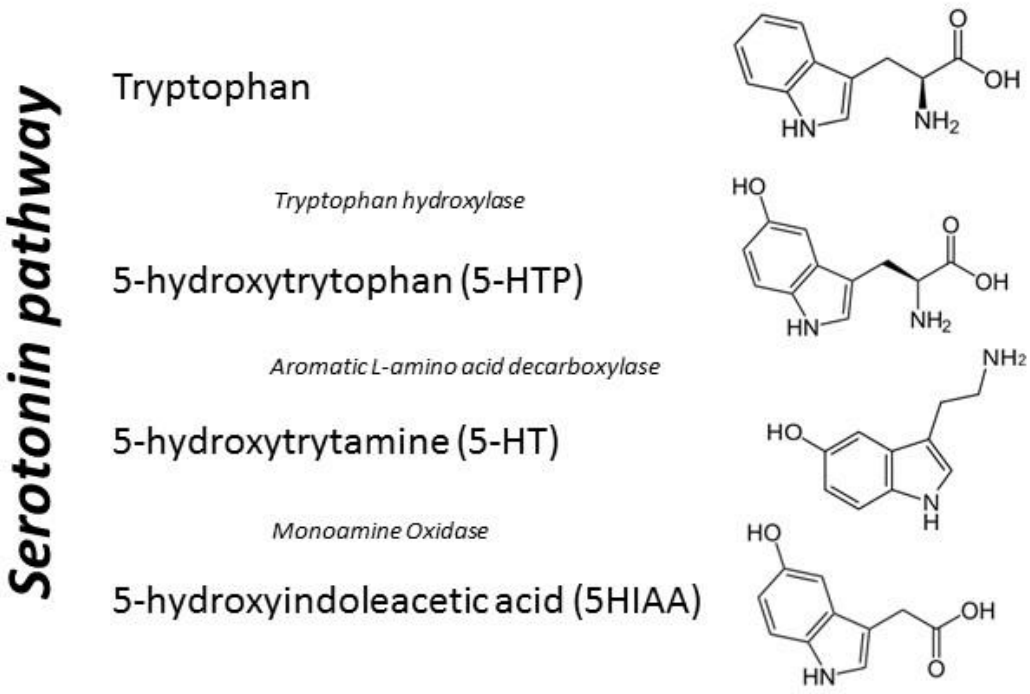


Figure 7. ***Serotonin production and metabolism****. Shown is the sequence of tryptophan conversion to serotonin with enzymes and structure.*

While further work is needed to clarify how serotonin enters the cardiovascular system, it is clear that platelets are the major storage site of serotonin within the blood (M. Berger, Gray, and Roth 2009; Apelseth, Nepstad, and Hervig 2012; Ziu et al. 2012; Hara et al. 2011; Dees et al. 2011). Serotonin that is not stored in platelets is quickly metabolized by the monoamine oxidase A (MAO-A) enzymes found in the liver (Figure 8) (Mészáros et al. 1998; Jernej et al. 2002). The liver is able to metabolize serotonin with extraordinary efficiency (Kim et al. 2005; Moore and Eiseman 2016). The initial step in serotonin metabolism is the decarboxylation by the enzyme L-aromatic amino acid decarboxylase. The majority of serotonin metabolism occurs through monoamine oxidase (MAO) (Figure 7). MAO-A is more selective for serotonin oxidation and metabolizes serotonin at a much

lower Km than MAO-B (Mészáros et al. 1998; Jernej et al. 2002; M. a Fox et al. 2007). However, platelets do express MAO-B in the portions of mitochondria that are within the platelet cell fragments (Mészáros et al. 1998). Therefore, the vast majority of serotonin measured in whole blood comes from the granular serotonin content found in platelets. This will be discussed in detail within the plasma section below.

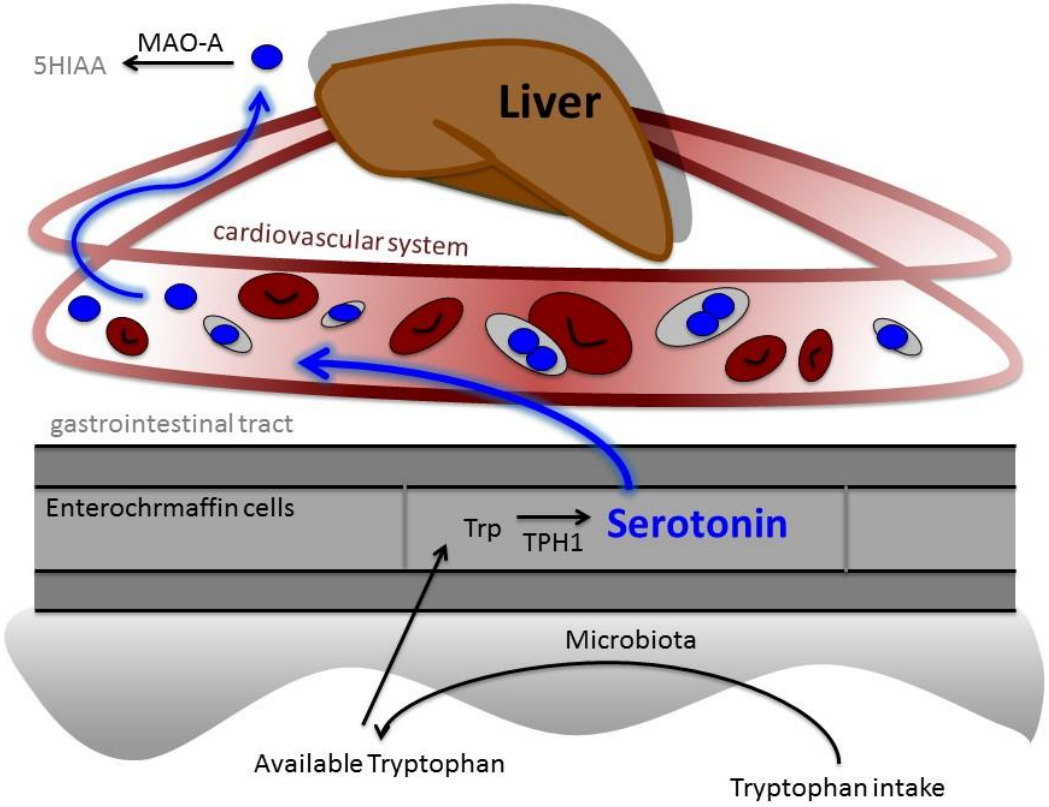


Figure 8. ***Serotonin in the blood****. Tryptophan is taken up via dietary input. The gut microbiota does metabolize some of the tryptophan from digestion that limits the amount of available tryptophan available for uptake. Once tryptophan is taken up by the enterochromaffin cells in the gastrointestinal tract, it is converted to serotonin as demonstrated enzymatically in Figure 7. Serotonin is then stored in granules where it is released and finds its way into the circulation. Once in the blood it is primarily stored in platelets. If serotonin remains in the plasma, it is quickly degraded by MAO-A liver metabolism.*

Cardiovascular function

The role of serotonin to induce vascular tone was initially described in the 1960s (Rapport and Virno 1952). Serotonin is also thought to play a major role in pathologies related to hypertension and cardiovascular disease (Hervé et al. 1995; Fraer and Kilic 2015; Brenner et al. 2007). The vasculature comes in contact with serotonin following release from platelets in both a pathological context and in normal hemostasis. Activated platelets release serotonin in substantial quantities causing vasoconstriction and recurrent aggregation of platelets (Dees et al. 2011; Heistad, Harrison, and Armstrong 1987; Watts, Priestley, and Thompson 2009b). Serotonin can also act as a growth factor leading to mitogenesis and migration of arterial smooth muscle cells (Dees et al. 2011). Serotonin promotes proliferation of vascular endothelial cells and directs endothelial injury in cell culture (Koba et al. 1999; Göoz et al. 2006). Physiologically, some studies suggest that serotonin is associated with coronary artery disease and the extent of the disease (Sanner, Frazier, and Udtha 2013). Serotonin can also lead to vascular permeability (Cloutier et al. 2012; Y. Li et al. 2016). While serotonin's ability to regulate vascular tone has been well established, serotonin can also potentially regulate plasma factors and platelets important for hemostasis.

Plasma serotonin

Serotonin, after it is synthesized in the EC cells in the GI tract, it is released into the plasma. The platelet serotonin represents the richest reservoir of peripheral serotonin (Beikmann et al. 2013; Toh 1956). However, it is only the extracellular or plasma serotonin that is able to signal to receptors. Extracellular or plasma levels of serotonin in virtually

absent are the plasma during non-platelet activating conditions, although this point remains controversial (Brand and Anderson 2011; G. M. Anderson and Cook 2016b).

Typically, serotonin in the plasma is around 100-200pg/mL while whole blood levels of serotonin are approximately 150ng/mL in healthy human samples, suggesting nearly a 1000-fold greater sequestration of serotonin in the platelet as compared to platelet poor plasma (G. M. Anderson and Cook 2016a; G. M. Anderson 2007). However, a wide range of values have been reported. Some have reported values in the 10-20ng/mL range (Bijl et al. 2015). The inconstancies seen in plasma levels, which may be derived from platelet and plasma purification techniques (Brand and Anderson 2011; G. M. Anderson 2007; G. M. Anderson and Cook 2016a).

Platelet function and serotonin

Acutely, serotonin can either act through the $5HT_{2A}$ receptors ($5HT_{2A}R$) or be taken up by the SERT. The acute uptake by SERT is distinct from the chronic loss of transporter function, which leads to loss of serotonergic tone. Chronic uptake by SERT is important in managing the extracellular versus intracellular (granular) serotonin concentration, and therefore serotonin hemostasis. When SERT is chronically blocked, serotonin whole blood levels are lost because serotonin metabolism is extremely efficient (Apelseth, Nepstad, and Hervig 2012; Malyszko et al. 1994; M. Berger, Gray, and Roth 2009). The acute action of SERT is suspected to play a role in platelet function (Ziu et al. 2012; Beikmann et al. 2013; Singh et al. 2013). Finally, activation of the $5HT_{2A}$ receptor, which is the only serotonergic receptor found on platelets, may also play a role in platelet function and activation(Miller, Mariano, and Cruz 1997; Przyklenk et al. 2010a; F. Cerrito et al. 1993a).

Aggregation and platelet serotonin

The effects of serotonin on platelet aggregation was initially recognized as early as the 1960's. In an article published by J.R. Obrien in 1964 in the Journal of Clinical Pathology entitled "A comparison of platelet aggregation produced by seven compounds and a comparison of their inhibitors", it was shown that "anti-serotonin" drugs were able to rescue the aggregation effects mediated by serotonin (O'Brien 1964). In this paper, they suggest that the administration of 50nM serotonin lead to minimal aggregation that was also reversible (O'Brien 1964). However, at 500nM-50μM more aggregation occurs in response to serotonin (O'Brien 1964). As compared to another agonist, serotonin was unique in that concentration of serotonin higher than 50μM lead to a reduced effect on platelet aggregation as compared to lower concentrations. This suggests a biphasic relationship between activation of serotonin and aggregation. It is important to note that all aggregation mediated by serotonin was relatively minor as compared to other agonists (O'Brien 1964).

One of the most interesting aspects of this study submits that a second administration of serotonin added either two minutes or thirty minutes post initial activation produced no additional activation response (O'Brien 1964). This would suggest that the mechanism by which serotonin initiated aggregation undergoes desensitization. If this were receptors, this would indicate that the initial administration of serotonin is able to cause desensitization of the receptor by preventing further signaling downstream of receptor activation or internalization of the receptor.

Initial aggregation-mediating effects of serotonin were reported by Mitchell and Sharp et al. 1964. In 1986, in order to determine how platelets contribute to occlusive

coronary artery thrombosis, a study performed by Benedict et al. examined *in vivo* thrombus formation in the coronary arteries of dogs (Benedict et al. 1986). They found that plasma serotonin levels significantly increased when flow current was reduced and was the highest immediate prior to full occlusion of the artery. These studies demonstrate that platelet aggregation is a significant factor in the evolution of occlusive coronary thrombosis and that serotonin is released during clot formation.

A crossroads between cardiovascular function and depression

Approximately 14.8 million American adults or about 6.7 percent of the U.S. population age 18 and older are diagnosed with Major Depressive Disorder (Lichtman et al. 2014). Importantly, depression increases the risk for CVD two-fold, potentially by contributing to thrombotic risk via increased platelet activity (Goldstein et al. 2015b; Musselman, Evans, and Nemeroff 1998; Frasure-Smith and Lespérance). Understanding the implication of serotonergic dysregulation, derived from depression treatments, within the CVS is imperative. Because over 90% of whole blood serotonin is stored within platelets, modulation of serotonin in platelets may have a consequence on thrombotic risk in depressed patients (Beikmann et al. 2013).

Current pharmacological therapies for the treatment of diseases, such as heart disease and stroke, often involve the use of sub-optimal drugs that do not reflect current scientific understanding of pathology. For example, some anti-platelet drugs do not sufficiently attenuate platelet activation while others can have a late onset and long durations of action leading to increased bleeding risk (Harrison et al. 2007). Anti-platelet therapeutics are used to treat a number of coronary diseases related to arterial thrombosis

including myocardial infarction, cerebral infarction and pulmonary embolism (Bismuth-Evenzal et al. 2012b). Therapeutic interventions have primarily targeted molecular interactions at the platelet membrane to impair aggregation including targeting the primary platelet integrin αIIbβ3 (GPIIbIIIa) (Gibbins 2004). Building our knowledge of this pathway could lead to novel targets or strategies for antiplatelet therapies in addition to altering clinical practices involving antidepressant prescriptions.

Drugs that alter serotonin and their consequences on hemostasis

Some have reported that platelet serotonin levels are decreased in patients with depression, however these results are very controversial (Greenberg et al. 1999; Maurer-Spurej, Pittendreigh, and Solomons 2004; Hara et al. 2011). SSRI treatment reduces blood serotonin significantly (Sherwin et al. 1986; Serebruany et al. 2003; Maurer-Spurej, Pittendreigh, and Solomons 2004). MAOI antidepressant treatment began in 1957 when iproniazid (Marsilid), introduced initially for the treatment of tuberculosis, was found to have pronounced euphoric effects that led to its use in the treatment of psychological depression (M. a Fox et al. 2007; F Artigas et al. 1996). However, there were many issues with MAOI treatment including multiple drug and dietary interactions. Many of the adverse reactions to MAOIs come from the development of serotonin syndrome that occurs due to increased serotonin signaling within the periphery (Gillman 2006). Serotonin syndrome, or serotonin toxicity, results as a consequence of excess serotonergic activity (Gillman 2006; M. a Fox et al. 2007).

Interestingly, platelets primarily contain MAO-B (5% MAO-A: 95% MAO-B), which has poor specificity to serotonin as compared to MAO-A (Mészáros et al. 1998).

Therefore, most degradation of serotonin in the periphery occurs through liver metabolism (55% MAO-A: 45% MAO-B). Most antidepressants and anxiolytics drugs target MAO-A, but novel drugs targeting MAO-B are currently being developed for Parkinson's disease and Alzheimer's disease. The effect of MAOIs on the periphery are distinctly opposed to the effects seen with SSRI treatment, because they actually increase platelet serotonin levels (Blazevic et al. 2015a).

SSRIs are the most commonly prescribed treatment for depression and are known to be well tolerated (Paraskevaidis et al. 2012). SSRIs have been shown to cause a bleeding phenotype through decreased aggregation of platelets stimulated with a number of platelet agonists (Kimmel et al. 2011; Apelseth, Nepstad, and Hervig 2012). This suggests a role for serotonin in the periphery and within primary hemostasis. There are multiple mechanisms by which serotonin regulates platelet function (Apelseth, Nepstad, and Hervig 2012; Diego J Walther et al. 2003; Guilluy et al. 2009).

The second role for serotonin in platelet function occurs through uptake by SERT (Ana Marin D Carneiro and Blakely 2006; Steiner, Carneiro, and Blakely 2008; Ahmed et al. 2008; Kimmel et al. 2011). After being transported into the platelet, serotonin has three main conduits. Serotonin can be repackaged into granules by vesicular monoamine transporter (VMAT), degraded by monoamine oxidase (MAO) or used as a substrate for serotonylation by transglutaminase (TGase) (Griffin, Casadio, and Bergamini 2002).

In order to better understand these mechanisms of serotonin in the periphery, a deeper understanding of the effects of SSRI and the role of serotonin in platelet function is required. The mechanisms by which SSRIs effects in the periphery, in terms of sustained versus acute inhibition of SERT, is needed to inform clinical discussion of depression

medication treatments. The current dissertation investigates the role of serotonin in platelet function by using both genetic and pharmacological models of acute and chronic inhibition of SERT. These studies will elucidate the novel implication of lost SERT function on platelet activation.

Dissertation overview

This dissertation explores the role of serotonin in modulating platelet activation. Within the introduction, I have discussed a foundational framework for the physiological role of platelets. The presence of serotonin within the platelet has been known for quite some time, but this body of work will shed new light on how serotonin modulates platelet activation within a physiologically relevant model of thrombus formation (Figure 3). The introduction has discussed some of the clinical relevance of serotonin function *in vivo* and suggests clinically relevant implications for serotonergic signaling in cardiovascular diseases. Chapter 2 discusses the techniques that were used to explore platelet function and signaling, expanding the technical foundation of platelet biology and pharmacology.

In chapters 3 through 5, I have separated the body of my work into three discrete sections. In chapter 3, I discuss the major gatekeeper of serotonin homeostasis, SERT. I will give a background on SERT and the role it plays in platelet function. Then I will discuss experiments that assess the outcomes of acute loss of SERT function in platelet biology via inhibition with SSRIs. Finally, I discuss these findings in relations to previously published reports and propose alternative mechanisms by which acutely altered SERT function may modulate platelet reactivity.

In chapter 4, I discuss in greater detail an equally important modulator of serotonin function, $5HT_{2A}R$, the only serotonergic receptor found on platelets. I introduce serotonin signaling via receptors and discuss the previous findings that link $5HT_{2A}R$ activation with *ex vivo* and *in vivo* platelet reactivity. I establish that $5HT_{2A}R$ activation is required for full ADP-mediated αIIbβ3 activation. I also relate that sustained loss of SERT leads to altered serotonin homeostasis paralleling disrupted surface $5HT_{2A}R$ levels on platelets. Finally, I

test the functional consequences of $5HT_{2A}R$ activation on platelet spreading. These findings lead to a provocative discussion on $5HT_{2A}R$ function in platelets and its phenotypic and hemostatic implications.

Finally, in chapter 5, I explore the opposing situation were alterations in αIIbβ3 function change serotonergic tone in the blood. I expand on the dynamic relationship between SERT and αIIbβ3 that has previously been established (Mazalouskas et al. 2015; Ana Marin D Carneiro et al. 2008). I examine this relationship in the context of a hyperactive αIIbβ3 polymorphism, Pro32Pro33. The functional consequences and mechanism of the Pro32Pro33 mutation is described (Oliver et al. 2014). Finally, I explore the serotonergic signaling conditions in the Pro32Pro33 mouse model and find that not only is this model hyperserotonergic, but that sustained loss of SERT function is able to rescue the hyperactive αIIbβ3 phenotype.

In total, this dissertation explores novel implications of serotonergic signaling in platelet function. Furthermore, I have expanded on the biological role of serotonin in the platelet and provide preliminary steps towards a serotonin homeostasis model. The model takes a systems biology approach employing pharmacokinetics and pharmacokinetic knowledge of serotonin metabolism and regulation in the periphery. Through this dissertation, I have explored the implications of modulating serotonin homeostasis on platelet function offering unique, intriguing opportunity to probe serotonin signaling in an innovative, but physiologically relevant, context.

CHAPTER 2

MATERIALS AND METHODS

Materials.

Reagents

For these studies, thrombin was purchased from Chronolog (Haventown, PA). Sterile saline, ADP, fibrinogen, ketanserin, 1-[2, 5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI), and citalopram were purchased from Sigma-Aldrich (St. Louis, MO). PAR4-activating peptide (PAR4-AP; AYPGKF) was acquired from GL Biochem (Shanghai, China). Flow cytometry antibodies anti–activated αIIbβ3 (JON/A-FITC) and anti–P-selectin–PE antibodies from EMFRET Analytics & Co. (KG, Würzburg, Germany).

Antibodies that were used in this study include Anti-serotonin transporter made in gp (Frontier), GAPDH antibody as well as others. Secondary antibodies that were used for detection include Goat anti-mouse-Cy2; goat anti-rabbit-Cy3; and mouse anti-rabbit and goat anti-mouse and rat anti-gp, all conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Staining antibodies Concanavalin A (Con A) conjugated to Alexa Flour 594 was purchased from Thermo Fisher Scientific (Grand Island, NY). Finally, radioactive isotopes ^{3H}ketanserin, ^{3H}serotonin, and ^{35S}GTPyS were purchased through (Perkin Elmer).

Buffers

One of the most important buffers that were used in these experiments was Tyrode's-HEPES buffer. This solution is used to keep the platelets in a solution that is roughly isotonic or the same osmotic potential, with intestinal fluid. Tyrode's is very

similar to other buffers such as the Ring's buffer, but also contain magnesium, sugar and uses bicarbonate and phosphate to buffer the solution. In this section, I will describe the components of the buffer and its importance within platelet functional assays.

Tyrode's Buffer

Sodium bicarbonate ($NaHCO_3$) is used to buffer the solution at an 11.9 mM concentration in Tyrode's Buffer. Sodium bicarbonate is found dissolved in many mineral springs and can be utilized as a food additive. Its role in the Tyrode's solution is to balance the pH. It is an amphoteric compound, meaning that it reacts with both acids and bases. Sodium bicarbonate reacts violently with acids, increasing the CO_2 gas as the reaction product. In cases of respiratory acidosis, the infused bicarbonate ion drives the carbonic acid/bicarbonate buffer of plasma to the left and raises the pH to a normal physiological pH of 7.4. It is for this reason that sodium bicarbonate is used in medically supervised cardiopulmonary resuscitation. Infusion of bicarbonate is indicated when the blood pH is markedly (<7.1–7.0) low.

To make Tyrode's buffer we also use 10 mM HEPES as a buffering agent. HEPES or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid is good at maintaining physiological pH despite changes in carbon dioxide concentrations. The dissociation of water decreases with falling temperature and the dissociation constant (pK) of many other buffers do not change much with temperature. HEPES is like water in that its dissociation decreases as the temperature decreases. This makes HEPES a more efficient buffering agent for maintaining enzyme structure and function at low temperatures. The final solution is brought to a pH of 7.4, or physiological relevance, using NaOH.

127.2 mM sodium chloride (salt, NaCl) is added to Tyrode's Buffer. NaCl is common salt, and its concentration in a fluid is described as the fluids salinity. This is a critical property for many biological systems because of its role in many processes such as nerve impulses, heart activity, metabolic function, electrolyte balance, and fluid balance. Too much or too little salt can lead to a various effect on a person physiologically including muscle cramps, dizziness, electrolyte distributed neurological function or death. For humans, the minimum physiological requirement for sodium is 500 milligrams/day. Normal levels are between 135-145mmol/L. anything less than 135mmol/L is considered hyponatremia. A sterile solution of NaCl called saline, are typically used for intravenous infusion, rinsing contact lenses, nasal irrigation, and cleaning a news piercing. Saline solutions are also used in cell biology, molecular biology, and biochemistry experiments. Normal saline (NSS, NS or N/S) is the commonly used phrase for a solution of 0.90% w/v of NaCl, 308 mOsm/L or 9.0g per liter.

5 mM potassium chloride (KCl) is also a component of Tyrode's buffer and is used in medicine, scientific applications, and food processing. Interestingly, it is also used to cause cardiac arrest as the third drug for executions by lethal injection. Potassium is vital in the human body, and oral potassium chloride is the common means to replenish it, although it can also be diluted and given intravenously. Medically, it is used in the treatment of hypokalemia and associated conditions as an electrolyte replenisher. Brand names include K-Dur, Klor-Con, Micro-K, Slow-K, Sando-K and Kaon Cl, most of which are extended release oral medicines. Oral and intravenous doses for adults are typically in the range of 8 to 20 mEq, which is 600 to 1500 mg of KCl. Side effects can include gastrointestinal discomfort, including nausea and vomiting, diarrhea, and bleeding of the

digestive tract. Overdoses cause hyperkalemia, which can lead to paresthesia, cardiac conduction blocks, fibrillation, arrhythmias, and sclerosis

Sodium phosphate monobasic (0.4 mM for Tyrode's Buffer), also called anhydrous monobasic sodium phosphate and sodium dihydrogen phosphate, is an inorganic compound of sodium with dihydrogen phosphate (H_2PO_4-) anion. Phosphate is one of the most abundant minerals in the body, and its serum levels are regulated by a complex set of processes occurring in the intestine, skeleton, and kidneys. The currently known central regulators of phosphate homeostasis include parathyroid hormone (PTH), calcitriol, and a number of peptides collectively referred to as the "phosphatonins" of which fibroblast growth factor-23 (FGF-23) has been best defined. Maintenance of extracellular and intracellular phosphate levels within a narrow range is necessary for many biological processes, including energy metabolism, cell signaling, and regulation of protein synthesis, skeletal development, and bone integrity.

Magnesium chloride hexahydrate (MgCl2: 1 mM for Tyrode's Buffer) is an essential element in biological systems. Magnesium appears typically as the Mg^{2+} ion. It is an essential mineral nutrient (i.e., element) for life and is present in every cell type in every organism. For example, ATP (adenosine triphosphate), the primary source of energy in cells, must be bound to a magnesium ion in order to be biologically active. What is called ATP is often actually Mg-ATP. As such, magnesium plays a role in the stability of all polyphosphate compounds in the cells, including those associated with the synthesis of DNA and RNA as well as other essential protein functions. Over 300 enzymes require the presence of magnesium ions for their catalytic action, including all enzymes utilizing or synthesizing ATP, or those that use other nucleotides to synthesize DNA and RNA.

Magnesium also plays an important for in integrin function, one of the primary receptors discussed in this dissertation. Increasing magnesium ion concentration increases cell migration, one major phenotype of integrin function, on type IV collagen in melanoma cells (Yoshinaga et al. 1994). This effect is also observed with calcium. However, when calcium is added to the magnesium the calcium actually inhibits the increased migrations seen in cancer cells. Additionally, it is important to note that manganese activates integrin leading to maturation and integrin clustering (Dormond et al. 2004). Manganese chloride (MnCl) can be used to induce integrin activation.

Finally, 5 mM d-glucose is used which is a simple monosaccharide sugar that serves as the primary source of energy and as an important metabolic substrate for most living things. Its chemical formula is $C_6H_{12}O_6$. Glucose is a hexose sugar since it contains six carbon atoms, one of which is part of an aldehyde group, hence, is referred to as an aldohexose. Glucose serves as an important metabolic intermediate of cellular respiration. In animals, an excess of glucose is stored as glycogen.

Methods

Animals and Housing.

All mice were group housed (2-5 per cage) in temperature- and humidity-controlled conditions under a 12-hour light/dark cycle with food and water available ad libitum. All studies were performed in accordance with humane guidelines established by the Vanderbilt Institutional Animal Care and Use Committee under an approved protocol. Age- and sex-matched mice were used in all experiments (8–25 weeks of age). The colony manager determined experimental cohorts, and experimenters were blinded to the

genotypes. All experiments were run with either wild-type ($SERT^{+/+}$), $SERT^{-/-}$, β-arrestin 2 KO mice, or Pro32Pro33 (KI) homozygous mice of both sexes. Littermate matched M172 and I172 mice were used.

Administration of citalopram.

Water treatment

Citalopram-treated drinking water was prepared based on average weight of the mice and an average consumption of approximately 7mL/day/mouse for a dosing of 15mg/kg/day. Citalopram-prepared water was added to a water dispenser and mice were given full access to either control or citalopram-treated water. After 6-days, mice were euthanized, and experiments were performed as indicated in the methods section.

IP-injection of citalopram

In a single study, to understand the effects of citalopram over time, IP-injection of citalopram were given based on a body weight injections (200µL for a 20g mouse). Mice were given the injections at the same time daily for a total dose of 15mg/kg/day.

Tail Bleed.

Experiments were carried out as previously described (Oliver et al. 2014). Briefly, mice were maintained under anesthesia (2% isoflurane and 1ml/min oxygen; JD Medical Distributing Co., Inc., Phoenix, AZ), and a transverse incision was made with a scalpel over a lateral vein. The tail was immersed in normal saline (37°C) in a hand-held test tube. The time from the incision to the cessation of bleeding beyond 30 seconds was recorded as

the bleeding time. Maximum time allowed for to cessation of bleeding was 6 minutes before manually stopping the bleeding.

Blood collection.

In vitro, platelet manipulation could pre-activate the platelet before experimentation. Therefore, we spent a significant amount of time optimizing our platelet isolation technique and validating that the platelets are in a basal state before experimentation. The overall amount of blood in an averaged size mouse (~25g) is ~1.8mL. The small size of the mouse leads to a higher risk of platelet activation during blood draws. There are many options for blood collection techniques while the mouse is under anesthesia. Based on a review of mouse platelet activation techniques, parenteral anesthesia has been reported to decrease platelet aggregation and inhibit the release reaction in both humans and animals. We decided to use isoflurane as the effect is considered negligible on platelets in most species (JIROUSKOVA, SHET, and JOHNSON 2007). We also used cardiac puncture with a 25-gauge needle/1-ml syringe containing sodium citrate 3.8%. The size of the needle and the anticoagulant, which chelates calcium to prevent platelet activation, are optimal for purifying platelets. Cardiac puncture allows for increased blood for experimentation as compared to alternative procedures. Additionally, we saw no difference between thrombin clotting time from blood collected via cardiac puncture versus *vena cava* collection, suggesting that this method is suitable for mouse whole blood collection and platelet purification.

To isolate the platelets, we use gel-filtered platelet preparation. One alternative approach to isolating platelets is to use washed platelet prep. There were no differences

observed in platelet activation between platelets prepared using gel-filtration and washed platelets (data not shown). Whole blood was diluted 1:1 in Tyrode's buffer and was layered onto 2 ml of Fico/Lite (Atlanta Biologicals, Inc., Lawrenceville, GA) and spun for 15 minutes at 350g to isolate platelets from other blood cells. We also use three other anti-platelet activation agents during our purification; ACD, an additional calcium chelator; apyrase, an enzyme that degrades ADP to prevent premature ADP activation during preparations; and PGE1, which stimulates adenyl cyclase activity in platelets and increases cyclic AMP concentrations. One of the most standard methods for measuring platelet activation is through the activation of integrin αIIbβ3. We compared baseline αIIbβ3 activation (via JON/A binding) in the non-stimulated condition and found that when activated, even with weak agonists such as ADP, there was a considerable increase in αIIbβ3 activation. Shown below is the concentration-response curve in wild-type platelets. If the platelets that we isolated were pre-activated, we would not be able to measure the relatively weak activation of the platelets with ADP. Basal JON/A and P-selectin binding are shown in most experiments to validate the baseline resting state of the platelets. Using baseline JON/A and P-selectin binding as internal controls as well as observing a concentration-dependent activation by both weak and strong agonists (Figure 9) we believe that our techniques are suitable for isolating platelets in the basal state before stimulation.

Alternative blood drawing techniques

Blood can be collected via a variety of different methods. This includes the retro-orbital venous plexus, the heart, the inferior vena cava, the jugular vein, the carotid artery, tail, or lateral saphenous vein. A collection of blood from the retro-orbital venous plexus

requires venipuncture with a ~1.5cm long glass capillary tube that may be coated with an anticoagulant. This will yield approximately ~0.5mL of blood. This is not suggested for platelet studying purposes because the glass can lead to platelet activation via the positive charge on the class and the interaction with phosphatidic serine lipid on the platelet surface.

A single milliliter of blood can be obtained from the mouse using cardiac puncture. The pitfalls of this approach are that the process is terminal, and tearing of the tissue factor-rich heart muscle during blood collection can cause thrombus formation and tissue activation. In order to perform a blood collection from the inferior vena cava, the surgical exposure of the inferior vena cava is required, a needle (22-27 gauge) is inserted into the widest part of the vessel. As much as 1.2mL of blood can be collected using this technique. However, in our lab typically 100-400µL is standard. Platelet collection via the inferior vena cava puncture most likely leads to least amount of platelet activation. A minuscule amount of blood can be obtained from the tail vein by either amputation of 0.5-1mm of the tailor by making a horizontal incision in the tail vein.

Platelet purification.

Platelets can be purified using two different methods. First, gel filtration of platelets from whole blood can be employed following blood isolation into sodium citrate 3.8% via cardiac puncture extraction (Figure 9). The whole blood is then diluted 1:1 with Tyrode's buffer. It is then layered onto either Fico/lite gradient gel or AccuPrep gel that separates the blood components based on size. The separation occurs during a 350g spin for 15 minutes where the platelet rich layer is formed within the gel. The platelet layer is then removed and placed in a new tube. ACD and apyrase are added to the new tube, and the

samples are diluted 1:5 in Tyrode's buffer. The platelets are allowed to rest for 10 minutes before the second spin occurs. During the second spin which is done at 1000g for 10 minutes, and then spun down to form a pellet. This pellet is then resuspended in Tyrode's buffer without ACD or apyrase. Platelets are then counted using a coulter counter.

An alternative method of platelet purification is centrifugation. This approach that has been used for human platelet studies have also been adapted for mouse purification. The most common method used to isolate platelets is through the preparation of platelet rich plasma (PRP), which can be centrifuged to obtain yield platelet pure fraction through separation by gel filtration. Anticoagulated blood is centrifuged at 200 g for 20 min, and the upper 2/3 of platelet-rich plasma removed by aspiration. The 1/10th volume of anticoagulant was added and platelets pelleted at 1,100g for 10 min. The platelets were washed by the addition of 1 mL Tyrode's buffer with anticoagulant and centrifuged at 1,100 g for 10 min. The platelets were resuspended in Tyrode's buffer with 1mM $CaCl_2$ immediately prior to experimentation. However, for all studies, gel-filtered platelets were used.

Whole-Blood Clotting Time.

Whole blood (90 μl) was added to a single well containing a small metal bead in the presence of 10 μl of $CaCl_2$ (16.4 mM), and the number of seconds to interruption of the small magnetic bead movement was recorded (Diagnostica Stago, Parsippany, NJ).

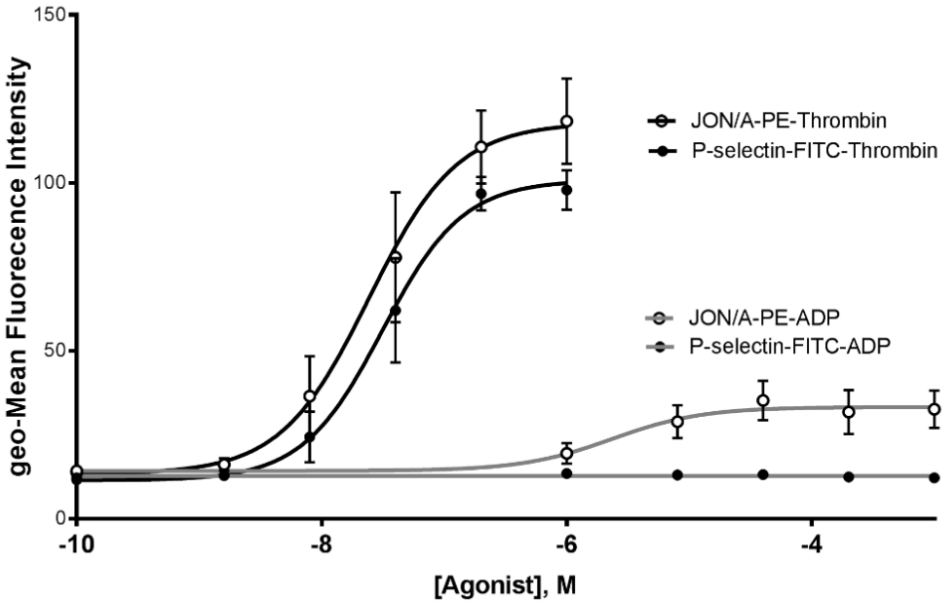


Figure 9. ***Platelet thrombin and ADP activation on purified platelets****. To ascertain if the platelet purification and activation methods was viable, platelets were isolated and activated with either the strong platelet agonist, thrombin, or the weak platelet agonist, ADP. Here we demonstrate that our platelets respond differently to the strong and weak agonist. The affinity of thrombin to activate platelet activation as measured by JON/A and P-selectin binding was much higher than that of ADP which only initiated JON/A but not P-selectin expression. These experiments help to validate our platelet purification and activation procedures.*

Nonlethal Thromboembolism.

The nonlethal systemic thrombosis method was used because it may reveal increases in platelet aggregation (Smyth et al. 2001). Mice were kept under anesthesia with 2% isoflurane and the right jugular vein exposed by a lateral neck incision for the collection of 100 μl of whole blood in sodium citrate. The left jugular vein was exposed to inject a coagulation solution containing 100 μg/ml ADP, 200 μg/ml collagen, and 200 μg/ml epinephrine in sterile saline at a dose of 5 μl/g during 10 seconds. One minute after the injection a sample of blood was collected into sodium citrate. Six minutes after injection mice were euthanized by rapid decapitation.

Whole-Blood Aggregation.

Electrical impedance was determined using multiple analyzers (Dynabyte GmbH, Munich, Germany) by adding 175 μl of 37°C 2× $CaCl_2$ to 175 μl of citrated whole blood, following agonist (200 μM PAR4-AP) addition. Aggregation and the velocity of aggregation were determined over a 6-minute period.

Aggregation in Washed Platelets.

Blood was spun for 10 minutes at 500*g*, and 500 μl of platelet-rich plasma was collected from the top layer of the supernatant. Pelleted platelets were suspended in Tyrodes-HEPES buffer and adjusted to a concentration of 3×10^8 platelets/ml. The change in light transmission was monitored with an aggregometer in the presence of 0.05 U/ml thrombin.

Flow Cytometry.

Whole blood.

Briefly, 250 ml of whole blood was mixed with 750 ml of Tyrodes-HEPES buffer and added to a tube containing a buffer. Antibodies (2.5 µl) were added to tubes, and activation was stopped by addition of 500 ml of 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.138 M NaCl, 0.0027 M KCl; pH 7.4) 15 minutes after activation. Samples were analyzed at the Nashville Veterans Affairs Medical Center Flow Cytometry Resource Center (Oliver et al. 2014; Przyklenk et al. 2010b) using geometric mean fluorescence intensity (gMFI) which gives the average fluorescence intensity of each fluorescence channel for the sample. The antibodies that were used include JON/A-PE and

P-selectin-FITC which stand for the fluorophores phycoerythrin (PE) and Fluorescein isothiocyanate (FITC), respectively.

Gel-filtered platelets.

An examples of a flow experiment is shown in Figure 9. After purifying platelets, platelet counts were determined and normalized to $1X10^8$/mL. 25µL of platelets were added and mixed with 5µL each of both EMFRET antibodies. Briefly, 250 ml of whole blood was mixed with 750 ml of Tyrodes-HEPES buffer and added to a tube containing a buffer. Antibodies (2.5 ml) were added to tubes, and activation was stopped by addition of 500 ml of 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.138 M NaCl, 0.0027 M KCl; pH 7.4) 15 minutes after activation. Samples were analyzed at the Nashville Veterans Affairs Medical Center Flow Cytometry Resource Center (Nashville, TN) (Michelson et al., 2000a).

Platelet Attachment and In-Cell Westerns.

In-cell Westerns were performed as described previously(Ana Marin D Carneiro et al. 2008).Whole blood was diluted 1:8 in Krebs-Ringer-HEPES (KRH) buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 g/l glucose, 10 mM HEPES; pH 7.4) or PBS and seeded onto 25 μg/ml fibrinogen-coated 96-well plates. After adding 45 μl diluted blood/well, $MnCl_2$ (0.2 mM), 200 μM PAR4-AP, or buffer was added to all wells and incubated at 37°C for 15 minutes. Wells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton for 10 minutes at room temperature. After blocking in 1% bovine serum albumin (BSA) and 5% normal goat serum for 30 minutes, primary antibodies were added at a 1:1000 dilution overnight at 4°C. Wells were

washed three times with PBS, and secondary antibodies (IRDye 800CW donkey anti-rabbit IgG and IRDye 680RD donkey anti-mouse IgG; LI-COR Biosciences, Lincoln, NE) were added at a 1:250 dilution for 1 hour at room temperature. Plates were washed three times with PBS and scanned in an Odyssey Infrared Imaging system (LI-COR Biosciences).

Platelet Spreading.

Washed platelets were resuspended in KRH ($1X10^8$/ml) and seeded onto 12-well (5-mm-diameter) glass printed slides (Thermo Scientific Cel-Line Specialty Printed Microscope Slides; SSG Braunschweig, Germany) previously coated with 25 μg/ml fibrinogen and blocked with 1% BSA. Each well received 4-9 μl of platelets and 1 μl of 1 mM PAR4-AP, 1 mM PAR4-AP + 0.5 μM SKI-606, KRH buffer, or other agonists and antagonists as described dissolved in KRH. Slides were incubated at 37°C for 15 minutes, washed once with 1× PBS, and fixed with 4% paraformaldehyde. Platelets were permeabilized with 0.2% Triton X-100 in PBS and blocked with 1% BSA and 5% normal goat serum. Slides were incubated with primary antibodies at 1:1000 dilution overnight at 4°C. Slides were washed with PBS and incubated with secondary antibodies and phalloidin-Cy5 or concovilin-A-Cy5 at 1:200 dilution in 1% BSA for 1 hour at room temperature. Slides were washed and mounted in Aqua Poly Mount (Polysciences, Inc., Warrington, PA). Images were captured with a Zeiss LSM510 META Inverted Confocal Microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) located at the Vanderbilt Cell Imaging Shared Resource. Images were obtained with a 63×/1.40 Plan-Apochromat oil lens using Zeiss Image Browser. Platelet number and area were quantified by a blinded experimenter using ImageJ analysis software in the talin channel (Cy2).

Western Blotting and Coimmunoprecipitations.

Washed platelets were resuspended in KRH or Tyrode's buffer, incubated at room temperature for 30 minutes, lysed by addition of 1 volume of 1% Triton X-100 in PBS (containing protease inhibitor; Roche, Indianapolis, IN), and clarified by centrifugation at 13,000*g* for 10 minutes at 4°C. Lysates were collected for input (10 μg), and 200μg of protein extract was incubated with 30 μl integrin β_3 antibody (2C9.G2 hamster anti-β_3; BioLegend) covalently attached to protein A magnetic beads (Dynabeads; Life Technologies/Invitrogen, Grand Island, NY) for 1 hour at 4°C. Beads were isolated magnetically and washed with 1 ml 0.5% Triton X-100 in PBS. Coimmunoprecipitated proteins were eluted with 1× NuPAGE lithium dodecyl sulfate sample buffer (Life Technologies/Invitrogen) and Western blot analysis performed. Proteins were detected by chemiluminescence and exposed to Hyperfilm through multiple exposures to ensure linear distribution of the signal. Films were scanned, and band densities were established using ImageJ software (NIH, Bethesda, MD).

Src In Vivo Inhibition.

A 10 mM stock of SKI-606 (Sigma-Aldrich) in dimethylsulfoxide was diluted in sterile saline (0.9% NaCl) to 0.1 mg/ml immediately prior to administration. Mice were maintained under anesthesia with 2% isoflurane during the whole procedure. The jugular vein was exposed for the collection of 300 μl of blood in sodium citrate for a pre–SKI-606 clotting time measurement. After this initial blood draw, SKI-606 was administered intraperitoneally at 1 mg/kg. After 30 minutes, the cardiac puncture was performed for

collection of blood samples (post–SKI-606). Blood samples were used to perform clotting time experiments and in-cell Westerns.

Serotonin, 5HIAA, and ADP levels

HPLC

5-HT and 5HIAA levels were measured by HPLC by the Vanderbilt Neurochemistry core. Blood was collected in 3.2% sodium citrate and stored at -80^{o}C until analyzed.

ELISA

Alternatively, serotonin levels were also measured using a serotonin ELISA using a serotonin EIA kit (Enzo life Sciences H #ADI-900-175) according to the manufacturer's instructions. The detection limit of the assay was 0.43 ng/ml, and samples were diluted 1:16 in order to remain within the linear range of the standard.

ADP ELISA

The ADP ELISA was performed according to manufacturer instructions. A standard curve was prepared for each assay to determine the concentration of ADP found within the whole blood samples (Abcam, United States, ab83359).

Radioligand binding and uptake

Platelet counts were normalized before analysis using a Coulter Counter at 5-3X10^{8} cells/mL. A saturation curve was done with increasing concentrations of ^{3H}ketanserin (0.625nM-20nM) incubated with 50µL of platelets in suspension and methysergide at 20µM to determine non-specific binding. Bmax values were calculated using a nonlinear

regression analysis for one-site specific binding (GraphPad Software, Inc., La Jolla, CA). G-protein activation was measured via ^{35S}GTPyS incorporation. Mouse platelets were pooled (n=2) per assay. Platelets were lysed by addition of ddH_2O and placed at -20^{o}C for a minimum of 3 hrs. Samples were thawed and spun at 20,000g for 20 minutes to collect membranes. Membranes were resuspended in membrane storage buffer (10 mM HEPES, pH 7.4, 1 mM EDTA). Samples were incubated in 50 µM GDP and 0.5 mM dithiothreitol for 15 minutes. 1 µM DOI and 0.2 nM ^{35S}GTPyS were incubated in samples for 60 minutes at room temperature. Counts were standardized by protein concentration of membrane prep. For non-specific ^{35S}GTPyS incorporation excess unlabeled-GTPyS (100 µM) was added. 5-HT_{2A} receptor specific ^{35S}GTPys was measured following normalized of $SERT^{+/+}$ DOI specific counts to non-DOI specific counts. The assay was terminated by filtration through polyethyleneimine-coated GF/B Whatman filters using a Brandel Cell Harvester (Brandel, Gaithersburg, MD), and final counts were measured using a scintillation counter. Finally, competitive binding with ^{3H}ADP to platelet membrane preparations was used to determine P_2Y_1 and P_2Y_{12} relative percentage of receptors. ^{3H}ADP at 20 nM was added to platelet membrane preparations (5-20µg total). Platelets were then incubated at the indicated concentration of either $P2Y_{12}$ (Ticagrelor) or $P2Y_1$ (MRS2179), or dual $P2Y_1$ and $P2Y_{12}$ antagonist (PIT, 2,2'-pyridylisatogen tosylate) to determine receptor specific binding.

Statistics.

All data were analyzed in Prism 4.0c (GraphPad Software, Inc., La Jolla, CA) using Student's *t* tests or two-way analysis of variance (ANOVA) with Bonferroni post-tests

where appropriate. Welch's correction parameters were employed in samples with unequal variances. Nonparametric *t* tests were used when normalized to the WT data (all WT = 100) or when the variation between samples sets was non-normally distributed. A *P* value of <0.05 was considered statistically significant. All data are shown as mean ± S.E.M., or median ± S.D. when appropriate based on statistical method used. Detailed statistics are given in the figure legend for each figure.

CHAPTER 3

ACUTE AND CHRONIC LOSS OF SEROTONIN TRANSPORTER FUNCTION IN PLATELETS

The serotonin transporter: pharmacology and protein interactions

The serotonin transporter (SERT)

Introduction

Although serotonin is known for its role as a neurotransmitter, it is often forgotten that serotonin was first characterized in the periphery and shown to regulate vascular tone (Ueno et al. 2011; Jedlitschky, Greinacher, and Kroemer 2012; M. Berger, Gray, and Roth 2009). When released into the extracellular space serotonin can initiate intracellular signaling cascades through both GPCRs and a ligand-gated ion channel. In the central nervous system, serotonin is involved in aggression, alcoholism, depression, and feeding behavior (Greenberg et al. 1999; Canli and Lesch 2007). These same GPCR-mediated signaling mechanisms result in serotonin's vasoactive properties stemming from endothelial cell activation and nitric oxide production (P M Vanhoutte 1987). However, serotonin does have the potential to signal through non-GPCR-mechanisms as discussed later in this chapter.

In the blood compartment, serotonin concentration is regulated via uptake and storage in platelets via the serotonin transporter (SERT). Within this chapter, I will separate the role of SERT in platelet function based on its acute and chronic functions. Acutely, SERT regulates the concentration of serotonin taken up by the platelet, and therefore also controls the extracellular concentration that is able to signal extracellularly. Long term, SERT controls the total amount of serotonin levels in the blood. Potentially this can alter

serotonin homeostasis and signaling by protecting it from metabolic degradation. SERT is responsible for the regulation of extracellular versus intracellular serotonin concentrations, which has distinct implication in both the acute and chronic contexts. Below, I will discuss SERT's role in platelet biology. I will also discuss known protein-protein interactions and describe a proposed non-GPCR function of serotonin that could be responsible for some acute effects of SERT inhibition.

The serotonin transporter: SERT

Regulating intercellular and extracellular serotonin levels, SERT exists in many different conformations that engage in specific interactions with molecules that alter intracellular signaling. Many groups have suggested that SERT has signaling capacities of its own within the intracellular environment (Monassier, Laplante, et al. 2010, Brenner et al. 2007; Lau et al. 2012). SERT, a protein consisting of 630 amino acids with 12 transmembrane domains, has the potential to be a hub of signaling mechanisms and a regulator of serotonin signaling.

SERT Protein Family

SERT belongs to the solute carrier 6-gene family (SCL6), which is also called the neurotransmitter-sodium-symporter family (Na^+/Cl^--dependent transporters) (He, Vasiliou, and Nebert 2009; Kristensen et al. 2011). There are a total of nine Na^+/Cl^--dependent plasma membrane transporters within this group, which include the neurotransmitter transporters for the monoamines serotonin, dopamine, and norepinephrine, and the amino acids GABA and glycine (Kristensen et al. 2011). Transport

requires energetically coupled mechanisms when the concertation gradient across the cell membrane for the solute is non-favorable. Examples of energetically coupled mechanisms include hydrolysis of ATP (e.g. ABC transporters) or the use of transmembrane ion gradients (e.g. SLC family). The SLC family specifically encompasses 350 transporters organized into 55 families (Borst and Elferink 2002; Vasiliou, Vasiliou, and Nebert 2009; Hediger et al. 2004; Dennis L Murphy et al. 2009; He, Vasiliou, and Nebert 2009). SLC6 is among the largest of these families containing 20 genes that encode a group of highly similar transport proteins. SLC6 transporters transport various amino acids and their derivatives into cells using co-transport of extracellular Na^+ and/or other ions (He, Vasiliou, and Nebert 2009). The favorable gradients of the co-transported ions energetically drives substrate translocation against a chemical gradient (Dennis L Murphy et al. 2009). The different types of transporters can be divided into different subgroups based on sequence similarity and substrate similarity. One subset includes the monoamine transporters (MATs) for dopamine (DAT), norepinephrine (NET), and serotonin (SERT) (Kristensen et al. 2011; Masson and Hamon 2009).

There are other classification which help to describe the transport mechanism of the SLC6 family members. SLC6 transporters can be categorized as secondary active transporters because they use the potential energy stored in the electrochemical gradient of Na^+, or other ions, to move their substrates (He, Vasiliou, and Nebert 2009; Kristensen et al. 2011). Additionally, they can be categorized as symporters because the movement of the monoamine is in the same direction as Na^+ (Manepalli et al. 2012; Nyola et al. 2010). However, in some cases but particularly for SERT (SLC6A4), there is also the antiport movement of potassium (K^+). K^+ performs the counter-transport of the Na^+ movement

moving the transporter back to a conformation allowing for another round of substrate internalization.

SERT Structure

SERT is the product made from the SLC6A4 gene, which is located within chromosome 17q11.2 and is composed of 14 exons that span approximately 40kb (GCID: GC17M030194). The sequence of the transcript predicts a protein made up of 630 amino acids with 12 transmembrane domains. Below, I will discuss a body of literature that has probed the SERT structure and binding sites for serotonin and antidepressants. Excitingly, the human SERT X-ray structure was recently published (Coleman, Green, and Gouaux 2016). However the preliminary work comparing SERT sequence to other homologous transporters including LeuT and DAT will also be discussed (Bulling et al. 2012; Khelashvili and Weinstein 2015).

The SERT ligand-binding pocket consists of residues from transmembrane domains 1,3,6, and 8, corresponding to the leucine-binding site of LeuT (Manepalli et al. 2012; Gabrielsen et al. 2011; Andersen et al. 2009). This comparison was preserved from a study in which LeuT and SERT substrate binding and antidepressant binding sites were compared (Z. Zhou et al. 2009). A smaller SERT side chains present in S1 relative to those that seen in LeuT may accommodate the larger serotonin substrate. The presence of the SERT TM 1 aspartate residue (D98) may be substantial for substrate interaction. The paired mutant–ligand analog complementary experiment has been used to study the binding of serotonin in the SERT S1 site (Kaufmann et al. 2009; Manepalli et al. 2012; Severinsen et al. 2012). Serotonin analogs were docked into each of three independently generated

recombinant human SERT models using an induced-fit method (Gabrielsen et al. 2012; Wildling et al. 2012). Different conformations were experimentally analyzed by characterizing 13 SERT point mutants with respect to binding of serotonin analogs. Interactions between serotonin and residues D98 (TM1), A173 (TM3), and T439 (TM8) were suggested as possible interaction sites between the transporter and serotonin (Kristensen et al. 2011; He, Vasiliou, and Nebert 2009; Hediger et al. 2004) .

SERT activity is dependent on relatively high extracellular concentrations of Na^+ and Cl^- (Beckman and Quick 2001; Mortensen et al. 1999; Demchyshyn et al. 1994). The driving force for the energetically unfavorable transport of serotonin is the Na^+ influx (Quick et al. 2003). The transporter moves Na^+ down its concentration gradient (Figure 10), which is maintained by the Na^+/K^+ pump (Na^+/K^+ ATPase). High concentrations of extracellular Cl^- is also necessary for SERT transport (Manepalli et al. 2012).

Although the SERT and $LeuT_{Aa}$ structure share only have 17% similarity, the comparision between the putative 5HT binding site raises comparability to 50% (Beuming et al. 2006; Kaufmann et al. 2009). However, novel crystal strucutres of the drosophila melanogaster dopamine transporter have been crystalized to 3.0A (Penmatsa, Wang, and Gouaux 2013). This model sheds new light on the Na^+/Cl^--coupled neurotransmitter uptake mechanisms including structure modulaion by lipids. One key lipid that could modulate substrate efflux is PIP_2 , which was shown to interacts with the N-terminal region of DAT (Khelashvili and Weinstein 2015).

It is currently thought that SSRIs inhibit SERT activity by binding to its primary high-affinity site, or orthosteric site. Additionally, the novel SERT crystal strucutre to 3.15A has demonstrated the allosteric binding site of (s)-citalorpam (Coleman, Green, and

Gouaux 2016). Two strucutres of SERT were examined: one was bound to (S)-citalopram, one bound to paroxetine. It was shown that the binding of both SSRIs locked SERT in a downward facing conformation (Coleman, Green, and Gouaux 2016). Both SSRIs were lodged in the center binding site located between helices 1,3,6,8 and 10. This is the site site in which serotonin would bind (Coleman, Green, and Gouaux 2016). The allosteric binding site of (s)-citalopram is defined by residues in TN1b, TM6a, TM10, and TM11 as well as EL4 and EL6 (Coleman, Green, and Gouaux 2016). This binding would directly compete with serotonin uptake. Overall, this site is very similar to the alloeseric site predicted by DAT comparative modeling.

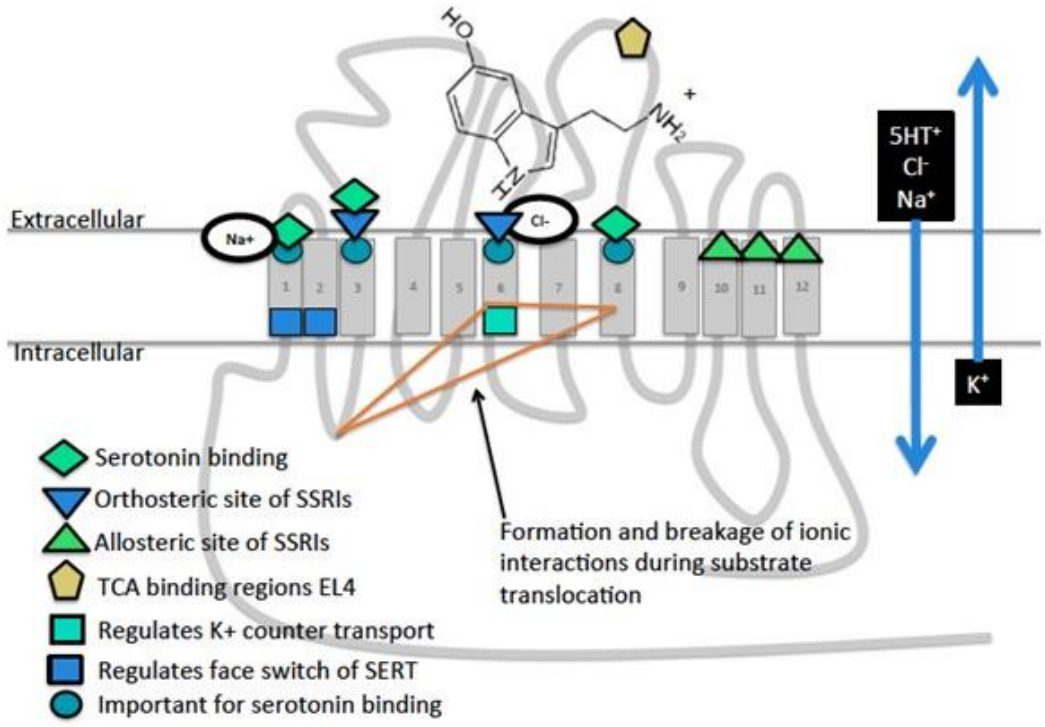


Figure 10. ***Binding sites of SSRIs on SERT****. Shown are the various localization of binding site on SERT with the proper arrangement of serotonin as it is transported by the transporter. The sites shown include serotonin, the orthosteric site, the allosteric site, and TCA binding.*

SERT as a signaling platform: known protein-transporter interactions

SERT-interacting proteins (SIPs) mediate trafficking, cytoskeletal dynamics and other non-canonical functions of these proteins (Figure 12). The primary role of SERT is serotonin uptake. Even though its uptake function can be readily demonstrated in recombinant expression systems, under physiological conditions the regulation of transport kinetics is vastly dependent on the intracellular and extracellular environment surrounding the transporter. SERT is part of a larger signaling complex, as evidenced by its dynamic regulation of activity and localization, as well as the numerous characterized protein-protein interactions with SIPs. Many of these proteins are either associated with vesicle fusion or endocytosis. Therefore there is still much to be determined about the role of SERT beyond serotonin uptake.

Phosphorylation is a major posttranslational modification that alters protein function and is regulated by the action and location of kinases and phosphatases. Protein kinases are an important aspect of SERT regulation. Activation of Protein Kinase C (PKC) through the phorbol ester activators β-PMA and β-PdBu rapidly inhibit serotonin uptake (within 30 mins) by SERT in HEK293 cells (Qian et al. 1997). This is likely due to a decrease of SERT at the plasma surface. PKA C-α activation may also regulate SERT uptake function (Vuorenpää et al. 2016). Other proteins that phosphorylate or modulate SERT include protein kinase B (Akt) and p38 mitogen-activated protein kinase (MAPK) (Vuorenpää et al. 2016). Other interactions that may also be important include PI_3K and Ca^{2+}/calmodulin-dependent kinase II which regulates SERT interaction with syntaxin 1A (Nazir et al. 2015; Ciccone et al. 2008).

Inhibitors that target protein phosphatase 2A (PP_{2A}) and protein phosphatase 1 (PP_1) lead to increased SERT phosphorylation (S. Ramamoorthy et al. 1998) and reduced SERT transport activity (Sakai et al. 2002). The interaction of PP2A with the SERT occurs through its catalytic subunit (PP2Ac), as shown in both transfected cells and brain tissues. (Daaka et al. 1997; Zhu et al. 2005). $PP_{1/2A}$ inhibitors, like okadaic acid and calyculin A can increase SERT phosphorylation and decrease its serotonin uptake function (Sakai et al. 2002; S. Ramamoorthy et al. 1998). The SERT phosphorylation site can be phosphorylated by both PKC and PKA within the serine/threonine sites. SERT displays moderate tonic phosphorylation, but this phosphorylation can be enhanced with activators of PKA and PKC, or reduced with inhibitors PP2A (Vuorenpää et al. 2016).

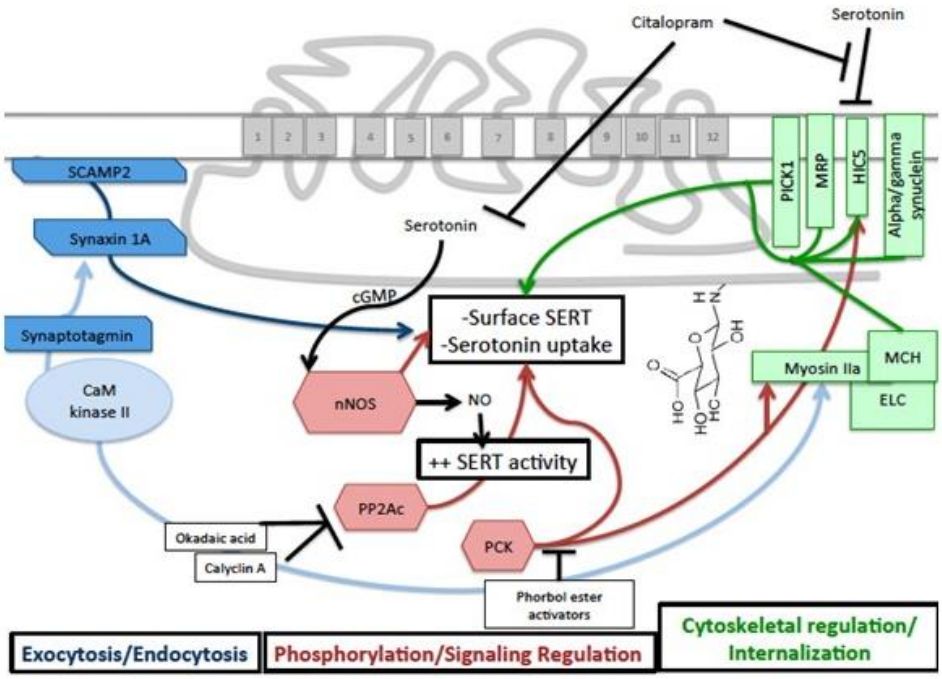


Figure 11. ***Signaling partners of SERT****. There are a number of difference signaling events that can occur which regulate and modulate SERT function. These signaling pathways include the endocytosis and exocytosis of SERT (shown in blue), the phosphorylation of SERT which alters it function (shown in red), and SERTs interaction with the cytoskeleton including that which leads to endocytosis (shown in green).*

SERT function is regulated by its membrane surface expression and catalytic activity. There are multiple intracellular signaling pathways that regulate these processes as discussed above (Sammanda Ramamoorthy, Shippenberg, and Jayanthi 2011; S. Ramamoorthy 1999; Steiner, Carneiro, and Blakely 2008). However there are a number of other SERT protein interactions that localize the SERT protein to the the cytoskeleton (Ana Marin D Carneiro and Blakely 2006; Scanlon, Williams, and Schloss 2001; Magnani et al. 2004). It has been suggest that cholesterol modulates the functional properties of SERT by stabilizing its structure (Scanlon, Williams, and Schloss 2001). The association of SERT with lipid rafts has been shown to decreased serotonin transport capacity (Vmax) of SERT (Magnani et al. 2004). Additionally the association of SERT with HIC-5, a LIM domain adaptor protein, may act as a shuttle or protein scaffold that mediates the transtion of SERT between lipid raft partitions (Ana Marin D Carneiro and Blakely 2006). Studies have shown that the interaction between SERT and myosin IIA is dependent upon N-linked glycosylation of SERT (Ozaslan et al. 2003). Association of the effector domain with membranes may be regulated by Ca^{2+}/calmodulin. Additionally, SERT may interact with Protein interacting with C kinase 1 (PICK1), which is an essential scaffolding protein that also regulates the function of DAT and NET (Torres et al. 2001).

SERT has also been demonstrated to interact with proteins that regulate endo- and exocytosis. Syntaxin 1A is a plasma membrane protein that is a critical part of the soluble N-ethylmaleimide- sensitive factor attachment protein receptor (SNARE) complex involved in the regulation of neurotransmitter release (Quick 2002; Haase et al. 2001). Syntaxin 1A interacts with the N-terminal region of SERT, as demonstrated by both co-IP and glutathione-s-transferase pull-down assays using recombinant systems. Syntaxins have

a C-terminal transmembrane domain, a SNARE domain, and a Habc N-terminal regulatory domain (Bracher et al. 2004). It has also been demonstrated that SERT interacts with other SNARE proteins including vesicle-associated membrane protein 2 (VAMP2) and synaptosome-associated protein of 25 kDa (SNAP25) (Kaastrup et al. 2014). Additionally, secretory carrier membrane protein 2 (SCAMP2) has also been shown to bind and directly interact with SERT (Müller, Wiborg, and Haase 2006). Co-expression of SCAMP2 with SERT caused a decrease in SERT surface density and a concomitant reduction in serotonin uptake. Mutation to the conserved E-peptide of SCAMP2 eliminated downregulation of SERT without affecting the interaction of SERT and SCAMP2 (Müller, Wiborg, and Haase 2006). In addition to the cytoskeletal interactions established above, these findings suggest that SERT regulates the vesicular movement of components within the membrane via direct interactions with vesicle-associated proteins.

Phenotype of SERT Knockout Mice

Serotonin is thought to play a significant role in numerous disorders. Therefore, generation of a SERT knockout mouse allowed for investigation into possible underlying mechanisms (Table 3) (J R Homberg et al. 2007; D L Murphy, Uhl, and Holmes 2003; M. a Fox et al. 2007; A. V Kalueff, Jensen, and Murphy 2007; Lynette C Daws 2009; a V Kalueff et al. 2010). Naturally occurring polymorphisms in recombinant inbred lines have been shown to modulate SERT function and lead to multiple phenotypes associated with SERT function (Ana M D Carneiro et al. 2009). Examples of serotonin-related neurological/psychiatric diseases include depression, bipolar disorder, anxiety disorders, eating disorders, autism, and ADHD (S. M. Stahl et al. 2013; Glatt et al. 2004; Nietzer et

al. 2011; Giannaccini et al. 2010). Peripheral disorders include myocardial infarction, pulmonary hypertension, irritable bowel syndrome and sudden infant death syndrome (SIDS) (Nazir et al. 2015; Gershon and Tack 2007; Kim et al. 2005). Although serotonin system dysregulation has been implicated in each of these disorders, the actual mechanisms behind these disorders have yet to be conclusively determined.

Serotonin uptake is profoundly decreased in the SERT KO mice (Dennis L Murphy and Lesch 2008). There are many different phenotypic traits associated with the loss of SERT. Mouse homozygous for loss of SERT, and to a lesser extent the heterozygous KO, exhibit diminished responses to serotonin receptor antagonists and other classes of drugs including MDMA, SSRIs, 8-OH-DPAT, and DOI (M. A. Fox et al. 2007). These findings suggests that the loss of one SLC6a4 allele leads to a decrease in transporter function, but a single copy of SLC6a4 is sufficient to maintain serotonin homeostasis (Dennis L Murphy and Lesch 2008; Lynette C Daws 2009).

SERT KO mice have been reported to have increased anxiety-like behaviors and altered neuroendocrine and sympathoadrenal responses (Adamec et al. 2006; Haenisch and Bönisch 2011; Araragi and Lesch 2013). These responses have been shown for multiple types of stimuli including minor stress. Furthermore the SERT knockout mice have a significant number of physiological changes, shown in Table 1. These characteristics suggest that the SERT KO mice would be useful in the study of serotonin and serotonin transporter in behavior and learning, obesity and type II diabetes, and other molecular consequences of SERT interactions (Dennis L Murphy and Lesch 2008; M. Berger, Gray, and Roth 2009)

Additionally, SERT knockout mice display serotonin concentrations reduced to

<10% of normal in most peripheral tissues where serotonin is not synthesized (Brenner et al. 2007; Dennis L Murphy and Lesch 2008; Kim et al. 2005). In all of these tissues, the reductions in tissue serotonin content seems to be a direct result of a failure of SERT-mediated uptake system, as serotonin synthesis is not affected by genetic loss of SERT, and may even be increased (Araragi and Lesch 2013). The minimal concentrations of serotonin still found in these tissues most likely arise by to compensation from other transporters. One example of this is the DAT-mediated accumulation of excess serotonin in dopaminergic neurons in the substantia nigra in SERT knockout mice (F. C. Zhou, Lesch, and Murphy 2002). Additionally, SERT knockout mice display increased expression of organic cation transporters OCT1 and OCT3, which are low-affinity transporters of monoamines (Kim et al. 2005).

At a biochemical level, SERT heterozygous knockout mice, have fewer specific SERT binding sites as determined with labeled SERT ligand (J R Homberg et al. 2007; Q. Li et al. 2000). They also show decreased serotonin clearance and elevated extracellular serotonin levels. However, it is important to note that they have unchanged tissue serotonin concentrations in the brain and in the periphery and have stable brain serotonin synthesis and turnover.

There are some interesting effects on serotoninergic receptors following the loss of SERT, which may explain sex-dependent responses. In the female SERT knockout mice the 5-HT_{1A} receptors in the brainstem raphe area are substantially (>60%) decreased, with smaller reductions in males and intermediate values in heterozygous mice (Dennis L Murphy et al. 2009; Q. Li et al. 2000; Fabre et al. 2000). Interestingly, female but not male SERT knockout mice also show modest reductions in 5-HT_{1A} receptors in the

hypothalamus and in some areas of the amygdala and septum but show no changes in the cortex or hippocampus (Q. Li et al. 2000). Downregulation of 5-HT_{1A} receptors at presynaptic somatodendritic sites for both SERT knockout and SERT homozygous mice may be a mechanism responsible for the insensitivity of these receptors and of 5-HT_{1A}-mediated neuroendocrine responses. Furthermore, in SERT knockout mice, 5-HT_{2A} receptors are decreased in the striatum, claustrum, and cortex, but increased in the septum and hypothalamus (Dennis L Murphy and Lesch 2008; Fabre et al. 2000). Diminished receptor-mediated responses are not attributable to abnormalities in G‑protein coupling (Q. Li et al. 2000). While there are some concerns using a constitutive model of lost SERT function, the $SERT^{-/-}$ mouse is still a powerful approach especially in isolated systems like the platelet.

SERT KO Phenotypes	References
Reduced SERT binding sites	(J R Homberg et al. 2007; Q. Li et al. 2000; Thakker et al. 2005; M. A. Fox et al. 2007)
Reduced serotonin uptake	(Adamec et al. 2006; Haenisch and Bönisch 2011; Araragi and Lesch 2013)
Reduced serotonin clearance	(Baganz et al. 2010)(L. C. Daws et al. 2006) (Mathews et al. 2004)
Decreased concentration of serotonin in brain and periphery	(Bengel et al. 1998)(G Gobbi et al. 2001)(Vogel et al. 2003)
Increased serotonin synthesis	(Kim et al. 2005)
Increased glucose, leptin, cholesterol, and triglycerides	(Haub et al. 2010)
Increased anxiety, learned fear, learned helplessness, acoustic startle	(A. V Kalueff, Jensen, and Murphy 2007; J R Homberg et al. 2007; Araragi and Lesch 2013)
Decreased aggregation, exploratory activity, rotarod agility, wire hang	(Monassier et al. 2010)
Increased stress response (ACTH, corticosterone, epinephrine, temperature and motor responses)	(Adamec et al. 2006; Adamec, Holmes, and Blundell 2008)
Increased gut motility and body weight	(J. J. Chen et al. 2001; Haub et al. 2010)
Decreased glucose tolerance, insulin sensitivity, brain glucose utilization, bone mass and strength	(Esaki et al. 2005)
Decreased nociception, bladder function	(L. L. Cornelissen et al. 2005)
Platelet serotonin decreased and plasma levels decreased	(ANDERSON et al. 1990; G. M. Anderson et al. 1987; G. M. Anderson and Cook 2016b)
Decreased SSRI effects, ipsapirone effects, MDMA effects, alcohol effects on 10-day treatment	(L. C. Daws et al. 2006)
Increased cocaine effects, alcohol effect on motor function	(Rocha 2003)

Table 3. ***SERT KO phenotypes****. The various phenotypes related to knockout of the serotonin transporter is reported.* (Amireault, Sibon, and Côté 2013)

The acute role of the serotonin transporter in platelet function

The serotonin transporter is not only the gate-keeper for the acute uptake of serotonin by platelets, but also long-term inhibition of SERT function regulates the serotonergic tone of the periphery (Brenner et al. 2007; Maurer-Spurej, Pittendreigh, and Solomons 2004). There are multiple ways by which acute uptake of serotonin by SERT could modulate platelet function. Some examples of how acute uptake by SERT to modulate cell function include changing the intracellular signaling hub of SERT or non-GPCR-mediated function of serotonin intracellularly. Additionally, uptake of serotonin could modify the oxidative state within the cell. One mechanism that has gained recognition in platelets for the non-GPCR-mediated role of serotonin intracellularly is serotonylation. Below I will discuss the details of serotonylation in platelets and the role of serotonylation in platelet function.

Serotonylation

Serotonylation is the post-translational modification of a glutamine residue on protein by the enzymatic addition of serotonin by a transglutaminase (Griffin, Casadio, and Bergamini 2002; Puszkins and Raghuraman 1985; Höltje, Winter, Pahner, et al. 2003; J. C.-Y. Lin et al. 2013; Diego J Walther et al. 2003; Dale et al. 2002). Serotonylation has been implicated in a number of other systems and pathological processes including fibrosis and pulmonary hypertension (J. C. Lin et al. 2013; Guilluy et al. 2009).

Importantly, there are two major transglutaminases (TGase) that could mediate serotonylation (Puszkins and Raghuraman 1985). One is FXIIIa, which is a member of the coagulation cascade and has been implicated in the formation of "coated platelets". Second

is tissue transglutaminase 2 (TG2), which has also been implicated in multiple diseases(Esposito and Caputo 2005). FXIII is found on the extracellular side of the membrane while TG2 is found intracellularly. Therefore, the possibility of serotonylation as a mechanism that regulates platelet function can happen on both the extracellular and intracellular side of the membrane. Knowledge regarding both intracellular and extracellular serotonin concentration, and how these levels are modulated by SERT, is vital in both settings.

Coated-platelets, formerly known as COAT-platelets, represent a subpopulation of cells that are observed following a dual stimulation of platelets with both collagen and thrombin (Szasz and Dale 2002; Jobe et al. 2005; Alberio et al. 2000). Similar to having a "coat" of extracellularly serotonylated glycoproteins, these platelets have increased phosphatidylserine surface expression that leads to greater prothrombinase activity. The proteins that are retained on the platelet surface include a number of α-granule proteins that are associated with a serotonin molecule. It is suggested that serotonylation of proteins lead to a modification that increases their retainment to the platelet surface through both fibrinogen and thrombospondin binding sites. This process is distinct from intracellular serotonylation, but the mechanism seen in extracellular serotonylation remains consistent (Jobe et al. 2005; Szasz and Dale 2002; Dale et al. 2002).

Intracellular serotonylation has been a proposed mechanism in a number of different cell types. This mechanism involves the modification of small GTPases including Ras and Rho. Also, GTPase modulates a number of intracellular events including granule exocytosis, spreading, contraction, etc. modification of their activity by serotonylation presents a major potential effect, which would be disrupted with lost SERT function.

Below, I will go into more detail about the mechanisms of serotonylation and discuss the current understanding of serotonylation in platelets.

Substrates for serotonylation

GTPase serotonylation is postulated to occur on a conserved glutamine residue within the GTP-hydrolyzing domain, thereby regulating GTP hydrolysis (Visvikis, Maddugoda, and Lemichez 2010; Schmidt et al. 1999; Schmidt et al. 1997; Hummerich et al. 2012; Diego J Walther et al. 2003). The GTP hydrolysis domain is shared by members of the Gα protein family and could be potential targets for transglutaminase-mediated serotonylation as well.

Given the vast number of proteins that could undergo serotonylation, RhoA is of particular interest, as a small GTPase. It has a known role in platelet function including stabilization of αIIbβ3 adhesions and clot retraction (Tabu and Yo 2001; I. Pleines et al. 2012; Article 2013). Constitutive activation of RhoA could be the boundary between reversible and irreversible platelet activation in platelets. Other GTP proteins have also been suggested substrates for serotonylation including Ras and Rab4 (J. C. Lin et al. 2013; Diego J Walther et al. 2003). It is believed that the interaction of Ras and Raf-1 is interrupted following serotonylation (J. C. Lin et al. 2013). While many have suggested a role for serotonylation in platelet function, the full implications of serotonylation have yet to be determined.

Serotonin in platelets: possible mechanisms explored through the work of Kilic et al.

The work of Kilic et al. comprises a significant effort to explore the role of serotonin in platelet function, including both the intracellular and extracellular modulation of platelet serotonin. Kilic's group focuses on how circulating serotonin is implicated in several disease states, including hypertension. In a Journal of Neurochemistry article written in 2007, Kilic studies the effects of serotonin on platelets and in patients with high blood pressure (Brenner et al. 2007). Serotonin uptake rates were reduced in hypertensive platelets with reduced Vmax but no difference in Km of SERT. This was due to a decrease in SERT surface expression and not whole expression levels. It was also found that platelet serotonin content decreased 33% in hypertensive patients as a result of reduced SERT surface expression. It is interesting to note that treatment with serotonin altered SERT uptake rates in a bi-phasic manner.

Expanding on these observation using CHO cells, Kilic et al demonstrates that extracellular serotonin treatment regulates surface SERT expression to show a role of intracellular serotonylation (Ahmed et al. 2008). They build from a previous observation from Walther et al. that suggests that acute uptake of serotonin can lead to receptor-independent function (Diego J Walther et al. 2003). In the work of Walther et al., it was reported that the stimulation of cells with high serotonin concentrations (15µM, [14C]-5HT) induces transamination of Rab4, a small GTPase, intracellularly (Diego J Walther et al. 2003). In Kilic et al. they demonstrates that treatment of serotonin leads to a significant reduction on surface SERT levels at high concentrations greater than 50µM (Ahmed et al. 2008). They conclude that association between Rab4-GTP is activated by sertotonylation and that activated Rab4 leads to SERT internalization (Ahmed et al. 2008). Many have

suggested similar mechanisms is other cell types (Muma and Mi 2015; Paulmann et al. 2009; Watts, Priestley, and Thompson 2009b).

One mechanisms to test serotonylation is through administration of SSRIs that block the acute uptake of serotonin. Kilic et al. demonstrate that SSRIs counteract the effect on elevated plasma serotonin levels (Mercado and Kilic 2010; Fraer and Kilic 2015; Ziu et al. 2012; Brenner et al. 2007; Singh et al. 2013). The reports published in Sci Rep 2014 entitled "Effect of serotonin on platelet function in cocaine-exposed blood", Kilic et al. report that cocaine which also downregulates SERT function leads to an increase in aggregation rates compared to control mice (Ziu et al. 2014). Interestingly, they show that infusion of serotonin increases aggregation while injection of paroxetine leads to a significant reduction in aggregation following stimulated with collagen. Additionally, the concentration of citalopram and paroxetine that they were using in the previously mentioned studies range from 50-100μM, far outside of the range necessary to block SERT-mediated uptake (Sauer, Berlin, and Kimmel 2003). Kilic et al. also indicate that acute loss of SERT leads to reduced platelet surface P-selectin levels with paroxetine but increased P-selectin levels with cocaine (Ziu et al. 2014). P-selectin is released to the platelet surface following α-granule exocytosis, and these finding would suggest that acute loss of SERT uptake leads to reduced granule exocytosis.

To understand modulation of serotonin Kilic et al attempts to separate the platelet and plasma levels of serotonin in whole blood. Whole blood serotonin levels is around 1ng/μL in the blood in saline treated mice while paroxetine-treated mice had approximated 3ng/μL of blood and cocaine treated mice have approximately 3.5ng/μL of blood serotonin levels and are closer to plasma levels typically seen (Ziu et al. 2014). This is about 10 times

lower than the typical levels of serotonin reported in the blood (Brenner et al. 2007; G. M. Anderson 2007; Anderson et al. 1990).

However, Kilic et al. have also suggest that serotonin could act through the $5HT_{2A}R$, the only receptor found on platelets (Lesch et al. 1993). Mice transfused with serotonin showed increased aggregation when stimulated with ADP (Ziu et al. 2012). Increased aggregation was also observed following stimulation with collagen at 3µg/mL. Both of these effects could be blocked with the Sarpogrelate, an antagonist of $5HT_{2A}R$ and $5HT_{2B}R$. The antagonist was injected 30mg/kg body weight at 18 hrs post infusion, and the platelets were purified at 24 hrs. These experiments would suggest that the increased aggregation in hyperserotonemia conditions occurs through a $5HT_{2A}R$ dependent manner, as $5HT_{2B}R$ is not expressed on platelets. This paper also suggests that plasma serotonin levels are increased, which is still very controversial blood (Brenner et al. 2007; G. M. Anderson 2007; Anderson et al. 1990). Furthermore, Kilic et al. indicate that treatment with paroxetine is able to rescue these effects through reduced serotonin concentration and attenuating platelet activation and aggregation. In total, this work suggests that further investigation of the role of serotonin in platelet activation and its mechanisms are reduced.

Finally, in Kilic et al. group also suggest an alternative mechanism of serotonin role in platelet activation. In the paper "A serotonin-induced N-glycan switch regulates platelet aggregation" they linked the increased levels of plasma serotonin to alter glycoprotein platelet surface content leading to increased aggregation(Mercado et al. 2013). This is a fascinating non-receptor, non-SERT mediated mechanism that could play a role in many extracellular processes (Jobe et al. 2005; Szasz and Dale 2002; Alberio et al. 2000). Further work is needed to understand the role of non-receptors and non-transporter mediated

function of serotonin in the periphery. This chapter looks to expand on these observations by examining the roles of acute versus chronic inhibition of SERT function. I establish pharmacologically relevant SSRI-treatment models that test the function of SERT while attempting to reduce the chances of off-target effects. We test for non-SERT mediated effect of citalopram using the citalopram insensitive SERT M172 mouse model (B. J. Thompson et al. 2011; Henry et al. 2006). Following these experiments, the role for serotonin in platelet activation and function will be discussed.

Acute SSRI treatment

Abstract

SERT is a regulator of the serotonergic signaling system in the periphery, and there are a few mechanisms of 5HT-mediated effects on platelet function that have been proposed. Most recently serotonylation, or the covalent addition ofserotoninto small GTPase via transglutamination, has been postulated to be one of the mechanisms by which SSRIs reduce platelet activation leading to altered platelet activation (Diego J Walther et al. 2003; Hummerich et al. 2012; Guilluy et al. 2009). Walter et al. demonstrated that serotonylation is important are α-granule exocytosis by using citalopram to block acute SERT uptake. To better understand the effects of citalopram and other SSRIs acute effects on platelet function, we use a novel mouse model that is insensitive to citalopram (Nackenoff et al. 2015; Judith R Homberg 2011) . It has previously been established that SERT levels and uptake, as well as serotonin levels, are normal, in the M172 mouse model, designed from drosophila SERT (Henry et al. 2006; B. J. Thompson et al. 2011). However, the SERT M172 mouse shows significantly reduced citalopram potency. We test the acute effect of blocking SERT function using platelets from the SERT M172 mouse model to determine if there is a non-SERT mediated effect of citalopram on platelet function. Through this work, we elucidate the acute role of SERT in platelet activation and function and test if acute treatment of SSRIs modulates platelet reactivity.

Objectives

- To determine if acute blockage of SERT with citalopram or other SSRIs alters platelet activation.

Results

Validation of the SERT M172 model in platelets. The M172 mouse model show reduced potency for SERT with citalopram, we examined platelet ^{3H}5HT uptake in platelets (Figure 14). We found that the wild-type SERT I172 mouse model demonstrated a logIC50 of -9.893. The SERT M172, however, show a significantly increased IC50 at -7.138. These results show over a 100 fold decrease in the sensitivity of citalopram to block the SERT M172 platelet ^{3H}5HT uptake. Previously, it has been determined that the SERT M172 mouse model responded similarly to paroxetine blockage (J R Homberg et al. 2007; B. J. Thompson et al. 2011).

, We decided to focus on two particular concentration of citalopram and paroxetine to examine the effect of citalopram and acute SERT inhibition on platelet function: 10nM and 10μM. We use 10nM for determining citalopram specific effect and 10μM to study platelet function using SSRI concentrations previously published in the literature. Using these two concentrations, we can distinguish the SERT-specific and non-SERT specific effect of SSRIs on platelet function.

Citalopram effects on platelet function. To examine the effects of SSRIs on platelet function we looked at two major markers of platelet activation. JON/A is used as a marker for αIIbβ3 activation following agonist stimulation while an anti-P-selectin antibody was used measure surface P-selectin expression following α-granule exocytosis. We also examined platelet activation with two different strength agonists: ADP, a relatively weak agonist, and thrombin, a strong platelet agonist.

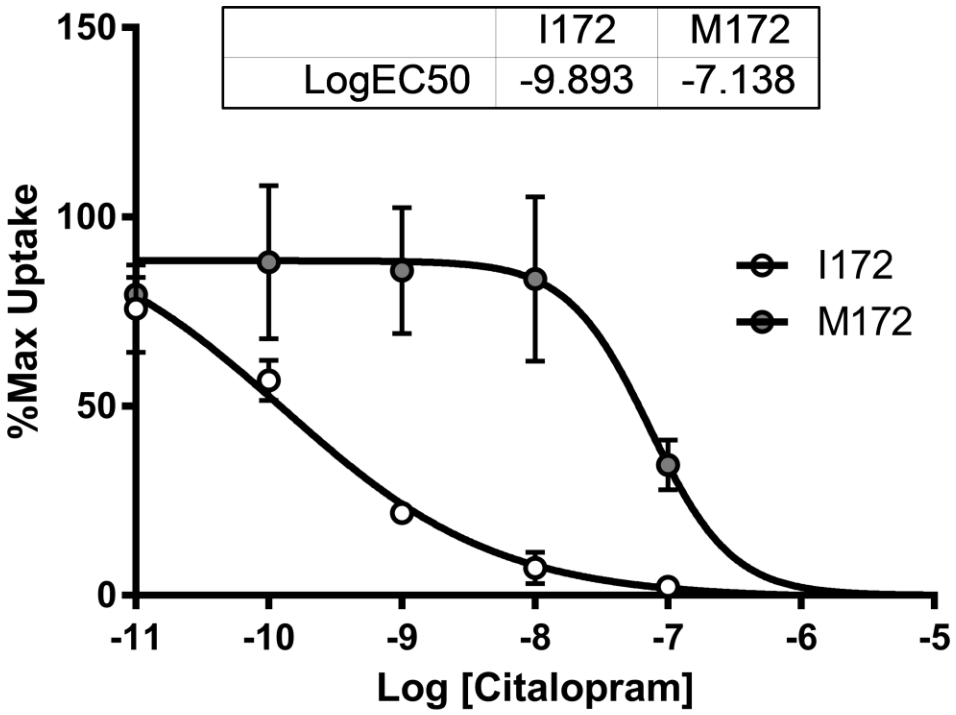
We found that there was no effect on P-selectin expression with the stimulation of thrombin at 10nM in the presence of 10nM citalopram (Figure 14). However, there was a minimal but significant decrease in JON/A binding when 10nM citalopram was present. This effect was only observed in the I172 platelets but not the M172 platelets. These findings show that citalopram has an effect on platelet function at 10nM citalopram concentration following 10nM thrombin activation that is not seen in the M172 SERT mutation and suggests that this effect is SERT-specific. There was also no effect on either P-selectin or JON/A with 10nM citalopram following activation with ADP.

Next, we examine platelet activation using the higher concentration of citalopram (10μM) that has been previously published in the literature and suggested to have an effect on platelet function (Figure 15). At this concentration of citalopram, which affects the M172 and I172 mouse platelets equally based on the curve shown in Figure 14, we see no effect on platelet reactivity with either thrombin or ADP (Figure 16). Additionally, we examined the effect of paroxetine on platelet activation using an alternative SSRI, paroxetine, which would affect both M172 and I172 SERT on platelets equally. We found that at either 10μM or 10nM paroxetine with either ADP or thrombin. These findings show no effect of paroxetine on platelet activation (Figure 17).

Finally, we looked at another aspect of platelet function, platelet spreading (Figure 18). We found that citalopram significantly reduced platelet spreading at high concentrations. However, it reduced platelet spreading in a non-genotypic manner between M172 and I172 SERT models. Both the M172 and I172 platelets show significantly reduced platelet spreading at 100nM and 1μM concentrations. These finding would suggest that citalopram has an effect on platelet spreading that is non-SERT mediated because there

is still a significant decrease in SERT specificity between the M172 and I172 SERT genotypes at 100nM citalopram.

To see if there are any differences between mice and human platelets responsiveness to blockage of SERT uptake, we examine acute SERT inhibition in purified human platelets (Figure 19). PAC-1, which binding to the human αIIbβ3 activate conformation similar to the JON/A antibody, showed no effect with either citalopram or paroxetine below 100μM concentrations, far beyond the physiological range of SSRI concentration. However, we also examined aggregation in human platelet following stimulation with ADP in the presence of increasing concentration of citalopram. We found that ADP-mediated aggregation was reduced at 0.45nM, 4.5nM and 45nM treatment with citalopram (Figure 18). These findings mimic the effects observed in mouse platelets such that inside-out αIIbβ3 activation is not altered in the presence of SSRIs, but that outside-in functional regulation of αIIbβ3 is disrupted with citalopram is present.



	I172	M172
LogEC50	-9.893	-7.138

	I172	M172
Goodness of Fit		
Degrees of Freedom	17	17
R square	0.9836	0.8804
Absolute Sum of Squares	476.6	3114
Sy.x	5.295	13.53

Figure 12. ***Reduced sensitivity to blocking uptake of ^{3H}5HT by M172 as compared to I172 SERT mouse platelets with citalopram****. Shown is the uptake rates of M172 and I172 platelets normalized to non-citalopram treated controls. Nonlinear regression was used to determine IC50 values which were determined to be 1.27X10^{-10} for WT platelets and 7.27X10^{-8} for M172 platelets. Experiments represent n=3 run in duplicate.*

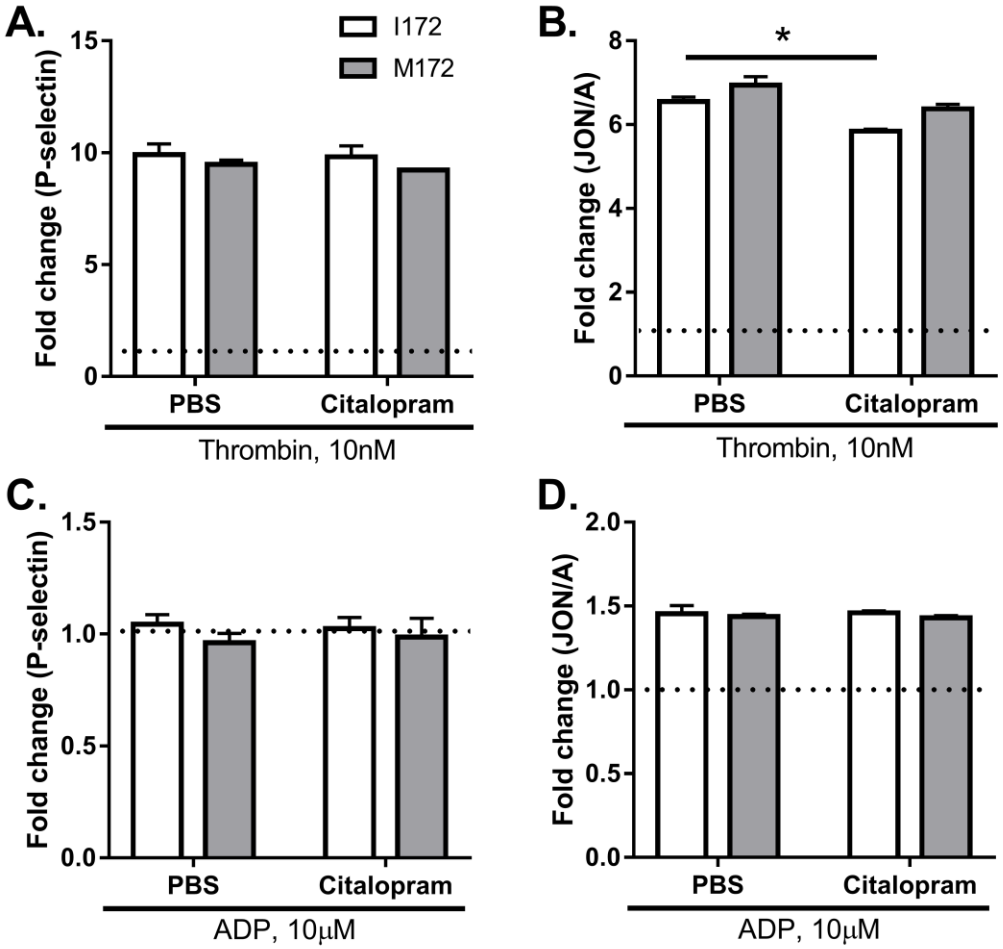


Figure 13. Acute treatment with 10nM citalopram reduces JON/A activation.Platelet were preincubated with citalopram for 5-10 minutes minutes prior to platelet acitivation with agonist. Tere was no difference in P-selectin expression +/- citalopram in either the M172 or I172 mouse models (A). Ther was a significant decrease in I172 citaloprma treated platelets with thrombin for JON.A binding (Figure B: two-way ANOVA: Interaction: n.s., SSRI P=0.0011, SERTm P=0.0069, PBS:I172 vs citalopram: I172 P=0.0261). No effect was seen with citalopram in either genotpe when activated with ADP (C ,D).*

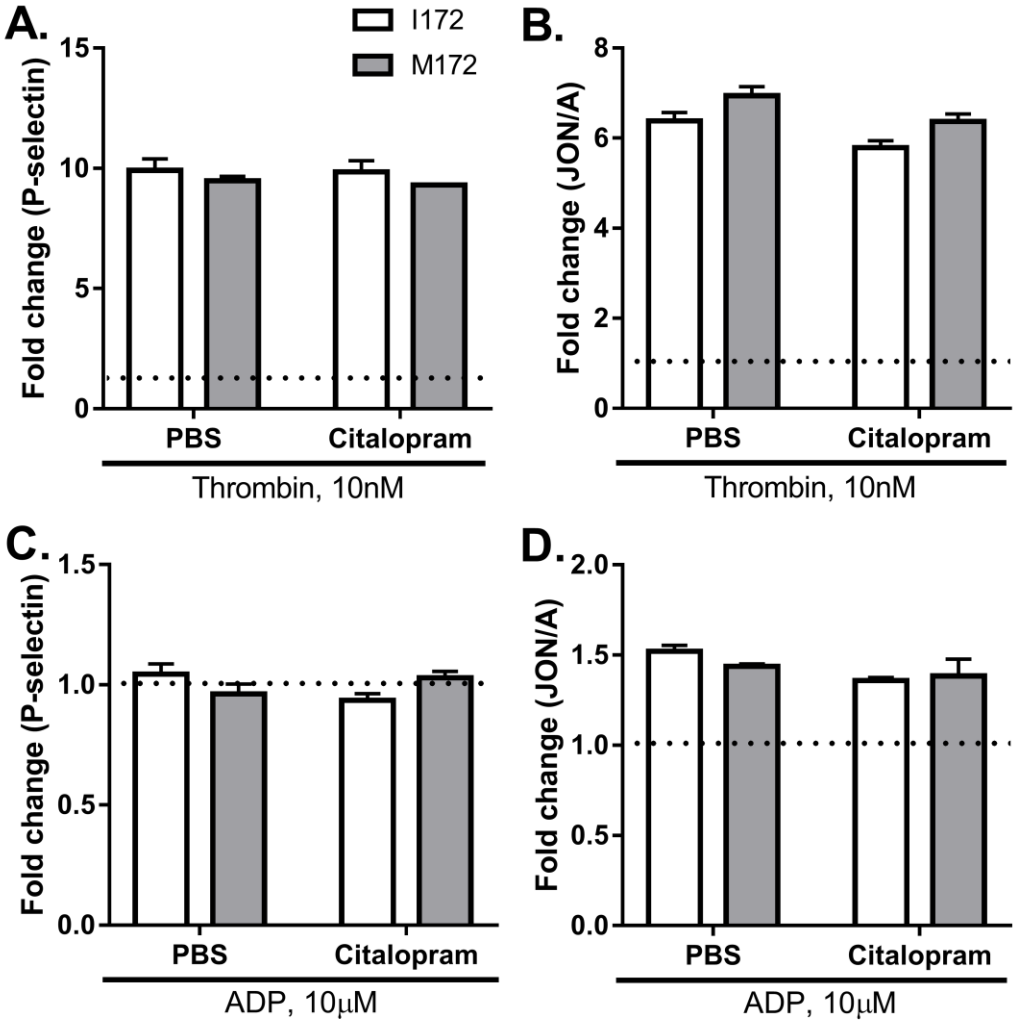


Figure 14. ***No effect of 10μM citalopram on platelet thrombin or ADP activation****. To examine the effect of citalopram at a higher concentration we pre-incubated the platelet for 5-10 minutes with citalopram before activation. We found no major difference in activation with either ADP or thrombin in the presence of 10μM citalopram. (Two-way ANOVA, N=3 run in duplicate).*

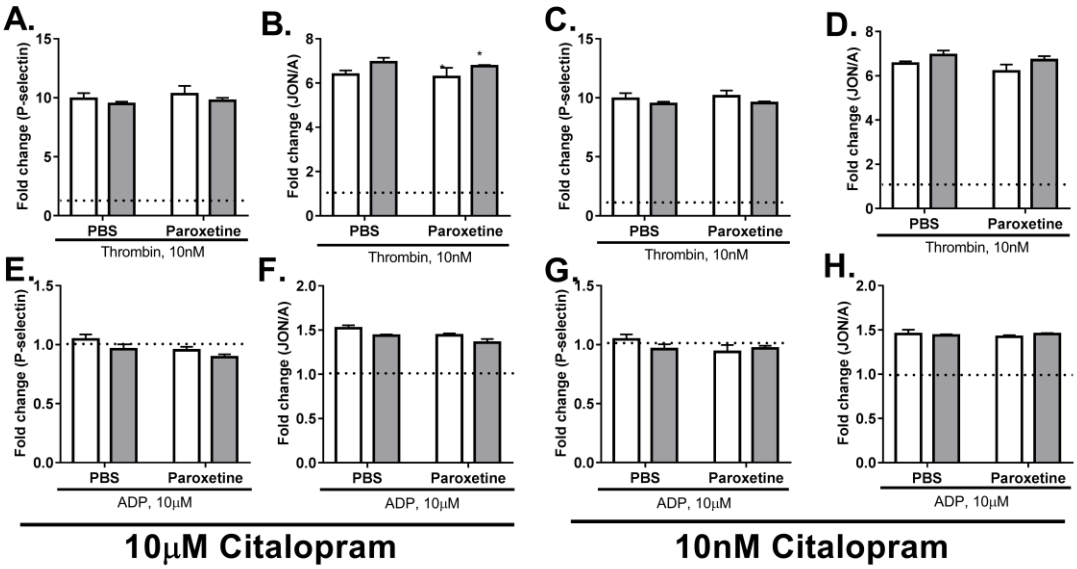


Figure 15. **Paroxetine at 10nM or 10μM has no effect on platelet activation with ADP or thrombin**. To examine the effect of paroxetine we preincubated the platelet for 5-10 minutes with either 10nM or 10μM paroxetine before activation. We found no major difference in activation with either ADP or thrombin in the presence of paroxetine. (Two-way ANOVA, n=3 run in duplicate).

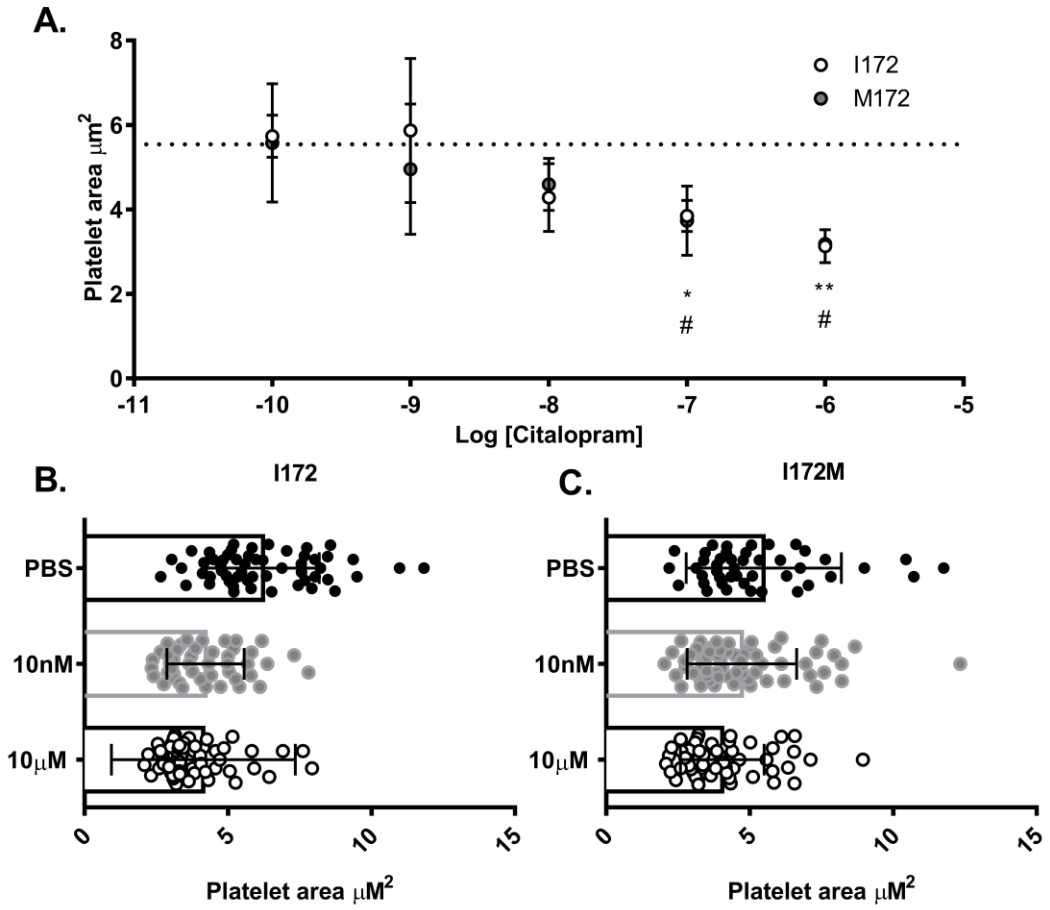


Figure 16. ***Citalopram reduces platelet spreading in both M172 and I172 SERT mouse platelets equally, suggesting a non-SERT-dependent mechanism****. A dose-response curve was done examining platelet spreading (Figure 20A). There was a statistically significant reduction in platelet spreading at 1X10^{-7} and 1X10^{-6} molar citalopram (Two-way ANOV: citalopram effect P,0.0001, PBS:I172 versus -7:I172 P=0.0379, PBS:M172 versus -7:M172 P=0.0246, PBS:I172 versus -6:I172 P=0.0019, PBS:M172 versus -7:M172 P=0.0318). This effect was repeated in a separate experiment comparing the platelet area in I172 (B) and M172 (C) respectively.*

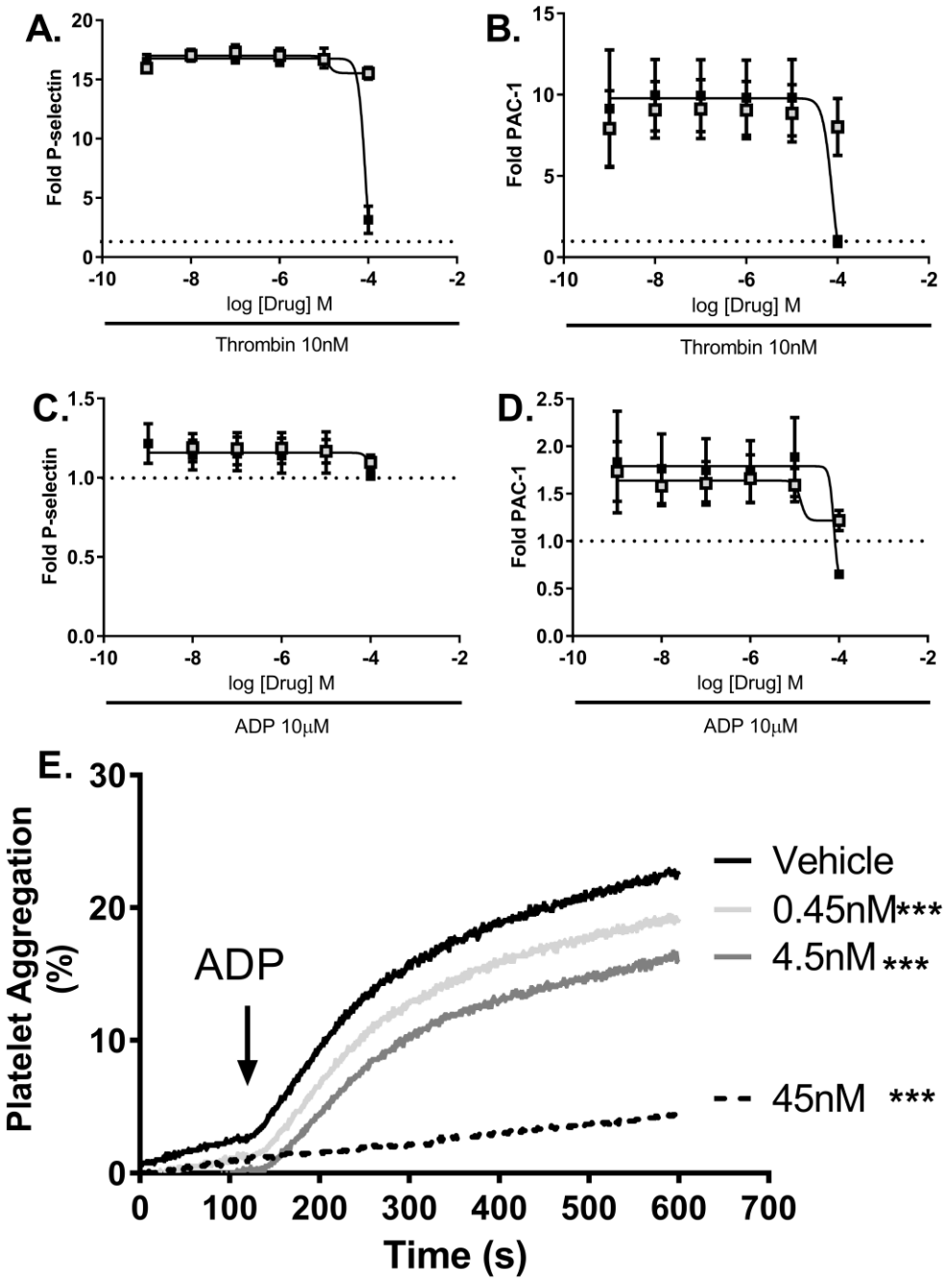


Figure 17. ***No effect of citalopram or paroxetine on human platelet activation put increasing concentrations of citalopram inhibit ADP-mediated platelet aggregation****. There was no effect of either Paroxetine or citalopram on human platelet activation within a reasonable concentration (>10μM: Two-way ANOVA). However, There was a significant decrease in ADP-mediated human platelet aggregation with all concentrations shown (n=4).*

Alternative effects of lost SERT function: Serotonylation of extracellular matrix proteins

Abstract

In the previous section, we establish that acute treatment of SSRIs do not alter platelet activation but that citalopram, on the type of SSRI, may have an off-target effect that reduces αIIbβ3-medaited outside-in signaling. Two alternatives possible mechanism that could explain the effect of sustained SSRI treatment on platelet function are 1) a role of serotonin in coagulation and 2) an alternative role of serotonin in platelet function. Here I will explore the first possibility by examining the serotonylation of peripheral proteins, in particular, the extracellular matrix protein fibronectin. We then discuss what these finding could mean for acute versus chronic blockage of SERT function and the role of serotonin.

Objectives

- To determine if serotonylation as a possible molecular mechanism that could regulate extracellular matrix proteins

Results

In the last section, we discussed how blocking acute uptake of SERT does not alter platelet activation but does seem to have an effect on platelet spreading. One possible alternative effect of blocked SERT uptake is increased extracellular serotonin concentrations. Relating back to serotonylation, we investigated if post-translational modification one extracellular matrix protein, fibrinogen. To study this effect we incubated increasing concentration of the enzyme TG2 with 100nM ^{3H}5HT and 20µg fibronectin. We found that there was an increase in incorporation of ^{3H}5HT onto fibrinogen with increasing levels of TG2 (Figure 18). We determined that this was an enzymatic process mediated by a transglutaminase by using the TGase inhibitor cystamine at 10mM concertation to block incorporation. These findings suggest that acute inhibition of SERT may increase extracellular serotonin concentration and possibly modify extracellular matrix proteins.

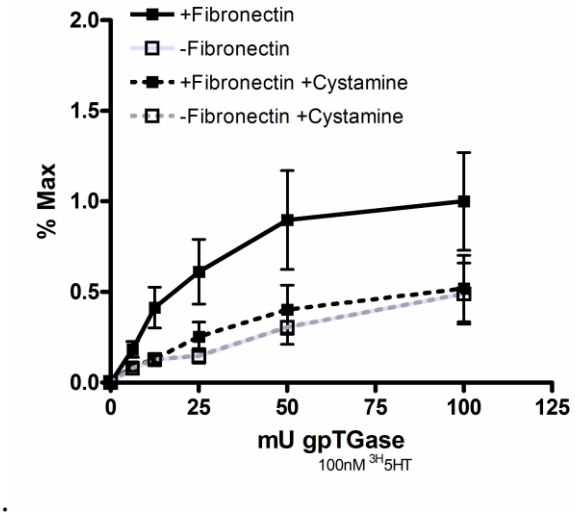


Figure 18. ***Serotonylation of fibronectin blocked by treatment with cystamine****. Incorporation of ^{3H}5HT in onto fibrinogen was measured. Samples were incubated with 20µM fibronectin and 100nM ^{3H}5HT. Increasing concentrations of TG2 was added to determine if the enzyme increased incorporation of the 3H5HT. We did see an increase of incorporation that was blocked with a TGase inhibitor cystamine (10mM).*

Conclusion

SSRIs and their acute versus chronic effect on platelet function

When patients are chronically taking SSRIs, there is a significant reduction in serotonin content (approximately 66% lower) and reduced ADP, collagen or epinephrine-induced platelet aggregation (10-51%) (Bismuth-Evenzal et al. 2012b). There are many contradictory findings that study the effects of long term SERT inhibition on platelet function (Tseng et al. 2010; Jedlitschky, Greinacher, and Kroemer 2012; Bismuth-Evenzal et al. 2012b; Schellander and Donnerer 2010; Ana Marin D Carneiro et al. 2008; Andrade et al. 2010; Maurer-Spurej, Pittendreigh, and Solomons 2004). In some reports, patients taking SSRIs do show a significant reduction in platelet aggregation and dense granule release in response to ADP when given acutely (Maurer-Spurej, Pittendreigh, and Solomons 2003). Acute treatment with citalopram showed reduced collagen-induced aggregation but not aggregation mediated by thrombin, U46619, and ionomycin (Tseng et al. 2010). These studies also found that acute treatment with citalopram also reduced α and dense granule exocytosis in response to collagen as measured by reduced p-selectin and adenosine triphosphate release, respectively. However, both of the previous studies mentioned use citalopram at concentrations ranging from 1μM-50μM, far outside the reported inhibitory concentration required to block SERT uptake (Tseng et al. 2010; Maurer-Spurej, Pittendreigh, and Solomons 2003; Tseng et al. 2013). A better pharmacological approach is required to elucidate the role of serotonin and SERT in platelet aggregation.

Acute inhibition of SERT functions in platelet activation

Our data explores the effects of SSRI and SERT on platelet function. Overall, our results suggests that acute treatment with an SSRI has little to no effect on P-selectin expression as a measure of α-granule exocytosis. We did find a subtle SERT-dependent effect on αIIbβ3 –activation with the addition of citalopram in the presence of thrombin stimulation. We believe that this effect is citalopram specific because the same phenotype was not observed in the paroxetine-treated samples. Furthermore, it is unclear how physiologically relevant these finding are because the difference in αIIbβ3-activation was minimal with citalopram pre-treatment. Similar to the mouse experiments, there was no observed effect on the human platelets with either paroxetine or citalopram. However, in both the human and mouse platelets, there was an effect of citalopram on platelet spreading, although this was far beyond the concentration needed to block SERT uptake. These findings suggest that citalopram may modulate platelet outside-in αIIbβ3 signaling and or function in such a way that it disrupts function in a non-SERT specific manner.

SSRIs are a major class of antidepressant drugs that are currently taken by 1 in 10 Americans ages 12 and over (Lichtman et al. 2014). There are a number of SSRIs that are currently on the market including citalopram (Celexa and Cipramil) and paroxetine (Paxil and Seroxat). These two compounds in particular show distinct structural feature that both block SERT uptake (Gabrielsen et al. 2012). Both drugs are typically administered orally with a once a day dose that leads to blood concentration ranging from 0.1-1μM levels (Maurer-Spurej, Pittendreigh, and Solomons 2004; Serebruany et al. 2003; Worm et al. 1998; Ostad Haji et al. 2011). Besides SSRIs central nervous system effects, it has been observed that SSRIs lead to a significant alteration in hemostasis (Andrade et al. 2010;

Schellander and Donnerer 2010; Wozniak et al. 2011; Bismuth-Evenzal et al. 2012b). From a pharmacological perspective, it is important to consider the effects of physiologically relevant concentrations of these SSRIs on platelet function to make an appropriate conclusion from experimental data. In particular, it is important to separate the role of lost serotonergic tone during platelet activation as compared to role of the SSRIs in blocking SERT function.

Some of the SSRIs effects of bleeding include increased bleeding risk, protection from recurrent thrombotic events, and reduced platelet aggregation (Andrade et al. 2010; Schellander and Donnerer 2010; Bismuth-Evenzal et al. 2012b). The mechanisms of these effects are unclear. However, it has been reported that acute uptake of serotonin by SERT leads to the transamination of small GTPase proteins that are important for mediating α-granule exocytosis (Diego J Walther et al. 2003). Our data suggest that blocking SERT as concentrations that inhibit uptake of serotonin does not alter α-granule exocytosis as determined by P-selectin surface expression. It has also been shown that citalopram inhibits collagen-induced platelet aggregation at 50μM when given acutely, but it is likely that this a non-SERT dependent mechanism based on our finding with the M172 citalopram-insensitive mouse model(Tseng et al. 2010). These findings suggest a non-SERT dependent effect on platelet function from high citalopram treatment occurs possibly due to an off-target effect of citalopram beyond the therapeutic range.

Possible off-target effects of citalopram

SSRIs and SNRIs are a pharmacological upgrade from the tricyclic antidepressant initially developed (M. J. Owens et al. 2001). However, these drugs still have numerous

and notorious off-target effects(Bonnin et al. 2012). Additionally the response rate or only ~50% and a remission rate of ~30% in the SSRI citalopram (Francesc Artigas 2013). As a group, SSRIs have a response rate of 60% ad a remission rate of 40% (Thase, Entsuah, and Rudolph 2001; Tollefson and Holman 1994).

One major off-target effect of citalopram that is non SERT-dependent of activation of sigma-1 receptors (Bonnin et al. 2012; Maurice and Su 2009). Currently, it is not known if sigma-1 receptors are expressed by platelets. Citalopram has little to no effect on noradrenergic receptors but does have the highest affinity for histamine H1 receptors(M. J. Owens et al. 2001). As additional consideration for *in vivo* experiments, citalopram has three active metabolites that many contribute to pharmacological effects (Bezchlibnyk-Butler, Aleksic, and Kennedy 2000). The dosing range for citalopram is no greater that 40mg/day for patients. This concentration was corrected from 60mg/day following the FDA announcements that citalopram causes dose-dependent QT interval prolongation. For citalopram efficacy, it is suggested that only ~8% of SERT much be occupied, and this requires a concentration of 50ng/mL (154.13 nM) (Ostad J Clin Psych 2011). This supports the *in vitro* evidence that citalopram as non-SERT targets *in vivo*.

Citalopram has a high affinity for SERT as compared to other transporters with a Ki value of 9.6nM as compared to NET, which is one off-target effect seen with citalopram (Ki of 5,029nM) (J. M. Owens, Knight, and Nemeroff 2016). There are a number of other receptors that citalopram can hit such as $5HT_{2C}$ receptor at 2,051nM (M. J. Owens et al. 2001; J. M. Owens, Knight, and Nemeroff 2016), the α1 receptor adrenergic receptors at 1,211nM (smooth muscle cells), the M1, muscarinic acetylcholine receptor (cholinergic receptor) receptors at 1430nM, and the H1 histamine receptor at 283nM (Besnard et al.

2012). Platelets express the α2 adrenergic receptor that participates in platelet aggregation which could explain some of the non-specific effects of citalopram (K.-P. Shen et al. 2008). Platelets do not appear to express M1 receptors but may express the histamine H1 receptor which has been shown to modulate platelet aggregation(Petríková et al. 2006; Masini et al. 1998). However, future experiments are required to examine this hypothesis.

In this chapter, we have attempted in this chapter to determine the SERT-dependent versus SERT-independent effects of SSRIs on platelet function. We have observed a minimal effect of SSRIs on platelet activation following agonist stimulation. These finding would suggest that at concentrations of SSRIs that are sufficient to block SERT uptake do not significantly alter platelet activation. Instead, we find that treatment with SSRIs, particularly at higher concentrations, lead to altered platelet spreading. Therefore in Chapter 2, we explore how long-term inhibition of SERT leads to an alteration in serotonergic signaling, through $5HT_{2A}R$ on platelets. However, in the following section, I will discuss some alternative mechanism through which serotonin may be acting related to serotonin.

Insight on the alternative mechanisms of serotonin

There are two major classes of proteins that can compose the extracellular matrix proteins (Watson 2009; Dees et al. 2011; Chavakis et al. 2002). One set of these proteins include the polysaccharide chains that fall within the group glycosaminoglycans (Rajtar et al. 1993). These proteins are typically found linked to other proteins in the form of proteoglycans and have shown susceptible to serotonylation (Mercado and Kilic 2010). The second form of extracellular matrix proteins includes fibrous proteins including

collagen, elastin, fibronectin, and laminin. Not only are these proteins found in the blood and vasculature but they also play a significant role in platelet function.

In a report published by Mercado et al. They show that increased plasma levels accelerate aggregation resulting in a hypercoagulable state (Mercado et al. 2013; Ziu et al. 2012). In the condition of increased plasma serotonin levels, there is also an increase in platelet surface proteins showing increased N-glycans. In these experiments, they infused the mice with serotonin in both the 5HT-infused mice and serotonin transporter KO mice and suggested that altered extracellular concentration could lead to increased Cytidine monophosphate-N-acetylneuraminate hydroxylase activity that promotes a change in the surface N-glycan content on the platelet surface.

Similar to these finding, serotonylation of extracellular matrix proteins like fibronectin could modulate platelet attachment and spreading. The connection between extracellular matrix serotonylation and platelet spreading could be one possible mechanism linking the SSRIs on platelet function in association with the protective effect for SSRIs in cardiovascular disease (Wozniak et al. 2011; Tseng et al. 2013; Tseng et al. 2010). We have demonstrated that fibronectin is able to undergo serotonylation with TG2 and is reversible with cystamine, a transglutaminase inhibitor. Further work in needed to clarify the role of extracellular serotonylation and its physiological consequences.

CHAPTER 4

ROLE OF THE $5HT_{2A}$ RECEPTOR IN PLATELET ACTIVATION

Introduction

Neurons and platelets store serotonin in vesicles (Nirenberg et al. 1995; Rudnick 1977; Höltje, Winter, Walther, et al. 2003). These vesicles can be released in a regulated manner leading to physiological effects via cell surface receptor or alternative mechanisms. Upon platelet activation, granules are secreted, potentiating aggregation and clot formation. In particular, serotonin, located within dense granules, can activate the serotonin receptor subtype 2A ($5HT_{2A}$) in platelets, which is a Gαq protein-coupled receptor, leading to Ca^{2+} influx in the cell (Offermanns 2006). In neurons, there are many different types of receptors with differential distribution. Furthermore, the distribution of serotonin receptor subtypes in the brain can change over time, for example with age (Lambe et al. 2011). One report showed that $5HT_{2A}$R binding changes with depression but also decreased significantly with age in multiple areas but less so in the hippocampus (Mintun et al. 2004). In addition to activating cell surface receptors, serotonin has been shown to have action intracellular through serotonylation, although the implication of this mechanism remains controversial (Diego J Walther et al. 2003). A common theme in both neurons and platelets is that serotonin is taken into the cell by SERT(Beikmann et al. 2013). SERT not only regulates the level of serotonin the extracellular space but secondarily the duration and/or presence of the serotonergic activation in both platelets and neurons. In this chapter, I will discuss the impact of the genetic deletion of SERT and the known phenotypic effects.

Serotonergic receptors

While serotonin levels are determined by SERT function, serotonin's action is dependent on recognition by plasma membrane receptors that transmit signals from the extracellular surface. Serotonin mediates its function through activation of a number of different receptors (M. Berger, Gray, and Roth 2009; Francesc Artigas 2013). In total there are seven different groups of serotonergic receptors (Barnes and Sharp 1999). Some of these groups have multiple subtypes for a total of thirteen different serotonergic receptors (M. Berger, Gray, and Roth 2009). Using a combination of approaches, it has been established that most of the serotonin receptors are GPCRs that span the membrane seven times and initiate their singling through binding to G-proteins. The one exception is $5HT_3$ which is a ligand-gated ion channel (Steiner, Carneiro, and Blakely 2008; S. M. Stahl et al. 2013). The extensive work on serotonergic receptor function as benefited from specific pharmacological activation or inhibition to delineate function. Below I will outline the different receptors that are implicated in serotonergic activation (Figure 19). However much more extensive reviews are available (Barnes and Sharp 1999).

Initially, the $5HT_1$ receptor was characterized by observing $^{3H}5HT$ binding that was partially blocked with spiperone (Peroutka and Snyder 1979). The $5HT_1$ receptor is a GPCR that is $G\alpha_{i/o}$ protein coupled. This leads to decreased cellular levels of cAMP and is typically considered to be inhibitory (S. M. Stahl et al. 2013). There are a number of different isoforms of the $5HT_1$ receptor. $5HT_{1a}$ receptor and the $5HT_{1b}$ receptor is found in blood vessels and CNS. Within the CNS is has a known role in addiction, aggregation, and anxiety phenotypes as an autoreceptor that inhibits further serotonin secretion from post-synaptic neurons. It modulates a number of other functions in the periphery including blood

pressure, cardiovascular function, heart rate, and vasoconstriction (M. Berger, Gray, and Roth 2009; S. M. Stahl et al. 2013). In the periphery, it has been demonstrated that activation of $5HT_{1A}$ leads to an increased in ACTH, corticosteroids and prolactin (Gilbert et al. 1988; Gartside et al. 1995). Other receptor isoforms include the $5HT_{1D}$ receptor is also found in blood vessels and CNS and is known to regulate anxiety vasoconstriction locomotion. Finally, the $5HT_{1E}$ receptor is found in blood vessels and CNS and serotonin$_{1F}$ receptor that are in the CNS and known to play a role in migraines.

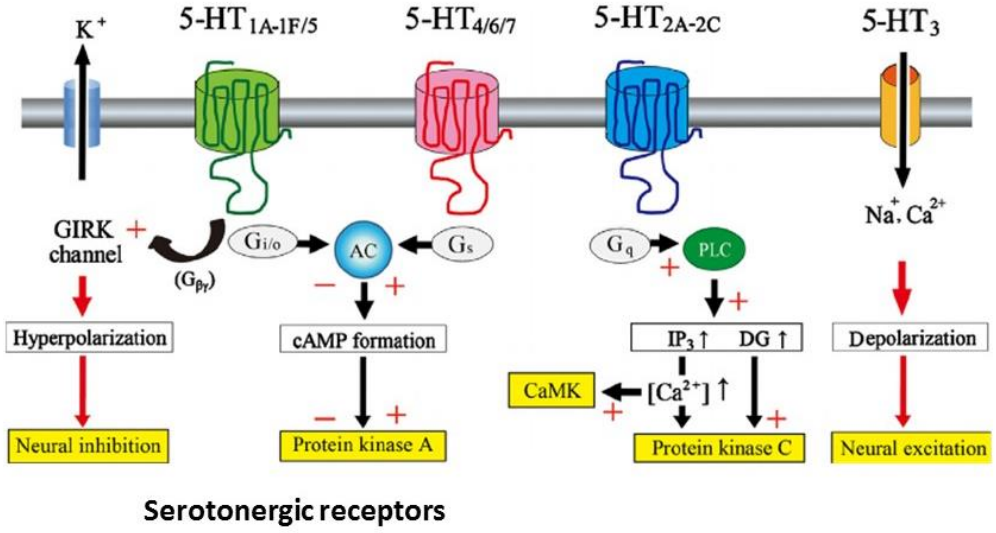


Figure 19. Types of serotonergic receptors and their signaling.(Ohno et al. 2015)

The $5HT_2$ receptor is also a GPCR but is a $G\alpha_{q/G11}$- protein coupled receptor (Miller, Mariano, and Cruz 1997; Przyklenk et al. 2010a; F. Cerrito et al. 1993a; S. M. Stahl et al. 2013). It is also known that it can also be coupled to $G\alpha_{13}$ or can signal through alternative pathways such as β-arrestin (Figure 20) (Schmid, Raehal, and Bohn 2008; Bohn and Schmid 2010; Schmid and Bohn 2010; E. L. Stahl et al. 2015; Coffa et al. 2011). Conical $G\alpha_q$ activation leads to increases in cellular levels of IP_3 and DAG that are typically

excitatory. The $5HT_{2A}R$ is found in blood vessels, CNS, GI tract, platelets, PNS, smooth muscle and have a known role in addiction, anxiety, appetite, cognition, imagination, learning, memory, mood, perception, sexual behavior, sleep, thermoregulation, vasoconstriction. For the serotonin$_{2A}$ receptor that is a number of different agonists including 251-NBOMe (full agonist), 2C-B, 5-MeO-DMT, BZP, Bufotenin, DMT, DOM, Ergonovine, Lisuride, LSD, mescaline, myristicin, PNU-22394 (partial agonist), Psilocin, Psilocybin, TFMPP (partial agonist or antagonist). There are also a number of antagonists that can act on the $5HT_{2A}$ receptor including aripiprazole, asenapine amitriptyline, clomipramine, cyproheptadine, eplivanserin, etoperidone, haloperidol, hydroxyzine, Ketanserin, Methysergide, Mianserin, Mirtazapine, Nefazodone, Pimavanserin, Pizotifen, Ritanserin, Trazodone, Yohimbine. However, as signaling bias between different ligands has been established for $5HT_{2A}R$ activation, as well as inhibition, future studies are necessary to explore the full signaling dynamics of the 5HT2A receptor. An overview of the agonists and antagonists that act on the $5HT_{2A}$ receptor can be found in a number of current reviews and articles (Francesc Artigas 2013; M. Berger, Gray, and Roth 2009; Schmid, Raehal, and Bohn 2008).

The vast array of different agonist and antagonist for the $5HT_{2A}$ receptor are important because some of this agonist and antagonists show biased signaling and divergent effects on the desensitization and internalization of the $5HT_{2A}$ receptor (Schmid, Raehal, and Bohn 2008; Bohn and Schmid 2010; Schmid and Bohn 2010). Additionally, there are atypical antipsychotics: clozapine, olanzapine, quetiapine. Risperidone, ziprasidone that acts on the $5HT_{2A}$ receptor and mediated clinical effects(Sauer, Berlin, and Kimmel 2003; S. M. Stahl et al. 2013). Atypical antipsychotics (antagonists), Psychedelics

(partial agonists), NaSSAs (antidepressants and anxiolytics; they serve as antagonists at this site), Treating serotonin syndrome (antagonists; e.g. cyproheptadine), Sleeping aid (antagonists; e.g. trazodone) all modulate the $5HT_{2A}$ receptor leading to a diverse array of physiological outcomes.

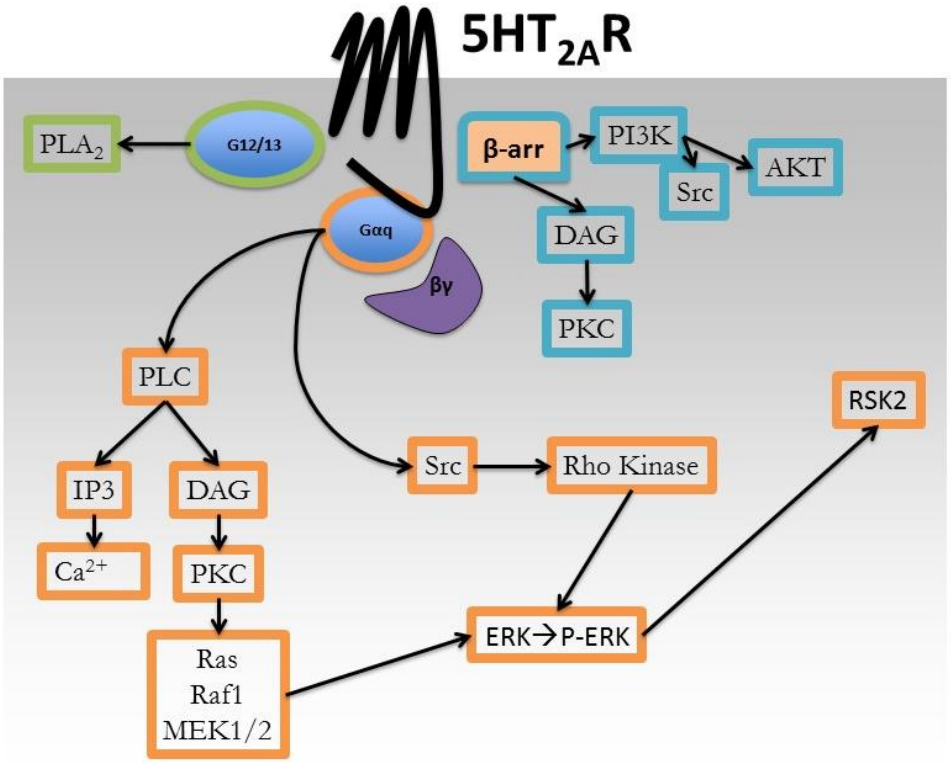


Figure 20. ***Alternative signaling from the $5HT_{2A}R$.*** *Alternative signaling downstream of $5HT_{2A}R$ activation is shown. $G_{12/13}$ signaling leads to PLA_2 activation as shown in green. Canonical Gq signaling leads to PLC activation and Src activation as shown in orange. PLA promotes IP3 and DAG production. Src activates Rho kinases as well as a number of other actions that leads to ERK phosphorylation. Finally, β-arrestin signaling following receptor activation leads to an alternative signaling cascade shown in blue. This pathway results in PKC and AKT activation.*

There is also the $5HT_{2B}$ receptor that is expressed in blood vessels, CNS, GI tract, PNS, smooth muscle. The $5HT_{2B}$ receptor is important in Anxiety, appetite, cardiovascular

Function, GI motility, sleep, vasoconstriction and migraine pathology (Barnes and Sharp 1999). The Agonists of the $5HT_{2B}$ receptor includes BW-723C86, Fenfluramine, MDMA, Norfenfluramine, PNU-22394 (Partial agonist), Ro60-0175. Antagonists and the serotonin$_{2B}$ receptor include Agomelatine, Asenapine, BZP, Ketanserin, Methysergide, Ritanserin, RS-127,445, Tegaserod, Yohimbine.

Finally, the $5HT_{2C}$ receptor that is found in blood vessels, CNS, GI tract, PNS, smooth muscle. Addiction. These receptors may have a role in modulating anxiety, appetite, GI motility. Furthermore, these receptors can also bind a number of other monoamines including norepinephrine and dopamine that mediated locomotion, mood, penile erection, sexual Behavior, sleep, thermoregulation, vasoconstriction (Barnes and Sharp 1999). Agonist of the $5HT_{2C}$ receptor agonists include A-372,159, AL-38022A, Aripiprazole, Ergonovine, Lorcaserin, PNU-22394 (Full agonist), Ro60-0175, TFMPP, Trazodone (hypnotic), YM-348. The $5HT_{2C}$ receptors antagonists include Agomelatine (antidepressant), Amitriptyline, Asenapine, Clomipramine, Clozapine (antipsychotic), Cyproheptadine, Dimebolin, Eltoprazine, Etoperidone, Fluoxetine, Haloperidol, Iloperidone, Ketanserin (antihypertensive), Lisuride, Methysergide, Mianserin, Mirtazapine, Nefazodone, Olanzapine, Paroxetine, Quetiapine, Risperidone, Ritanserin, Tramadol, Trazodone, and Ziprasidone.

The remaining serotonin receptors include the $5HT_3$, which is a ligand-gated ion channel. This ion channel, which is found in the CNS, GI, and PNS, is a ligand-mediated gate that allows Na^+ and K^+ cation channel. This leads to the depolarization of the plasma membrane and an excitatory effect. There is also the $5HT_4$ receptor, which is a GPCR that is $G\alpha_s$-protein coupled leading to increased cellular cAMP levels and is typically excitatory

and found in the CNS, GI, PNS. The $5HT_5$ receptor is also a GPCR this is a $G\alpha_{i/o}$ leading to decreased cellular levels of cAMP and is inhibitory. The $5HT_{5A}$ receptor in the CNS and the $5HT_{5B}$ receptor that is only found in rodents, the serotonin$_6$ receptor which is a GPCR-$G\alpha_s$ increasing cellular cAMP level, excitatory in the CNS, the $5HT_7$ receptor that is also $G\alpha_s$-protein coupled and found in blood vessels, CNS, and the GI.

Platelet serotonergic receptor: $5HT_{2A}R$

The $5HT_{2A}$ receptor is the only serotonergic receptor found on platelets. Its inhibition has proved beneficial in a number of circumstances to reduce platelet function(Bush et al. 1984). It has been long known that $5HT_{2A}$ receptor antagonist inhibits serotonin-dependent platelet aggregation and in cyclic flow for stenosed arteries (Ashton et al. 1986; Przyklenk et al. 2010b). However, they have not been introduced as an anti-platelet drug in the clinic, although many have been developed(Adams et al. 2009; Przyklenk et al. 2010a; Pawlak et al. 1998; Ogawa et al. 2002; Herbert et al. 1993; N. Li et al. 1997; De Clerck et al. 1990). $5HT_{2A}$ antagonist has been shown to inhibit serotonin-potentiated platelet aggregation in PRP in humans and many other species *in vitro* and *ex vivo* and reduced serotonin-stimulates thrombus formation in mice *in vivo*. These studies show that aggregation induced by physiologically relevant agonists such as collagen, ADP, thrombin, and epinephrine can be reduced by $5HT_{2A}$ receptor antagonists *in vitro* and *ex vivo* in a model of coronary occlusion in dogs. However, these results are conflicting (F. Cerrito et al. 1993b; van EE and van Oene 1991). Some have shown that $5HT_{2A}R$ inhibitors like ketanserin have been able to block aggregation and are protective for coronary patency in a model of recurrent thrombosis while other show no effect (Przyklenk et al. 2010a; van

EE and van Oene 1991). In order to better understand the role of serotonin in platelet function further analysis and experimentation are required.

Loss of SERT and the consequences on $5HT_{2A}R$

In the periphery, serotonin is produced by enterochromaffin cells in the gastrointestinal tract, released into the plasma, and quickly taken up by platelets via the serotonin transporter (SERT). Following uptake, serotonin is stored in dense granules by the actions of the vesicular monoamine transporter 2 (VMAT2) (Brenner et al. 2007; Matondo et al. 2009; Beikmann et al. 2013; Bismuth-Evenzal et al. 2012b). Chronic inhibition of SERT through selective serotonin reuptake inhibitors (SSRIs) (e.g. citalopram and paroxetine) leads to dramatically reduced platelet serotonin granule content (Ana Marin D Carneiro et al. 2008; Abdelmalik et al. 2008). This leads to altered peripheral serotonin homeostasis and potentially modifying multiple physiological processes including hemostasis (Namkung, Kim, and Park 2015; Hranilovic et al. 2011; Blazevic et al. 2015b; Amireault, Sibon, and Côté 2013). Clinically, increased bleeding risk has been observed in patients taking SSRIs, and platelet aggregation are disrupted (Tseng et al. 2010; Ana Marin D Carneiro et al. 2008). Within this chapter, I will discuss our characterization of two distinct mouse models of lost SERT function, with results suggesting that sustained loss of SERT function affects the mechanisms of hemostasis.

Dense-granules contain serotonin along with other platelet agonists including adenosine diphosphate (ADP), thromboxane (TXA2), and histamine. Appropriate platelet activation depends on the timely release of these factors (Ana Marin D Carneiro et al. 2008; E. Cerrito et al. 1993; Bismuth-Evenzal et al. 2012b). Platelet aggregation is an crucial

early step in thrombus formation (Ana Marin D Carneiro et al. 2008; Thompson, Scrutton, and Wallis 1986; Bismuth-Eventuzal et al. 2012). Aggregation, which is the bridging of platelet-platelet contacts, requires a conformational switch in αIIbβ3 leading to activation and fibrinogen binding. serotonin has been shown to enhance aggregation in a $5HT_{2A}$ receptor-dependent manner (Mercado et al. 2013; Bismuth-Evenzal et al. 2012b; O. a. Lin et al. 2014; Adams et al. 2009; Przyklenk et al. 2010a). The $5HT_{2A}R$ is the only serotonergic receptor found on platelets and potentiates platelet responses to weak agonists like ADP (N. Li et al. 1997). Sub-threshold concentrations of two different platelet agonists can exert a synergistic effect on platelet activation. One example includes dual ADP and serotonin activation leading to increases in cytosolic [Ca^{2+}] (Thompson NT, Scrutton MC 1986). However, the role of serotonin during *in vivo* hemostasis remain unclear, particularly in the context of chronic SERT inhibition.

In order to elucidate the underlying mechanisms of SSRI effects on platelet aggregation, a better understanding of acute versus chronic inhibition of SERT function during platelet activation is required (Chapter 3). Acute and chronic blockage of SERT function result in distinct scenarios in terms of the effects on serotonin homeostasis. Acute inhibition of SERT blocks the amount of serotonin carried into the cytosol during platelet activation while chronic blockage of SERT slowly depletes platelet serotonin levels resulting in reduced whole blood serotonin, a loss of serotonin secretion, and serotonin signaling during platelet activation. Some have shown that acute SERT-mediated serotonin uptake modulates platelet function(Diego J Walther et al. 2003; Hummerich and Schloss 2010), in part through the transamination of small GTPase proteins essential for platelet α-granule exocytosis. However, our results suggest that there is little to no effect of acute

SSRI treatment on platelet activation. However, citalopram may have an off-target effect that modulates platelet spreading. Therefore, in the current chapter, I will explore the results of long-term (6-day) citalopram treatment on platelet activation. These studies examine how altered serotonin homeostasis alters platelet reactivity.

In this study, we investigated the mechanisms by which chronic inhibition of SERT alters hemostatic function using two independent models ($SERT^{-/-}$ and 6-day citalopram treatment). We hypothesize that the bleeding effects noted with the disruption of SERT function are due to altered $5HT_{2A}R$ signaling during platelet activation. Indeed, we found that mice that are lacking SERT function display a bleeding phenotype that can be rescued by the addition of wild-type platelets. Furthermore, we found that ADP-mediated αIIbβ3 activation was reduced in $SERT^{-/-}$ and citalopram-treated platelets due to loss of $5HT_{2A}R$ signaling and surface expression.

Loss of serotonin transporter function alters ADP-mediated αIIbβ3 activation through dysregulation of $5HT_{2A}$ receptor.

Abstract

Reduced platelet aggregation and a mild bleeding phenotype have been observed in patients chronically taking selective serotonin reuptake inhibitors (SSRIs) that block the serotonin transporter (SERT). Here we report that both $SERT^{-/-}$ mice and mice treated with citalopram for 6-days have similar bleeding phenotypes. Following transfusion of wild-type (WT) gel-filtered platelets into $SERT^{-/-}$ mice, $SERT^{-/-}$ bleeding times were reduced to WT levels suggesting defects in platelet function were contributing to the bleeding phenotypes. We examined platelet function by αIIbβ3 activation (JON/A binding) and P-selectin expression (CD62p binding). ADP-mediated αIIbβ3 activation was reduced in $SERT^{-/-}$ platelets. Acute treatment of platelets with SSRIs (paroxetine and citalopram) to prevent serotonin uptake during activation did not alter ADP-mediated αIIbβ3 activation. $5HT_{2A}R$ antagonists (ketanserin and methysergide), on the other hand, significantly reduced ADP-mediated αIIbβ3 activation. These findings suggest that serotonin synergizes with ADP through $5HT_{2A}R$ activation, but not acute SERT uptake. Compared with WT platelets, $SERT^{-/-}$ platelets displayed reduced serotonin enhancement of ADP-mediated αIIbβ3 activation, likely due to reduced $5HT_{2A}R$ cell surface levels. These data suggest that acute function of SERT does not alter ADP-mediated αIIbβ3 activation, but that sustained loss of SERT function leads to reduce surface expression of $5HT_{2A}R$ and ultimately reduced $serotonin_{2A}R$ mediated synergy with ADP-mediated αIIbβ3 activation. Our data suggests that the bleeding phenotypes in $SERT^{-/-}$ and 6-day citalopram-treated mice results from reduced $5HT_{2A}R$ surface expression. Additionally, this data advocates that the

reduced platelet aggregation seen in patients treated with SSRIs could arise from the loss of $5HT_{2A}R$ synergy with ADP warranting investigation of the $5HT_{2A}R$ as a potential antithrombotic target.

Objectives

- To determine the effects of sustained SERT inhibition on platelet function
- To determine how serotonin modulates platelet activation

Results

Decreased serotonin content in platelets isolated from SERT$^{-/-}$ and citalopram-treated mice parallels bleeding phenotype.

Mice treated with citalopram for 6 days have reduced whole blood serotonin levels and increased tail-bleed time. To determine if SSRIs alter blood serotonin homeostasis, mice were exposed to citalopram-treated water for 6 days, one day beyond the lifetime of a circulating platelet in a mouse (Malyszko et al. 1994). We used high-performance liquid chromatography (HPLC) to measure whole blood levels of serotonin and its major metabolite 5-HIAA (5-Hydroxyindoleacetic acid). Whole blood from wild-type mice undergoing citalopram-treatment (Cit-t) showed reduced serotonin levels as compared to water-treated (Water-t) controls (Figure 21A). No significant difference in 5-HIAA levels was found between Water-t and Cit-t samples (Figure 21B). To determine if citalopram treatment alters hemostasis, we performed a tail bleed assay. We discovered that citalopram-treated mice exhibited a significantly longer tail-bleed time as compared to water-treated controls (Figure 21C). These findings indicate a phenotypic association between reduced serotonin levels and increased bleeding time in mice treated with SSRIs.

Reduced serotonin whole blood levels and increased tail-bleed times in SERT$^{-/-}$. We observed very minimal measurable serotonin in SERT$^{-/-}$ platelets (Figure 21D.). Similar to whole blood samples isolated from citalopram-treated mice, there was no significant difference in 5-HIAA between SERT$^{+/+}$ and SERT$^{-/-}$ mice (Figure 21E.). We found that bleeding time was significantly increased in SERT$^{-/-}$ mice as compared to SERT$^{+/+}$ (Figure 21F). These data show that SERT function modulates whole blood serotonin levels and that SERT inhibition is associated with increased bleeding time.

Bleeding in SERT$^{-/-}$ mice is rescued by transfusion of SERT$^{+/+}$ platelets

As serotonin potentiates platelet activation and previous studies have shown that SERT function alters platelet aggregation (Ana Marin D Carneiro et al. 2008), we investigated whether the SERT$^{-/-}$ bleeding phenotype resulted from platelet functional defects. We first measured the number of platelets in SERT$^{-/-}$ and SERT$^{+/+}$ whole blood samples. No significant difference in the number of platelets was observed between genotypes (Figure 22A). To determine if the addition of SERT$^{+/+}$ platelets rescued the SERT$^{-/-}$ bleeding phenotype, mice were transfused with gel-filtered platelets resuspended in saline via jugular vein injection. Tail-bleed was performed three minutes after transfusion of SERT$^{+/+}$ platelets to SERT$^{+/+}$ and SERT$^{-/-}$ mice (Figure 22B). In control experiments, saline-transfused SERT$^{-/-}$ mice showed a significantly longer tail bleed time as compared to SERT$^{+/+}$ mice. We found that addition of gel-purified platelets ($2X10^7$ in 200μL of saline) to SERT$^{+/+}$ mice did not significantly alter tail-bleed time. The difference in SERT$^{+/+}$ and SERT$^{-/-}$ bleeding time was abolished following platelet transfusion, indicating hemostasis was rescued in SERT$^{-/-}$ mice. These findings support the conclusion that the bleeding phenotype of the SERT$^{-/-}$ mice results from a defect in platelet function.

ADP-mediated αIIbβ3 activation is reduced in SERT$^{-/-}$ platelets.

SERT$^{-/-}$ platelets respond normally to thrombin. Gel-filtered platelets from SERT$^{+/+}$ and SERT$^{-/-}$ mice exposed to 10nM and 200nM thrombin had similar αIIbβ3 activation as measured by JON/A binding (Figure 23A). Following thrombin stimulation, there were no significant differences between the SERT$^{-/-}$ and SERT$^{+/+}$ platelet P-selectin expression

(CD62p binding) following post-tests analysis (Figure 23B). These findings reveal that platelet activation with thrombin is not altered in SERT$^{-/-}$ platelets.

SERT$^{-/-}$ platelets have reduced ADP-mediated αIIbβ3 activation. We next tested ADP platelet activation in SERT$^{-/-}$ platelets. We found that 10μM, but not 1μM ADP-mediated αIIbβ3 activation is significantly reduced in the SERT$^{-/-}$ platelets (Figure 23C). No difference in P-selectin expression was observed in SERT$^{-/-}$ or SERT$^{+/+}$ platelets (Figure 23D). These data demonstrate that loss of SERT function leads to a deficiency in the ADP-mediated inside-out signaling that triggers activation of αIIbβ3.

5-HT$_{2A}$R stimulation enhances ADP-mediated αIIbβ3 activation.

To examine the role of serotonin in ADP-mediated platelet activation, we distinguished the role of two plasma membrane proteins that are responsive to serotonin in platelets: SERT and the 5-HT$_{2A}$R. We performed a dose-response curve with citalopram and paroxetine, two structurally distinct SSRIs, and found that they block intact ^{3H}5-HT platelet uptake with an IC_{50} of $1.5X10^{-11}$ and $1.3X10^{-11}$, respectively (Figure 24A). Platelet activation with 1μM and 10μM ADP was examined with the addition of either 5-HT$_{2A}$R antagonists (ketanserin and methysergide) or SERT inhibitors (citalopram and paroxetine) 5 minutes before ADP activation. Both ketanserin and methysergide treatment significantly reduced ADP-mediated activation at 1μM (Figure 24B). Acute inhibition of serotonin uptake by SERT with citalopram (10nM) or paroxetine (10nM) had no effect on ADP-mediated αIIbβ3 activation despite effectively blocking SERT-mediated serotonin uptake. These data reveal a role for 5-HT$_{2A}$R activation, but not serotonin uptake, in ADP-mediated αIIbβ3 inside-out activation.

SERT$^{-/-}$ platelets have reduced ADP-5-HT synergy. To explore the role of 5-HT$_{2A}$R signaling in the context of the SERT$^{-/-}$ model, we examined JON/A binding to activated αIIbβ3 in the context of simultaneous serotonin and ADP receptor stimulation. serotonin alone was unable to elicit activation of the αIIbβ3 integrins at either 1μM or 10μM concentration in either SERT$^{+/+}$ or SERT$^{-/-}$ platelets (Figure 24C). However, using a submaximal concentration of ADP (1μM), we observed that 10μM serotonin potentiated the ADP-mediated activation of αIIbβ3 in SERT$^{+/+}$ platelets. serotonin was unable to potentiate ADP-mediated platelet activation in SERT$^{-/-}$ platelets (Figure 24D). In a separate experiment we found that incubation with ketanserin prevented the 5-HT-dependent potentiation of αIIbβ3 activation in SERT$^{+/+}$ platelets (Figure 24E). These results demonstrate that submaximal stimulation of ADP receptors is potentiated by co-stimulation of 5-HT$_{2A}$R, which synergizes to mediate αIIbβ3 inside-out activation. Additionally, these data indicate a defect in 5-HT$_{2A}$R signaling within SERT$^{-/-}$ platelet.

SERT$^{-/-}$ platelets have reduced 5-HT$_{2A}$R surface expression.

ADP levels and ADP receptor expression are retained in SERT$^{-/-}$ platelets. We first explored whether the loss of ADP-mediated αIIbβ3-activation was due to a deficit in ADP-dependent signaling. We examined ADP receptor (P2Y$_1$ and P2Y$_{12}$) expression levels via ^{3H}ADP binding that was competitively blocked with ADP-receptor specific antagonists. This assay was optimized in SERT$^{+/+}$ platelet membrane preparations and shown as percent of total ^{3H}ADP binding blocked in the presence of PIT (2,2'-pyridylisatogen tosylate) a P2Y antagonist, (Figure 25A). Competitive inhibition with MRS2179 (P2Y$_1$ specific antagonist), and ticagrelor (P2Y$_{12}$ specific antagonist, were used to determine relative

receptor binding in $SERT^{+/+}$ and $SERT^{-/-}$ platelet membrane preps (Figure 26B, C). $P2Y_1$ accounted for approximately 46.42% of ^{3H}ADP binding while $P2Y_{12}$ accounted for approximately 55.89% of total ^{3H}ADP binding. Inhibition at 1μM for both MRS2179 and ticagrelor was used to determine %^{3H}ADP binding to $P2Y_1$ to platelet membranes. We found no statistically significant difference in either $P2Y_{12}$ (Figure 25E) or $P2Y_1$ (Figure 25F) binding between $SERT^{+/+}$ and $SERT^{-/-}$ membrane preparations. Additionally, we measured whole blood ADP levels using an ELISA and observed no difference between $SERT^{+/+}$ or $SERT^{-/-}$ preparations (Figure 25G). These data demonstrate that the ADP whole blood levels and receptor numbers are not different in $SERT^{-/-}$ platelets.

5-HT_{2A}R G-protein activation and surface expression levels are reduced in $SERT^{-/-}$ platelets. Based on our findings, the 5-HT_{2A}R plays an important role in ADP-mediated αIIbβ3 activation and this effect is reduced in $SERT^{-/-}$ platelets. Therefore, we purified platelet membranes and measured ^{35S}GTPyS incorporation to 5-HT_{2A}R following activation with DOI, a 5-HT_{2A}R-specific agonist. We observed a significant reduction in ^{35S}GTPyS incorporation to $SERT^{-/-}$ membrane preparations as compared to $SERT^{+/+}$ (Figure 25. n=8, P=0.0002: Mann-Whitney). To determine if this reduction in G-protein activation was caused by reduced 5-HT_{2A}R surface expression we used radiolabeled ^{3H}ketanserin to measure surface levels of the 5-HT_{2A}R on intact platelets. A saturation curve was performed to examine the number of surface receptors (Bmax) of 5HT_{2A}R (Figure 25). $SERT^{-/-}$ platelets showed significantly reduced Bmax for 5-HT_{2A}R surface levels as compared to those observed in $SERT^{+/+}$ platelets (Figure 26J). These results show reduced 5-HT_{2A}R signaling in $SERT^{-/-}$ platelets, likely resulting from reduced 5-HT_{2A}R surface expression.

Citalopram-treated platelets recapitulate the SERT$^{-/-}$ platelet phenotype.

Citalopram-treated platelets have reduced ADP-mediated αIIbβ3 activation and reduced 5-HT$_{2A}$R surface expression. To determine if we could recapitulate the loss of 5-HT$_{2A}$R surface expression using an alternative model of lost SERT function we treated mice with chronically with citalopram for 6-days. Similar to SERT$^{-/-}$ platelets, citalopram-treated platelets showed reduced ADP-dependent αIIbβ3 activation compared to water-treated controls (Figure 26A). In control, water-treated mouse platelets αIIbβ3 activation by ADP was enhanced by 5-HT, whereas serotonin potentiation of ADP signaling was absent in citalopram-treated mouse platelets (Figure 26B). Furthermore, 5-HT$_{2A}$R levels were reduced in platelets isolated from citalopram-treated mice (Figure 26C). These findings establish that 6-day treatment with SSRI is sufficient to reduce platelet expression of 5-HT$_{2A}$R and decrease the capacity of platelets to be synergistically activated by ADP and 5-HT. Additionally, these findings demonstrate that the loss of 5-HT$_{2A}$R surface expression and platelet function in SERT$^{-/-}$ mice is not due to loss of the SERT protein but rather, results from sustained loss of SERT function. Finally, to test if premature exposure to serotonin could alter ADP-mediated αIIbβ3 activation, wild-type platelets were incubated with serotonin (10μM) for 15 minutes, washed and resuspended. They were then activated with 10μM ADP, and JON/A binding was measured (Figure 26D). We found that platelets pre-treated with serotonin for 15 minutes lost ADP-mediated αIIbβ3 activation similar to both SERT$^{-/-}$ and citalopram-treated platelets. These results suggest that the pre-activation or possible desensitization of the 5HT$_{2A}$R reduces platelet ADP-mediated αIIbβ3 activation.

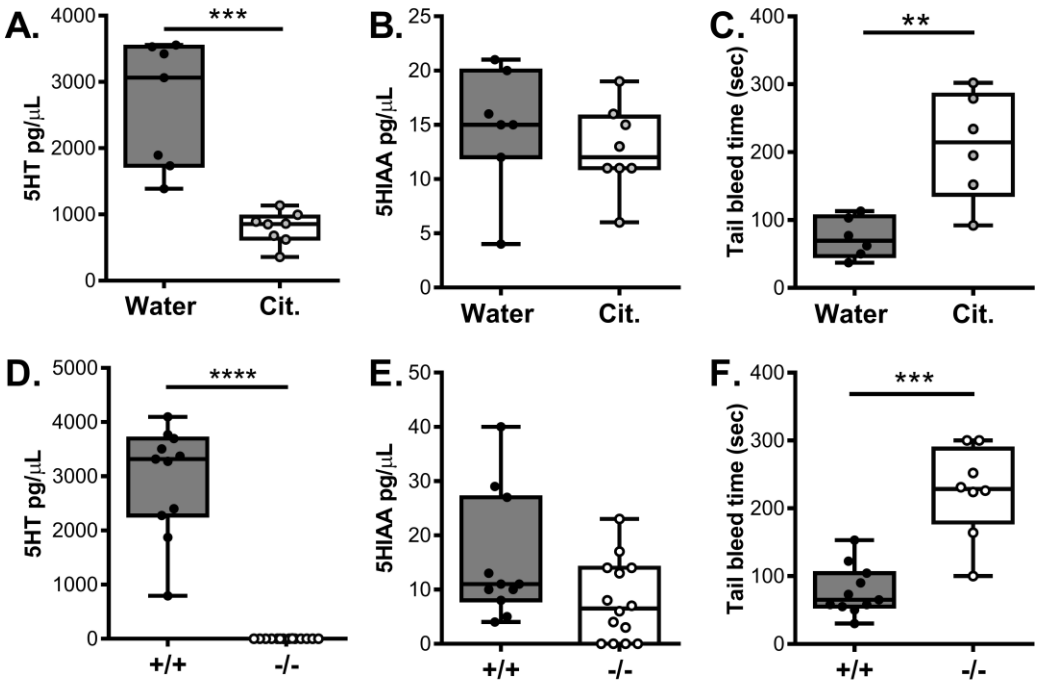


Figure 21. ***Decreased serotonin content in SERT$^{-/-}$ and citalopram-treated mice associated with bleeding phenotype.*** *serotonin and 5-HIAA were measured using HPLC from whole blood samples in both citalopram-treated platelet (Water n=7: Cit. n=8,* ***A, B****) and SERT$^{-/-}$ mice (SERT$^{+/+}$n=11: SERT$^{-/-}$ n=14,* ***D, E****). Both citalopram-treated (P=0.0003***; Mann-Whitney test) and SERT$^{-/-}$ mice (P<0.0001****; Mann-Whitney test) have a significant reduction in serotonin levels but no significant change in 5-HIAA. Tail-bleed time* ***(C, F)*** *was also performed in citalopram-treated (Water n=6: Cit. n=6) and SERT$^{-/-}$ mice (SERT$^{+/+}$ n=11: SERT$^{-/-}$n=8). Both citalopram-treated mice (P=0.0087**; Mann-Whitney test) and SERT$^{-/-}$ mice (P=0.0002***; Mann-Whitney test) show increased tail-bleed time as compared to controls. Data is shown with median, range, and individual points.*

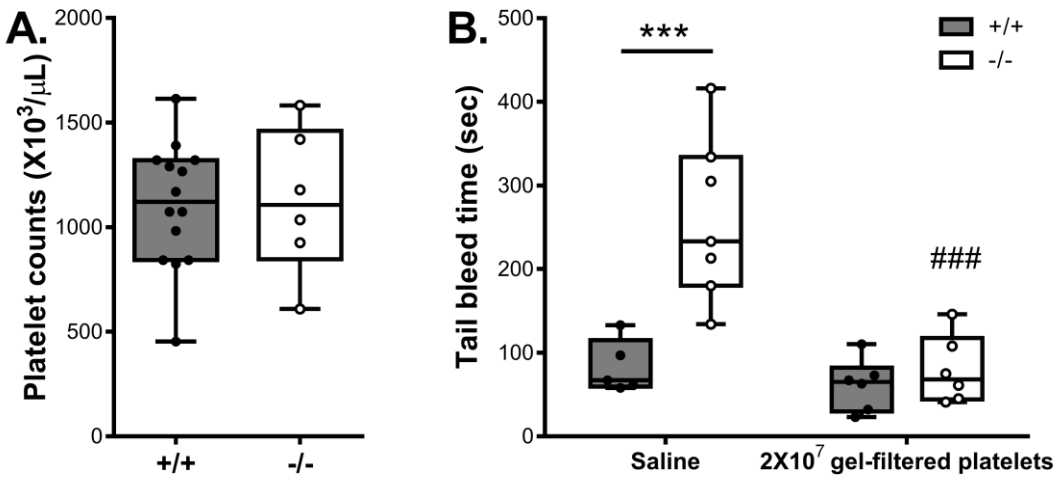


Figure 22. ***Bleeding phenotype can be rescued with transfusion of wild-type platelets. (A)*** *Platelet counts were performed in $SERT^{+/+}$ (n=14) and $SERT^{-/-}$ mice (n=6). There was no significant difference in the number of platelets (P=0.8890, Mann-Whitney).* ***(B)*** *Tail-bleed time was performed three minutes after to transfusion of wild-type mouse platelets and was measured in $SERT^{+/+}$ and $SERT^{-/-}$ mice with the addition of either saline ($SERT^{+/+}$ n=5, $SERT^{-/-}$ n=6) or platelets ($SERT^{+/+}$ n=6, $SERT^{-/-}$ n=6). $SERT^{-/-}$ saline transfused tail-bleed time was significantly increased from $SERT^{+/+}$ (P=0.0039***) and were rescued to $SERT^{+/+}$ bleeding time following platelet transfusion ($_{saline:}SERT^{-/-}$ vs. $_{platelet:}SERT^{-/-}$ $P=0.002^{\#\#}$, $_{saline;}SERT^{+/+}$ vs. $_{platelet:}SERT^{-/-}$ P=0.9893, $_{saline:}SERT^{+/+}$ vs. $_{platelet}SERT^{+/+}$ P=0.9837: Tukey's multiple comparison test: 2-way ANOVA). Data is shown with median, range, and individual points.*

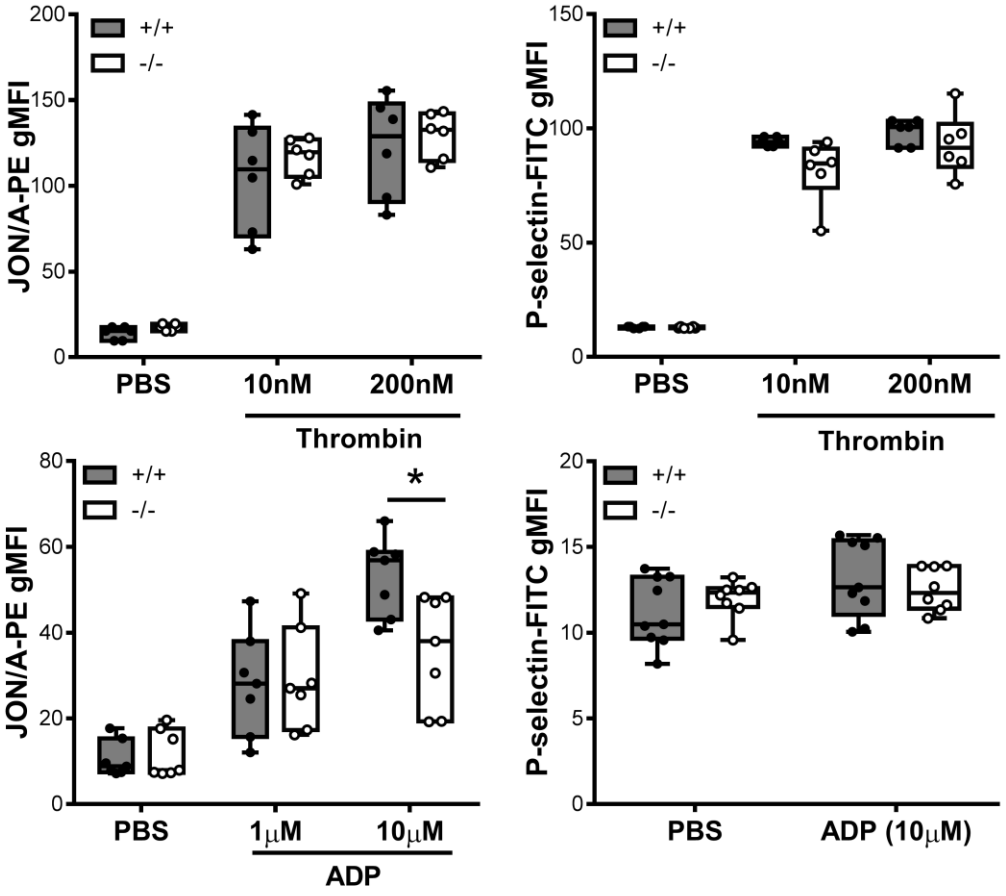


Figure 23. ***Platelet ADP-mediated αIIbβ3 activation is reduced in SERT$^{-/-}$ mice. (A)*** *JON/A binding in SERT$^{-/-}$ (n=6) and SERT$^{+/+}$ (n=6) mice with 10nM and 200nM thrombin show no difference based on genotype (Tukey's: 2-way ANOVA: Thrombin P<0.0001).* ***(B)*** *P-selective binding did account for some variation (2-way ANOVA: Thrombin P<0.0001, Genotype P=0.032) but did not show any difference following post-test (Tukey's multiple comparisons).* ***(C)*** *ADP-mediated JON/A binding is significantly reduced in the SERT$^{-/-}$ platelets at 10μM ADP ($_{10\mu M:}$SERT$^{+/+}$ vs. $_{10\mu M:}$SERT$^{-/-}$ P=0.0354*) but not at 1μM ADP (2-Way ANOVA: Interaction P=0.026, ADP P<0.0001 $_{1\mu M:}$SERT$^{+/+}$ vs. $_{1\mu M:}$SERT$^{-/-}$ P>0.999: Tukey's multiple comparisons).* ***(D)*** *There is no difference in P-selectin expression in SERT$^{-/-}$ and SERT$^{+/+}$ mice (2-way ANOVA ADP P=0.0473: Tukey's multiple comparisons: N.S.). Data is shown with median, range, and individual points.*

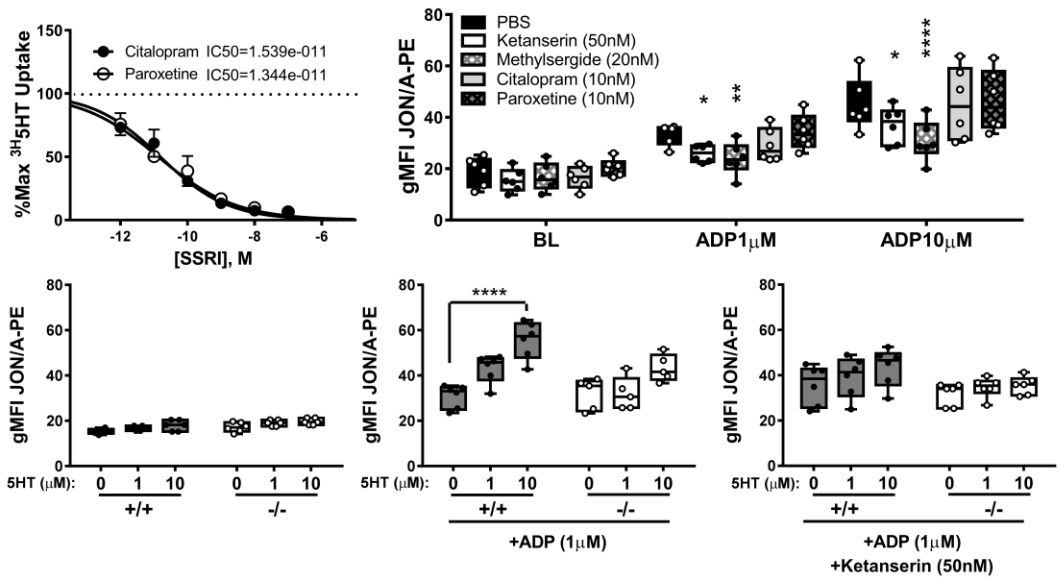


Figure 24. ***ADP requires 5-HT$_{2A}$ receptor activation for ADP-5-HT-mediated αIIbβ3 synergy, which is disrupted in SERT$^{-/-}$ platelets.*** ***(A)*** *Isolated platelet uptake of ^{3H}5-HT blocked with either citalopram or paroxetine (Log (inhibition) vs. normalized response-Variable slope: citalopram-Robust sum of squares 20.58, paroxetine robust sum of squares 22.92).* ***(B)*** *JON/A binding following 15 minutes ADP activation was measured using gMFI following a 5 minutes pre-incubation with either 5-HT$_{2A}$ receptor antagonists, ketanserin or methysergide, or selective serotonin reuptake inhibitors, citalopram or paroxetine, on the same platelet preparation (n=6). Both ketanserin and methysergide treatment significantly reduced ADP αIIbβ3-activation at both 1μM (ketanserin P=0.0409*, methysergide P=0.0041**) and 10μM (ketanserin P=0.0187*, methysergide P<0.0001****: Dunnett's: 2-way RM ANOVA: interaction P=0.0103, ADP P<0.0001, Inhibitors P<0.0001, Subjects (matching) P<0.0001). Non-parallel experiments examined JON/A binding following incubation with* ***(C) serotonin*** *alone,* ***(D)*** *ADP1μM + serotonin (P<0.0001***), or (**E**) ADP-5-HT following a 5-minute incubation with ketanserin (Sidak's: Two-way ANOVA). Data is shown with median, range, and individual points*

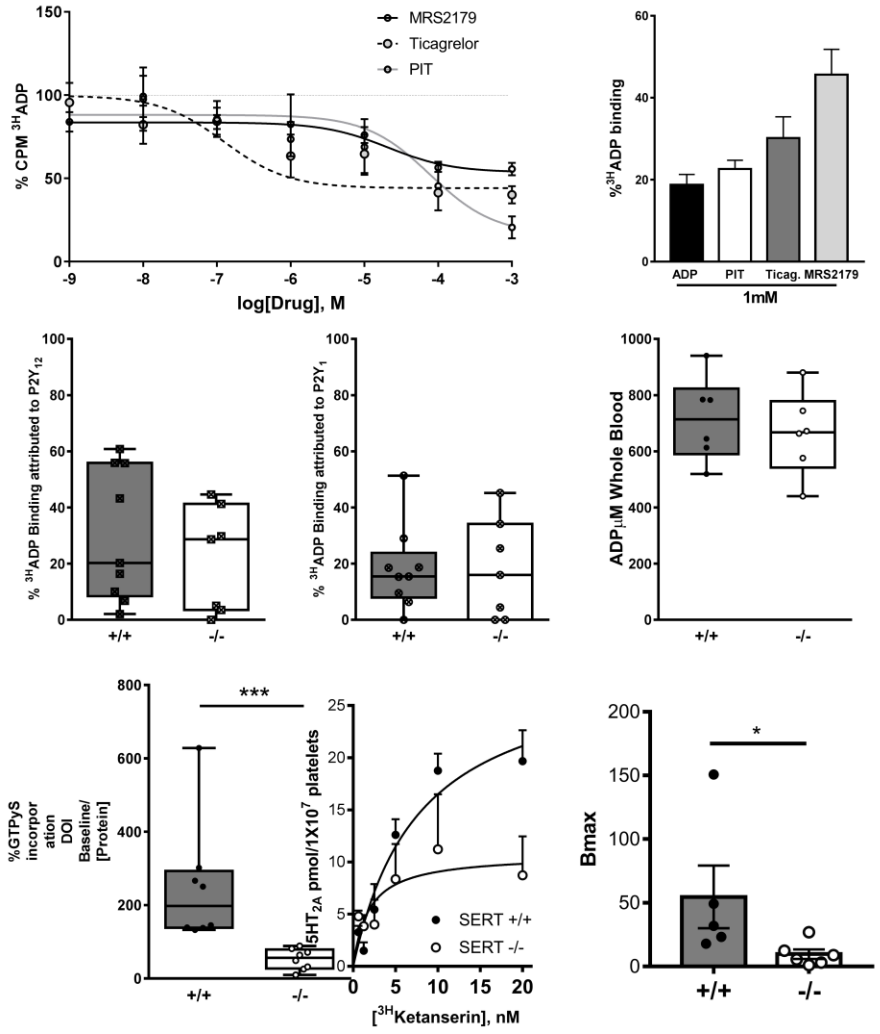


Figure 25. ***SERT$^{-/-}$ platelets have reduced 5-HT$_{2A}$ receptor surface expression but no change in P$_2$Y$_1$ and P$_2$Y$_{12}$. (A) (B)*** *Competitive inhibition with PIT (dual P2Y$_1$ and P2Y$_{12}$, IC50=7.80X10^{-5}, 83.71%), MRS2179 (P2Y$_1$ specific antagonist, IC50=2.08X10^{-5}, 46.42%) and Ticagrelor (P2Y$_{12}$ specific antagonist, IC50=1.25X10^{-7}, 55.89%) was used with ^{3H}ADP to determine relative of receptors. Single point inhibition was used to determine %^{3H}ADP binding for **(C)** P2Y$_{12}$ receptors (n=9 SERT$^{+/+}$, n=7 SERT$^{-/-}$, P=0.4592: Mann-Whitney) **(D)** P2Y$_1$ (n=9 SERT$^{+/+}$, n=7 SERT$^{-/-}$, P=0.9646: Mann-Whitney) and in SERT$^{+/+}$ and SERT$^{-/-}$ platelets. Data is shown with median, range, and individual points. **(E)** ADP whole blood levels were determined by ELISA (n=6, P=0.675, Mann-Whitney). **(F)** ^{35S}GTPγS incorporation was used to measure 5-HT$_{2A}$ receptor specific G-protein activation in purified platelet membranes (n=8, P=0.0002***: Mann-Whitney). **(G)** 5HT$_{2A}$R specific saturation curve with ^{3H}ketanserin is shown for both SERT$^{+/+}$ and SERT$^{-/-}$ platelets. **(H)** Calculated Bmax values are significantly reduced in SERT$^{-/-}$ platelets (n=5 SERT$^{+/+}$, n=6 SERT$^{-/-}$ P=0.0173*, Mann-Whitney).*

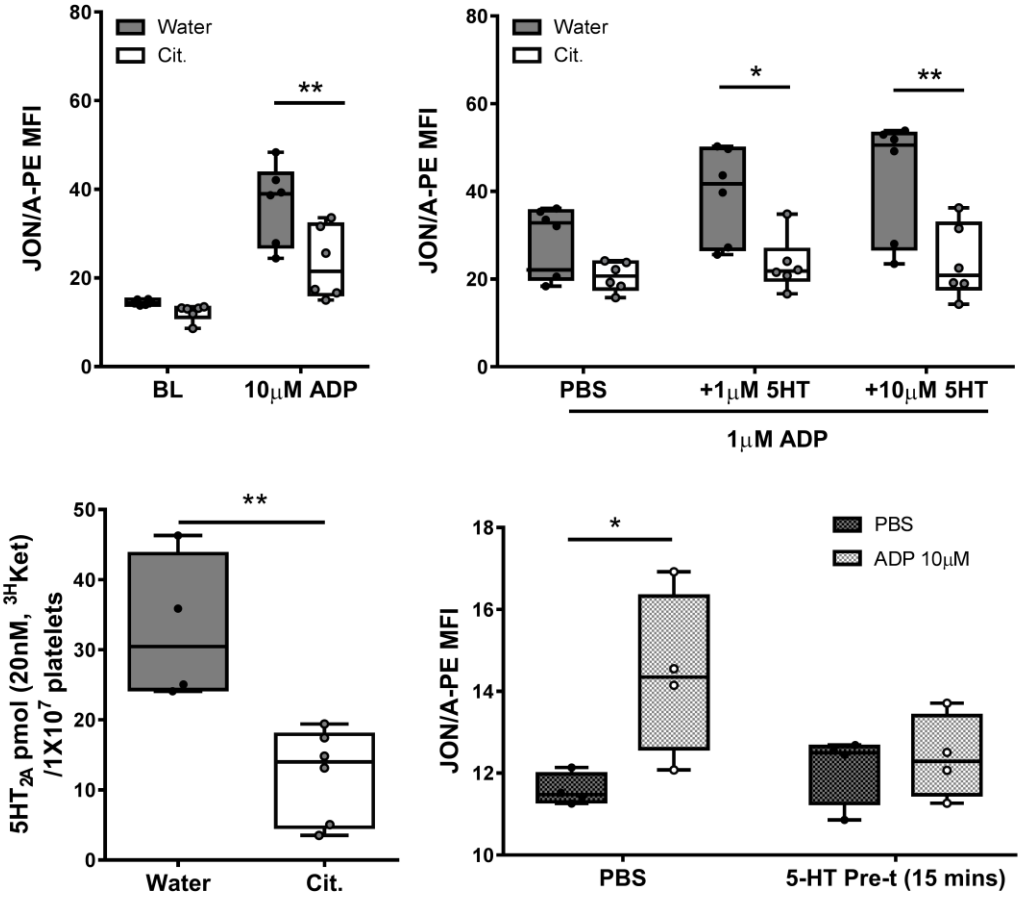


Figure 26. ***Citalopram-treated mice have reduced ADP-5-HT synergy and reduced 5-HT$_{2A}$ surface expression.*** ***(A)*** *Citalopram ADP-mediated JON/A binding was probed (n=6, 2-wayANOVA: Tukey's Post-test: $_{BL:}$Water vs $_{10\mu MADP:}$Water P<0.0001, $_{BL:}$Cit. vs. $_{10\mu MADP:}$Cit P=0.025, $_{10\mu MADP:}$Water vs. $_{10\mu M:}$Cit P=0.0056**).* ***(B)*** *ADP-5-HT synergy toward JON/A binding was also probed in citalopram-treated mice (n=6, 2-way ANOVA: Tukey's Post-test:$_{PBS:}$Water vs $_{PBS:}$Cit P=0.5501, $_{1\mu M5\text{-}HT:}$Water vs $_{1\mu M5\text{-}HT:}$Cit P=0.0452*, $_{10\mu M5\text{-}HT:}$Water vs $_{10\mu M:}$Cit P=0.009**).* ***(C)*** *Surface 5-HT$_{2A}$ receptor levels were also determined using 20nM ^{3H}ketanserin binding on intact platelets (Water n=4, Cit-t n=6: P=0.0095**, Mann-Whitney).* ***(D)*** *Wild-type platelets that were pre-treated with serotonin for 15 minutes washed, and activated with 10μM ADP show decreased JON/A binding. Data is shown with median, range, and individual points.*

Spreading and $5HT_{2A}R$ activation

Abstract

In the previous section, we show that long-term inhibition of SERT leads to an alteration in $5HT_{2A}R$ signaling. We also found that the activation of the $5HT_{2A}R$ is necessary for full ADP-mediated αIIbβ3 inside-out activation. In this next section we explore the outside-in activation of αIIbβ3 and its relationship with altered SERT function.

Objectives

- To determine if loss of the SERT protein alters platelet function, particularly platelet spreading
- To determine if platelet spreading is altered with the addition of serotonin and through which mechanism is it acting

Results

In order to determine if platelet spreading is altered with the sustained loss of SERT function, we used the $SERT^{-/-}$ mouse model. We found that when platelets are spreading onto 25µg/mL fibrinogen-coated plates, $SERT^{-/-}$ platelets spread significantly less than wild-type platelets (Figure 27).

Next we examine how serotonin modulates platelet spreading through examination of the two principle platelet serotonergic modulation mechanisms: $5HT_{2A}R$ activation and SERT uptake (Figure 28). In wild-type $SERT^{+/+}$ platelets treatment with ketanserin 50nM significantly reduced spreading in non-treated platelet but this effect was lost with the addition of 10µM serotonin. Also, there was no effect of acute citalopram treatment on platelet spreading with or without the addition of serotonin. In $SERT^{-/-}$ platelets, the addition of serotonin again increased platelet spreading. Treatment of $SERT^{-/-}$ platelets with ketanserin had no effect on non-treated $SERT^{-/-}$ spreading as compared to $SERT^{+/+}$. However, the acute addition of ketanserin blocked the rescued spreading phenotype seen when $SERT^{-/-}$ platelets were treated with ketanserin. There was no difference between PBS-treated platelets with or without serotonin when the platelets were acutely treated with citalopram. These data suggest that the addition of serotonin is enough to rescue the $SERT^{-/-}$ platelet spreading deficiency and that it acts through $5HT_{2A}R$ activation.

Finally, to confirm that activation of the $5HT_{2A}R$ is enough to rescue the $SERT^{-/-}$ platelet spreading phenotype we treated the platelet with 1-[2,5-dimethoxy-4-idophenyl]-2-aminopropane (DOI), a $5HT_{2A}R$ agonist. This treatment resulted in no difference in spreading between $SERT^{+/+}$ and $SERT^{-/-}$ platelets suggesting that activation of $5HT_{2A}R$ restores $SERT^{-/-}$ platelet spreading.

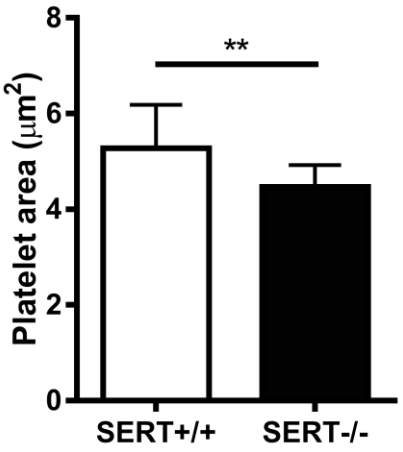


Figure 27. ***Spreading phenotype in SERT$^{-/-}$ platelets****. Platelet spreading was analyzed by staining platelets with concovilin-A and measuring spreading on 25μg/mL coated fibrinogen. SERT$^{-/-}$ platelets have significantly reduces platelet spreading as compared to SERT$^{+/+}$ platelets (Mann-Whitney, P=0.0024**).*

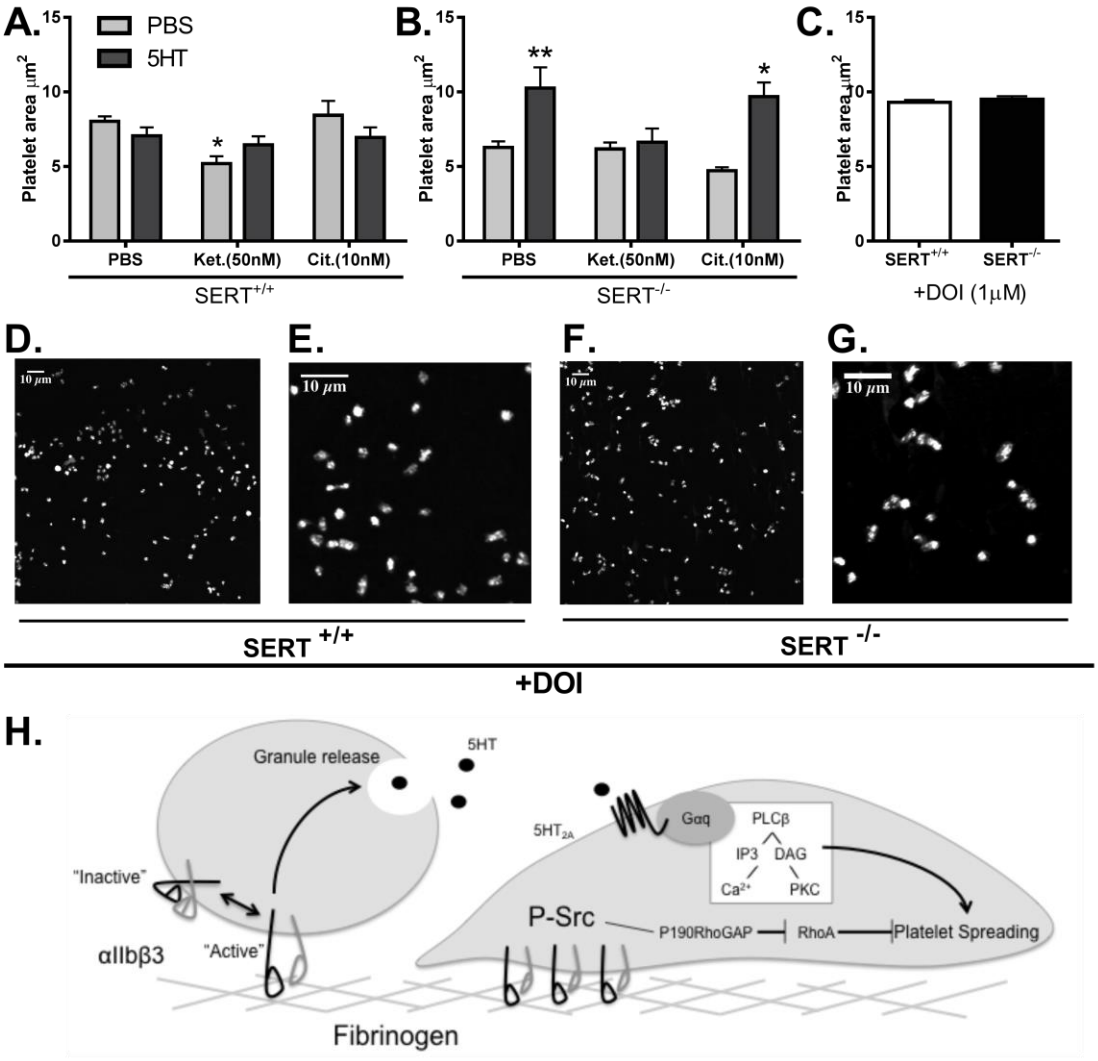


Figure 28. ***Spreading phenotype of SERT$^{-/-}$ platelets is rescued with serotonin through 5HT$_{2A}$R activation****. In order to examine the effects of serotonin on platelet spreading we analyzed separately SERT$^{+/+}$ and SERT$^{-/-}$ platelet spreading with the addition of either citalopram (block acute SERT function) or ketanserin (block 5HT$_{2A}$R activation). In SERT$^{+/+}$ platelets, there was a significant reduction in platelet spreading in the presence of ketanserin but this effect was lost with the addition of serotonin (A: Two-way ANOVA: Interaction P=N.S.., Inhibitors P=0.0083, serotonin P=N.S.: Pbs:PBS vs Ket:PBS P=0.0129*). In SERT$^{-/-}$ platelets, the addition of serotonin was able to increase spreading, and this effect was blocked with the addition of ketanserin but not with citalopram (B: Two-way ANOVA: Interaction P=0.0176, Inhibitors =N.S., serotonin P<0.000: PBS versus PBS: serotonin P=0.0032**, PBS:PBS vs. Cit (10nM):5HT P=0.0134*). The addition of DOI, a 5HT$_{2A}$R agonist, lost the genotype effect seen previously (C). Representative platelets from SERT$^{+/+}$ and SERT$^{-/-}$ platelets in the presence of DOI is shown (D-G). Finally, in Figure H, a schematic of serotonin role in platelet spreading is shown.*

Conclusion

The alteration in 5HT$_{2A}$R surface expression with sustained inhibition of SERT and its effects on ADP-mediated αIIbβ3 activation.

Our research has demonstrated how loss of SERT function leads to platelet dysfunction providing mechanistic insight into the increased bleeding times previously observed in patients taking SSRIs (Andrade et al. 2010; Bismuth-Evenzal et al. 2012b). We characterized a bleeding phenotype in two independent models of lost SERT function, genetic and pharmacologic. This effect was rescued by addition of purified platelets with intact SERT function, indicating that the actions of SSRIs are mediated primarily via alteration of platelet function. Sustained loss of SERT function reduces 5-HT$_{2A}$R surface expression leading to reduced ADP-mediated αIIbβ3 activation. Therefore, our findings support a novel mechanism for the reduced aggregation in SSRI-treated patients: reduced 5-HT$_{2A}$R surface expression and defective ADP-mediated αIIbβ3 activation.

There are three possibilities by which SSRIs may mediate their effects on platelets: (1) acute blockage of SERT function and serotonin uptake, (2) depletion of granule serotonin and loss of serotonin secretion during activation, and (3) increased in extracellular serotonin levels. Many studies concluding that acute uptake of serotonin contributes to platelet function used SSRIs as tool compounds to acutely inhibit serotonin uptake. However, these studies utilized SSRIs at high concentrations (>10μM) that are known to exert off-target effects (e.g. the σ1 receptor) (Maurice and Su 2009; Tseng et al. 2010). Comparatively, our study uses concentrations of SSRIs that block platelet-mediated SERT uptake but are far below the reported concentrations resulting in off-target effects (10nM, Figure 29). Here, we observed no change in ADP-mediated αIIbβ3 activation following

acute SSRI treatment, suggesting that acute serotonin uptake is not required for inside-out activation of αIIbβ3 by weak agonists. However, we observed reduced ADP-mediated αIIbβ3 activation following chronic SSRI treatment.

Others have suggested that acute uptake of serotonin by SERT leads to a receptor-independent signaling pathway via the post-translational modification of intracellular proteins (Diego J Walther et al. 2003). This process, known as serotonylation, involves the covalent attachment of serotonin molecules to proteins mediated by the enzyme transglutaminase, resulting in transamination of small GTPases important for platelet α-granule secretion (Ahmed et al. 2008; Diego J Walther et al. 2003). In our experiments, we demonstrate no difference in P-selectin antibody as a measure for α-granule exocytosis in $SERT^{-/-}$ platelet following thrombin activation. These data would suggest that α-granule exocytosis is intact following the chronic loss of SERT function. Instead, we observed that chronic loss of SERT function led to reduced 5-HT_{2A}R signaling and reduced ADP-mediated αIIbβ3 activation, which is necessary for proper aggregation *in vivo*.

In conjunction with depleting platelet granule serotonin levels, one would expect that chronic SERT inhibition would increase plasma concentrations of serotonin as suggested by the effects of SSRIs in the CNS (Gartside et al. 1995; F Artigas et al. 1996). However, because 5-HT_{2A}R stimulation by serotonin alone does not lead to platelet activation, loss of SERT function likely leads to local increases in plasma serotonin levels within the portal vein and indirectly triggers internalization of the 5-HT_{2A}R. It has been demonstrated that serotonin induces internalization of 5-HT_{2A}R in a β-arrestin dependent manner within 30 minutes of initial exposure (Schmid, Raehal, and Bohn 2008; Bohn and Schmid 2010). $5HT_{2A}$R desensitization has been shown in the in the brain following prolonged exposure

to elevated levels of serotonin in citalopram- treated mice(Schaff et al. 2012; Yamauchi et al. 2006). However, it has yet to be validated that extracellular plasma serotonin levels increase following SSRI treatment. Evaluating changes in plasma (i.e. extracellular) serotonin levels as compared to platelet (i.e. intracellular) serotonin levels following SSRI treatment has been challenging. Measuring plasma serotonin levels has proven difficult due to very low concentration by extraordinarily efficient liver metabolism as well as the free diffusion of serotonin out of circulation (Moore and Eiseman 2016; G. M. Anderson et al. 2005; G. M. Anderson and Cook 2016a). Furthermore, levels of plasma serotonin can vary greatly possibly due to contamination by platelet granule release during plasma purification (Brenner et al. 2007; Matondo et al. 2009; Lau et al. 2012; G. M. Anderson and Cook 2016a). Reported plasma serotonin levels fluctuate between investigators and preparations, but our data clearly demonstrate that serotonin homeostasis is altered by loss of SERT function (Jernej et al. 2002; Singh et al. 2013; D. J. Walther 2003). Furthermore, our data demonstrates that improper exposure of platelets to serotonin likely desensitizes the receptor leading to reduced ADP-mediated αIIbβ3 activation (Figure). Our findings indicate that chronic loss of SERT function reduces 5-HT_{2A}R surface expression and signaling in each of two models of altered serotonin homeostasis.

It has previously been established that serotonin plays a synergistic role with ADP activation during platelet aggregation (J. S. Berger et al. 2013; HirshRokach et al. 2015; E. Cerrito et al. 1993; Thompson NT, Scrutton MC 1986). Here we demonstrate that 5-HT_{2A}R stimulation potentiates ADP-dependent signaling to activate αIIbβ3 (i.e. JON/A binding) leading to aggregation. While 5-HT_{2A}R stimulation alone did not influence αIIbβ3-activation, dual stimulation of 5-HT_{2A}R with a submaximal concentration of ADP

enhanced JON/A binding. 5-HT_{2A}R stimulation may be required to enhance $P2Y_1$ Gq-mediated signaling and Gi-mediated $P2Y_{12}$ activation converging on αIIbβ3 inside-out activation. It is well known that the 5-HT_{2A}R can signal through multiple non-G protein pathways including β-arrestins (Schmid, Raehal, and Bohn 2008; E. L. Stahl et al. 2015; Schmid and Bohn 2010). Therefore, while canonical 5-HT_{2A}R signaling would be redundant relative to ADP-mediated $P2Y_1$ activation, alternative signaling pathways downstream of β-arrestins, such as ERK1/2 and c-Src could culminate in αIIbβ3 activation(Z. Li et al. 2010; E. L. Stahl et al. 2015; Schmid, Raehal, and Bohn 2008; Schmid and Bohn 2010). Additionally, 5-HT_{2A}R signaling through arachidonic acid (AA), 2-arachidonylglycerol (2-AG), calmodulin, or AKT could also synergize with ADP signaling (Quinn et al. 2002; Johnson-Farley et al. 2005; Göoz et al. 2006; Miller, Mariano, and Cruz 1997). Further experiments are required to determine what signaling pathways are necessary and sufficient for maximal αIIbβ3 activation via ADP/serotonin synergy.

Clinically, targeted inhibition of the 5-HT_{2A}R has been efficacious (Przyklenk et al. 2010b). APD791, an inverse agonist of 5-HT_{2A}R, is currently in clinical trials and attenuated recurrent thrombosis irrespective of the time of treatment (Adams et al. 2009; Przyklenk et al. 2010a). No increase in bleeding time in the presence of APD791 was observed as compared to other anti-platelet therapies. Additionally, APD791 was able to block serotonin-dependent platelet activation over a short time scale (2hrs) (Adams et al. 2009; Przyklenk et al. 2010a). This data provides mechanistic insight into the reduced aggregation previously observed in SSRI-treated patients and supports explorations of peripherally restricted, well-tolerated SSRIs or 5-HT_{2A}R antagonists as an antiplatelet therapeutic approach. The therapeutic implications of this work suggest a novel approach

targeting the serotonin signaling systems via SERT-inhibition leading to reduced 5-HT_{2A}R-mediated platelet aggregation.

$5HT_{2A}$R and spreading

Platelet activation is distinct from platelet spreading because it falls within a difference phase of αIIbβ3 function. Spreading is related to αIIbβ3 outside-in activation (Z. Li et al. 2010; Joseph E. Aslan et al. 2012; J E Aslan and McCarty 2013). Within this dataset we observe no difference in platelet attachment between $SERT^{+/+}$ and $SERT^{-/-}$ platelets suggesting that the baseline status of αIIbβ3 activate conformation is not different between $SERT^{+/+}$ and $SERT^{-/-}$ platelets based on conformational activation of the αIIbβ3 integrin (Oliver et al. 2014; Watson 2009). However, we do observe significantly reduced platelet spreading in the $SERT^{-/-}$ platelets suggesting that signaling downstream of αIIbβ3 binding is disrupted in the $SERT^{-/-}$ platelets. Our data suggest that the rescued spreading phenotype observed is a results of lost serotonergic tone and activation of the $5HT_{2A}$R.

This data set is distinct from the data set observed in the previous section because serotonin was able to rescue the reduced platelet spreading but was not able to rescue ADP-medaited αIIbβ3 activation. Previously, we demonstrate that ADP-mediated αIIbβ3 activation could not be rescued with the addition of 5HT. We subsequently demonstrate the $5HT_{2A}$R surface expression is reduced in the $SERT^{-/-}$ and citalopram treatment model that causes the ADP-mediated αIIbβ3 activation phenotype not to be rescued simple with the addition of serotonin. As we consider these results alongside the spreading data, one could postulate that $5HT_{2A}$R receptor surface expression is restored during platelet

spreading. It is clear that platelet area increases during platelet spreading but further experiments are required to test this hypothesis(Sakurai et al. 2015; Peters et al. 2012).

There are multiple signaling pathways that relate $5HT_{2A}R$ activation to platelet spreading. The simplest of these pathways is Gαq activation leading to DAG that positively regulates platelet spreading (Canault et al. 2014). In the $SERT^{-/-}$ model, where serotonergic tone is lost, this signaling pathway could be reduced leading to insufficient DAG production and spreading. Further experiments would be required to elucidate the signaling pathways involved.

CHAPTER 5

INTEGRIN MODULATION OF SEROTONERGIC SIGNALING

Introduction

It was initially characterized that the β3-subunit (ITGB3) regulated the serotonergic system through an interaction with the SERT (Ana Marin D Carneiro et al. 2008). Additionally, as it has been noted that there are integrin-required, adhesion-dependent changes in SERT localization in human platelets (Steiner, Carneiro, and Blakely 2008). There exists an intriguing genetic relationship between SERT and β3 (ITGB3) where single nucleotide polymorphisms in ITGB3 parallel alteration in SERT platelet proteins levels (Mazalouskas et al. 2015). Additionally, blood serotonin has been used historically as a characterization markers for a number of diseases including hypertension and depression (Brenner et al. 2007; Serebruany et al. 2003; DeLisi et al. 1981; Hammock et al. 2012). Which this chapter we test that hypothesis that alterations in αIIbβ3 could disturb SERT function and peripheral serotonin homeostasis. In this chapter, the Pro32Pro33 (KI) mouse model will be used to determine if alterations to the integrin also alter serotonergic regulation in platelets.

PI^{A2} polymorphism and platelet function

The platelet membrane integrin αIIbβ3 or GPIIbIIIa binds to von Willebrand factor, which results in platelet binding to normal endothelium. It also can bind to fibrinogen that results in bivalent crosslinking of platelets leading to aggregation. A single substitution in αIIbβ3 results in a dynamic change in the functional regulation of the integrin. One common mutation is a substitution of leucine in PI^{A1} to proline in PI^{A2} at position 33 in the

GPIIIa subunits (Santiago-Germán et al. 2012). This polymorphism, $PI^{A1/A2}$ influences both the activation of αIIbβ3 and the aggregation of platelets (Feng et al. 1999).

The name of this polymorphism stems from the PI^{A} alloantigen, related to immune processes involving immune recognition of the opposing mutation (Newman, Derbes, and Aster, n.d.). There are two antigen determinants, $PI^{A1,}$ and PI^{A2}, located in the 17-23kD fragment of glycoprotein IIIa(β3). Clinically, this polymorphism leads to antigens production against the PSI (plexin-semaphorin-integrin) region of the integrin seen in two disorders: post-transfusion purpura (PTP) and neonatal alloimmune thrombocytopenic purpura (NATP). The frequency of these two alleles has been calculated to make up 85% A1 and 15% A2(Shulman et al. 1961). However, several studies debate the distribution of this polymorphism is different ethnic groups (Santiago-Germán et al. 2012).

Studies suggest that the $PI^{A1/A2}$ polymorphism of β3 is associated with stent thrombosis (Kastrati et al. 1999), stent restenosis(Walter et al. 1997), bypass occlusion (Zotz et al. 2000), coronary artery disease (Gardemann et al. 1998), and myocardial infarction (Weiss et al. 2009; J. L. Anderson et al. 1999). Although these findings are controversial, some studies have also shown that the PI^{A2} polymorphism is associated with increased platelet adhesion and aggregation (Sentí et al. 1998). However, the debate on whether or not the PI^{A2} polymorphism acts as a risk factor for cardiovascular disease is still under discussion. However, it is clear that fibrinogen is a major ligand of platelet αIIbβ3, and its function is a significant determinate in appropriate hemostasis. I have built upon the αIIbβ3 and SERT dynamics relationship demonstrating the sustained loss of SERT function reduces αIIbβ3 ADP-mediated activation. It has previously been established that SERT mediates αIIbβ3 directly (Ana Marin D Carneiro et al. 2008). In the current study,

we first characterize the platelet function in a mouse model that mimics the PI^{A2} polymorphism and then ask if this lead to an alteration is serotonergic handling and signaling.

Mouse model of PI^{A2} and platelet functional consequences

Platelet hyperaggregability is a critical factor influencing risk for arterial thrombosis (Lippi et al., 2011). The platelet integrin αIIbβ3 (glycoprotein IIbIIIa), the functional receptor for fibrinogen, mediates platelet aggregation through fibrinogen-dependent platelet cross-linking, a critical step in thrombus formation (Calvete, 1994; Ruggeri, 2002). While several polymorphisms in the integrin β3 subunit (*ITGB3* gene) have been associated with impaired platelet function (Wang et al., 1993; Wang and Newman, 1998), the presence of one allele for the β3 alloantigen Pl^{A2} has been reported in some studies to be associated with increased risk for coronary event, atherosclerotic plaque rupture and myocardial infarction (Kunicki and Nugent, 2002; Knowles et al., 2007). The Pl^{A2} antigen corresponds to a missense substitution of leucine to proline at residue 33 of the mature integrin β3, located in a hydrophobic pocket the β3 extracellular PSI (plexin-semaphorin-integrin) domain (Leu33Pro; rs5918, also known as the HPA-1 or Zw system) (Newman et al., 1989). While the number of studies assessing the influence of the Pro33 allele on platelet function is significant, findings are inconsistent due to the small number of homozygous Pro33 subjects studied or possibly due to population stratification (Michelson et al., 2000b; Undas et al., 2001; Vijayan et al., 2003b; Angiolillo et al., 2004; Dropinski et al., 2005; Lev et al., 2007). Therefore, other *in vivo* models must be developed

to determine the contributions of structural modifications in the PSI domain to platelet aggregation and thrombosis risk.

Structurally, the Leu33Pro substitution generates a Pro32Pro33 sequence, which may increase the flexibility of integrin αIIbβ3 extracellular domains (Xiong et al., 2004; Jallu et al., 2012). Several studies suggest that the increased platelet function in Pro33 carriers may result from a facilitation of integrin-mediated intracellular signaling (Goodall et al., 1999), (Vijayan et al., 2003a; Vijayan et al., 2003b; Vijayan et al., 2005). To achieve platelet activation, integrin αIIbβ3 undergoes conformational changes that involve disruption of αIIb-β3 interactions and extension of the cytoplasmic domain of integrin β3 (Yang et al., 2009). This extension can be achieved by extracellular matrix (ECM) binding under high flow conditions (outside-in activation) or by the agonist-dependent translocation of talin or kindlin 3 to the plasma membrane and binding to the β3 subunit (inside-out activation) (Vinogradova et al., 2000; Tadokoro et al., 2003; Wegener et al., 2007; Moser et al., 2008). These events trigger phosphorylation of tyrosine residues in the β3 tail and expose domains necessary for the interaction of focal adhesion kinase (FAK), Src and Hic-5 (Osada et al., 2001; Nieswandt et al., 2007; Kim-Kaneyama et al., 2012). Alternatively, $G\alpha_{13}$ downstream of thrombin (PAR-1/4) receptors can directly bind to the β3 cytoplasmic domain and activate Src (Gong et al., 2010). Upon αIIb/β3 separations, the αIIb cytoplasmic tail also can interact with signaling proteins, such as the calcium and integrin-binding protein (CIB), the serine/threonine protein phosphatase PP1 (Vijayan et al., 2003b; Vijayan et al., 2004). Although several studies suggest inside-out dependent increases in integrin-dependent signaling in cells expressing Pro33 integrin αIIbβ3, the

mechanism by which this extracellular PSI domain mutation influences integrin outside-in signaling remains unknown.

In the present study, we generated a new knock-in (KI) transgenic mouse model where the Pro32Pro33 isoform is expressed from the endogenous integrin β3 loci and examined the effects of this sequence variation to platelet function, integrin αIIbβ3 activation and outside-in signaling. In these mice, we demonstrate increased clotting time, enhanced fibrinogen-mediated platelet adhesion and elevated basal outside-in signaling without full αIIbβ3 integrin activation. Importantly, we show that early signaling events linked to Src activation dictate the proaggregatory phenotype in the KI mice.

Pro32Pro33 mutation in the integrin β3 domain result in αIIbβ3 priming and enhanced adhesion: Reversal of the hypercoagulability phenotype by Src inhibitor SKI-606

Abstract

The plasma membrane integrin αIIbβ3 (CD41/CD61, GPIIbIIIa) is a primary functional receptor in platelets during clotting. A common isoform of integrin β3 (Pro32Pro33) has been suggested to increase the risk of coronary thrombosis and stroke, however, the findings remain controversial. To better understand the molecular mechanisms by which this sequence variation modifies αIIbβ3-mediated platelet function we produced a transgenic, knock-in mice expressing the Pro32Pro33. Consistent with reports in human platelets, we found bleeding and clotting times to be significantly decreased in Pro32Pro33 homozygous mice. There was also an increase in platelet adhesion in vivo based on non-fatal venous thromboembolism. Activation with PAR4-AP, the main thrombin signaling receptor in mice, showed no significant difference in activation of Pro32Pro33 mice as compared to controls, suggesting that inside-out signing remains intact. However, increases in attachment and spreading of platelets onto fibrinogen are increased in Pro32Pro33 proposing enhances in integrin αIIbβ3 ligand-mediated functions (Outside-in signaling). We also observed elevated Src phosphorylation and Talin association in Pro32Pro33 platelets in unstimulated platelets. JON/An (active extracellular conformation of αIIbβ3) binding was not different in Pro32Pro33 platelets as compared to controls. These findings suggest that the intracellular domains of the Pro32Pro33 integrin are primed for activation while the extracellular domain remains unchanged. Enhanced priming of intracellular αIIbβ3, which leads to increase Src singling, rescued Pro33

clotting time to wild-type levels with acute in vivo administration of a clinically relevant inhibitor of Src, SKI-606. Together, our data establish that the Pro32Pro33 structural alteration modifies the function of integrin αIIbβ3, priming the integrin for outside-in signaling, ultimately leading to hypercoagulability. Additionally, these finding suggest that targeting Src signaling pathways may be of beneficial in reducing the risk for a cardiovascular disease mediated through enhanced αIIbβ3 activity.

Objectives

- To characterize the Pro32Pro33 mouse model's hemostatic condition and relate it to altered platelet function
- To evaluate αIIbβ3 function and particularly P-Src signaling related to spreading
- To attempt to rescue the Pro32Pro33 phenotype

Results

The introduction of the Pro32Pro33 residues in the mouse integrin β3. Alignment of the mature human and mouse integrin β3 protein sequences reveals a lack of conservation at residues 32 and 33 (Figure 29 A, corresponding to residues 58 and 59 in the immature protein, respectively. The human sequence contains a Pro32 and Leu33, modified to Pro32Pro33 by the Leu33Pro polymorphism, which introduces a structural flexibility associated with the functional changes observed in platelets (Jallu et al., 2012). Therefore, we designed the targeting construct to change Ser32Gln33 to Pro32Pro33 (Figure 35B, details on the targeting construct can be found in the methods section). C57BL/6 embryonic stem cells were screened for homologous recombination by two complementary PCRs (Figure 29 C and D) and correct targeting was confirmed by Southern blotting. Germline transmission of the Pro32Pro33 allele and excision of the Cre/Neo cassette was confirmed by PCR (Figure 29 E) and sequencing of the final targeted locus (KI). Pro32Pro33 KI mice were born at Mendelian ratios, independently of the genotype of the parents, and were fertile with no overt developmental or behavioral effects.

Enhanced clot formation and aggregation in KI mice. Mice expressing the Pro32Pro33 integrin β3 had normal platelet production and blood cell count. To establish the physiological consequences of the Pro32Pro33 integrin β3 substitutions, we measured platelet function using *in vivo* and *ex vivo* paradigms. Clotting time was significantly decreased in KI mice when measured by tail bleed (Figure 30 A) or whole blood clot formation (Figure 30 B). To test whether the increased clotting could influence thrombosis *in vivo*, we implemented a model of *in vivo* non-fatal thromboembolism. In this model, we

injected a solution containing weak agonists (0.5mg/kg ADP, 100μ/kg epinephrine and 1mg/kg collagen) to prevent a ceiling fatal effect, which would prevent us from detecting increases in thromboembolism in KI mice. We collected blood from mice before and one minute after the injection of agonists and counted the number of platelets in each sample. Repeated measures ANOVA of the data revealed a significant reduction in the number of circulating platelets in KI mice only (Figure 30 C). We then measured *ex vivo* platelet aggregation by two methods. Whole blood aggregation in the presence of the protease-activated receptor 4 activating peptide (PAR4 stimulation), led to a significant increase in the velocity of clot formation in KI mice when compared to WT controls (Figure 30 D, E). These changes were also recapitulated in aggregation experiments using washed platelets, suggesting that the proaggregatory phenotype derives from enhanced platelet function (Figure 30 F).

Enhanced adhesion and spreading in KI platelets. To examine the consequences of the Pro32Pro33 mutation on integrin αIIbβ3 functions we examined platelet adhesion *ex vivo*. Platelet adhesion depends on both integrin affinity (determined by ligand binding) and avidity (determined by integrin crosslinking), which can be assessed by adhesion to immobilized fibrinogen. Although basal binding to fibrinogen was not significantly different between genotypes, homozygous KI platelets had increased adhesion to fibrinogen in the presence of 0.2mM $MnCl_2$ (Figure 31 A). We then measured platelet adhesion to Arginine-Glycine-Aspartic Acid (RGD) peptides, which do not induce clustering of the receptor. We observed similar levels of platelet attachment to wells coated

with RGD (Figure 31 B), suggesting that the Pro32Pro33 mutation does not alter the affinity of αIIbβ3 of the ligand-binding domain to RGD.

Adhesion comprises two integrin-initiated events, attachment and spreading (Arias-Salgado et al., 2005; Lawson and Schlaepfer, 2012). We utilized confocal microscopy to determine platelet number and surface area after adhesion to 25μg/ml fibrinogen (Figure 31 C). We found that talin staining better represented the spreading of cells onto fibrinogen-coated slides compared to phalloidin and observed significant increases in the number and mean area of attached KI platelets when compared to WT platelets (Figure 31 D: Platelet number. Figure 31 E: Platelet area). The significant increases in spreading led us to examine proximal intracellular signaling cascades, including Src and FAK in the context of platelet adhesion to fibrinogen. In-cell Western analyses revealed increases in Src (Tyr^{416}), but not FAK(Tyr^{397}) or ERK phosphorylation in adhered KI platelets (Figure 31 F). Confocal imaging of pSrc (Tyr^{416}) staining in platelets adhered onto fibrinogen suggests that the increases in Src phosphorylation occur at adhesion sites (Figure 31 G). The significant increases in attachment and spreading in KI platelets are indicative of enhanced integrin αIIbβ3 function, likely due to increased integrin clustering and ligand-induced propagation of intracellular signals.

Enhanced basal talin binding to integrin αIIbβ3 is independent of conformational changes in the ligand-binding domain in KI platelets. The integrin β3 Pro32Pro33 mutations may influence αIIbβ3 functions by altering receptor expression at the plasma membrane, by facilitating changes in the ligand-binding domain and consequent integrin activation, or by facilitating changes in the conformation of the transmembrane

and intracellular domains of αIIbβ3 and thus promoting outside-in signaling. Flow cytometry using extracellular epitope antibodies revealed comparable plasma membrane expression levels of both αIIb and β3 subunits between genotypes (Figures 32 A and 38B, respectively). Changes in affinity in the integrin αIIbβ3 ligand-binding domain were assayed using an antibody that recognizes the active conformation of αIIbβ3 (JON/A, (Bergmeier et al., 2002). No changes were observed in JON/A binding to WT, KI, and *Itgb3*$^{-/-}$ platelets, indicating the absence of fully-activated integrin αIIbβ3 in resting platelets (Figure 32 C). Because we observed adhesion-dependent increases in Src phosphorylation, we examined whether the Pro32Pro33 mutation could modulate Src activation in unstimulated conditions. Western blot analysis of unstimulated platelets revealed no significant alterations in the expression levels of integrin αIIbβ3 or any proximal signaling proteins such as talin, Src and FAK (Figure 32 D). We observed, however, a significant increase in Src(Tyr^{416}) phosphorylation with a concomitant decrease in FAK(Tyr^{397}) phosphorylation in KI platelets, while no significant differences in ERK phosphorylation were observed (Figure 32 E). Co-immunoprecipitation of talin and Src with integrin αIIbβ3, using an extracellular epitope anti-integrin β3 antibodies revealed increased basal association of talin to β3 KI platelets (Figure 38F, top panel). In contrast, Src associations with β3 remained unchanged in KI platelets (co-immunoprecipitated integrin αIIb is also shown in Figure 32 F). Together, these data demonstrate that the integrin β3 Pro32Pro33 mutations increased talin binding to the intracellular domains of αIIbβ3, concomitant with increased pSrc and decreased pFAK levels in unstimulated platelets. Most remarkably, these changes in second messenger kinases occur without observed activation of the ligand-binding domain of αIIbβ3.

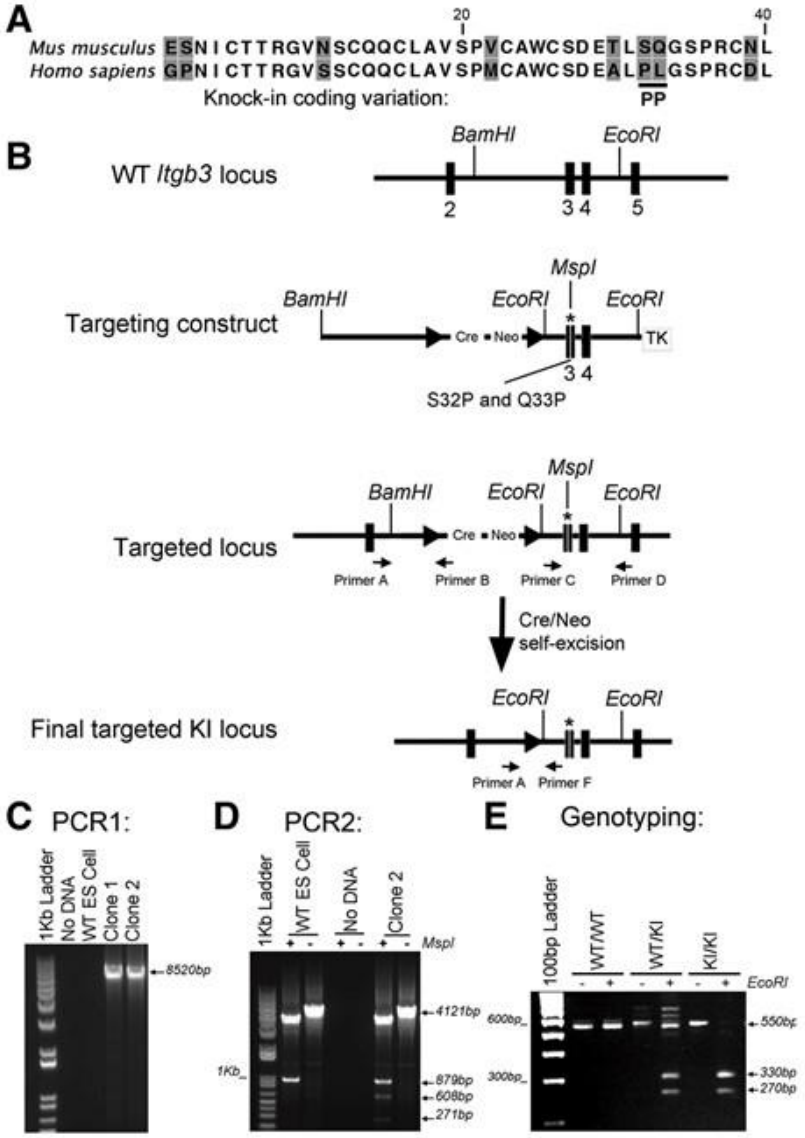


Figure 29. ***Production of the Pro32Pro33 (KI) mouse****. Generation of mice bearing the *Pro32Pro33 mutation (KI mice). (A) Sequence alignment of mouse and human mature $\beta_3$integrin. The Pro32Pro33 mutation introduced in the KI mouse model is shown below the alignment. (B) Targeting strategy to generate the KI mice, where exon 3 contains the S32P, Q33P substitution. The self-excising Cre/Neo cassette, flanked by LoxP sites, is located 5' of exon 3. Two complementary PCR strategies were used to screen embryonic stem (ES) cells. (C) PCR1 using primers A and B showing presence of the Cre/Neo cassette in recombinant ES cells. (D) PCR2 using primers C and D followed by MspI digestion showing successful targeting of the KI allele in clone 2. (E) Genotyping by PCR confirms excision of the Cre/Neo cassette, and EcoRI digestion reveals fragments measuring 330 and 270 bp identifying KI mice.*

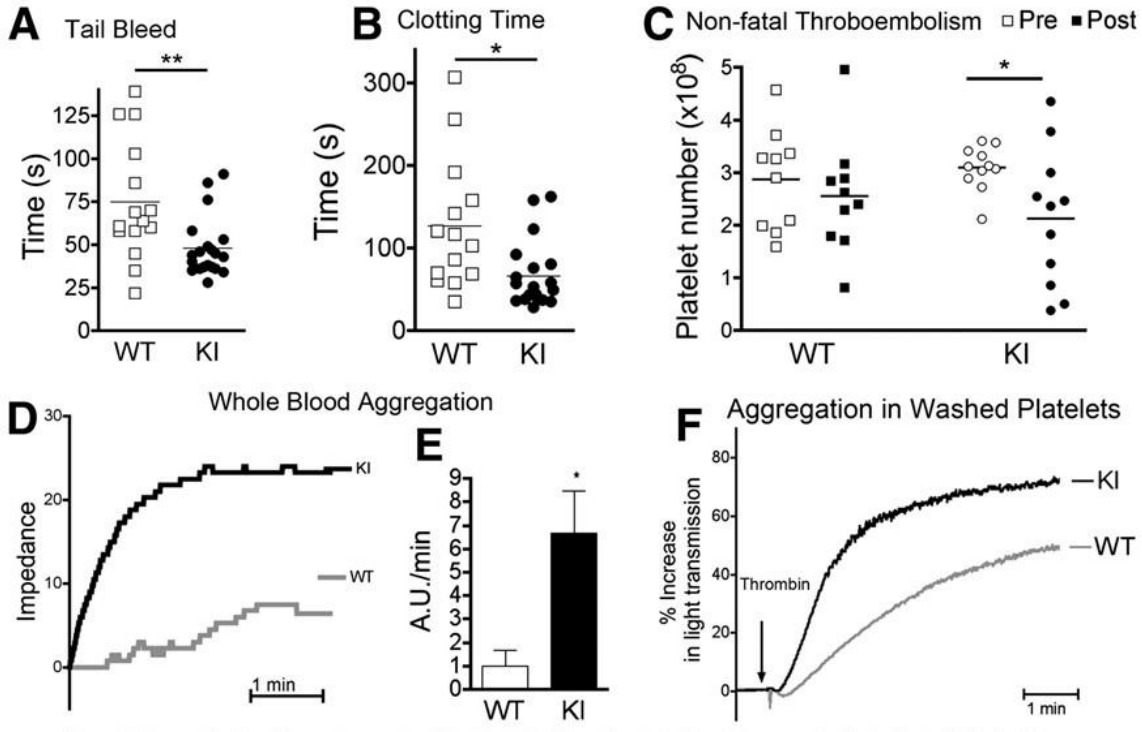


Figure 30. ***KI mice have increased aggregation as compared to WTs****. Enhanced clot formation and aggregation in KI mice. (A) Tail bleed time is significantly decreased in KI mice (Student's t test: **P = 0.0049; WT = 15; KI = 20). (B) Clotting time is significantly decreased in KI mice (Student's t test: *P = 0.0164; WT = 14; KI = 20). (C) Thromboembolism experiment showing equivalent initial number of circulating platelets between WT and KI samples (Pre). After administration of agonist in vivo (Post), the number of circulating platelets significantly decreases in KI mice but not in WT control mice (two-way ANOVA agonist effect: P = 0.0319; Bonferroni post-test, KI_{Pre} versus KI_{Post}: *P < 0.05; WT = 10; KI = 11). (D) Whole-blood aggregation stimulated with 200 μM PAR4-AP. (E) Aggregation velocity [arbitrary units (A.U.)/min] is significantly increased in KI mice (unpaired t test with Welch's correction: *P = 0.0267; WT = 6; KI = 6). (F) Aggregation in washed platelets. Representative plot showing increased KI aggregation in platelets stimulated by 0.05 U/ml thrombin. Similar results were observed in six independent experiments.*

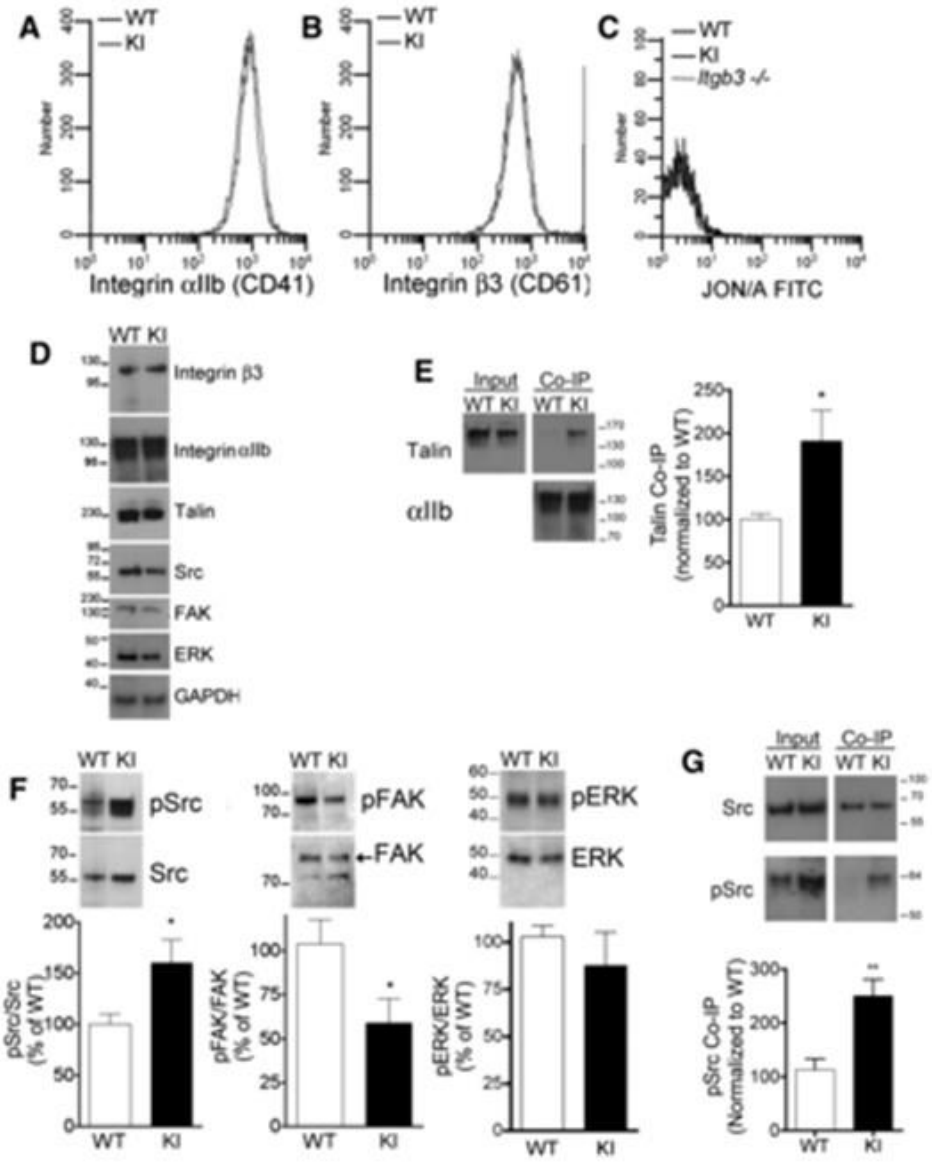


*Figure 31**. KI mice have increased P-Src at BL**. Increased spreading and adhesion mediated by outside-in signaling in KI platelets. Platelet adhesion was monitored by in-cell Western blot ofβ-actin on platelets bound to increased concentrations of immobilized fibrinogen (A) or RGD peptides (B) in the presence of 0.2 mM $MnCl_2$[(A) two-way ANOVA, fibrinogen: P < 0.0001; genotype: P = 0.0070; Bonferroni post-test, KI versus WT at 25 μg/ml: *P < 0.05; (B) two-way ANOVA, RGD: P < 0.0001]. (C) Platelets were allowed to adhere to fibrinogen-coated (25 μg/ml) coverslips (incubation time, 15 minutes). Representative confocal images of platelets stained with talin are shown. Scale bar, 10 μm. (D) Number of platelets attached per image was quantified in WT and KI samples (unpaired t test with Welch's correction: **P = 0.0091; number of images: WT = 8; KI = 6). (E) Platelet spreading was assessed by quantification of platelet area (in arbitrary*

*units) in WT and KI samples (unpaired t test with Welch's correction: ***P < 0.0001; number of platelets: WT = 86; KI = 111). (F) Platelets were allowed to adhere to 25 μg/ml fibrinogen for 15 minutes, washed, and fixed with 4% paraformaldehyde to assess kinase phosphorylation by in-cell Western blot. In-cell Western data were normalized for each WT/KI pair (pSrc/Src, nonparametric t test: *P = 0.0304; WT = 10; KI = 10).*

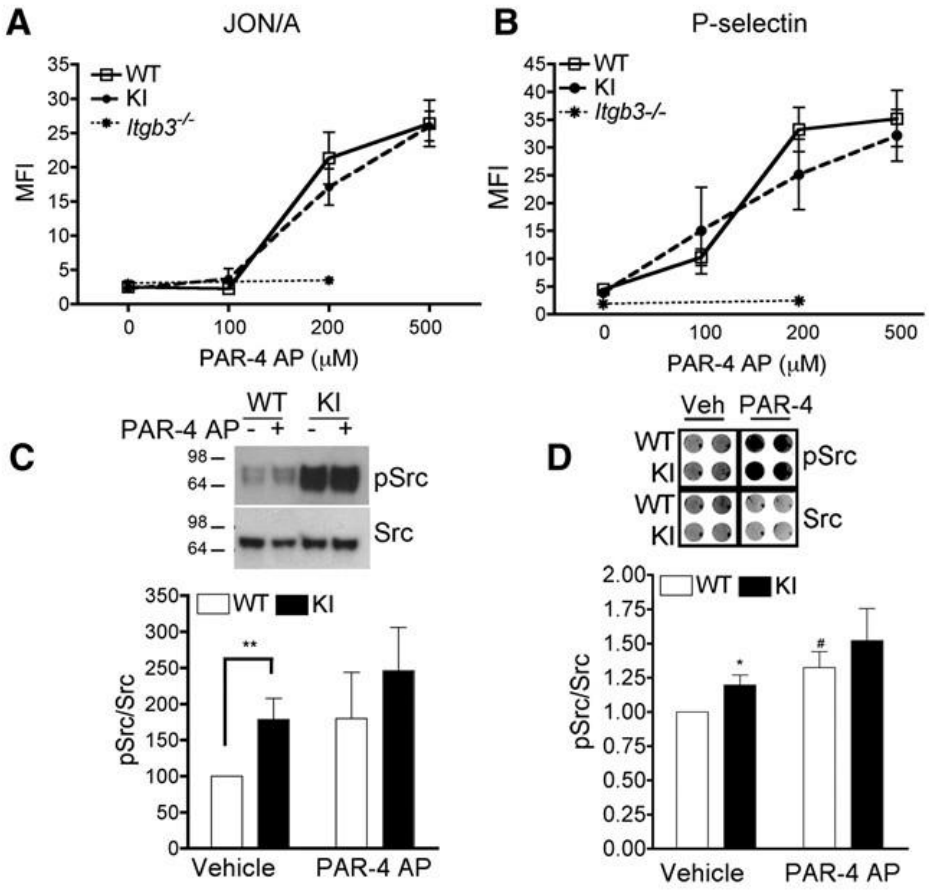


Figure 32. ***There is no difference in outside inactivation of the αIIbβ3 integrin in KI mice****. Activation of PAR4 signaling partially normalizes WT and KI genotype differences in Src phosphorylation. (A) Integrin $\alpha IIb\beta_3$ activation by PAR4 was determined by flow cytometry. (B) P-selectin plasma membrane levels are elevated by PAR4 activation in both WT and KI samples. Traces show data from WT, KI, and Itgb3$^{-/-}$ platelets. (C) Platelets in suspension were stimulated with PAR4-activating peptide followed by Western blot analysis of Src phosphorylation. PAR4 activation does not significantly increase Src phosphorylation in either genotype (nonparametric t tests for pSrc/Src ratios, $WT_{vehicle}$ versus $KI_{vehicle}$: $^{**}P < 0.01$; $WT_{vehicle}$ N = 14, WT_{PAR4} N = 7, $KI_{vehicle}$ N = 14, KI_{PAR4} N = 8). (D) PAR4 activation in attached platelets differentially influences Src phosphorylation. PAR4 stimulation significantly increases pSrc levels in WT platelets (nonparametric t tests, $WT_{vehicle}$ versus $KI_{vehicle}$: $^{*}P < 0.05$; $WT_{vehicle}$ versus WT_{PAR4}: $^{\#}P < 0.05$; WT = 6, KI = 6). MFI, mean fluorescence intensity.*

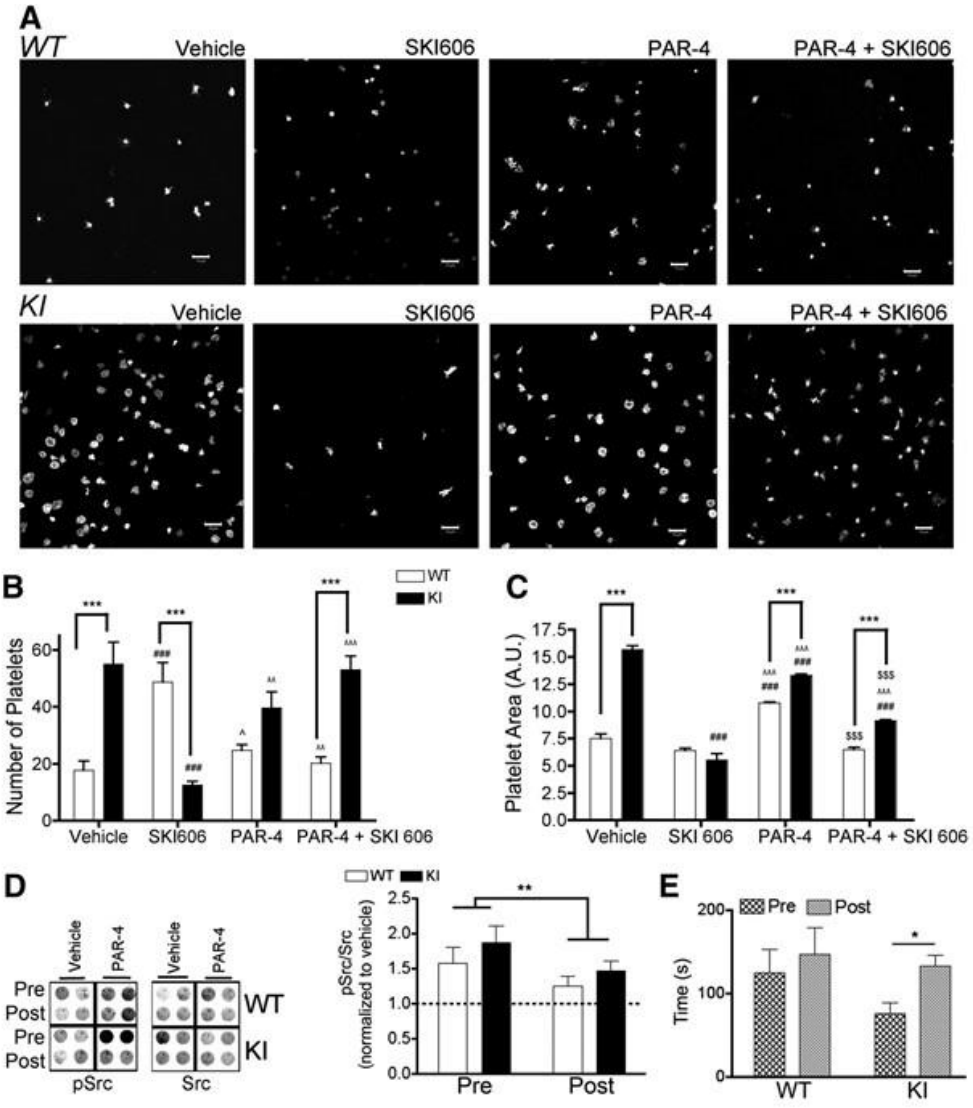


Figure 33. ***Increased spreading in KI mice****. Inhibition of Src rescues the spreading and clotting phenotypes observed in KI mice. (A–D) Ex vivo inhibition of Src. Platelets were resuspended in KRH, seeded onto fibrinogen-coated slides (25 μg/ml), and incubated with different agonists for quantification of cell adhesion. (A) Representative confocal images of talin staining. (Left to right) Vehicle, Src/Abl tyrosine kinase inhibitor SKI-606, PAR4-AP, PAR4-AP + SKI-606. (Top) WT platelets; (bottom) KI platelets. Scale bar, 10 μm. (B) Platelet attachment. SKI-606 elicits opposing effects in WT and KI platelets, increasing adhesion in WT while decreasing adhesion in KI samples. PAR4-AP activation does not significantly enhance platelet attachment. PAR4-AP activation normalizes the effects of SKI-606 on platelet attachment in both genotypes (two-way ANOVA, interaction* $F_{(34,3)} = 22.51$: $P < 0.0001$; *genotype* $F_{(34,1)} = 11.80$: $P = 0.0016$; *Bonferroni post-tests:* $WT_{vehicle}$ *versus* $KI_{vehicle}$: $^{***}P < 0.001$; $WT_{SKI\text{-}606}$ *versus* $KI_{SKI\text{-}606}$: $^{***}P < 0.001$; $WT_{PAR4\text{-}AP+SKI\text{-}606}$ *versus* $KI_{PAR4\text{-}AP+SKI\text{-}606}$: $^{***}P < 0.001$; $WT_{vehicle}$ *versus* $WT_{SKI\text{-}606}$: $^{\#\#\#}P < 0.001$; $KI_{vehicle}$ *versus* $KI_{SKI\text{-}606}$: $^{\#\#\#}P < 0.001$; $WT_{SKI\text{-}606}$ *versus* $WT_{PAR4\text{-}AP}$: $^{\wedge}P < 0.05$; $KI_{SKI\text{-}606}$ *versus* $KI_{PAR4\text{-}AP}$: $^{\wedge\wedge}P < 0.01$; $WT_{SKI\text{-}606}$ *versus* $WT_{PAR4\text{-}AP+SKI\text{-}606}$: $^{\wedge\wedge}P < 0.01$; $KI_{SKI\text{-}}$

*$_{606}$ versus KI$_{PAR4\text{-}AP+SKI\text{-}606}$: $^{\wedge\wedge\wedge}$P < 0.001; number of images: vehicle: WT = 9, KI = 6; SKI-606: WT = 3, KI = 4; PAR4-AP: WT = 5, KI = 5; PAR4-AP + SKI-606: WT = 5, KI = 5). (C) Platelet spreading. SKI-606 alone normalized platelet spreading of KI platelets to WT levels. PAR4-AP activation significantly enhanced spreading in WT platelets, while reducing KI platelet spreading. Additionally, SKI-606 significantly reduced PAR4-AP–stimulated platelet spreading in both genotypes (two-way ANOVA, interaction $F_{(1516,\ 3)}$ = 39.72: P < 0.0001; genotype $F_{(1516,\ 1)}$ = 110.5: P < 0.0001; treatment $F_{(1516,\ 3)}$ = 99.10: P < 0.0001; Bonferroni post-tests: WT$_{vehicle}$ versus KI$_{vehicle}$: ***P < 0.001; WT$_{PAR4\text{-}AP}$ versus KI$_{PAR4\text{-}AP}$: ***P < 0.001; WT$_{PAR4\text{-}AP+SKI\text{-}606}$ versus KI$_{PAR4\text{-}AP+SKI\text{-}606}$: ***P < 0.001; KI$_{vehicle}$ versus KI$_{SKI\text{-}606}$: $^{\#\#\#}$P < 0.001; WT$_{vehicle}$ versus WT$_{PAR4\text{-}AP}$: $^{\#\#\#}$P < 0.001; WT$_{PAR4\text{-}AP}$versus WT$_{PAR4\text{-}AP+SKI\text{-}606}$: $^{\$\$\$}$P < 0.001; WT$_{SKI\text{-}606}$ versus WT$_{PAR4\text{-}AP}$: $^{\wedge\wedge\wedge}$P < 0.001; KI$_{SKI\text{-}606}$ versus KI$_{PAR4}$: $^{\wedge\wedge\wedge}$P < 0.001; KI$_{SKI\text{-}606}$ versus KI$_{PAR4+SKI\text{-}606}$: $^{\wedge\wedge\wedge}$P < 0.001; KI$_{vehicle}$ versus KI$_{PAR4\text{-}AP}$: $^{\#\#\#}$P < 0.001; KI$_{vehicle}$ versus KI$_{PAR4\text{-}AP+SKI\text{-}606}$: $^{\#\#\#}$P < 0.001; KI$_{PAR4\text{-}AP}$ versus KI$_{PAR4\text{-}AP+SKI\text{-}606}$: $^{\$\$\$}$P < 0.001; number of platelets: vehicle: WT = 159, KI = 480; SKI-606: WT = 146, KI = 51; PAR4-AP: WT = 104, KI = 198; PAR4-AP + SKI-606: WT = 101, KI = 265). (D and E) In vivo inhibition of Src. Blood was collected before (Pre) and after (Post) administration of 1 mg/kg SKI-606 to anesthetized mice. Blood was diluted 1:20 and seeded onto fibrinogen-coated (25 μg/ml) 96-well plates. (D) In-cell Western of platelets adhered to fibrinogen. Src phosphorylation was assessed by staining platelets with total Src or pSrc at Tyr^{416}. As there was variability in the volume of blood collected before and after SKI-606 administration, data were normalized to vehicle (dotted line in right panel) to assess PAR4-induced Src phosphorylation. SKI-606 significantly decreases PAR4-AP–induced Src phosphorylation in both KI and WT samples (repeated-measures ANOVA, SKI-606: **P = 0.0228; WT = 6, KI = 6). (E) Clotting time is significantly increased in KI mice treated with SKI-606 (repeated-measures ANOVA, SKI-606: *P = 0.0103; Bonferroni post-test, KI$_{vehicle}$ versus KI$_{SKI\text{-}606}$: *P < 0.05; WT = 6, KI = 6).*

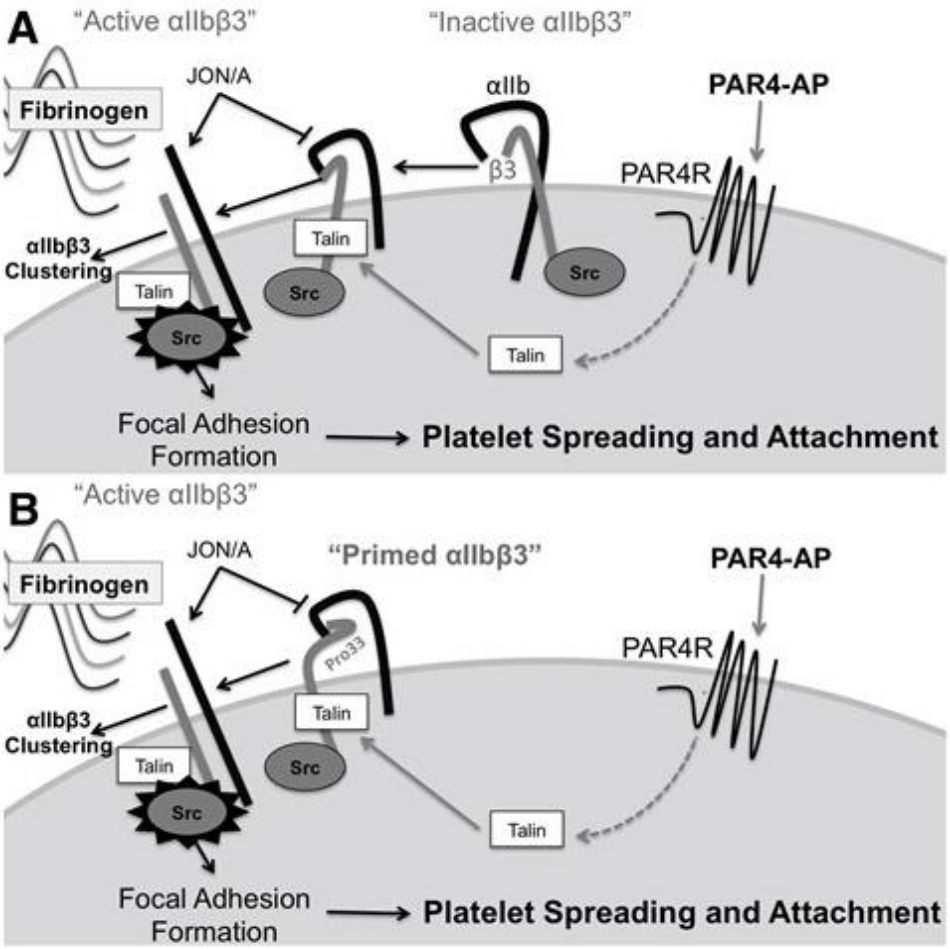


Figure 34. ***Graphics of KI mutation on platelet activation****. Mechanism of enhanced outside-in signaling in KI mice. (A) In wild-type mice, αIIbβ₃ integrin is activated by talin binding upon activation of inside-out signaling, represented here by PAR4 activation. Talin binding separates the intracellular domains of αIIb and β₃, resulting in the opening of the extracellular domains of αIIbβ₃ and exposing the ligand-binding domain, recognized by the antibody JON/A. These conformational changes initiated by talin binding also provide a platform for protein associations. While Src binding to β₃ is independent of its phosphorylation state, Src/β₃ interactions are sufficient to trigger Src autophosporylation. (B) In KI mice, a mutation within the PSI domain ofβ₃ allows talin binding in nonstimulated conditions. The increased talin binding likely enhances the probability of Src associations and phosphorylation, thus enhancing platelet activation downstream of fibrinogen binding. These molecular changes may have reached "ceiling levels" in KI platelets, as activation of PAR4 signaling does not further enhance Src phosphorylation.*

Activation of inside-out signaling normalizes some, but not all genotype-specific differences between KI and WT platelets. Although our data reveal enhanced basal Src activation in unstimulated platelets, several studies have provided evidence for agonist-induced enhancement of FAK and ERK signaling in platelets and cells expressing the human Pro33 integrin β3 (Vijayan et al., 2003b; Vijayan et al., 2005; Carneiro et al., 2008). As Src activation is one of the many signaling pathways influenced by integrin αIIbβ3 activations, we examined whether PAR4 activation modifies the cellular phenotypes observed in KI platelets. Flow cytometry experiments revealed comparable levels of JON/An antibody binding to "active" αIIbβ3 in both WT and KI platelets, upon PAR4 stimulation (Figure 33 A). PAR4 also did not differentially stimulate P-selectin plasma membrane expression, indicating similar platelet activation upon strong agonist stimulation in both WT and KI platelets (Figure 33 B). PAR4 activation did not significantly increase Src(Tyr^{416}) phosphorylation in platelets in suspension, regardless of genotype (Figure 39C). However, upon adhesion, PAR4 stimulation significantly increased Src phosphorylation in WT but not in KI platelets (Figure 33 D). Because baseline Src phosphorylation in the KI platelets may have reached ceiling levels, activation of inside-out signaling does not further increase Src phosphorylation. This data, along with the findings in Figure 38, demonstrate that structural changes elicited by the Pro32Pro33 mutation are independent of inside-out signaling.

Src activation is the dominant step driving the increased clotting in KI platelets. To test whether Src activation is necessary for the enhanced adhesion and clotting phenotypes observed in KI mice, we exposed platelets to the dual Src/Abl tyrosine kinase

inhibitor SKI-606 (Bosutinib). SKI-606 is an orally available inhibitor of Src, Fgr, and Lyn (Boschelli et al., 2001; Remsing Rix et al., 2009). With platelet activation via both PAR4 and fibrinogen, SKI-606 (0.1μM) fully inhibited Src phosphorylation but had no influence on platelet attachment (Figure 34 B). PAR4 activation significantly enhanced spreading in WT platelets and induced a small reduction in spreading in KI samples (Figure 34 C). SKI-606 significantly reduced platelet spreading in both WT and KI platelets, normalizing spreading to un-stimulated, WT levels (Figure 34 C). These data demonstrate that Src is involved in the spreading, but not attachment, of platelets onto fibrinogen, and inhibition of Src signaling may counteract the basal activated state of KI platelets (Figure 34 A).

We then examined whether *in vivo* administration of SKI-606 can normalize KI clotting *ex vivo*. Mice were anesthetized and blood collected before and after intraperitoneal administration of 1mg/kg SKI-606. In-cell Western analysis confirmed that SKI-606 significantly reduced adhesion-dependent Tyr^{416} Src phosphorylation in the presence of PAR4 AP (Figure 34 D). At this concentration SKI-606 significantly increased clotting time in KI mice, whereas no significant differences were observed in WT (Figure 34 E). This differential effect was concentration-dependent, as we found increased clotting times after injection of higher concentrations of SKI-606, albeit with larger increases in KI. These data demonstrate that the integrin β3 Pro32Pro33 mutation is sufficient to induce increased talin association to αIIbβ3 and enhanced basal Src phosphorylation, which are responsible for facilitated platelet spreading and a prothrombotic phenotype in KI mice.

Serotonergic modulation of Pro32Pro33 mice

Abstract

The connection between SERT activity and αIIbβ3 function has been noted previously (Ana Marin D Carneiro et al. 2008). It has been shown that fibrinogen, the activator or ligand for the αIIbβ3 integrin can enhance SERT activity in human platelets (Ana Marin D Carneiro et al. 2008). Furthermore, it has been demonstrated that the integrin acts directly with the C-terminus of SERT (Ana Marin D Carneiro et al. 2008). When the integrin is knocked-out in β3 KO mice, the activity of SERT is significantly reduced. However when the integrin is in it active conformation there is increased SERT activity in stably transfected HEK293 cells (Wildling et al. 2012; Wong et al. 2012). This also coincided with an increase in SERT surface expression when the integrin was in its active conformation. In total, this work establishes has established an association between the function of the αIIbβ3 integrin and SERT. In the following set of experiments, we test if the Pro32Pro33 mouse model has altered serotonergic tone. Furthermore, we test if reducing serotonergic tone with 6-day citalopram water treatment can rescue the Pro32Pro33 hypercoagulability mouse model.

Objectives

- Determine if the Pro32Pro33 alters serotonergic signaling
- Determine if the Pro32Pro33 hypercoagulability phenotype can be rescued with sustained inhibition of SERT

Results

In order to test the association between the Pro32Pro33 mouse model and SERT function, we chronically inhibited SERT by giving mice citalopram in their drinking water at 15mg/kg/day for 6-days. The 6-day treatment model was used because it blocks SERT function over the lifespan of a mouse platelet and would, therefore, recapitulate chronic SERT inhibition within the. Additionally, this model allows for the continued administration of citalopram to the animal as compared to IP injections, where metabolism of the drug can lead to a cyclic inhibition of SERT function.

We found that KI mice have a significant increase in whole blood serotonin levels as compared to WT mice (Figure 35). However, following citalopram treatment, both WT and KI mice have significant reduced serotonin levels suggesting that the storage of serotonin is significantly reduced. Furthermore, there were no significant changes in 5HIAA suggesting that the overall production of serotonin was not altered.

In order to determine if the citalopram treatment alters the hypercoagulability phenotype in KI mice, both clotting time and thrombin clotting time measurements were performed. Pro32Pro33 mouse clotting time and thrombin clotting time are significantly altered with the addition of sub-chronic SSRI treatment reducing serotonergic signaling in platelets. There was a significant citalopram effect on clotting time (Figure 35). While there was a reduction in KI and WT clotting time when compared directly using a t-test (P=0.03), this significance was lost when performing a 2-way ANOVA test due to multiplicity (P=0.70). Pro32Pro33 mice had a significant increase in clotting time after citalopram treatment. This effect was not seen in the WT mice. Thrombin time also had an overall

citalopram effect. Following post-test analysis, a significant increase in thrombin clotting time was observed in Pro32Pro33 mice.

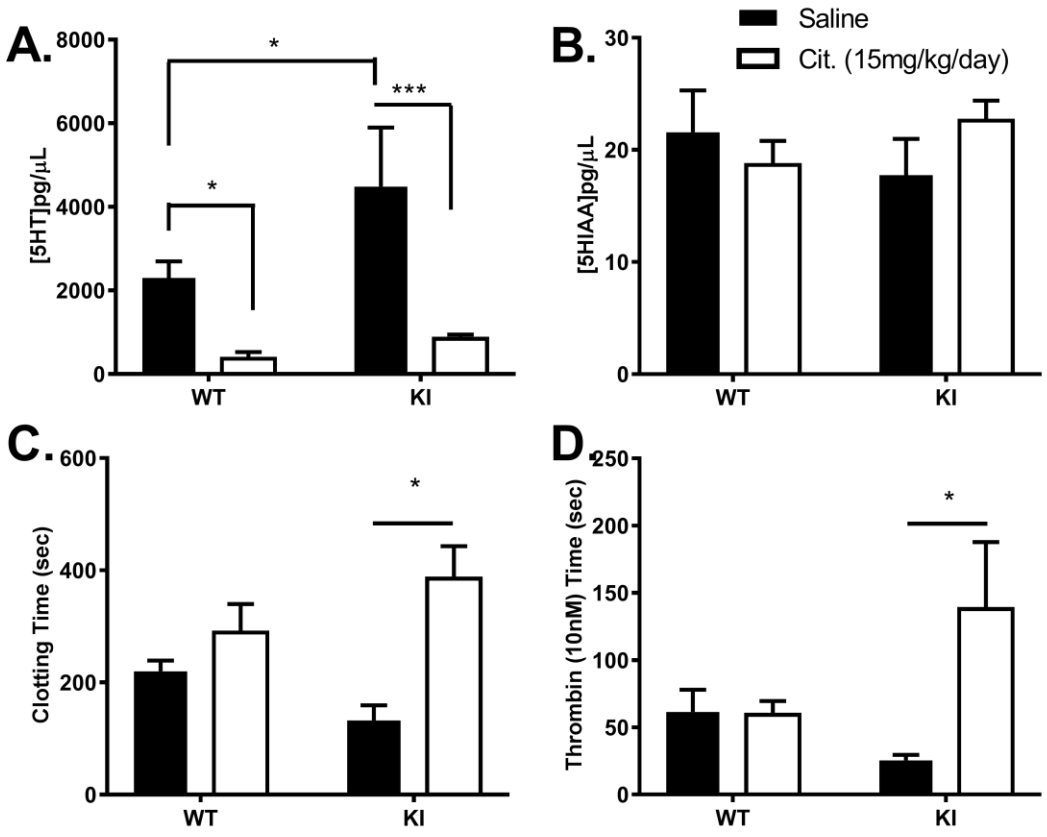


Figure 35. ***6-day citalopram treatment rescues KI hypercoagulability****. Serotonin whole blood levels were measured in WT and KI platelet with and without citalopram-treated water treatment. KI mice had a significantly increased level of blood serotonin as compared to WT blood (Two-way ANOVA: genotype P=0.01, treatment P<0.0001: WT: saline vs KI: saline P=0.0338*, WT:saline vs WT:cit P=0.027*, KI:saline vs KI:Cit P=0.0002***). However both WT and KI whole blood serotonin levels were significantly reduced with citalopram treatment. There was no change in 5HIAA levels in wither WT or KI whole blood with or without citalopram (Two-way ANOVA). Similar to previous studies there was reduced clotting time observed in KI blood as compared to WT blood when compared using a t-test (P=0.03. However, these effects were not signaifcant in the two-way ANOVA analysis. There was an increase in clotting time when KI mice were treated with citalopram and this effect was not seen in WT samples (Two-way ANOVA, interaction P=0.03, Treatment P=0.0006, Genotype P=0.91: KI:saline vs KI:cit P=0.0023*) . Again, this effect was observed in thrombin clotting time assay (Two-way ANOVA interaction P=0.0219, treatment P=0.0268, Genotype P=0.37: KI:saline vs KI: cit P=0.02*5).*

Conclusion

Pro32Pro33 leads to a preactivated αIIbβ3 integrin

Platelet aggregation is a tightly controlled event, essential for the maintenance of thrombosis and hemostasis. Here we focused on the study of a common integrin β3 coding polymorphism, Leu33Pro within the αIIbβ3 integrin (Newman et al., 1989). Flow cytometry studies utilizing anti-Leu33 β3 antisera from Pro33 thrombocytopenia patients reveal conformational changes in an epitope located between residues 9 to 50, likely due to the formation of a two proline sequence in the PSI domain (Barron-Casella et al., 1999; Bougie et al., 2012). To mimic the structural changes induced by the Leu33Pro mutation in humans, we introduced the Pro32Pro33 sequence in the mouse *Itgb3* locus, replacing the endogenous Ser32Gln33. Pro32Pro33 mice have altered adhesion, increased velocity in aggregation, resulting in a proaggregatory phenotype observed in tail bleeding and non-fatal thromboembolism. Therefore, our data demonstrate that the Pro32Pro33 KI mouse model, despite not being a fully humanized allele, replicates the phenotypes observed in the human Pro33 platelets.

In accordance with data obtained from Pro33 human samples, no changes in plasma membrane integrin αIIbβ3 expressions were seen in KI mice (Goodall et al., 1999). JON/A binding to resting platelets indicates that the murine Pro32Pro33 αIIbβ3 is not in an open conformation. This finding, however, does not exclude the possibility that other conformational changes have taken place, or that the presence of two successive proline residues increases the flexibility of the PSI/hybrid domains, thus facilitating extension and the opening of the αIIbβ3 heads. In fact, our findings are consistent with the Pro32Pro33 mutation maintaining the ligand-binding domain in the closed conformation, while

modifying the transmembrane domains, thus enhancing talin association with the β3 intracellular carboxy-terminal tail and initiating outside-in signaling. This unique phenotype confers a gain-of-function to the receptor without exerting a dramatic deleterious effect, as observed in another gain-of-function integrin β3 polymorphisms (Ruiz et al., 2001; Mor-Cohen et al., 2007; Ghevaert et al., 2008). The D723H mutation results in constitutive activation of integrin αIIbβ3, increased cell adhesion with no effects on platelet aggregation *in vitro*. This mutation has dramatic effects on platelet size and is found in Glanzmann thrombasthenia patients, suggesting that constitutive activation of αIIbβ3 is deleterious for clotting (Ghevaert et al., 2008). Other mutations disrupting disulfide bridges in the extracellular domains, lead to high-affinity binding to soluble fibrinogen and thus result in a loss of platelet aggregation *in vitro* (Fang et al., 2013). These polymorphisms differ from our KI model because they display increased fibrinogen binding/affinity and represent a constitutively active receptor. The Pro32Pro33 integrin β3, though showing enhanced priming and basal Src signaling, remains sensitive to modulation by inside-out signaling, and does not present increased JON/A binding or adhesion onto RGD peptides and thus represents a "facilitated" receptor, but not one that is constitutively active.

Regardless of the molecular mechanism, KI platelets exhibit specific alterations in intracellular Src and FAK signaling. These signaling changes drive clotting and spreading phenotypes in KI platelets, as both were normalized to wild-type levels in the presence of the Src inhibitor SKI-606. The role of Src (c-Src specifically) in platelet spreading has been established in several studies (Obergfell et al., 2002; Arias-Salgado et al., 2003). Deletion of Src in mouse platelets reduces spreading on fibrinogen independently of other Src-

family kinases, but Src is not necessary for the aggregation of platelets under flow conditions (Severin et al., 2012). Src activation through Tyr^{416} phosphorylation can be achieved by several mechanisms, although we have excluded enhanced interactions with the β3 cytoplasmic domain (Arias-Salgado et al., 2003). A second mechanism worthy of consideration is that Src activation, through its association with FAK, can inhibit RhoA and increase spreading (Panetti, 2002; Serrels and Frame, 2012). Although possible, we feel that this explanation is unlikely to be the mechanism driving the KI phenotypes, as we detected reduced FAK Tyr^{379} phosphorylation under basal conditions, a necessary step during the formation of the FAK/Src/integrin αIIbβ3 complex (Chan et al., 1994; Cobb et al., 1994; Schaller et al., 1994; Xing et al., 1994). Finally, Src could also be engaged through $G\alpha_{13}$ activations (Gong et al., 2010). Platelets lacking $G\alpha_{13}$ have no adhesion-dependent Src Tyr^{416} phosphorylation and reduced spreading. It is possible that $G\alpha_{13}$ activations through PAR4 stimulation is involved in the spreading of WT samples, and could dampen constitutively activated Src in KI mice, as suggested by the significant statistical drug by genotype interactions in the presence of PAR4 AP.

Although several other signaling events may be altered in KI platelets, we capitalized on an FDA-approved Src kinase inhibitor to reverse the Pro32Pro33-induced hypercoagulable state. SKI-606 (bosutinib, Bosulif®) is an orally available tyrosine kinase inhibitor approved for the treatment of chronic myelogenous leukemia with low bleeding risk (Remsing Rix et al., 2009)(Quintas-Cardama et al., 2009). Orally available anti-platelet therapies include warfarin, aspirin, clopidogrel and ticagrelor (Varon and Spectre, 2009). Most of these pharmacotherapies present multiple drug-drug interactions and increased risk for bleeding (Phillips et al., 2005; Varon and Spectre, 2009). Additionally,

in the context of stent placement, the human Pro33 allele remains a risk factor for thrombosis and death, even in the presence of dual anti-platelet therapy (Goldschmidt-Clermont et al., 2000; Motovska et al., 2009). Our study is a major step towards identifying patient-specific, safe and efficacious therapies as we were able to normalize, but not dramatically reduce, clotting times specifically in Pro32Pro33 platelets. SKI-606 normalized Pro32Pro33 platelet response to platelet spreading with fibrinogen and PAR4 AP activation, partially representative of thrombus formation.

In conclusion, our data demonstrate that (1) a functional link between the Pro32Pro33 structural modification in the extracellular PSI domain and cytoplasmic alterations that result in the activation of Src signaling underlying platelet spreading, clotting and thrombus formation *in vivo*, and (2) confirm potential of novel, tailored therapeutic strategies targeting Src to reduce thrombotic risk.

Association between αIIbβ3 and serotonergic signaling

These findings suggest that sub-chronic, 6-day SSRI treatment reduces serotonergic tone in the blood as previous described (Maurer-Spurej, Pittendreigh, and Solomons 2004; Andrade et al. 2010; Bismuth-Evenzal et al. 2012b). Furthermore, it appears that this alteration to serotonergic tone has a greater impact in a hyperactive and primed integrin mutation (Pro32Pro33) as compared to baseline conditions. These finding further support the role of the integrin within the SERT regulatory complex and suggest that alterations in SERT function could have implication on αIIbβ3 activation in platelets.

It has previously been shown that the Pro33 polymorphism does not alter the association between SERT and αIIbβ3 complex (Ana Marin D Carneiro et al. 2008).

Additionally, there is no alteration between the association of PP2A with the SERT complex with either the Leu33 or the Pro33 mutation (Oliver et al. 2014; Ana Marin D Carneiro et al. 2008). However, there in an increase in the uptake of serotonin in the Pro33 mutation (Ana Marin D Carneiro et al. 2008). This can be reduced following inhibition of with serine/threonine phosphatases using either okadaic acid or fostriecin. Uptake by SERT was also reduced following blockage of p38 MAPK signaling preferentially in the Pro33 mutation, which showed increased phosphorylation of p-38 MAPK at baseline.

Previously published data, including this dataset, investigates how a polymorphism in the integrin alters the function of SERT and SERT surface expression (Mazalouskas et al. 2015; Steiner, Carneiro, and Blakely 2008; Ana Marin D Carneiro et al. 2008). The role of SERT and the surface expression of SERT can have both short and long term consequences on platelet function. These data clearly demonstrate a compelling relationship between altered αIIbβ3 function and SERT regulation, however future studies are required to elucidate the molecular mechanism. While SSRI treatment was able to restore clotting time and thrombin time in the KI mouse model, both of these assays have complex molecular underpinning that require further dissection. It is possible that SERT and αIIbβ3 interact within the same platelet lipid-raft and thereby modulate each other's function in either a direct or indirect manner (Ana Marin D Carneiro and Blakely 2006). However, it is also possible that the increased levels of serotonin change the dynamins of platelet reactivity via platelet activation or alternative mechanisms. Future experiments can separate these mechanisms in more detail.

CHAPTER 6

CONCLUSION

Overview of serotonin in platelet function and future directions

In summation, this work has explored several molecular mechanisms of serotonin's actions *in vivo*, with a particular focus on serotonin's role in modulating αIIbβ3-dependnet activation in platelet function. This work suggests that acute inhibition of SERT function does not play a significant role in platelet αIIbβ3-activation. Furthermore, this work further substantiates the functional interaction between integrins and SERT function (Steiner, Carneiro, and Blakely 2008; Ana Marin D Carneiro et al. 2008; Ana M D Carneiro 2010). Considering the broad array of functional roles of integrins, including cell migration, cell adhesion, and metastasis, the implications and clarification of a SERT- or serotonin-dependent component to integrin function is vital. Pathologically, integrins are considered to have an emerging role in neuropsychiatric disorders, cardiovascular disorders and others (Bennett, Berger, and Billings 2009; Zarbock, Polanowska-Grabowska, and Ley 2007; Gibbins 2004; Ana M D Carneiro 2010), therefore it is crucial to gain a better understanding of their many functions.

Two major questions have been addressed by this dissertation: (1) how do SSRIs, which block SERT uptake, alter platelet function? and (2) how do changes in serotonin levels regulation by SERT uptake affect the platelet? In particular, we were interested in monitoring serotonin's effects on αIIbβ3-mediated platelet activation. This question was broken down into three sections: first, does acute blockage of SERT alter platelet activation and/or spreading, second, does sustained inhibition of SERT alter platelet activation and/or spreading, and, third, does an alteration in the αIIbβ3 integrin (Pro32Pro33) change

serotonin blood levels. Each of these sub-questions are addressed in chapters 3, 4, and 5 respectively.

In chapter 3, we directly examined the function of acute serotonin uptake via SERT in platelet activation using pharmacology relevant concentrations of SSRIs that block SERT uptake in platelets. We found minimal effects on platelet αIIbβ3-activation when SERT was blocked acutely. While there was no difference in αIIbβ3 inside-out activation following acute blockade of SERT uptake, we did find that citalopram altered platelet spreading in a SERT-independent manner. Furthermore, I have discussed possible non-SERT specific targets for citalopram's effects on platelet spreading.

The major implications resulting from this chapter are twofold. First, this chapter stresses the importance of using pharmacologically validated concentration of drugs to limit off-target, non-specific effects to understand specific biological mechanisms. Second, validating that the concentrations of drug used are appropriate for the intended biological assumption. In this case, we were interested in determine who acute SERT uptake modulates platelet function, and therefore use concentrations that are sufficient to block platelet serotonin uptake. Previous studies have suggested that SSRIs, including citalopram, or loss of acute SERT function, alter platelet function (Bismuth-Evenzal et al. 2012a; HirshRokach et al. 2015; Tseng et al. 2013; Abdelmalik et al. 2008; Diego J Walther et al. 2003). However, these studies failed to separate the acute role of SERT versus the long term effects of lost SERT function (lost serotonin in whole blood). In some cases, the concentrations of SSRIs that were used in the studies fell outside the range of therapeutic use and far beyond the concentrations needed to inhibit SERT (Tseng et al. 2010; Ziu et al. 2014; Ziu et al. 2012). Pharmacology is a powerful tool to explore the biological

mechanisms but it is important to consider both the function and chemistry of the drug in use.

In chapter 4, we tested how the extended loss of SERT function alters αIIbβ3 activation. We observed that mice that lack SERT ($SERT^{-/-}$) had reduced ADP-mediated αIIbβ3 activation. Furthermore, we found that serotonin no longer synergized with ADP to enhance αIIbβ3 activation. We tested if acute uptake or sustained blockage of SERT alters platelet function, in particular, αIIbβ3 activation. We found that serotonin modulates integrin activation through triggering the $5HT_{2A}$ receptor, but not via acute uptake by SERT. These findings suggest that the granular serotonin content is paramount in mediating platelet activation through its release and activation of the serotonergic receptor, $5HT_{2A}R$. In order to explore why the $SERT^{-/-}$ platelets had reduced serotonin-dependent activation, we measured $5HT_{2A}R$-medaited GTPyS incorporation and $5HT_{2A}R$ surface expression. We found that $5HT_{2A}R$ surface expression was reduced in $SERT^{-/-}$ platelets. This was recapitulated in 6-day citalopram-treated mice. We speculate that the loss of $5HT_{2A}R$ surface expression and function arises through altered serotonin homeostasis that ensues following sustained inhibition of SERT function. Using the serotonin homeostasis model discussed at the end of chapter 4, it would be possible to test this hypothesis. However, our experimental findings suggest that long-term inhibition of SERT leads to a shift in serotonergic signaling to a desensitized, internalized $5HT_{2A}R$.

The experiments discussed in chapter 4 attempt to distinguish the mechanism behind the reduced ADP-mediated αIIbβ3 activation platelet function seen when SERT inhibition is sustained. We used the 6-day treatment model in an attempt to minimize the central nervous systems effects of SSRIs from modulation of the peripheral serotonin

system. Additionally, as the platelet life span in circulation is 5-6 days in a mouse, this treatment ensured that all platelets underwent an impairment SERT function and loss of granule serotonin. This study demonstrated that activation of serotonin, which is not acutely taken up by SERT, potentiated ADP-mediated αIIbβ3 activation. In terms of platelet spreading, both $SERT^{-/-}$ and citalopram-treated platelets showed reduced spreading. However, further work is required to separate the effects of SERT versus serotonin in this experimental design. It appears that addition of serotonin is able to rescue the spreading phenotype in the $SERT^{-/-}$ model, however, as seen in chapter 2, citalopram may exert an off-target effect that modulates spreading in a non-SERT dependent manner. Further characterization and experimentation is required to define and distinguish the roles of SERT and $5HT_{2A}R$-medaited serotonin activation in platelet spreading.

Finally, in chapter 5, the Pro32Pro33 hypercoagulability phenotype mouse model was characterized and the effect that this mutation had on platelet serotonin uptake and storage based on whole blood serotonin levels was determined. It was initially characterized that the β3-subunit (ITGB3) regulated the serotonergic system through an interaction with SERT (Ana Marin D Carneiro et al. 2008). Additionally, there are integrin-required, adhesion-dependent changes in SERT localization in human platelets (Steiner, Carneiro, and Blakely 2008). The Pro32Pro33 (KI) mutation is hypercoaguable due to a pre-primed αIIbβ3 integrin downstream of a single mutated the PSI domain of the β3 integrin (Hummerich et al. 2012). While this mutation leads to increased platelet spreading and P-Src signaling, a hyper-serotonergic phenotype was also observed. As platelets are the major uptake and storage site for serotonin in the blood, we tested if sustained inhibition of SERT using the 6-day citalopram treatment model could alter clotting time and thrombin

time in Pro32Pro33 mouse model. Indeed, we found that loss of serotonergic tone leads to the rescue of the hypercoagulability phenotype in a Pro32Pro33 mouse model. These findings further highlight two distinct points. First, it establishes a connection between bleeding times and serotonin homeostasis, and second suggests that not only can integrins regulate SERT based on serotonin levels, but that modulation of the serotonergic system can restore integrin function.

In total, this work has demonstrated that SERT plays an important role in platelet function by regulates both platelet serotonin uptake and serotonin storage . However, it is imperative to separate the role of acute versus sustained effects of SERT function when designing and considering experimental design. To do this, background knowledge about the drugs being used is required, including efficacy range, physiological concentrations, and potential off-target effects. Overall, this dissertation explores a systems biology approach to study the molecular mechanisms of serotonin in platelet function. For example, we have shown a novel implication of lost SERT function in platelet biology through reduced $5HT_{2A}R$ surface expression. We hypothesis that reduced $5HT_{2A}R$ surface expression is related to the ability of SERT to regulate serotonin homeostasis. Loss of SERT function alters serotonin homeostasis by increasing extracellular serotonin concentrations, and we speculate that this change leads to the pre-activation and desensitization of the $5HT_{2A}R$. This dissertation advocates future experiments utilizing a systems approach to understand complex disorders and to unveil multifaceted molecular mechanisms. Although there remains a sizable amount of future directions to this work, this dissertation has utilized multiple approaches and specific experimental design to address scientific questions that tease apart complicated biological systems.

Below, I will detail additional experiments that could be done to expand on this body of work including a simplified model of peripheral serotonin hemostasis that can be used to approximate serotonin concentrations in varying body compartments.

Peripherally restricted SSRIs

One promising future direction of this work is the use of peripherally restricted SSRIs that avoid central nervous system effects for the treatment of peripheral diseases related to hyperplatelet function. It is well known that SSRIs act within the central nervous system to affect mood (Wozniak et al. 2011). Changes in extracellular serotonin levels are believed to be required for this effect, however, the exact mechanisms remain unknown (Schellander and Donnerer 2010; S. M. Stahl et al. 2013). In order to separate the role of peripheral versus central SERT function, introducing peripherally restricted SSRIs could be used. In this case, only SERT found within the periphery would be blocked.

A similar approach has been applied to cannabionoid-1 receptor (CB1) blockers (Chorvat 2013). Similar to our observation with peripheral serotonin modulation, peripheral CB1 has showed promise as a target for new therapeutics for controlling obesity and metabolism, without the undesired side effects of engaging central receptors. As most drug discovery efforts have difficulty developing drugs that penetrate the blood-brain barrier, creating SSRIs that are non-brain pematrating is relatively straightforward. Using a structure-activity relationship, agents with a low propensity to penetrate the blood brain barrier (BBB) can be designed based on a current compound that is known to have low or no brain penetrance. Some of these characteristics include lipophilicity, hydrogen bonds, and polar surface area. One example of a peripherally restricted SSRIs approach could be

adding a PEGylated addition to the SSRI. PEGylation is the process of both covalent and non-covalent attachment of amalgamation of polyethylene glycol (PEG) (Jevsevar, Kunstelj, and Porekar 2010). The downside to this approach is that the drug would need to be administered via injection because PEGylation prevents absorption within the gastrointestinal tract. However, this process could be used to establish proof of concept.

Some additional aspects that should be considered are that the attachment of a PEG group to a drug or protein should "mask" the agent from the host's immune system. This could be beneficial in that it would prevent phagocytosis of the compound and initiation of immune response following drug administration (Jevsevar, Kunstelj, and Porekar 2010; Chorvat 2013). However, this can also lead to altering metabolism of the compound which could complicate known pharmacokinetic and pharmacodynamics values. Furthermore, PEGylation in particular increases the hydrodynamic size of the agent which could lead to the prolonged circulation of the drug by reducing renal clearance.

One would expect that with a peripherally restricted SSRI, the same responses would be observed following 6-day SSRI treatment within our models. Specifically, one would predict that the homeostasis of serotonin in the blood would be altered, leading to decrease storage of serotonin within the dense granules of platelets. If the overall amount of serotonin production did not change, then one would expect that extracellular concentration of serotonin would increase. It is possible that the increase in extracellular, or plasma levels of serotonin, could occur locally within the portal vein prior to degradation by the liver.

In the blood, we have established an association between decreased serotonin whole blood levels, i.e. platelet stored serotonin, and decreased $5HT_{2A}R$ expression. We predict

that the missing link between these two observations is a brief increase in plasma serotonin levels within the portal vein leading to $5HT_{2A}R$ receptor desensitization. One would predict that a peripherally restricted SSRI would cause a similar effect. Furthermore, we believe that this approach is preferable than a $5HT_{2A}R$ antagonist, because SSRI treatment potentially desensitizes $5HT_{2A}R$ signaling, and likely engages other signaling cascades through β-arrestin and other intracellular proteins. Further work is needed to test these hypotheses.

Serotonin homeostasis: bridging physiology and pharmacology

Serotonin homeostasis model

To better understand the role of SERT and serotonin in the periphery, it would be beneficial to model the production and metabolism of serotonin using system biology and computational model approaches. The production of a serotonin homeostasis model may shed light on the possible effect of lost SERT function within the periphery. Additionally, these models could be used to predict and characterize other serotonin-related pathological conditions such as hypertension, IBS, or even health concerns involving obesity associated diabetes (M. Berger, Gray, and Roth 2009; Hervé et al. 1995; Dennis L Murphy and Lesch 2008; Gershon and Tack 2007). Finally, an accurate serotonin homeostasis model could help predict how serotonergic drugs would behave *in vivo*. Generating and validating a holistic homeostasis model is no simple undertaking, however. To create a homeostasis model one needs an in-depth knowledge of all aspect of the pharmacokinetics and pharmacodynamics of the molecule in question. Fortunately, as discussed in chapter 3, there is much known about serotonin homeostasis. Production of this preliminary model

shed will light on the weakness in our understanding of physiological mechanisms that remain unresolved.

A broad conceptual understanding of serotonin homeostasis through a pharmacology lens will elucidate the levels of serotonin and potential mechanisms for peripheral conditions such as platelet activation mechanisms involved in a number of cardiovascular diseases such as hypertension and acute coronary syndrome (ACS), irritable bowel disease (IBS), and diabetes (Lichtman et al. 2014; Nazir et al. 2015; Gershon and Tack 2007; Malyszko et al. 1994). We are particularly interested in the regulation of serotonin in the blood component and will discuss the regulation of serotonin within the blood compartment.

Typically, serotonin is associated with mood, appetite, and sleep, through its described role as a neurotransmitter (Park et al. 2014; Wozniak et al. 2011; Ohno et al. 2015). However, its regulatory role in multiple disease states suggests a substantial role in peripheral function. The serotonin system can be conceptualized as an endocrine-like system that modulates the function of various systems including cardiovascular vascular responses (Musumeci et al. 2015; Fraer and Kilic 2015), gastrointestinal functions, inflammation, as well as platelets activation (J. S. Berger et al. 2013; Bismuth-Evenzal et al. 2012b; Ziu et al. 2012). The major location and movement of serotonin occur within the blood component where it is stored within platelet granules. This is in addition to the central nervous system effects which is strongly linked to impairments in serotonin neurotransmission (Fakhoury 2015; F Artigas et al. 1996; Park et al. 2014; Wozniak et al. 2011). Serotonin neurons also play a significant role in bladder function (Burgard, Fraser, and Thor 2003), gastrointestinal mechanical modulation (Mawe and Hoffman 2013;

Gershon 2013), and lung function (Dawson et al. 1987; Sherwin et al. 1986). Because serotonin is unable to cross the blood-brain barrier (BBB), this suggests separate and distinct serotonergic regulation mechanisms in the peripheral and central system.

To understand the pharmacokinetic and pharmacodynamics of serotonin, knowledge of tryptophan (Trp) is crucial. Trp is an essential amino acid attained only through diet (Gershon and Tack 2007; Mangge et al. 2014; Musumeci et al. 2015). The nutritional intake of L-Trp is therefore crucial for serotonin homeostasis. In several studies, the reduction or absence of L-Trp in the daily diet was demonstrated to cause a decrease in the levels of brain serotonin (Fernstrom 2013). The goal is to determine if a model of peripheral regulation of serotonin homeostasis is able to predict and elucidate pathological mechanisms involved in disease.

First, this means producing a schematic model of serotonin homeostasis (Figure 36), that could be used to ascertain whole blood serotonin levels based on input of tryptophan. Secondly, this model would incorporate the mechanisms of pharmacokinetics and pharmacodynamics of serotonin and tryptophan as well as regulatory controls. This includes kinetics values for the enzymes that produce and metabolize serotonin as well as the rates of the transporter for the vast array for transporters for both tryptophan and serotonin. Furthermore, we have included a circuit-like map of the serotonin homeostasis model to orient the viewer to the location of serotonin or its related molecules (Figure 36). To produce this map requires a systems biology approach that incorporates multiple organ dynamics and functions. To make this model we have also utilized the core principles of pharmacokinetics and pharmacodynamics.

Future work with build a computation model when specific enzymatic or uptake rates can be modified and their effects on serotonin homeostasis can be determined. Many have tried to correlated whole blood serotonin levels to a variety of pathologies, but these finding are controversial (Malyszko et al. 1994; Janusonis 2008; DeLisi et al. 1981). Using our model, we can better predict how specific mutations on key enzymes or transporters could potentially disrupt serotonin homeostasis (Greenberg et al. 1999). Furthermore, we test how SSRI treatment affects either local or total levels of extracellular or plasma serotonin.

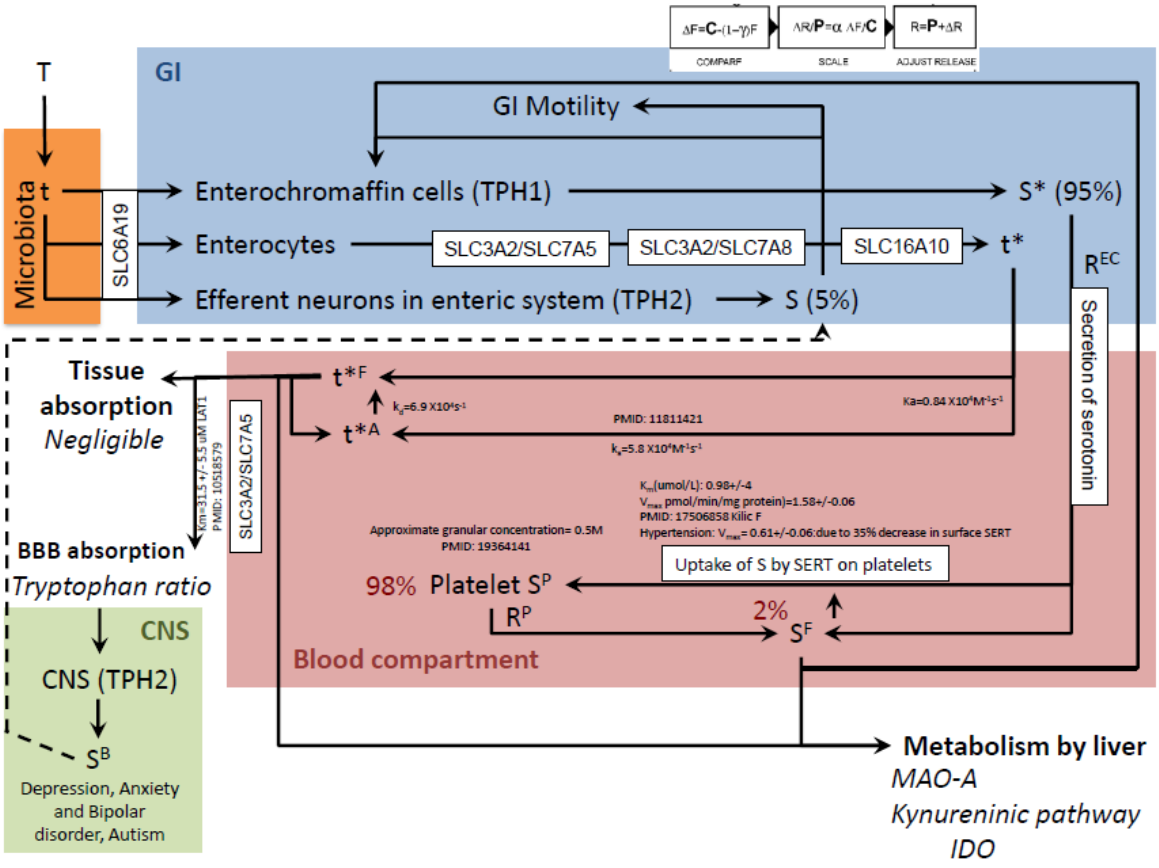


Figure 36. The initial model of serotonin homeostasis.

REFERENCES

Abdelmalik, N, H G Ruhé, K Barwari, E-J van den Dool, J C M Meijers, S Middeldorp, H R Büller, a H Schene, and P W Kamphuisen. 2008. "Effect of the Selective Serotonin Reuptake Inhibitor Paroxetine on Platelet Function Is Modified by a SLC6A4 Serotonin Transporter Polymorphism." *Journal of Thrombosis and Haemostasis : JTH* 6 (12): 2168–74. doi:10.1111/j.1538-7836.2008.03196.x.

Adamec, Robert, Paul Burton, Jacqueline Blundell, Dennis L. Murphy, and Andrew Holmes. 2006. "Vulnerability to Mild Predator Stress in Serotonin Transporter Knockout Mice." *Behavioural Brain Research* 170 (1): 126–40. doi:10.1016/j.bbr.2006.02.012.

Adamec, Robert, Andrew Holmes, and Jacqueline Blundell. 2008. "Vulnerability to Lasting Anxiogenic Effects of Brief Exposure to Predator Stimuli: Sex, Serotonin and Other Factors-Relevance to PTSD." *Neuroscience and Biobehavioral Reviews* 32 (7). NIH Public Access: 1287–92. doi:10.1016/j.neubiorev.2008.05.005.

Adams, John W, Juan Ramirez, Yunqing Shi, William Thomsen, John Frazer, Michael Morgan, Jeffrey E Edwards, et al. 2009. "APD791, 3-Methoxy-N-(3-(1-Methyl-1h-Pyrazol-5-Yl)-4-(2-Morpholinoethoxy)phenyl)benzamide, a Novel 5-Hydroxytryptamine 2A Receptor Antagonist: Pharmacological Profile, Pharmacokinetics, Platelet Activity and Vascular Biology." *The Journal of Pharmacology and Experimental Therapeutics* 331 (1): 96–103. doi:10.1124/jpet.109.153189.

Ádány, Róza, D Ph, and D Sc. 1996. "Intracellular Factor XIII : Cellular Distribution of Factor XIII Subunit a in Humans" 22 (5).

Agbani, Ejaife O, Marion T J van den Bosch, Ed Brown, Christopher M Williams, Nadine J A Mattheij, Judith M E M Cosemans, Peter W Collins, Johan W M Heemskerk, Ingeborg Hers, and Alastair W Poole. 2015. "Coordinated Membrane Ballooning and Procoagulant Spreading in Human Platelets." *Circulation* 132 (15): 1414–24. doi:10.1161/CIRCULATIONAHA.114.015036.

Ahmed, Billow a, Brandon C Jeffus, Syed I a Bukhari, Justin T Harney, Resat Unal, Vladimir V Lupashin, Peter van der Sluijs, and Fusun Kilic. 2008. "Serotonin Transamidates Rab4 and Facilitates Its Binding to the C Terminus of Serotonin Transporter." *The Journal of Biological Chemistry* 283 (14): 9388–98. doi:10.1074/jbc.M706367200.

Alberio, L, O Safa, K J Clemetson, C T Esmon, and G L Dale. 2000. "Surface Expression and Functional Characterization of Alpha-Granule Factor V in Human Platelets: Effects of Ionophore A23187, Thrombin, Collagen, and Convulxin." *Blood* 95 (5): 1694–1702. http://www.ncbi.nlm.nih.gov/pubmed/10688826.

Ambrosio, Andrea L, Judith A Boyle, and Santiago M Di Pietro. 2012. "Mechanism of Platelet Dense Granule Biogenesis: Study of Cargo Transport and Function of

Rab32 and Rab38 in a Model System." *Blood* 120 (19): 4072–81. doi:10.1182/blood-2012-04-420745.

Amireault, Pascal, David Sibon, and Francine Côté. 2013. "Life without Peripheral Serotonin: Insights from Tryptophan Hydroxylase 1 Knockout Mice Reveal the Existence of Paracrine/autocrine Serotonergic Networks." *ACS Chemical Neuroscience* 4 (1): 64–71. doi:10.1021/cn300154j.

Andersen, Jacob, Olivier Taboureau, Kasper B Hansen, Lars Olsen, Jan Egebjerg, Kristian Strømgaard, and Anders S Kristensen. 2009. "Location of the Antidepressant Binding Site in the Serotonin Transporter: Importance of Ser-438 in Recognition of Citalopram and Tricyclic Antidepressants." *The Journal of Biological Chemistry* 284 (15). American Society for Biochemistry and Molecular Biology: 10276–84. doi:10.1074/jbc.M806907200.

Anderson, George M, Christina S Barr, Stephen Lindell, Amy C Durham, Ilya Shifrovich, and J Dee Higley. 2005. "Time Course of the Effects of the Serotonin-Selective Reuptake Inhibitor Sertraline on Central and Peripheral Serotonin Neurochemistry in the Rhesus Monkey." *Psychopharmacology* 178 (2-3): 339–46. doi:10.1007/s00213-004-2011-7.

Anderson, George M, and Edwin H Cook. 2016a. "Commentary on "Platelet Studies in Autism Spectrum Disorder Patients and First-Degree Relatives"." *Molecular Autism* 7: 20. doi:10.1186/s13229-016-0086-8.

Anderson, George M. 2007. "Measurement of Plasma Serotonin in Autism." *Pediatric Neurology*. doi:10.1016/j.pediatrneurol.2006.11.007.

Anderson, George M., and Edwin H. Cook. 2016b. "Commentary on 'Platelet Studies in Autism Spectrum Disorder Patients and First-Degree Relatives.'" *Molecular Autism* 7 (1): 20. doi:10.1186/s13229-016-0086-8.

Anderson, George M., Daniel X. Freedman, Donald J. Cohen, Fred R. Volkmar, E. Lawrence Hoder, Peter McPhedran, Ruud B. Minderaa, Carl R. Hansen, and J. Gerald Young. 1987. "WHOLE BLOOD SEROTONIN IN AUTISTIC AND NORMAL SUBJECTS." *Journal of Child Psychology and Psychiatry* 28 (6). Blackwell Publishing Ltd: 885–900. doi:10.1111/j.1469-7610.1987.tb00677.x.

Anderson, George M., Willian C. Horne, Diptendu Chatterjee, and Donald J. Cohen. 1990. "The Hyperserotonemia of Autism." *Annals of the New York Academy of Sciences* 600 (1 The Neurophar): 331–40. doi:10.1111/j.1749-6632.1990.tb16893.x.

Anderson, Jeffrey L, Gretchen J King, Tami L Bair, Sidney P Elmer, Joseph B Muhlestein, Jessica Habashi, and John F Carlquist. 1999. "Associations between a Polymorphism in the Gene Encoding Glycoprotein IIIa and Myocardial Infarction or Coronary Artery Disease." *Journal of the American College of Cardiology* 33 (3): 727–33. doi:10.1016/S0735-1097(98)00603-2.

Andrade, Chittaranjan, Surya Sandarsh, Kumar B Chethan, and Koregala S Nagesh. 2010. “Serotonin Reuptake Inhibitor Antidepressants and Abnormal Bleeding: A Review for Clinicians and a Reconsideration of Mechanisms.” *The Journal of Clinical Psychiatry* 71 (12). United States: 1565–75. doi:10.4088/JCP.09r05786blu.

Angiolillo, Dominick J, Davide Capodanno, and Shinya Goto. 2010. “Platelet Thrombin Receptor Antagonism and Atherothrombosis.” *European Heart Journal* 31 (1): 17–28. doi:10.1093/eurheartj/ehp504.

Antkowiak, A, J Viaud, S Severin, M Zanoun, L Ceccato, G Chicanne, C Strassel, et al. 2016. “Cdc42-Dependent F-Actin Dynamics Drive Structuration of the Demarcation Membrane System in Megakaryocytes.” *Journal of Thrombosis and Haemostasis : JTH* 14 (6): 1268–84. doi:10.1111/jth.13318.

Apelseth, Torunn Oveland, I N A Nepstad, and T O R Audun Hervig. 2012. “The Effects of Selective Serotonin Reuptake Inhibitors on Platelet Function in Whole Blood and Platelet Concentrates” 23 (March 2011): 299–308. doi:10.3109/09537104.2011.618852.

Araragi, Naozumi, and Klaus-Peter Lesch. 2013. “Serotonin (5-HT) in the Regulation of Depression-Related Emotionality: Insight from 5-HT Transporter and Tryptophan Hydroxylase-2 Knockout Mouse Models.” *Current Drug Targets* 14 (5): 549–70. http://www.ncbi.nlm.nih.gov/pubmed/23547810.

Ariëns, Robert A S, Thung-Shenq Lai, John W Weisel, Charles S Greenberg, and Peter J Grant. 2002. “Role of Factor XIII in Fibrin Clot Formation and Effects of Genetic Polymorphisms.” *Blood* 100 (3): 743–54. http://www.ncbi.nlm.nih.gov/pubmed/12130481.

Article, Review. 2013. “Rho GTPases in Platelet Function,” 35–46. doi:10.1111/jth.12051.

Artigas, F, L Romero, C de Montigny, and P Blier. 1996. “Acceleration of the Effect of Selected Antidepressant Drugs in Major Depression by 5-HT1A Antagonists.” *Trends in Neurosciences* 19 (9): 378–83. doi:10.1016/S0166-2236(96)10037-0.

Artigas, Francesc. 2013. “Future Directions for Serotonin and Antidepressants.” *ACS Chemical Neuroscience* 4 (1). American Chemical Society: 5–8. doi:10.1021/cn3001125.

Ashton, J H, C R Benedict, C Fitzgerald, S Raheja, A Taylor, W B Campbell, L M Buja, and J T Willerson. 1986. “Serotonin as a Mediator of Cyclic Flow Variations in Stenosed Canine Coronary Arteries.” *Circulation* 73 (3): 572–78. http://www.ncbi.nlm.nih.gov/pubmed/2936531.

Aslan, J E, and O J T McCarty. 2013. “Rho GTPases in Platelet Function.” *Journal of Thrombosis and Haemostasis : JTH* 11 (1): 35–46. doi:10.1111/jth.12051.

Aslan, Joseph E., Asako Itakura, Jacqueline M. Gertz, and Owen J. T. McCarty. 2012. "Platelet Shape Change and Spreading." In , 91–100. doi:10.1007/978-1-61779-307-3_7.

Baganz, Nicole, Rebecca Horton, Kathryn Martin, Andrew Holmes, and Lynette C Daws. 2010. "Repeated Swim Impairs Serotonin Clearance via a Corticosterone-Sensitive Mechanism: Organic Cation Transporter 3, the Smoking Gun." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 30 (45). NIH Public Access: 15185–95. doi:10.1523/JNEUROSCI.2740-10.2010.

Baker-Groberg, S M, S Lattimore, M Recht, O J T McCarty, and K M Haley. 2016. "Assessment of Neonatal Platelet Adhesion, Activation, and Aggregation." *Journal of Thrombosis and Haemostasis : JTH* 14 (4): 815–27. doi:10.1111/jth.13270.

Barnes, Nicholas M., and Trevor Sharp. 1999. "A Review of Central 5-HT Receptors and Their Function." *Neuropharmacology* 38 (8): 1083–1152. doi:10.1016/S0028-3908(99)00010-6.

Beckman, Matthew L., and Michael W. Quick. 2001. "Substrates and Temperature Differentiate Ion Flux from Serotonin Flux in a Serotonin Transporter." *Neuropharmacology* 40 (4): 526–35. doi:10.1016/S0028-3908(00)00191-X.

Beikmann, Brendan S, Ian D Tomlinson, Sandra J Rosenthal, and Anne Milasincic Andrews. 2013. "Serotonin Uptake Is Largely Mediated by Platelets versus Lymphocytes in Peripheral Blood Cells." *ACS Chemical Neuroscience* 4 (1): 161–70. doi:10.1021/cn300146w.

Benedict, C. R., B. Mathew, K. A. Rex, J. Cartwright, and L. A. Sordahl. 1986. "Correlation of Plasma Serotonin Changes with Platelet Aggregation in an in Vivo Dog Model of Spontaneous Occlusive Coronary Thrombus Formation." *Circulation Research* 58 (1): 58–67. doi:10.1161/01.RES.58.1.58.

Bengel, D, D L Murphy, a M Andrews, C H Wichems, D Feltner, a Heils, R Mössner, H Westphal, and K P Lesch. 1998. "Altered Brain Serotonin Homeostasis and Locomotor Insensitivity to 3, 4-Methylenedioxymethamphetamine ('Ecstasy') in Serotonin Transporter-Deficient Mice." *Molecular Pharmacology* 53 (4): 649–55. http://www.ncbi.nlm.nih.gov/pubmed/9547354.

Bennett, J S, B W Berger, and P C Billings. 2009. "The Structure and Function of Platelet Integrins." *Journal of Thrombosis and Haemostasis : JTH* 7 Suppl 1 (July): 200–205. doi:10.1111/j.1538-7836.2009.03378.x.

Berger, G, J P Caen, M C Berndt, and E M Cramer. 1993. "Ultrastructural Demonstration of CD36 in the Alpha-Granule Membrane of Human Platelets and Megakaryocytes." *Blood* 82 (10): 3034–44. http://www.ncbi.nlm.nih.gov/pubmed/7693034.

Berger, Jeffrey S., Richard C. Becker, Cynthia Kuhn, Michael J. Helms, Thomas L. Ortel, and Redford Williams. 2013. "Hyperreactive Platelet Phenotypes:

Relationship to Altered Serotonin Transporter Number, Transport Kinetics and Intrinsic Response to Adrenergic Co-Stimulation." *Thrombosis and Haemostasis* 109 (1): 85–92. doi:10.1160/TH12-03-0202.

Berger, Miles, John a Gray, and Bryan L Roth. 2009. "The Expanded Biology of Serotonin." *Annual Review of Medicine* 60 (January): 355–66. doi:10.1146/annurev.med.60.042307.110802.

Bergmeier, W., and R. O. Hynes. 2012. "Extracellular Matrix Proteins in Hemostasis and Thrombosis." *Cold Spring Harbor Perspectives in Biology* 4 (2). Cold Spring Harbor Lab: a005132–a005132. doi:10.1101/cshperspect.a005132.

Bergmeier, Wolfgang, Valerie Schulte, Gero Brockhoff, Ulrich Bier, Hubert Zirngibl, and Bernhard Nieswandt. 2002. "Flow Cytometric Detection of Activated Mouse Integrin alphaIIbbeta3 with a Novel Monoclonal Antibody." *Cytometry* 48 (2). United States: 80–86. doi:10.1002/cyto.10114.

Bernatowicz, M S, C E Klimas, K S Hartl, M Peluso, N J Allegretto, and S M Seiler. 1996. "Development of Potent Thrombin Receptor Antagonist Peptides." *Journal of Medicinal Chemistry* 39 (25): 4879–87. doi:10.1021/jm960455s.

Besnard, Jérémy, Gian Filippo Ruda, Vincent Setola, Keren Abecassis, Ramona M Rodriguiz, Xi-Ping Huang, Suzanne Norval, et al. 2012. "Automated Design of Ligands to Polypharmacological Profiles." *Nature* 492 (7428): 215–20. doi:10.1038/nature11691.

Best, Janet, H Frederik Nijhout, and Michael Reed. 2010. "Serotonin Synthesis, Release and Reuptake in Terminals: A Mathematical Model." *Theoretical Biology & Medical Modelling* 7: 34. doi:10.1186/1742-4682-7-34.

Beuming, Thijs, Lei Shi, Jonathan A Javitch, and Harel Weinstein. 2006. "A Comprehensive Structure-Based Alignment of Prokaryotic and Eukaryotic neurotransmitter/Na+ Symporters (NSS) Aids in the Use of the LeuT Structure to Probe NSS Structure and Function." *Molecular Pharmacology* 70 (5): 1630–42. doi:10.1124/mol.106.026120.

Bezchlibnyk-Butler, K, I Aleksic, and S H Kennedy. 2000. "Citalopram--a Review of Pharmacological and Clinical Effects." *Journal of Psychiatry & Neuroscience : JPN* 25 (3). Canadian Medical Association: 241–54. http://www.ncbi.nlm.nih.gov/pubmed/10863884.

Bijl, Nora, Chantal Thys, Christine Wittevrongel, Wouter De la Marche, Koenraad Devriendt, Hilde Peeters, Chris Van Geet, and Kathleen Freson. 2015. "Platelet Studies in Autism Spectrum Disorder Patients and First-Degree Relatives." *Molecular Autism* 6 (1): 57. doi:10.1186/s13229-015-0051-y.

Bing, RJ, and M Hammond. 1949. "Coronary Blood Flow, Cardiac Oxygen Consumption and Cardiac Efficiency in Man." *Bulletin of the Johns Hopkins Hospital* 84 (4): 396–

400. http://www.ncbi.nlm.nih.gov/pubmed/18118608.

Bismuth-Evenzal, Yona, Yehudit Gonopolsky, David Gurwitz, Iulian Iancu, Abraham Weizman, and Moshe Rehavi. 2012a. "Decreased Serotonin Content and Reduced Agonist-Induced Aggregation in Platelets of Patients Chronically Medicated with SSRI Drugs." *Journal of Affective Disorders* 136 (1-2): 99–103. doi:10.1016/j.jad.2011.08.013.

Bismuth-Evenzal, Yona, Y. Gonopolsky, D. Gurwitz, I. Iancu, A. Weizman, M. Rehavi. 2012b. "Decreased Serotonin Content and Reduced Agonist-Induced Aggregation in Platelets of Patients Chronically Medicated with SSRI Drugs." *Journal of Affective Disorders* 136 (1-2). Elsevier B.V.: 99–103. doi:10.1016/j.jad.2011.08.013.

Blackmer, Trillium, Eric C Larsen, Cheryl Bartleson, Judith A Kowalchyk, Eun-Ja Yoon, Anita M Preininger, Simon Alford, Heidi E Hamm, and Thomas F J Martin. 2005. "G Protein Betagamma Directly Regulates SNARE Protein Fusion Machinery for Secretory Granule Exocytosis." *Nature Neuroscience* 8 (4): 421–25. doi:10.1038/nn1423.

Blazevic, S, I Erjavec, M Brizic, S Vukicevic, and D Hranilovic. 2015a. "Molecular Background and Physiological Consequences of Altered Peripheral Serotonin Homeostasis in Adult Rats Perinatally Treated with Tranylcypromine." *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society* 66 (4): 529–37. http://www.ncbi.nlm.nih.gov/pubmed/26348077.

Blazevic, S., I. Erjavec, M. Brizic, S. Vukicevic, and Dubravka Hranilovic. 2015b. "Molecular Background and Physiological Consequences of Altered Peripheral Serotonin Homeostasis in Adult Rats Perinatally Treated with Tranylcypromine." *Journal of Physiology and Pharmacology* 66 (4): 529–37.

Bodary, S C, M A Napier, and J W McLean. 1989. "Expression of Recombinant Platelet Glycoprotein IIbIIIa Results in a Functional Fibrinogen-Binding Complex." *The Journal of Biological Chemistry* 264 (32): 18859–62. http://www.ncbi.nlm.nih.gov/pubmed/2808396.

Bohn, Laura M, and Cullen L Schmid. 2010. "Serotonin Receptor Signaling and Regulation via β-Arrestins." *Critical Reviews in Biochemistry and Molecular Biology* 45 (6): 555–66. doi:10.3109/10409238.2010.516741.

Bonnefoy, A., R. Hantgan, C. Legrand, and M. M. Frojmovic. 2001. "A Model of Platelet Aggregation Involving Multiple Interactions of Thrombospondin-1, Fibrinogen, and GPIIbIIIa Receptor." *Journal of Biological Chemistry* 276 (8). American Society for Biochemistry and Molecular Biology: 5605–12. doi:10.1074/jbc.M010091200.

Bonnin, Alexandre, Le Zhang, Randy D Blakely, and Pat Levitt. 2012. "The SSRI Citalopram Affects Fetal Thalamic Axon Responsiveness to Netrin-1 in Vitro Independently of SERT Antagonism." *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology* 37 (8). Nature

Publishing Group: 1879–84. doi:10.1038/npp.2012.35.

Borst, P, and R Oude Elferink. 2002. “Mammalian ABC Transporters in Health and Disease.” *Annual Review of Biochemistry* 71 (January): 537–92. doi:10.1146/annurev.biochem.71.102301.093055.

Bracher, Andreas, Winfried Weissenhorn, R Jahn, TC Südhof, R Fukuda, JA McNew, T Weber, et al. 2004. “Crystal Structure of the Habc Domain of Neuronal Syntaxin from the Squid Loligo Pealei Reveals Conformational Plasticity at Its C-Terminus.” *BMC Structural Biology* 4 (1). BioMed Central: 6. doi:10.1186/1472-6807-4-6.

Brand, T., and G. M. Anderson. 2011. “The Measurement of Platelet-Poor Plasma Serotonin:A Systematic Review of Prior Reports and Recommendations for Improved Analysis.” *Clinical Chemistry* 57 (10): 1376–86. doi:10.1373/clinchem.2011.163824.

Brenner, B, J T Harney, B a Ahmed, B C Jeffus, R Unal, J L Mehta, and F Kilic. 2007. “Plasma Serotonin Levels and the Platelet Serotonin Transporter.” *Journal of Neurochemistry* 102 (1): 206–15. doi:10.1111/j.1471-4159.2007.04542.x.

Bulling, Simon, Klaus Schicker, Yuan-Wei Zhang, Thomas Steinkellner, Thomas Stockner, Christian W Gruber, Stefan Boehm, et al. 2012. “The Mechanistic Basis for Noncompetitive Ibogaine Inhibition of Serotonin and Dopamine Transporters.” *The Journal of Biological Chemistry* 287 (22): 18524–34. doi:10.1074/jbc.M112.343681.

Burgard, Edward C., Mathew O. Fraser, and Karl B. Thor. 2003. “Serotonergic Modulation of Bladder Afferent Pathways.” *Urology* 62 (4 SUPPL. 1): 10–15. doi:10.1016/S0090-4295(03)00590-9.

Bush, L R, W B Campbell, K Kern, G D Tilton, P Apprill, J Ashton, J Schmitz, L M Buja, and J T Willerson. 1984. “The Effects of Alpha 2-Adrenergic and Serotonergic Receptor Antagonists on Cyclic Blood Flow Alterations in Stenosed Canine Coronary Arteries.” *Circulation Research* 55 (5): 642–52. http://www.ncbi.nlm.nih.gov/pubmed/6488486.

Bynagari-Settipalli, Yamini S, Ivo Cornelissen, Daniel Palmer, Daniel Duong, Cherry Concengco, Jerry Ware, and Shaun R Coughlin. 2014. “Redundancy and Interaction of Thrombin- and Collagen-Mediated Platelet Activation in Tail Bleeding and Carotid Thrombosis in Mice.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 34 (12): 2563–69. doi:10.1161/ATVBAHA.114.304244.

Canault, Matthias, Dorsaf Ghalloussi, Charlotte Grosdidier, Marie Guinier, Claire Perret, Nadjim Chelghoum, Marine Germain, et al. 2014. “Human CalDAG-GEFI Gene (RASGRP2) Mutation Affects Platelet Function and Causes Severe Bleeding.” *The Journal of Experimental Medicine* 211 (7): 1349–62. doi:10.1084/jem.20130477.

Canli, Turhan, and Klaus-Peter Lesch. 2007. “Long Story Short: The Serotonin

Transporter in Emotion Regulation and Social Cognition." *Nature Neuroscience* 10 (9): 1103–9. doi:10.1038/nn1964.

Carneiro, Ana M D. 2010. "The Emerging Role of Integrins in Neuropsychiatric Disorders." *Neuropsychopharmacology* 35 (1). Nature Publishing Group: 338–39. doi:10.1038/npp.2009.134.

Carneiro, Ana M D, David C Airey, Brent Thompson, Chong-Bin Zhu, Lu Lu, Elissa J Chesler, Keith M Erikson, and Randy D Blakely. 2009. "Functional Coding Variation in Recombinant Inbred Mouse Lines Reveals Multiple Serotonin Transporter-Associated Phenotypes." *Proceedings of the National Academy of Sciences of the United States of America* 106 (6): 2047–52. doi:10.1073/pnas.0809449106.

Carneiro, Ana Marin D, and Randy D Blakely. 2006. "Serotonin-, Protein Kinase C-, and Hic-5-Associated Redistribution of the Platelet Serotonin Transporter." *The Journal of Biological Chemistry* 281 (34): 24769–80. doi:10.1074/jbc.M603877200.

Carneiro, Ana Marin D, Edwin H Cook, Dennis L Murphy, and Randy D Blakely. 2008. "Interactions between Integrin αIIbβ3 and the Serotonin Transporter Regulate Serotonin Transport and Platelet Aggregation in Mice and Humans" 118 (4). doi:10.1172/JCI33374.1544.

Cerrito, E., M.P. Lazzaro, E. Gaudio, P. Arminio, and G. Aloisi. 1993. "5HT2-Receptors and Serotonin Release: Their Role in Human Platelet Aggregation." *Life Sciences* 53 (3): 209–15. doi:10.1016/0024-3205(93)90671-O.

Cerrito, F, M P Lazzaro, E Gaudio, P Arminio, and G Aloisi. 1993a. "5HT2-Receptors and Serotonin Release: Their Role in Human Platelet Aggregation." *Life Sciences* 53 (3): 209–15. http://www.ncbi.nlm.nih.gov/pubmed/8321084.

Champy, C. A Fiehrer, and M. Demay. 1952. "[Microscopic Modifications of the Fibrinous Clot in Relation to the Plasma Modifications; Variations in the Thrombin-Fibrinogen Reaction]." *La Presse Médicale* 60 (52): 1107–9. http://www.ncbi.nlm.nih.gov/pubmed/13026904.

Chavakis, Triantafyllos, Nicole Boeckel, Sentot Santoso, Reinhard Voss, Irma Isordia-Salas, Robin A. Pixley, Eberhard Morgenstern, Robert W. Colman, and Klaus T. Preissner. 2002. "Inhibition of Platelet Adhesion and Aggregation by a Defined Region (Gly-486-Lys-502) of High Molecular Weight Kininogen." *J. Biol. Chem.* 277 (26): 23157–64.

Chen, J, M Ishii, L Wang, K Ishii, and S R Coughlin. 1994. "Thrombin Receptor Activation. Confirmation of the Intramolecular Tethered Liganding Hypothesis and Discovery of an Alternative Intermolecular Liganding Mode." *The Journal of Biological Chemistry* 269 (23): 16041–45. http://www.ncbi.nlm.nih.gov/pubmed/8206902.

Chen, J J, Z Li, H Pan, D L Murphy, H Tamir, H Koepsell, and M D Gershon. 2001. "Maintenance of Serotonin in the Intestinal Mucosa and Ganglia of Mice That Lack the High-Affinity Serotonin Transporter: Abnormal Intestinal Motility and the Expression of Cation Transporters." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 21 (16): 6348–61. http://www.ncbi.nlm.nih.gov/pubmed/11487658.

Chorvat, Robert J. 2013. "Peripherally Restricted CB1 Receptor Blockers." *Bioorganic & Medicinal Chemistry Letters* 23 (17): 4751–60. doi:10.1016/j.bmcl.2013.06.066.

Ciccone, Marcia A, Miranda Timmons, Anthony Phillips, and Michael W Quick. 2008. "Calcium/calmodulin-Dependent Kinase II Regulates the Interaction between the Serotonin Transporter and Syntaxin 1A." *Neuropharmacology* 55 (5). NIH Public Access: 763–70. doi:10.1016/j.neuropharm.2008.06.018.

Clemetson, Kenneth J., and Jeannine M. Clemetson. 2001. "Platelet Collagen Receptors." *Thrombosis and Haemostasis* 86 (1). Schattauer Publishers: 189–97.

Cloutier, Nathalie, Alexandre Paré, Richard W Farndale, H Ralph Schumacher, Peter a Nigrovic, Steve Lacroix, and Eric Boilard. 2012. "Platelets Can Enhance Vascular Permeability." *Blood* 120 (6): 1334–43. doi:10.1182/blood-2012-02-413047.

Coffa, Sergio, Maya Breitman, Susan M Hanson, Kari Callaway, Seunghyi Kook, Kevin N Dalby, and Vsevolod V Gurevich. 2011. "The Effect of Arrestin Conformation on the Recruitment of c-Raf1, MEK1, and ERK1/2 Activation." *PloS One* 6 (12): e28723. doi:10.1371/journal.pone.0028723.

Coleman, Jonathan A, Evan M Green, and Eric Gouaux. 2016. "X-Ray Structures and Mechanism of the Human Serotonin Transporter." *Nature* 532 (7599): 334–39. doi:10.1038/nature17629.

Coller, B S. 1992. "Erythrocytes and Thrombo-Erythrocytes as Target Specific Agents." Google Patents. http://www.google.com/patents/WO1992008804A1?cl=en.

Cornelissen, Ivo, Daniel Palmer, Tovo David, Lisa Wilsbacher, Cherry Concengco, Pamela Conley, Anjali Pandey, and Shaun R Coughlin. 2010. "Roles and Interactions among Protease-Activated Receptors and P2ry12 in Hemostasis and Thrombosis." *Proceedings of the National Academy of Sciences of the United States of America* 107 (43): 18605–10. doi:10.1073/pnas.1013309107.

Cornelissen, L L, D P Brooks, A Wibberley, P. Abrams, L. Cardozo, M. Fall, D. Griffiths, et al. 2005. "Female, but Not Male, Serotonin Reuptake Transporter (5-HTT) Knockout Mice Exhibit Bladder Instability." *Autonomic Neuroscience : Basic & Clinical* 122 (1-2). Elsevier: 107–10. doi:10.1016/j.autneu.2005.06.005.

Covic, L, a L Gresser, and a Kuliopulos. 2000. "Biphasic Kinetics of Activation and Signaling for PAR1 and PAR4 Thrombin Receptors in Platelets." *Biochemistry* 39 (18): 5458–67. http://www.ncbi.nlm.nih.gov/pubmed/10820018.

Cramer, E M, J Breton-Gorius, J E Beesley, and J F Martin. 1988. "Ultrastructural Demonstration of Tubular Inclusions Coinciding with von Willebrand Factor in Pig Megakaryocytes." *Blood* 71 (6): 1533–38. http://www.ncbi.nlm.nih.gov/pubmed/3285907.

Cramer, E M, N Debili, J F Martin, A M Gladwin, J Breton-Gorius, P Harrison, G F Savidge, and W Vainchenker. 1989. "Uncoordinated Expression of Fibrinogen Compared with Thrombospondin and von Willebrand Factor in Maturing Human Megakaryocytes." *Blood* 73 (5): 1123–29. http://www.ncbi.nlm.nih.gov/pubmed/2649167.

Daaka, Y., Yehia Daaka, Julie A. Pitcher, and Robert J. Lefkowitz. 1997. "The Role of Sequestration in G Protein-Coupled Receptor Resensitization." *Journal of Biological Chemistry* 272 (1). American Society for Biochemistry and Molecular Biology: 5–8. doi:10.1074/jbc.272.1.5.

Dale, George L, Paul Friese, Peter Batar, Stephen F Hamilton, Guy L Reed, Kenneth W Jackson, Kenneth J Clemetson, and Lorenzo Alberio. 2002. "Stimulated Platelets Use Serotonin to Enhance Their Retention of Procoagulant Proteins on the Cell Surface." *Nature* 415 (6868): 175–79. doi:10.1038/415175a.

Dalgliesh, CE, and R W Dutton. 1957. "Biogenesis of 5-Hydroxytryptophan." *British Journal of Cancer* 11 (2). Nature Publishing Group: 296–309. http://www.ncbi.nlm.nih.gov/pubmed/13460154.

Davì, G, C Giammaresi, I Catalano, A Calà, M Prosdocimi, S Alvino, S Piva, and A Notarbartolo. 1994. "Platelet Aggregation, ATP Release and Cytoplasmic Ca2+ Movement: The Effects of Cloricromene." *Thrombosis Research* 76 (2): 121–31. http://www.ncbi.nlm.nih.gov/pubmed/7863464.

Daws, L. C., S. Montanez, J. L. Munn, W. A. Owens, N. L. Baganz, J. M. Boyce-Rustay, R. A. Millstein, L. M. Wiedholz, D. L. Murphy, and A. Holmes. 2006. "Ethanol Inhibits Clearance of Brain Serotonin by a Serotonin Transporter-Independent Mechanism." *Journal of Neuroscience* 26 (24). Society for Neuroscience: 6431–38. doi:10.1523/JNEUROSCI.4050-05.2006.

Daws, Lynette C. 2009. "Unfaithful Neurotransmitter Transporters: Focus on Serotonin Uptake and Implications for Antidepressant Efficacy." *Pharmacology & Therapeutics* 121 (1). Elsevier Inc.: 89–99. doi:10.1016/j.pharmthera.2008.10.004.

Dawson, C A, J H Linehan, D A Rickaby, and T A Bronikowski. 1987. "Kinetics of Serotonin Uptake in the Intact Lung." *Annals of Biomedical Engineering* 15 (2). UNITED STATES: 217–27.

De Clerck, F, W Loots, Y Somers, J Beetens, L Wouters, J Wynants, and P A Janssen. 1990. "5-Hydroxytryptamine and Arachidonic Acid Metabolites Modulate Extensive Platelet Activation Induced by Collagen in Cats in Vivo." *British Journal of Pharmacology* 99 (4): 631–36. http://www.ncbi.nlm.nih.gov/pubmed/2113823.

Dees, Clara, Alfiya Akhmetshina, Pawel Zerr, Nicole Reich, Katrin Palumbo, Angelika Horn, Astrid Jüngel, et al. 2011. "Platelet-Derived Serotonin Links Vascular Disease and Tissue Fibrosis." *The Journal of Experimental Medicine* 208 (5): 961–72. doi:10.1084/jem.20101629.

Delaney, M Keegan, Junling Liu, Yi Zheng, Michael C Berndt, and Xiaoping Du. 2012. "The Role of Rac1 in Glycoprotein Ib-IX-Mediated Signal Transduction and Integrin Activation." *Arteriosclerosis, Thrombosis, and Vascular Biology* 32 (11): 2761–68. doi:10.1161/ATVBAHA.112.254920.

DeLisi, Lynn E., Leonard M. Neckers, Daniel R. Weinberger, Richard Jed Wyatt, Gillin JC, Neff N, et al Garelis E, Tait AC, Marshall EF, et al Todrick A, Vergani E, Gaira S Torre M, et al. 1981. "Increased Whole Blood Serotonin Concentrations in Chronic Schizophrenic Patients." *Archives of General Psychiatry* 38 (6). American Medical Association: 647. doi:10.1001/archpsyc.1981.01780310047004.

Demchyshyn, L L, Z B Pristupa, K S Sugamori, E L Barker, R D Blakely, W J Wolfgang, M A Forte, and H B Niznik. 1994. "Cloning, Expression, and Localization of a Chloride-Facilitated, Cocaine-Sensitive Serotonin Transporter from Drosophila Melanogaster." *Proceedings of the National Academy of Sciences of the United States of America* 91 (11): 5158–62. http://www.ncbi.nlm.nih.gov/pubmed/8197200.

Dormond, Olivier, Lionel Ponsonnet, Meriem Hasmim, Alessandro Foletti, and Curzio Rüegg. 2004. "Manganese-Induced Integrin Affinity Maturation Promotes Recruitment of Alpha V Beta 3 Integrin to Focal Adhesions in Endothelial Cells: Evidence for a Role of Phosphatidylinositol 3-Kinase and Src." *Thrombosis and Haemostasis* 92 (1): 151–61. doi:10.1160/TH03-11-0728.

Dürk, Thorsten, Daniel Duerschmied, Tobias Müller, Melanie Grimm, Sebastian Reuter, Rodolfo Paula Vieira, Korcan Ayata, et al. 2013. "Production of Serotonin by Tryptophan Hydroxylase 1 and Release via Platelets Contribute to Allergic Airway Inflammation." *American Journal of Respiratory and Critical Care Medicine* 187 (5): 476–85. doi:10.1164/rccm.201208-1440OC.

Duvernay, Matthew T, Anton Matafonov, Craig W Lindsley, and Heidi E Hamm. 2015. "Platelet Lipidomic Profiling: Novel Insight into Cytosolic Phospholipase A2α Activity and Its Role in Human Platelet Activation." *Biochemistry* 54 (36): 5578–88. doi:10.1021/acs.biochem.5b00549.

Duvernay, Matthew, Summer Young, David Gailani, Jonathan Schoenecker, Heidi E Hamm, and Heidi Hamm. 2013. "Protease-Activated Receptor (PAR) 1 and PAR4 Differentially Regulate Factor V Expression from Human Platelets." *Molecular Pharmacology* 83 (4): 781–92. doi:10.1124/mol.112.083477.

Esaki, Takanori, Michelle Cook, Kazuaki Shimoji, Dennis L Murphy, Louis Sokoloff, and Andrew Holmes. 2005. "Developmental Disruption of Serotonin Transporter Function Impairs Cerebral Responses to Whisker Stimulation in Mice." *Proceedings of the National Academy of Sciences of the United States of America* 102 (15).

National Academy of Sciences: 5582–87. doi:10.1073/pnas.0501509102.

Esposito, Carla, and Ivana Caputo. 2005. “Mammalian Transglutaminases. Identification of Substrates as a Key to Physiological Function and Physiopathological Relevance.” *The FEBS Journal* 272 (3): 615–31. doi:10.1111/j.1742-4658.2004.04476.x.

Fabre, V, C Beaufour, a Evrard, a Rioux, N Hanoun, K P Lesch, D L Murphy, L Lanfumey, M Hamon, and M P Martres. 2000. “Altered Expression and Functions of Serotonin 5-HT1A and 5-HT1B Receptors in Knock-out Mice Lacking the 5-HT Transporter.” *The European Journal of Neuroscience* 12 (7): 2299–2310. http://www.ncbi.nlm.nih.gov/pubmed/10947809.

Fakhoury, Marc. 2015. “Revisiting the Serotonin Hypothesis: Implications for Major Depressive Disorders.” *Molecular Neurobiology*. doi:10.1007/s12035-015-9152-z.

Feng, D, K Lindpaintner, M G Larson, V S Rao, C J O’Donnell, I Lipinska, C Schmitz, et al. 1999. “Increased Platelet Aggregability Associated with Platelet GPIIIa PlA2 Polymorphism: The Framingham Offspring Study.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 19 (4): 1142–47. http://www.ncbi.nlm.nih.gov/pubmed/10195947.

Fernstrom, John D. 2013. “Large Neutral Amino Acids: Dietaryeffects on Brain Neurochemistry and Function.” *Amino Acids* 45 (3): 419–30. doi:10.1007/s00726-012-1330-y.

Flaumenhaft, Robert. 2012a. “Monitoring Granule Traffic in Megakaryocytes.” *Blood* 120 (19): 3869–70. doi:10.1182/blood-2012-09-455121.

Flaumenhaft, Robert. 2012b. “A-Granules: A Story in the Making.” *Blood* 120 (25): 4908–9. doi:10.1182/blood-2012-10-459115.

Flaumenhaft, Robert, James R Dilks, Nataliya Rozenvayn, Rita A Monahan-Earley, Dian Feng, and Ann M Dvorak. 2005. “The Actin Cytoskeleton Differentially Regulates Platelet Alpha-Granule and Dense-Granule Secretion.” *Blood* 105 (10). American Society of Hematology: 3879–87. doi:10.1182/blood-2004-04-1392.

Ford, C E, N P Skiba, H Bae, Y Daaka, E Reuveny, L R Shekter, R Rosal, et al. 1998. “Molecular Basis for Interactions of G Protein Betagamma Subunits with Effectors.” *Science (New York, N.Y.)* 280 (5367): 1271–74. http://www.ncbi.nlm.nih.gov/pubmed/9596582.

Fox, Meredith A, Anne M Andrews, Jens R Wendland, Klaus-Peter Lesch, Andrew Holmes, and Dennis L Murphy. 2007. “A Pharmacological Analysis of Mice with a Targeted Disruption of the Serotonin Transporter.” *Psychopharmacology* 195 (2): 147–66. doi:10.1007/s00213-007-0910-0.

Fox, Meredith a, Catherine L Jensen, Pamela S Gallagher, and Dennis L Murphy. 2007.

"Receptor Mediation of Exaggerated Responses to Serotonin-Enhancing Drugs in Serotonin Transporter (SERT)-Deficient Mice." *Neuropharmacology* 53 (5): 643–56. doi:10.1016/j.neuropharm.2007.07.009.

Fraer, M., and F. Kilic. 2015. "Serotonin:A Different Player in Hypertension-Associated Thrombosis." *Hypertension* 65 (5): 942–48. doi:10.1161/HYPERTENSIONAHA.114.05061.

Frasure-Smith, Nancy, and François Lespérance. "Reflections on Depression as a Cardiac Risk Factor." *Psychosomatic Medicine* 67 Suppl 1 (January): S19–25. doi:10.1097/01.psy.0000162253.07959.db.

Friedman, Eitan A, Luisa Texeira, Jessica Delaney, Peter E Weeke, Donald R Lynch, Ehab Kasasbeh, Yanna Song, et al. 2016. "Evaluation of the F2R IVS-14A/T PAR1 Polymorphism with Subsequent Cardiovascular Events and Bleeding in Patients Who Have Undergone Percutaneous Coronary Intervention." *Journal of Thrombosis and Thrombolysis* 41 (4): 656–62. doi:10.1007/s11239-015-1285-4.

Gabrielsen, Mari, Rafa1 Kurczab, Aina W Ravna, Irina Kufareva, Ruben Abagyan, Zdzis1aw Chilmonczyk, Andrzej J Bojarski, and Ingebrigt Sylte. 2011. "Molecular Mechanism of Serotonin Transporter Inhibition Elucidated by a New Flexible Docking Protocol." *European Journal of Medicinal Chemistry*. doi:10.1016/j.ejmech.2011.09.056.

Gabrielsen, Mari, Aina Westrheim Ravna, Kurt Kristiansen, and Ingebrigt Sylte. 2012. "Substrate Binding and Translocation of the Serotonin Transporter Studied by Docking and Molecular Dynamics Simulations." *Journal of Molecular Modeling* 18 (3): 1073–85. doi:10.1007/s00894-011-1133-1.

Gardemann, A, J Humme, J Stricker, Q D Nguyen, N Katz, M Philipp, H Tillmanns, F W Hehrlein, M Rau, and W Haberbosch. 1998. "Association of the Platelet Glycoprotein IIIa PlA1/A2 Gene Polymorphism to Coronary Artery Disease but Not to Nonfatal Myocardial Infarction in Low Risk Patients." *Thrombosis and Haemostasis* 80 (2): 214–17. http://www.ncbi.nlm.nih.gov/pubmed/9716139.

Gartside, S.E., V. Umbers, M. Hajós, and T. Sharp. 1995. "Interaction between a Selective 5-HT1A Receptor Antagonist and an SSRI in Vivo: Effects on 5-HT Cell Firing and Extracellular 5-HT." *British Journal of Pharmacology* 115 (6): 1064–70. doi:10.1111/j.1476-5381.1995.tb15919.x.

Gershon, Michael D. 2013. "5-Hydroxytryptamine (Serotonin) in the Gastrointestinal Tract." *Current Opinion in Endocrinology, Diabetes, and Obesity* 20 (1). NIH Public Access: 14–21. doi:10.1097/MED.0b013e32835bc703.

Gershon, Michael D., and Jan Tack. 2007. "The Serotonin Signaling System: From Basic Understanding To Drug Development for Functional GI Disorders." *Gastroenterology* 132 (1): 397–414. doi:10.1053/j.gastro.2006.11.002.

Giannaccini, Gino, Laura Betti, Lionella Palego, Lara Schmid, Laura Fabbrini, Caterina Pelosini, Claudia Gargini, et al. 2010. "Human Serotonin Transporter Expression during Megakaryocytic Differentiation of MEG-01 Cells." *Neurochemical Research* 35 (4): 628–35. doi:10.1007/s11064-009-0112-8.

Gibbins, Jonathan M. 2004. "Platelet Adhesion Signalling and the Regulation of Thrombus Formation." *Journal of Cell Science* 117 (Pt 16): 3415–25. doi:10.1242/jcs.01325.

Gilbert, François, Celia Brazell, Mark D. Tricklebank, and Stephen M. Stahl. 1988. "Activation of the 5-HT1A Receptor Subtype Increases Rat Plasma ACTH Concentration." *European Journal of Pharmacology* 147 (3). Elsevier: 431–39. doi:10.1016/0014-2999(88)90178-1.

Gillman, P Ken. 2006. "A Review of Serotonin Toxicity Data: Implications for the Mechanisms of Antidepressant Drug Action." *Biological Psychiatry* 59 (11): 1046–51. doi:10.1016/j.biopsych.2005.11.016.

Glatt, Charles E, Maricel Tampilic, Carroll Christie, Joe DeYoung, and Nelson B Freimer. 2004. "Re-Screening Serotonin Receptors for Genetic Variants Identifies Population and Molecular Genetic Complexity." *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics* 124B (1): 92–100. doi:10.1002/ajmg.b.20056.

Gobbi, G, D L Murphy, K Lesch, and P Blier. 2001. "Modifications of the Serotonergic System in Mice Lacking Serotonin Transporters: An in Vivo Electrophysiological Study." *The Journal of Pharmacology and Experimental Therapeutics* 296 (3): 987–95. http://www.ncbi.nlm.nih.gov/pubmed/11181933.

Gobbi, Giuliana, Prisco Mirandola, Pier Luigi Tazzari, Francesca Ricci, Luigi Caimi, Antonio Cacchioli, Stefano Papa, Roberto Conte, and Marco Vitale. 2003. "Flow Cytometry Detection of Serotonin Content and Release in Resting and Activated Platelets." *British Journal of Haematology* 121 (6): 892–96. doi:10.1046/j.1365-2141.2003.04369.x.

Gohla, A., Rainer Harhammer, and Günter Schultz. 1998. "The G-Protein G13 but Not G12 Mediates Signaling from Lysophosphatidic Acid Receptor via Epidermal Growth Factor Receptor to Rho." *Journal of Biological Chemistry* 273 (8). American Society for Biochemistry and Molecular Biology: 4653–59. doi:10.1074/jbc.273.8.4653.

Goldstein, Benjamin I, Mercedes R Carnethon, Karen A Matthews, Roger S McIntyre, Gregory E Miller, Geetha Raghuveer, Catherine M Stoney, Hank Wasiak, and Brian W McCrindle. 2015a. "Major Depressive Disorder and Bipolar Disorder Predispose Youth to Accelerated Atherosclerosis and Early Cardiovascular Disease: A Scientific Statement From the American Heart Association." *Circulation* 132 (10): 965–86. doi:10.1161/CIR.0000000000000229.

Goldstein, Benjamin I., Mercedes R. Carnethon, Karen A. Matthews, Roger S. McIntyre, Gregory E. Miller, Geetha Raghuveer, Catherine M. Stoney, Hank Wasiak, and Brian W. McCrindle. 2015b. "Major Depressive Disorder and Bipolar Disorder Predispose Youth to Accelerated Atherosclerosis and Early Cardiovascular Disease." *Circulation* 132 (10): 965–86. doi:10.1161/CIR.0000000000000229.

Golebiewska, Ewelina M., and Alastair W. Poole. 2014. "Secrets of Platelet Exocytosis - What Do We Really Know about Platelet Secretion Mechanisms?" *British Journal of Haematology* 165 (2): 204–16. doi:10.1111/bjh.12682.

Göoz, Monika, Pal Göoz, Louis M Luttrell, and John R Raymond. 2006. "5-HT2A Receptor Induces ERK Phosphorylation and Proliferation through ADAM-17 Tumor Necrosis Factor-Alpha-Converting Enzyme (TACE) Activation and Heparin-Bound Epidermal Growth Factor-like Growth Factor (HB-EGF) Shedding in Mesangial Cells." *The Journal of Biological Chemistry* 281 (30): 21004–12. doi:10.1074/jbc.M512096200.

Greenberg, B D, T J Tolliver, S J Huang, Q Li, D Bengel, and D L Murphy. 1999. "Genetic Variation in the Serotonin Transporter Promoter Region Affects Serotonin Uptake in Human Blood Platelets." *American Journal of Medical Genetics* 88 (1): 83–87. http://www.ncbi.nlm.nih.gov/pubmed/10050973.

Griffin, Martin, Rita Casadio, and Carlo M Bergamini. 2002. "Transglutaminases : Nature ' S Biological Glues" 396: 377–96.

Gryglewski, R J, A Dembínska-Kieć, and R Korbut. 1978. "A Possible Role of Thromboxane A2 (TXA2) and Prostacyclin (PGI2) in Circulation." *Acta Biologica et Medica Germanica* 37 (5-6): 715–23. http://www.ncbi.nlm.nih.gov/pubmed/369254.

Guilluy, Christophe, Saadia Eddahibi, Christian Agard, Christophe Guignabert, Mohamed Izikki, Ly Tu, Laurent Savale, et al. 2009. "RhoA and Rho Kinase Activation in Human Pulmonary Hypertension: Role of 5-HT Signaling." *American Journal of Respiratory and Critical Care Medicine* 179 (12): 1151–58. doi:10.1164/rccm.200805-691OC.

Haase, J, A M Killian, F Magnani, and C Williams. 2001. "Regulation of the Serotonin Transporter by Interacting Proteins." *Biochemical Society Transactions* 29 (Pt 6): 722–28. http://www.ncbi.nlm.nih.gov/pubmed/11709063.

Haddy, FJ, P Gordon, and D A Emanuel. 1959. "The Influence of Tone upon Responses of Small and Large Vessels to Serotonin." *Circulation Research* 7 (1): 123–30. http://www.ncbi.nlm.nih.gov/pubmed/13619051.

Haenisch, Britta, and Heinz Bönisch. 2011. "Depression and Antidepressants: Insights from Knockout of Dopamine, Serotonin or Noradrenaline Re-Uptake Transporters." *Pharmacology & Therapeutics* 129 (3): 352–68. doi:10.1016/j.pharmthera.2010.12.002.

Hammock, Elizabeth, Jeremy Veenstra-VanderWeele, Zhongyu Yan, Travis M. Kerr, Marianna Morris, George M. Anderson, C. Sue Carter, Edwin H. Cook, and Suma Jacob. 2012. "Examining Autism Spectrum Disorders by Biomarkers: Example From the Oxytocin and Serotonin Systems." *Journal of the American Academy of Child & Adolescent Psychiatry* 51 (7): 712–21.e1. doi:10.1016/j.jaac.2012.04.010.

Hara, Katsuko, Yuji Hirowatari, Yuko Shimura, and Hakuo Takahashi. 2011. "Serotonin Levels in Platelet-Poor Plasma and Whole Blood in People with Type 2 Diabetes with Chronic Kidney Disease." *Diabetes Research and Clinical Practice* 94 (2): 167–71. doi:10.1016/j.diabres.2011.06.020.

Harrison, Paul, a L Frelinger, Mark I Furman, and Alan D Michelson. 2007. "Measuring Antiplatelet Drug Effects in the Laboratory." *Thrombosis Research* 120 (3): 323–36. doi:10.1016/j.thromres.2006.11.012.

Harrison, Paul, and Alison H Goodall. 2008. ""Message in the Platelet"--More than Just Vestigial mRNA!" *Platelets* 19 (6): 395–404. doi:10.1080/09537100801990582.

Haub, Synia, Giridhar Kanuri, Valentina Volynets, Thomas Brune, Stephan C Bischoff, and Ina Bergheim. 2010. "Serotonin Reuptake Transporter (SERT) Plays a Critical Role in the Onset of Fructose-Induced Hepatic Steatosis in Mice." *American Journal of Physiology. Gastrointestinal and Liver Physiology* 298 (3): G335–44. doi:10.1152/ajpgi.00088.2009.

Hayward, C P, E Furmaniak-Kazmierczak, A M Cieutat, J C Moore, D F Bainton, M E Nesheim, J G Kelton, and G Côté. 1995. "Factor V Is Complexed with Multimerin in Resting Platelet Lysates and Colocalizes with Multimerin in Platelet Alpha-Granules." *The Journal of Biological Chemistry* 270 (33): 19217–24. http://www.ncbi.nlm.nih.gov/pubmed/7642592.

He, Lei, Konstandinos Vasiliou, and Daniel W Nebert. 2009. "Analysis and Update of the Human Solute Carrier (SLC) Gene Superfamily." *Human Genomics* 3 (2): 195–206. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2752037&tool=pmcentrez&rendertype=abstract.

Hediger, Matthias a, Michael F Romero, Ji-Bin Peng, Andreas Rolfs, Hitomi Takanaga, and Elspeth a Bruford. 2004. "The ABCs of Solute Carriers: Physiological, Pathological and Therapeutic Implications of Human Membrane Transport proteinsIntroduction." *Pflügers Archiv : European Journal of Physiology* 447 (5): 465–68. doi:10.1007/s00424-003-1192-y.

Heemskerk, Johan W M, Edouard M Bevers, and Theo Lindhout. 2002. "Platelet Activation and Blood Coagulation." *Thrombosis and Haemostasis* 88 (2): 186–93. http://www.ncbi.nlm.nih.gov/pubmed/12195687.

Heistad, D D, D G Harrison, and M L Armstrong. 1987. "Serotonin and Experimental Vascular Disease." *International Journal of Cardiology* 14 (2): 205–12.

http://www.ncbi.nlm.nih.gov/pubmed/3818136.

Henry, L Keith, Julie R Field, Erika M Adkins, M Laura Parnas, Roxanne A Vaughan, Mu-Fa Zou, Amy H Newman, and Randy D Blakely. 2006. "Tyr-95 and Ile-172 in Transmembrane Segments 1 and 3 of Human Serotonin Transporters Interact to Establish High Affinity Recognition of Antidepressants." *The Journal of Biological Chemistry* 281 (4): 2012–23. doi:10.1074/jbc.M505055200.

Herbert, J M, A Bernat, G Barthelemy, F Dol, and M Rinaldi. 1993. "Antithrombotic Activity of SR 46349, a Novel, Potent and Selective 5-HT2 Receptor Antagonist." *Thrombosis and Haemostasis* 69 (3): 262–67. http://www.ncbi.nlm.nih.gov/pubmed/8470049.

Hervé, Philipe, Jean-Marie Launay, Marie-Laurène Scrobohaci, François Brenot, Gérald Simonneau, Patrick Petitpretz, Patrice Poubeau, Jacques Cerrina, Pierre Duroux, and Ludovic Drouet. 1995. "Increased Plasma Serotonin in Primary Pulmonary Hypertension." *The American Journal of Medicine* 99 (3): 249–54. doi:10.1016/S0002-9343(99)80156-9.

Hirano, Katsuya, Namie Nomoto, Mayumi Hirano, Fumi Momota, Akiko Hanada, and Hideo Kanaide. 2007. "Distinct Ca2+ Requirement for NO Production between Proteinase-Activated Receptor 1 and 4 (PAR1 and PAR4) in Vascular Endothelial Cells." *The Journal of Pharmacology and Experimental Therapeutics* 322 (2): 668–77. doi:10.1124/jpet.107.121038.

HirshRokach, Bruria, Galia Spectre, Ela Shai, Amit Lotan, Amit Ritter, Fadiea Al-Aieshy, Rickard E. Malmström, David Varon, and Ronny Alcalai. 2015. "Differential Impact of Selective Serotonin Reuptake Inhibitors on Platelet Response to Clopidogrel: A Randomized, Double-Blind, Crossover Trial." *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 35 (2): 140–47. doi:10.1002/phar.1542.

Holinstat, Michael, Bryan Voss, Matthew L Bilodeau, Joseph N McLaughlin, John Cleator, and Heidi E Hamm. 2006. "PAR4, but Not PAR1, Signals Human Platelet Aggregation via Ca2+ Mobilization and Synergistic P2Y12 Receptor Activation." *The Journal of Biological Chemistry* 281 (36): 26665–74. doi:10.1074/jbc.M602174200.

Höltje, Markus, Sandra Winter, Ingrid Pahner, Heide Hörtnagl, Petter Ottersen, and Michael Bader. 2003. "TRANSDUCTION : The Vesicular Monoamine Content Regulates VMAT2 Activity through G α Q in Mouse Platelets : evidence for auoregulation of vesicular transmitter uptake. The Vesicular Monoamine Content Regulates VMAT2 Activity through Ga Q in Mouse Platelet." doi:10.1074/jbc.M212816200.

Höltje, Markus, Sandra Winter, Diego Walther, Ingrid Pahner, Heide Hörtnagl, Ole Petter Ottersen, Michael Bader, and Gudrun Ahnert-Hilger. 2003. "The Vesicular Monoamine Content Regulates VMAT2 Activity through Galphaq in Mouse

Platelets. Evidence for Autoregulation of Vesicular Transmitter Uptake." *The Journal of Biological Chemistry* 278 (18): 15850–58. doi:10.1074/jbc.M212816200.

Hom, Sarah, Li Chen, Tony Wang, Berhane Ghebrehiwet, Wei Yin, and David A. Rubenstein. 2016. "Platelet Activation, Adhesion, Inflammation, and Aggregation Potential Are Altered in the Presence of Electronic Cigarette Extracts of Variable Nicotine Concentrations." *http://dx.doi.org/10.3109/09537104.2016.1158403*. Taylor & Francis.

Homberg, J R, J D a Olivier, B M G Smits, J D Mul, J Mudde, M Verheul, O F M Nieuwenhuizen, et al. 2007. "Characterization of the Serotonin Transporter Knockout Rat: A Selective Change in the Functioning of the Serotonergic System." *Neuroscience* 146 (4): 1662–76. doi:10.1016/j.neuroscience.2007.03.030.

Homberg, Judith R. 2011. "A Mouse Model to Address Unresolved Antidepressant Issues." *Proceedings of the National Academy of Sciences of the United States of America* 108 (9). National Academy of Sciences: 3463–64. doi:10.1073/pnas.1100757108.

Hranilovic, Dubravka, Sofia Blazevic, Nedjeljka Ivica, Lipa Cicin-Sain, and Darko Oreskovic. 2011. "The Effects of the Perinatal Treatment with 5-Hydroxytryptophan or Tranylcypromine on the Peripheral and Central Serotonin Homeostasis in Adult Rats." *Neurochemistry International* 59 (2): 202–7. doi:10.1016/j.neuint.2011.05.003.

Huang, Jin-Sheng, Lanlan Dong, Tohru Kozasa, and Guy C Le Breton. 2007. "Signaling through G(alpha)13 Switch Region I Is Essential for Protease-Activated Receptor 1-Mediated Human Platelet Shape Change, Aggregation, and Secretion." *The Journal of Biological Chemistry* 282 (14): 10210–22. doi:10.1074/jbc.M605678200.

Hummerich, René, and Patrick Schloss. 2010. "Serotonin--More than a Neurotransmitter: Transglutaminase-Mediated Serotonylation of C6 Glioma Cells and Fibronectin." *Neurochemistry International* 57 (1): 67–75. doi:10.1016/j.neuint.2010.04.020.

Hummerich, René, Jörg-Oliver Thumfart, Peter Findeisen, Dusan Bartsch, and Patrick Schloss. 2012. "Transglutaminase-Mediated Transamidation of Serotonin, Dopamine and Noradrenaline to Fibronectin: Evidence for a General Mechanism of Monoaminylation." *FEBS Letters* 586 (19): 3421–28. doi:10.1016/j.febslet.2012.07.062.

Jackson, Shaun P. 2007. "The Growing Complexity of Platelet Aggregation." *Blood* 109 (12): 5087–95. doi:10.1182/blood-2006-12-027698.

Janusonis, Skirmantas. 2008. "Origin of the Blood Hyperserotonemia of Autism." *Theoretical Biology & Medical Modelling* 5 (January): 10. doi:10.1186/1742-4682-5-10.

Jedlitschky, Gabriele, Andreas Greinacher, and Heyo K Kroemer. 2012. "Transporters in

Human Platelets: Physiologic Function and Impact for Pharmacotherapy." *Blood* 119 (15): 3394–3402. doi:10.1182/blood-2011-09-336933.

Jernej, Branimir, Anton Vladić, Lipa Cicin-Sain, Dubravka Hranilović, Miroslav Banović, Melita Balija, Ervina Bilić, Zvonimir Sucić, Stjepan Vukadin, and Damir Grgicević. 2002. "Platelet Serotonin Measures in Migraine." *Headache* 42 (7): 588–95.

Jevsì□evar, Simona, Mencì□i Kunstelj, and Vladka Gaberc Porekar. 2010. "PEGylation of Therapeutic Proteins." *Biotechnology Journal* 5 (1). WILEY- VCH Verlag: 113–28. doi:10.1002/biot.200900218.

Jirouskova, M, A S Shet, and G. J. Johnson. 2007. "A Guide to Murine Platelet Structure, Function, Assays, and Genetic Alterations." *Journal of Thrombosis and Haemostasis* 5 (4). Blackwell Publishing Ltd: 661–69. doi:10.1111/j.1538-7836.2007.02407.x.

Jobe, Shawn M, Lorie Leo, Joshua S Eastvold, Gerhard Dickneite, Timothy L Ratliff, Steven R Lentz, and Jorge Di Paola. 2005. "Role of FcRgamma and Factor XIIIA in Coated Platelet Formation." *Blood* 106 (13): 4146–51. doi:10.1182/blood-2005-03-1223.

Johnson-Farley, Nadine N, Sylvia B Kertesy, George R Dubyak, and Daniel S Cowen. 2005. "Enhanced Activation of Akt and Extracellular-Regulated Kinase Pathways by Simultaneous Occupancy of Gq-Coupled 5-HT2A Receptors and Gs-Coupled 5-HT7A Receptors in PC12 Cells." *Journal of Neurochemistry* 92 (1): 72–82. doi:10.1111/j.1471-4159.2004.02832.x.

Kaastrup, Heidi, Mü Ller, Marie Kragballe, Anja Winther Fjorback, and Ove Wiborg. 2014. "Differential Regulation of the Serotonin Transporter by Vesicle-Associated Membrane Protein 2 in Cells of Neuronal versus Non-Neuronal Origin." *PLoS ONE* 9 (5). doi:10.1371/journal.pone.0097540.

Kahn, M L, Y W Zheng, W Huang, V Bigornia, D Zeng, S Moff, R V Farese, C Tam, and S R Coughlin. 1998. "A Dual Thrombin Receptor System for Platelet Activation." *Nature* 394 (6694): 690–94. doi:10.1038/29325.

Kalueff, a V, J D a Olivier, L J P Nonkes, and J R Homberg. 2010. "Conserved Role for the Serotonin Transporter Gene in Rat and Mouse Neurobehavioral Endophenotypes." *Neuroscience and Biobehavioral Reviews* 34 (3): 373–86. doi:10.1016/j.neubiorev.2009.08.003.

Kalueff, Allan V, Catherine L Jensen, and Dennis L Murphy. 2007. "Locomotory Patterns, Spatiotemporal Organization of Exploration and Spatial Memory in Serotonin Transporter Knockout Mice." *Brain Research* 1169 (September): 87–97. doi:10.1016/j.brainres.2007.07.009.

Kastrati, A, A Schömig, M Seyfarth, W Koch, S Elezi, C Böttiger, J Mehilli, K Schömig,

and N von Beckerath. 1999. “PlA Polymorphism of Platelet Glycoprotein IIIa and Risk of Restenosis after Coronary Stent Placement.” *Circulation* 99 (8): 1005–10. http://www.ncbi.nlm.nih.gov/pubmed/10051292.

Kaufmann, Kristian W, Eric S Dawson, L Keith Henry, Julie R Field, Randy D Blakely, and Jens Meiler. 2009. “Structural Determinants of Species-Selective Substrate Recognition in Human and Drosophila Serotonin Transporters Revealed through Computational Docking Studies.” *Proteins* 74 (3). NIH Public Access: 630–42. doi:10.1002/prot.22178.

Kerdjoudj, Halima, Nicolas Berthelemy, Fouzia Boulmedais, Jean-François Stoltz, Patrick Menu, Jean Claude Voegel, G. Arnulf, et al. 2010. “Multilayered Polyelectrolyte Films: A Tool for Arteries and Vessel Repair.” *Soft Matter* 6 (16). The Royal Society of Chemistry: 3722. doi:10.1039/b920729e.

Keszthelyi, D., F. J. Troost, and a. a M Masclee. 2009. “Understanding the Role of Tryptophan and Serotonin Metabolism in Gastrointestinal Function.” *Neurogastroenterology and Motility* 21 (12): 1239–49. doi:10.1111/j.1365-2982.2009.01370.x.

Khelashvili, George, and Harel Weinstein. 2015. “Functional Mechanisms of Neurotransmitter Transporters Regulated by Lipid–protein Interactions of Their Terminal Loops.” *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1848 (9): 1765–74. doi:10.1016/j.bbamem.2015.03.025.

Kiesselbach, T H, and R H Wagner. 1972. “Demonstration of Factor XIII in Human Megakaryocytes by a Fluorescent Antibody Technique.” *Annals of the New York Academy of Sciences* 202 (December): 318–28. http://www.ncbi.nlm.nih.gov/pubmed/4629554.

Kim, Dong-Kyu, Teresa J Tolliver, Su-Jan Huang, Bradley J Martin, Anne M Andrews, Christine Wichems, Andrew Holmes, Klaus-Peter Lesch, and Dennis L Murphy. 2005. “Altered Serotonin Synthesis, Turnover and Dynamic Regulation in Multiple Brain Regions of Mice Lacking the Serotonin Transporter.” *Neuropharmacology* 49 (6): 798–810. doi:10.1016/j.neuropharm.2005.08.010.

Kimmel, Stephen E, Hedi Schelleman, Jesse a Berlin, David W Oslin, Rachel B Weinstein, Judith L Kinman, William H Sauer, and James D Lewis. 2011. “The Effect of Selective Serotonin Re-Uptake Inhibitors on the Risk of Myocardial Infarction in a Cohort of Patients with Depression.” *British Journal of Clinical Pharmacology* 72 (3): 514–17. doi:10.1111/j.1365-2125.2011.04008.x.

Klages, Birgit, Ursula Brandt, Melvin I. Simon, Günter Schultz, and Stefan Offermanns. 1999. “Activation of G_{12} /G_{13} Results in Shape Change and Rho/Rho-Kinase–mediated Myosin Light Chain Phosphorylation in Mouse Platelets.” *The Journal of Cell Biology* 144 (4). Rockefeller Univ Press: 745–54. doi:10.1083/jcb.144.4.745.

Koba, Shinji, Rajbabu Pakala, Takuya Watanabe, Takashi Katagiri, and Claude R

Benedict. 1999. “Vascular Smooth Muscle Proliferation.” *Journal of the American College of Cardiology* 34 (5): 1644–51. doi:10.1016/S0735-1097(99)00349-6.

Komanasin, N, a J Catto, T S Futers, a van Hylckama Vlieg, F R Rosendaal, and R a S Ariëns. 2005. “A Novel Polymorphism in the Factor XIII B-Subunit (His95Arg): Relationship to Subunit Dissociation and Venous Thrombosis.” *Journal of Thrombosis and Haemostasis : JTH* 3 (11): 2487–96. doi:10.1111/j.1538-7836.2005.01624.x.

Kristensen, Anders S, Jacob Andersen, Trine N Jørgensen, Lena Sørensen, Jacob Eriksen, Claus J Loland, and Kristian Strømgaard. 2011. “SLC6 Neurotransmitter Transporters : Structure , Function , and Regulation” 63 (3): 585–640. doi:10.1124/pr.108.000869.585.

Kulkarni, Suhasini, and Shaun P Jackson. 2004. “Platelet Factor XIII and Calpain Negatively Regulate Integrin alphaIIbbeta3 Adhesive Function and Thrombus Growth.” *The Journal of Biological Chemistry* 279 (29): 30697–706. doi:10.1074/jbc.M403559200.

Kuwahara, Mitsuhiro, Mitsuhiko Sugimoto, Shizuko Tsuji, Hideto Matsui, Tomohiro Mizuno, Shigeki Miyata, and Akira Yoshioka. 2002. “Platelet Shape Changes and Adhesion under High Shear Flow.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 22 (2): 329–34. http://www.ncbi.nlm.nih.gov/pubmed/11834537.

L Shen, L Lorand. 1983. “Contribution of Fibrin Stabilization to Clot Strength. Supplementation of Factor XIII-Deficient Plasma with the Purified Zymogen.” *Journal of Clinical Investigation* 71 (5). American Society for Clinical Investigation: 1336.

Lambe, Evelyn K, Stu G Fillman, Maree J Webster, and Cynthia Shannon Weickert. 2011. “Serotonin Receptor Expression in Human Prefrontal Cortex: Balancing Excitation and Inhibition across Postnatal Development.” *PloS One* 6 (7): e22799. doi:10.1371/journal.pone.0022799.

Lau, Way K W, Moira M W Chan-Yeung, Benjamin H K Yip, Amy H K Cheung, Mary S M Ip, and Judith C W Mak. 2012. “The Role of Circulating Serotonin in the Development of Chronic Obstructive Pulmonary Disease.” *PloS One* 7 (2): e31617. doi:10.1371/journal.pone.0031617.

Lee, K N, C S Lee, W C Tae, K W Jackson, V J Christiansen, and P a McKee. 2000. “Cross-Linking of Wild-Type and Mutant Alpha 2-Antiplasmins to Fibrin by Activated Factor XIII and by a Tissue Transglutaminase.” *The Journal of Biological Chemistry* 275 (48): 37382–89. doi:10.1074/jbc.M003375200.

Legate, Kyle R, and Reinhard Fässler. 2009. “Mechanisms That Regulate Adaptor Binding to Beta-Integrin Cytoplasmic Tails.” *Journal of Cell Science* 122 (Pt 2): 187–98. doi:10.1242/jcs.041624.

Lesch, K P, B L Wolozin, D L Murphy, and P Reiderer. 1993. “Primary Structure of the Human Platelet Serotonin Uptake Site: Identity with the Brain Serotonin Transporter.” *Journal of Neurochemistry* 60 (6): 2319–22. http://www.ncbi.nlm.nih.gov/pubmed/7684072.

Levin, Jack. 1997. “The Evolution of Mammalian Platelets.” In *Thrombopoiesis and Thrombopoietins*, 63–78. Totowa, NJ: Humana Press. doi:10.1007/978-1-4612-3958-1_3.

Li, N, N H Wallen, M Ladjevardi, and P Hjemdahl. 1997. “Effects of Serotonin on Platelet Activation in Whole Blood.” *Blood Coagulation & Fibrinolysis : An International Journal in Haemostasis and Thrombosis* 8 (8). ENGLAND: 517–23.

Li, Q, C Wichems, a Heils, K P Lesch, and D L Murphy. 2000. “Reduction in the Density and Expression, but Not G-Protein Coupling, of Serotonin Receptors (5-HT1A) in 5-HT Transporter Knock-out Mice: Gender and Brain Region Differences.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20 (21): 7888–95. http://www.ncbi.nlm.nih.gov/pubmed/11050108.

Li, Renhao, Karin M. Hoffmeister, and Hervé Falet. 2016. “Glycans and the Platelet Life Cycle.” *Platelets*, May, 1–7. doi:10.3109/09537104.2016.1171304.

Li, Yicong, Coedy Hadden, Anthonya Cooper, Asli Ahmed, Hong Wu, Vladimir V Lupashin, Philip R Mayeux, and Fusun Kilic. 2016. “Sepsis-Induced Elevation in Plasma Serotonin Facilitates Endothelial Hyperpermeability.” *Scientific Reports* 6: 22747. doi:10.1038/srep22747.

Li, Zhenyu, M Keegan Delaney, Kelly A O’Brien, and Xiaoping Du. 2010. “Signaling during Platelet Adhesion and Activation.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 30 (12): 2341–49. doi:10.1161/ATVBAHA.110.207522.

Lichtman, Judith H., Erika S. Froelicher, James a. Blumenthal, Robert M. Carney, Lynn V. Doering, Nancy Frasure-Smith, Kenneth E. Freedland, et al. 2014. “Depression as a Risk Factor for Poor Prognosis among Patients with Acute Coronary Syndrome: Systematic Review and Recommendations: A Scientific Statement from the American Heart Association.” *Circulation* 129 (12): 1350–69. doi:10.1161/CIR.0000000000000019.

Lin, Jason Ching-yao, Chi-chi Chou, Shijay Gao, and Shang-chuen Wu. 2013. “An in Vivo Tagging Method Reveals That Ras Undergoes Sustained Activation upon Transglutaminase-Mediated Protein Serotonylation” 10617: 813–17. doi:10.1002/cbic.201300050.

Lin, Jason Ching-Yao, Chi-Chi Chou, Shijay Gao, Shang-Chuen Wu, Kay-Hooi Khoo, and Chun-Hung Lin. 2013. “An in Vivo Tagging Method Reveals That Ras Undergoes Sustained Activation upon Transglutaminase-Mediated Protein Serotonylation.” *Chembiochem : A European Journal of Chemical Biology* 14 (7): 813–17. doi:10.1002/cbic.201300050.

Lin, Olivia a., Zubair a. Karim, Hari Priya Vemana, Enma V P Espinosa, and Fadi T. Khasawneh. 2014. "The Antidepressant 5-HT2A Receptor Antagonists Pizotifen and Cyproheptadine Inhibit Serotonin-Enhanced Platelet Function." *PLoS ONE* 9 (1). doi:10.1371/journal.pone.0087026.

Lister, J. 1891. "On the Coagulation of the Blood in Its Practical Aspects." *British Medical Journal* 1 (1585): 1057–60. http://www.ncbi.nlm.nih.gov/pubmed/20753319.

Machlus, Kellie R., and Joseph E. Italiano. 2013. "The Incredible Journey: From Megakaryocyte Development to Platelet Formation." *The Journal of Cell Biology* 201 (6). Rockefeller Univ Press: 785–96. doi:10.1083/jcb.201304054.

Mackman, Nigel. 2009. "The Role of Tissue Factor and Factor VIIa in Hemostasis." *Anesthesia and Analgesia* 108 (5). NIH Public Access: 1447–52. doi:10.1213/ane.0b013e31819bceb1.

Magnani, Francesca, Christopher G Tate, Samantha Wynne, Clive Williams, and Jana Haase. 2004. "Partitioning of the Serotonin Transporter into Lipid Microdomains Modulates Transport of Serotonin." *The Journal of Biological Chemistry* 279 (37): 38770–78. doi:10.1074/jbc.M400831200.

Malyszko, J, T Urano, R Knofler, a Taminato, T Yoshimi, Y Takada, and a Takada. 1994. "Daily Variations of Platelet Aggregation in Relation to Blood and Plasma Serotonin in Diabetes." *Thrombosis Research* 75 (5): 569–76. http://www.ncbi.nlm.nih.gov/pubmed/7992257.

Manepalli, Sankar, Christopher K Surratt, Jeffry D Madura, and Tammy L Nolan. 2012. "Monoamine Transporter Structure, Function, Dynamics, and Drug Discovery: A Computational Perspective." *The AAPS Journal* 14 (4): 820–31. doi:10.1208/s12248-012-9391-0.

Mangge, Harald, Kelli L Summers, Andreas Meinitzer, Sieglinde Zelzer, Gunter Almer, Ruth Prassl, Wolfgang J Schnedl, et al. 2014. "Obesity-Related Dysregulation of the Tryptophan-Kynureninemetabolism: Roleof Age and Parameters of the Metabolic Syndrome." *Obesity (Silver Spring, Md.)* 22 (1): 195–201. doi:10.1002/oby.20491.

Masini, E, M G Di Bello, S Raspanti, J F Ndisang, J Fomusi Ndisang, R Baronti, P Cappugi, and P F Mannaioni. 1998. "The Role of Histamine in Platelet Aggregation by Physiological and Immunological Stimuli." *Inflammation Research : Official Journal of the European Histamine Research Society ... [et Al.]* 47 (5): 211–20. http://www.ncbi.nlm.nih.gov/pubmed/9657253.

Masson, J, and M Hamon. 2009. "Monoamine Transporters : Focus on the Regulation of Serotonin Transporter by Cytokines," 921–29.

Mathews, Tiffany A., Denise E. Fedele, Francesca M. Coppelli, Amy M. Avila, Dennis L. Murphy, and Anne M. Andrews. 2004. "Gene Dose-Dependent Alterations in

Extraneuronal Serotonin but Not Dopamine in Mice with Reduced Serotonin Transporter Expression." *Journal of Neuroscience Methods* 140 (1): 169–81. doi:10.1016/j.jneumeth.2004.05.017.

Matondo, Ramadhan B, Carine Punt, Judith Homberg, Mathilda J M Toussaint, Ronald Kisjes, Suzanne J a Korporaal, Jan Willem N Akkerman, Edwin Cuppen, and Alain de Bruin. 2009. "Deletion of the Serotonin Transporter in Rats Disturbs Serotonin Homeostasis without Impairing Liver Regeneration." *American Journal of Physiology. Gastrointestinal and Liver Physiology* 296 (4): G963–68. doi:10.1152/ajpgi.90709.2008.

Maurer-Spurej, Elisabeth, Cheryl Pittendreigh, and Kevin Solomons. 2003. "The Influence of Selective Serotonin Reuptake Inhibitors on Serotonin Metabolism in Human Platelets." *Thrombosis and Haemostasis*, November, 119–28. doi:10.1160/TH03-05-0330.

Maurer-Spurej, Elisabeth, Cheryl Pittendreigh, and Kevin Solomons. 2004. "The Influence of Selective Serotonin Reuptake Inhibitors on Human Platelet Serotonin." *Thrombosis and Haemostasis* 91 (1): 119–28. doi:10.1160/TH03-05-0330.

Maurice, Tangui, and Tsung-Ping Su. 2009. "The Pharmacology of Sigma-1 Receptors." *Pharmacology & Therapeutics* 124 (2): 195–206. doi:10.1016/j.pharmthera.2009.07.001.

Mawe, Gary M, and Jill M Hoffman. 2013. "Serotonin Signalling in the Gut--Functions, Dysfunctions and Therapeutic Targets." *Nature Reviews. Gastroenterology & Hepatology* 10 (8): 473–86. doi:10.1038/nrgastro.2013.105.

Maynard, D M, H F G Heijnen, M K Horne, J G White, and W A Gahl. 2007. "Proteomic Analysis of Platelet Alpha-Granules Using Mass Spectrometry." *Journal of Thrombosis and Haemostasis : JTH* 5 (9): 1945–55. doi:10.1111/j.1538-7836.2007.02690.x.

Mazalouskas, Matthew, Tammy Jessen, Seth Varney, James S Sutcliffe, Jeremy Veenstra-VanderWeele, Edwin H Cook, and Ana M D Carneiro. 2015. "Integrin β3 Haploinsufficiency Modulates Serotonin Transport and Antidepressant-Sensitive Behavior in Mice." *Neuropsychopharmacology* 40 (8). Nature Publishing Group: 2015–24. doi:10.1038/npp.2015.51.

McManus, David D., and Jane E. Freedman. 2015. "MicroRNAs in Platelet Function and Cardiovascular Disease." *Nature Reviews Cardiology* 12 (12). Nature Publishing Group: 711–17. doi:10.1038/nrcardio.2015.101.

Mercado, Charles P, and Fusun Kilic. 2010. "Charles P. Mercado and Fusun Kilic" 10 (4): 231–41.

Mercado, Charles P, Maritza V Quintero, Yicong Li, Preeti Singh, Alicia K Byrd, Krajang Talabnin, Mayumi Ishihara, et al. 2013. "A Serotonin-Induced N-Glycan

Switch Regulates Platelet Aggregation." *Scientific Reports* 3: 2795. doi:10.1038/srep02795.

Mészáros, Z, D Borcsiczky, M Máté, J Tarcali, T Szombathy, K Tekes, and K Magyar. 1998. "Platelet MAO-B Activity and Serotonin Content in Patients with Dementia: Effect of Age, Medication, and Disease." *Neurochemical Research* 23 (6): 863–68. http://www.ncbi.nlm.nih.gov/pubmed/9572675.

Meyers, K M, H Holmsen, and C L Seachord. 1982. "Comparative Study of Platelet Dense Granule Constituents." *The American Journal of Physiology* 243 (3): R454–61. http://www.ncbi.nlm.nih.gov/pubmed/7114301.

Miller, K J, C L Mariano, and W R Cruz. 1997. "Serotonin 5HT2A Receptor Activation Inhibits Inducible Nitric Oxide Synthase Activity in C6 Glioma Cells." *Life Sciences* 61 (18): 1819–27.

Mintun, Mark a, Yvette I Sheline, Stephen M Moerlein, Andrei G Vlassenko, Yiyun Huang, and Abraham Z Snyder. 2004. "Decreased Hippocampal 5-HT2A Receptor Binding in Major Depressive Disorder: In Vivo Measurement with [18F]altanserin Positron Emission Tomography." *Biological Psychiatry* 55 (3): 217–24. doi:10.1016/j.biopsych.2003.08.015.

Monassier, Laurent, Marc André Laplante, Tarak Ayadi, Stéphane Doly, and Luc Maroteaux. 2010. "Contribution of Gene-Modified Mice and Rats to Our Understanding of the Cardiovascular Pharmacology of Serotonin." *Pharmacology & Therapeutics* 128 (3). Elsevier Inc.: 559–67. doi:10.1016/j.pharmthera.2010.08.004.

Monroe, D. M., and Maureane Hoffman. 2006. "What Does It Take to Make the Perfect Clot?" *Arteriosclerosis, Thrombosis, and Vascular Biology* 26 (1). Lippincott Williams & Wilkins: 41–48. doi:10.1161/01.ATV.0000193624.28251.83.

Moore, Thomas C, and Ben Eiseman. 2016. "Serotonin Metabolism in the Isolated Perfused Canine Liver." *Surgery* 59 (5). Elsevier: 765–69. doi:10.5555/uri:pii:0039606066902406.

MOREL, F F. 1950. "[Measurement of Transcapillary Diffusion with Radioactive Tracers]." *Helvetica Physiologica et Pharmacologica Acta* 8 (2): 146–68. http://www.ncbi.nlm.nih.gov/pubmed/15435966.

Mortensen, Ole V, Anders S Kristensen, Gary Rudnick, and Ove Wiborg. 1999. "Molecular Cloning, Expression and Characterization of a Bovine Serotonin Transporter." *Molecular Brain Research* 71 (1): 120–26. doi:10.1016/S0169-328X(99)00178-3.

Muehlschlegel, Jochen D, Tjörvi E Perry, Kuang-Yu Liu, Amanda A Fox, Shane A Smith, Peter Lichtner, Charles D Collard, et al. 2012. "Polymorphism in the Protease-Activated Receptor-4 Gene Region Associates with Platelet Activation and Perioperative Myocardial Injury." *American Journal of Hematology* 87 (2): 161–66.

doi:10.1002/ajh.22244.

Müller, Heidi Kaastrup, Ove Wiborg, and Jana Haase. 2006. “Subcellular Redistribution of the Serotonin Transporter by Secretory Carrier Membrane Protein 2.” *The Journal of Biological Chemistry* 281 (39): 28901–9. doi:10.1074/jbc.M602848200.

Muma, Nancy A, and Zhen Mi. 2015. “Serotonylation and Transamidation of Other Monoamines.” doi:10.1021/cn500329r.

Murphy, D L, G R Uhl, and A Holmes. 2003. “Experimental Gene Interaction Studies with SERT Mutant Mice as Models for Human Polygenic and Epistatic Traits and Disorders,” 350–64. doi:10.1046/j.1601-183X.2003.00049.x.

Murphy, Dennis L, Meredith A Fox, Kiara R Timpano, Pablo Moya, Renee Ren-, Anne M Andrews, Andrew Holmes, Klaus-peter Lesch, and R Jens. 2009. “How the Serotonin Sotry Is Being Rewritten by New Gene-Based Discoveries Principally Related to SLC6A4, the Serotonin Transporter Gene, Which Functions to Influence All Cellular Serotonin Systems” 55 (6): 932–60. doi:10.1016/j.neuropharm.2008.08.034.How.

Murphy, Dennis L, and Klaus-Peter Lesch. 2008. “Targeting the Murine Serotonin Transporter: Insights into Human Neurobiology.” *Nature Reviews. Neuroscience* 9 (2): 85–96. doi:10.1038/nrn2284.

Musselman, D L, D L Evans, and C B Nemeroff. 1998. “The Relationship of Depression to Cardiovascular Disease: Epidemiology, Biology, and Treatment.” *Archives of General Psychiatry* 55 (7): 580–92.

Musumeci, Giuseppe, Rosa Imbesi, Francesca Maria Trovato, Marta Anna Szychlinska, Flavia Concetta Aiello, Pietro Buffa, and Paola Castrogiovanni. 2015. “Importance of Serotonin (5-HT) and Its Precursor L-Tryptophan for Homeostasis and Function of Skeletal Muscle in Rats. A Morphological and Endocrinological Study.” *Acta Histochemica*, March. Elsevier GmbH. doi:10.1016/j.acthis.2015.03.003.

Nackenoff, Alex, Linda Simmler, Nicole Baganz, Krista Paffenroth, Gregg Stanwood, Alan Pehrson, Connie Sánchez, and Randy Blakely. 2015. “Serotonin Transporter-Independent Actions of the Antidepressant Vortioxetine as Revealed Using the SERT M172 Mouse.” *The FASEB Journal* 29 (1 Supplement). Federation of American Societies for Experimental Biology: 932.5.

Nahrendorf, Matthias, Kai Hu, Stefan Frantz, Farouc a Jaffer, Ching-Hsuan Tung, Karl-Heinz Hiller, Sabine Voll, et al. 2006. “Factor XIII Deficiency Causes Cardiac Rupture, Impairs Wound Healing, and Aggravates Cardiac Remodeling in Mice with Myocardial Infarction.” *Circulation* 113 (9): 1196–1202. doi:10.1161/CIRCULATIONAHA.105.602094.

Namkung, Jun, Hail Kim, and Sangkyu Park. 2015. “Peripheral Serotonin: A New Player in Systemic Energy Homeostasis.” *Molecules and Cells* 38 (9): 1–6.

doi:10.14348/molcells.2015.0258.

Nazir, Saad, Anoop Kumar, Ishita Chatterjee, Arivarasu N Anbazhagan, Tarunmeet Gujral, Shubha Priyamvada, Seema Saksena, Waddah A Alrefai, Pradeep K Dudeja, and Ravinder K Gill. 2015. "Mechanisms of Intestinal Serotonin Transporter (SERT) Upregulation by TGF-β1 Induced Non-Smad Pathways." *PloS One* 10 (5): e0120447. doi:10.1371/journal.pone.0120447.

Newman, Peter J, Rebecca S Derbes, and Richard H Aster. n.d. "Rapid Publication The Human Platelet Alloantigens, p1Al and pIA2 Are Associated with a Leucine33/Proline33 Amino Acid Polymorphism in Membrane Glycoprotein Ilia, and Are Distinguishable by DNA Typing."

Nietzer, S L, M Bonn, F Jansen, R S Heiming, L Lewejohann, N Sachser, E S Asan, K P Lesch, and a G Schmitt. 2011. "Serotonin Transporter Knockout and Repeated Social Defeat Stress: Impact on Neuronal Morphology and Plasticity in Limbic Brain Areas." *Behavioural Brain Research* 220 (1). Elsevier B.V.: 42–54. doi:10.1016/j.bbr.2011.01.011.

Niiya, K, E Hodson, R Bader, V Byers-Ward, J A Koziol, E F Plow, and Z M Ruggeri. 1987. "Increased Surface Expression of the Membrane Glycoprotein IIb/IIIa Complex Induced by Platelet Activation. Relationship to the Binding of Fibrinogen and Platelet Aggregation." *Blood* 70 (2): 475–83. http://www.ncbi.nlm.nih.gov/pubmed/3607284.

Nirenberg, M J, Y Liu, D Peter, R H Edwards, and V M Pickel. 1995. "The Vesicular Monoamine Transporter 2 Is Present in Small Synaptic Vesicles and Preferentially Localizes to Large Dense Core Vesicles in Rat Solitary Tract Nuclei." *Proceedings of the National Academy of Sciences of the United States of America* 92 (19): 8773–77. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=41049&tool=pmcentrez&rendertype=abstract.

Nurden, A, and P Nurden. 2011. "Advances in Our Understanding of the Molecular Basis of Disorders of Platelet Function." *Journal of Thrombosis and Haemostasis : JTH* 9 Suppl 1 (July): 76–91. doi:10.1111/j.1538-7836.2011.04274.x.

Nurden, Paquita, Martine Jandrot-Perrus, Robert Combrié, Joelle Winckler, Veronique Arocas, Christelle Lecut, Jean-Max Pasquet, Thomas J Kunicki, and Alan T Nurden. 2004. "Severe Deficiency of Glycoprotein VI in a Patient with Gray Platelet Syndrome." *Blood* 104 (1): 107–14. doi:10.1182/blood-2003-11-3842.

Nyola, Ajeeta, Nathan K Karpowich, Juan Zhen, Jennifer Marden, Maarten E Reith, and Da-Neng Wang. 2010. "Substrate and Drug Binding Sites in LeuT." *Current Opinion in Structural Biology* 20 (4). Elsevier Ltd: 415–22. doi:10.1016/j.sbi.2010.05.007.

O'Brien, J R. 1964. "A Comparison of Platelet Aggregation Produced By Seven

Compounds and a Comparison of Their Inhibitors." *Journal of Clinical Pathology* 17: 275–81. doi:10.1136/jcp.17.3.275.

Obydennyy, Sergey I, Anastasia N Sveshnikova, Fazly I Ataullakhanov, and Mikhail A Panteleev. 2016. "Dynamics of Calcium Spiking, Mitochondrial Collapse and Phosphatidylserine Exposure in Platelet Subpopulations during Activation." *Journal of Thrombosis and Haemostasis : JTH*, June. doi:10.1111/jth.13395.

Offermanns, Stefan. 2006. "Activation of Platelet Function through G Protein-Coupled Receptors." *Circulation Research* 99 (12): 1293–1304. doi:10.1161/01.RES.0000251742.71301.16.

Offermanns, Stefan, and Stefan Offermanns. 2013. "Activation of Platelet Function Through G Protein – Coupled Receptors," 1293–1304. doi:10.1161/01.RES.0000251742.71301.16.

Ogawa, Taketoshi, Atsuhiro Sugidachi, Naoki Tanaka, Koichi Fujimoto, and Fumitoshi Asai. 2002. "Pharmacological Profiles of R-96544, the Active Form of a Novel 5-HT2A Receptor Antagonist R-102444." *European Journal of Pharmacology* 457 (2-3): 107–14. http://www.ncbi.nlm.nih.gov/pubmed/12464356.

Ohlmann, Philippe, Anna Lecchi, Ali El-Tayeb, Christa E Müller, Marco Cattaneo, and Christian Gachet. 2013. "The Platelet P2Y(12) Receptor under Normal and Pathological Conditions. Assessment with the Radiolabeled Selective Antagonist [(3)H]PSB-0413." *Purinergic Signalling* 9 (1): 59–66. doi:10.1007/s11302-012-9329-0.

Ohno, Yukihiro, Saki Shimizu, Kentaro Tokudome, Naofumi Kunisawa, and Masashi Sasa. 2015. "New Insight into the Therapeutic Role of the Serotonergic System in Parkinson's Disease." *Progress in Neurobiology* 134: 104–21. doi:10.1016/j.pneurobio.2015.09.005.

Oliver, Kendra H, Tammy Jessen, Emily L Crawford, Chang Y Chung, James S Sutcliffe, and Ana M Carneiro. 2014. "Pro32Pro33 Mutations in the Integrin β3 PSI Domain Result in αIIbβ3 Priming and Enhanced Adhesion: Reversal of the Hypercoagulability Phenotype by the Src Inhibitor SKI-606." *Molecular Pharmacology* 85 (6): 921–31. doi:10.1124/mol.114.091736.

Ono, Akiko, Erik Westein, Sarah Hsiao, Warwick S Nesbitt, Justin R Hamilton, Simone M Schoenwaelder, and Shaun P Jackson. 2008. "Identification of a Fibrin-Independent Platelet Contractile Mechanism Regulating Primary Hemostasis and Thrombus Growth." *Blood* 112 (1): 90–99. doi:10.1182/blood-2007-12-127001.

Ostad Haji, Elnaz, André Tadić, Stefanie Wagner, Aleksandra Dragicevic, Matthias J Müller, Katja Boland, Marie-Luise Rao, Miriam Fric, Gerd Laux, and Christoph Hiemke. 2011. "Association between Citalopram Serum Levels and Clinical Improvement of Patients with Major Depression." *Journal of Clinical Psychopharmacology* 31 (3): 281–86. doi:10.1097/JCP.0b013e318218f503.

Owens, J M, D L Knight, and C B Nemeroff. 2016. "[Second Generation SSRIS: Human Monoamine Transporter Binding Profile of Escitalopram and R-Fluoxetine]." *L'Encéphale* 28 (4): 350–55. Accessed July 9. http://www.ncbi.nlm.nih.gov/pubmed/12232544.

Owens, Michael J, David L Knight, Charles B Nemeroff, N.M Barnes, T Sharp, C Bolden-Watson, E Richelson, et al. 2001. "Second-Generation SSRIs: Human Monoamine Transporter Binding Profile of Escitalopram and R-Fluoxetine." *Biological Psychiatry* 50 (5). Elsevier: 345–50. doi:10.1016/S0006-3223(01)01145-3.

Ozaslan, Deniz, Sophie Wang, Billow a Ahmed, Arif M Kocabas, John C McCastlain, Anca Bene, and Fusun Kilic. 2003. "Glycosyl Modification Facilitates Homo- and Hetero-Oligomerization of the Serotonin Transporter. A Specific Role for Sialic Acid Residues." *The Journal of Biological Chemistry* 278 (45): 43991–0. doi:10.1074/jbc.M306360200.

Packham, Marian A, and Margaret L Rand. 2011. "Historical Perspective on ADP-Induced Platelet Activation." *Purinergic Signalling* 7 (3): 283–92. doi:10.1007/s11302-011-9227-x.

Palta, Sanjeev, Richa Saroa, and Anshu Palta. 2014. "Overview of the Coagulation System." *Indian Journal of Anaesthesia* 58 (5): 515–23. doi:10.4103/0019-5049.144643.

Paraskevaidis, Ioannis, John Palios, John Parissis, Gerasimos Filippatos, and Maria Anastasiou-Nana. 2012. "Treating Depression in Coronary Artery Disease and Chronic Heart Failure: What's New in Using Selective Serotonin Re-Uptake Inhibitors?" *Cardiovascular & Hematological Agents in Medicinal Chemistry* 10 (2): 109–15.

Park, Hyung-Jin, Sang-Eun Lee, Jung-Hyun Oh, Kyoung-Won Seo, Kun-Ho Song, EM Lund, PJ Armstrong, et al. 2014. "Leptin, Adiponectin and Serotonin Levels in Lean and Obese Dogs." *BMC Veterinary Research* 10 (1). BioMed Central: 113. doi:10.1186/1746-6148-10-113.

Paul, B. Z. S., J. L. Daniel, and S. P. Kunapuli. 1999. "Platelet Shape Change Is Mediated by Both Calcium-Dependent and -Independent Signaling Pathways: ROLE OF p160 Rho-ASSOCIATED COILED-COIL-CONTAINING PROTEIN KINASE IN PLATELET SHAPE CHANGE." *Journal of Biological Chemistry* 274 (40): 28293–300. doi:10.1074/jbc.274.40.28293.

Paulmann, Nils, Maik Grohmann, Jörg-Peter Voigt, Bettina Bert, Jakob Vowinckel, Michael Bader, Masa Skelin, et al. 2009. "Intracellular Serotonin Modulates Insulin Secretion from Pancreatic Beta-Cells by Protein Serotonylation." *PLoS Biology* 7 (10): e1000229. doi:10.1371/journal.pbio.1000229.

Pawlak, D, K Pawlak, E Chabielska, J Małyszko, A Takada, M Myśliwiec, and W

Buczko. 1998. “A Potent 5-Hydroxytryptamine Receptor (5-HT2A) Antagonist, DV-7028, Delays Arterial Thrombosis Development in Rats.” *Thrombosis Research* 90 (6): 259–70. http://www.ncbi.nlm.nih.gov/pubmed/9700856.

Penmatsa, Aravind, Kevin H Wang, and Eric Gouaux. 2013. “X-Ray Structure of Dopamine Transporter Elucidates Antidepressant Mechanism.” *Nature* 503 (7474): 85–90. doi:10.1038/nature12533.

Peroutka, S J, and S H Snyder. 1979. “Multiple Serotonin Receptors: Differential Binding of [3H]5-Hydroxytryptamine, [3H]lysergic Acid Diethylamide and [3H]spiroperidol.” *Molecular Pharmacology* 16 (3): 687–99. http://www.ncbi.nlm.nih.gov/pubmed/530254.

Peters, Christian G, Alan D Michelson, Robert Flaumenhaft, RT. Swank, EK. Novak, MP. McGarry, ME. Rusiniak, et al. 2012. “Granule Exocytosis Is Required for Platelet Spreading: Differential Sorting of α-Granules Expressing VAMP-7.” *Blood* 120 (1). American Society of Hematology: 199–206. doi:10.1182/blood-2011-10-389247.

Petríková, M, V Jancinová, R Nosál, M Májeková, and D Holománová. 2006. “H(1)-Antihistamines and Activated Blood Platelets.” *Inflammation Research : Official Journal of the European Histamine Research Society ... [et Al.]* 55 Suppl 1 (April): S51–52. doi:10.1007/s00011-005-0038-9.

Pleines, I., I. Hagedorn, S. Gupta, F. May, L. Chakarova, J. van Hengel, S. Offermanns, et al. 2012. “Megakaryocyte-Specific RhoA Deficiency Causes Macrothrombocytopenia and Defective Platelet Activation in Hemostasis and Thrombosis.” *Blood* 119 (4): 1054–63. doi:10.1182/blood-2011-08-372193.

Pleines, Irina, Ina Hagedorn, Shuchi Gupta, Frauke May, Lidija Chakarova, Jolanda van Hengel, Stefan Offermanns, et al. 2012. “Megakaryocyte-Specific RhoA Deficiency Causes Macrothrombocytopenia and Defective Platelet Activation in Hemostasis and Thrombosis.” *Blood* 119 (4): 1054–63. doi:10.1182/blood-2011-08-372193.

Potočnjak, Ines, Vesna Degoricija, Dina Vukičević Baudoin, Josip Čulig, and Miro Jakovljević. 2016. “Cardiovascular Side Effects of Psychopharmacologic Therapy.” *International Journal of Cardiology* 219: 367–72. doi:10.1016/j.ijcard.2016.06.057.

Preininger, A M, L G Henage, W M Oldham, E J Yoon, H E Hamm, and H A Brown. 2006. “Direct Modulation of Phospholipase D Activity by Gbetagamma.” *Molecular Pharmacology* 70 (1): 311–18. doi:10.1124/mol.105.021451.

Przyklenk, K, A L Frelinger, M D Linden, P Whittaker, Y Li, M R Barnard, J Adams, M Morgan, H Al-Shamma, and A D Michelson. 2010a. “Targeted Inhibition of the Serotonin 5HT2A Receptor Improves Coronary Patency in an in Vivo Model of Recurrent Thrombosis.” *Journal of Thrombosis and Haemostasis : JTH* 8 (2): 331–40. doi:10.1111/j.1538-7836.2009.03693.x.

Przyklenk, K, a L Frelinger, M D Linden, P Whittaker, Y Li, M R Barnard, J Adams, M Morgan, H Al-Shamma, and a D Michelson. 2010b. "Targeted Inhibition of the Serotonin 5HT2A Receptor Improves Coronary Patency in an in Vivo Model of Recurrent Thrombosis." *Journal of Thrombosis and Haemostasis : JTH* 8 (2): 331–40. doi:10.1111/j.1538-7836.2009.03693.x.

Puszkins, Elena G, and Vijayalakshmi Raghuraman. 1985. "Catalytic Properties of a Calmodulin-Regulated Transglutaminase from Human Platelet and Chicken Gizzard *" 260 (29): 16012–20.

Qian, Y, A Galli, S Ramamoorthy, S Risso, L J DeFelice, and R D Blakely. 1997. "Protein Kinase C Activation Regulates Human Serotonin Transporters in HEK-293 Cells via Altered Cell Surface Expression." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 17 (1): 45–57. http://www.ncbi.nlm.nih.gov/pubmed/8987735.

Qiu, Yongzhi, Ashley C. Brown, David R. Myers, Yumiko Sakurai, Robert G. Mannino, Reginald Tran, Byungwook Ahn, et al. 2014. "Platelet Mechanosensing of Substrate Stiffness during Clot Formation Mediates Adhesion, Spreading, and Activation." *Proceedings of the National Academy of Sciences* 111 (40). National Academy of Sciences: 14430–35. doi:10.1073/pnas.1322917111.

Quick, Michael W. 2002. "Role of Syntaxin 1A on Serotonin Transporter Expression in Developing Thalamocortical Neurons." *International Journal of Developmental Neuroscience* 20 (3): 219–24. doi:10.1016/S0736-5748(02)00021-7.

Quick, Michael W, S.V Adams, L.J DeFelice, M.K Bennett, J.E García-Arrarás, L.A Elferink, K Peterson, et al. 2003. "Regulating the Conducting States of a Mammalian Serotonin Transporter." *Neuron* 40 (3). Elsevier: 537–49. doi:10.1016/S0896-6273(03)00605-6.

Quinn, John C, Nadine N Johnson-Farley, JiYoung Yoon, and Daniel S Cowen. 2002. "Activation of Extracellular-Regulated Kinase by 5-hydroxytryptamine(2A) Receptors in PC12 Cells Is Protein Kinase C-Independent and Requires Calmodulin and Tyrosine Kinases." *The Journal of Pharmacology and Experimental Therapeutics* 303 (2): 746–52. doi:10.1124/jpet.102.038083.

Racke, K, A Reimann, H Schworer, and H Kilbinger. 1996. "Regulation of 5-HT Release from Enterochromaffin Cells." *Behavioural Brain Research* 73 (1-2). NETHERLANDS: 83–87.

Rajtar, Grazyna, Egidio Marchi, Giovanni de Gaetano, and Chiara Cerletti. 1993. "Effects of Glycosaminoglycans on Platelet and Leucocyte Function: Role of N-Sulfation." *Biochemical Pharmacology* 46 (5). Elsevier: 958–60. doi:10.1016/0006-2952(93)90507-S.

Ramamoorthy, S. 1999. "Phosphorylation and Sequestration of Serotonin Transporters Differentially Modulated by Psychostimulants." *Science* 285 (5428): 763–66.

doi:10.1126/science.285.5428.763.

Ramamoorthy, S., E. Giovanetti, Y. Qian, and R. D. Blakely. 1998. “Phosphorylation and Regulation of Antidepressant-Sensitive Serotonin Transporters.” *Journal of Biological Chemistry* 273 (4). American Society for Biochemistry and Molecular Biology: 2458–66. doi:10.1074/jbc.273.4.2458.

Ramamoorthy, Sammanda, Toni S Shippenberg, and Lankupalle D Jayanthi. 2011. “Regulation of Monoamine Transporters: Role of Transporter Phosphorylation.” *Pharmacology & Therapeutics* 129 (2). Elsevier Inc.: 220–38. doi:10.1016/j.pharmthera.2010.09.009.

Randriamboavonjy, Voahanginirina, Johann Isaak, Amro Elgheznawy, Frank Pistrosch, Timo Frömel, Xiaoke Yin, Klaus Badenhoop, Heinrich Heide, Manuel Mayr, and Ingrid Fleming. 2012. “Calpain Inhibition Stabilizes the Platelet Proteome and Reactivity in Diabetes.” *Blood* 120 (2): 415–23. doi:10.1182/blood-2011-12-399980.

Rapport, M M, and M Virno. 1952. “Metabolic Effect of Serotonin in the Rat.” *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* 81 (1): 203–5. http://www.ncbi.nlm.nih.gov/pubmed/13047357.

Rasmussen, Søren G. F., Brian T. DeVree, Yaozhong Zou, Andrew C. Kruse, Ka Young Chung, Tong Sun Kobilka, Foon Sun Thian, et al. 2011. “Crystal Structure of the β2 Adrenergic receptor–Gs Protein Complex.” *Nature* 477 (7366). Nature Publishing Group: 549–55. doi:10.1038/nature10361.

Rayes, Julie, Martine J Hollestelle, Paulette Legendre, Isabelle Marx, Philip G de Groot, Olivier D Christophe, Peter J Lenting, and Cécile V Denis. 2010. “Mutation and ADAMTS13-Dependent Modulation of Disease Severity in a Mouse Model for von Willebrand Disease Type 2B.” *Blood* 115 (23): 4870–77. doi:10.1182/blood-2009-11-254193.

Rendu, F, and B Brohard-Bohn. 2001. “The Platelet Release Reaction: Granules’ Constituents, Secretion and Functions.” *Platelets* 12 (5): 261–73. doi:10.1080/09537100120068170.

Rios-Avila, Luisa, H Frederik Nijhout, Michael C Reed, Harry S Sitren, and Jesse F Gregory. 2013. “A Mathematical Model of Tryptophan Metabolism via the Kynurenine Pathway Provides Insights into the Effects of Vitamin B-6Deficiency,Tryptophan Loading, and Induction of Tryptophan 2,3-Dioxygenase on TryptophanMetabolites.” *The Journal of Nutrition*. doi:10.3945/jn.113.174599.

Rocha, Beatriz A. 2003. “Stimulant and Reinforcing Effects of Cocaine in Monoamine Transporter Knockout Mice.” *European Journal of Pharmacology* 479 (1): 107–15. doi:10.1016/j.ejphar.2003.08.061.

Rowley, Jesse W, Hansjörg Schwertz, and Andrew S Weyrich. 2012. "Platelet mRNA: The Meaning behind the Message." *Current Opinion in Hematology* 19 (5): 385–91. doi:10.1097/MOH.0b013e328357010e.

Rudnick, G. 1977. "Active Transport of 5-Hydroxytryptamine by Plasma Membrane Vesicles Isolated from Human Blood Platelets." *The Journal of Biological Chemistry* 252 (7): 2170–74.

Sakai, Norio, Keiko Sasaki, Mayumi Nakashita, Shigeru Honda, Natsu Ikegaki, and Naoaki Saito. 2002. "Modulation of Serotonin Transporter Activity by a Protein Kinase C Activator and an Inhibitor of Type 1 and 2A Serine/Threonine Phosphatases." *Journal of Neurochemistry* 68 (6). Blackwell Science Ltd: 2618–24. doi:10.1046/j.1471-4159.1997.68062618.x.

Sakurai, Yumiko, Jennifer L Fitch-Tewfik, Yongzhi Qiu, Byungwook Ahn, David R Myers, Reginald Tran, Meredith E Fay, et al. 2015. "Platelet Geometry Sensing Spatially Regulates α-Granule Secretion to Enable Matrix Self-Deposition." *Blood* 126 (4). American Society of Hematology: 531–38. doi:10.1182/blood-2014-11-607614.

Sanner, Jennifer E, Lorraine Frazier, and Malini Udtha. 2013. "The Role of Platelet Serotonin and Depression in the Acute Coronary Syndrome Population." *The Yale Journal of Biology and Medicine* 86 (1): 5–13.

Santiago-Germán, David, Alfredo Leaños-Miranda, Ethel García-Latorre, Gabriela Borrayo-Sánchez, Abraham Majluf-Cruz, and Irma Isordia-Salas. 2012. "Platelet Glycoprotein IIIA PIA2 Polymorphism Is Associated with ST Elevation Acute Myocardial Infarction in Young Mexican Population." *Journal of Thrombosis and Thrombolysis* 33 (4). Springer US: 389–96. doi:10.1007/s11239-012-0714-x.

Sauer, William H., Jesse a. Berlin, and Stephen E. Kimmel. 2003. "Effect of Antidepressants and Their Relative Affinity for the Serotonin Transporter on the Risk of Myocardial Infarction." *Circulation* 108 (1): 32–36. doi:10.1161/01.CIR.0000079172.43229.CD.

Scanlon, S M, D C Williams, and P Schloss. 2001. "Membrane Cholesterol Modulates Serotonin Transporter Activity." *Biochemistry* 40 (35): 10507–13. http://www.ncbi.nlm.nih.gov/pubmed/11523992.

Schaff, M, N Receveur, C Bourdon, P Ohlmann, F Lanza, C Gachet, and P H Mangin. 2012. "β-Arrestin-1 Participates in Thrombosis and Regulates Integrin aIIbβ3 Signalling without Affecting P2Y Receptors Desensitisation and Function." *Thrombosis and Haemostasis* 107 (4): 735–48. doi:10.1160/TH11-06-0430.

Schellander, Rene, and Josef Donnerer. 2010. "Antidepressants: Clinically Relevant Drug Interactions to Be Considered." *Pharmacology* 86 (4): 203–15. doi:10.1159/000319744.

Schmid, Cullen L, and Laura M Bohn. 2010. “Serotonin, but Not N-Methyltryptamines, Activates the Serotonin 2A Receptor via a SS-arrestin2/Src/Akt Signaling Complex in Vivo.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 30 (40): 13513–24. doi:10.1523/JNEUROSCI.1665-10.2010.

Schmid, Cullen L, Kirsten M Raehal, and Laura M Bohn. 2008. “Agonist-Directed Signaling of the Serotonin 2A Receptor Depends on Beta-Arrestin-2 Interactions in Vivo.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (3): 1079–84. doi:10.1073/pnas.0708862105.

Schmidt, Gudula, Udo-michael Goehring, Maria Lerm, and Klaus Aktories. 1999. “ENZYMOLOGY : Identification of the C-Terminal Part of Bordetella Dermonecrotic Toxin as a Transglutaminase for Rho GTPases Identification of the C-Terminal Part of Bordetella Dermonecrotic Toxin as a Transglutaminase for Rho GTPases *.” doi:10.1074/jbc.274.45.31875.

Schmidt, Gudula, Peter Sehr, Matthias Wilm, Matthias Mann, and Klaus Aktories. 1997. “Letters to Nature Gln 63 of Rho Is Deamidated by Escherichia Coli Cytotoxic Necrotizing Factor-1” 387 (June): 725–29.

Schubert, Sebastian, Andrew S Weyrich, and Jesse W Rowley. 2014. “A Tour through the Transcriptional Landscape of Platelets.” *Blood* 124 (4): 493–502. doi:10.1182/blood-2014-04-512756.

Sentí, Mariano, Clara Aubó, Mireia Bosch, Marco Pavesi, Araceli Pena, Rafel Masiá, and Jaume Marrugat. 1998. “Platelet Glycoprotein IIb/IIIa Genetic Polymorphism Is Associated with Plasma Fibrinogen Levels in Myocardial Infarction Patients.” *Clinical Biochemistry* 31 (8): 647–51. doi:10.1016/S0009-9120(98)00077-0.

Serebruany, Victor L, Alexander H Glassman, Alex I Malinin, Charles B Nemeroff, Dominique L Musselman, Louis T van Zyl, Mitchell S Finkel, et al. 2003. “Platelet/endothelial Biomarkers in Depressed Patients Treated with the Selective Serotonin Reuptake Inhibitor Sertraline after Acute Coronary Events: The Sertraline AntiDepressant Heart Attack Randomized Trial (SADHART) Platelet Substudy.” *Circulation* 108 (8): 939–44. doi:10.1161/01.CIR.0000085163.21752.0A.

Severinsen, Kasper, Johan F Kraft, Heidi Koldsø, Katrine A Vinberg, Richard B Rothman, John S Partilla, Ove Wiborg, Bruce Blough, Birgit Schiøtt, and Steffen Sinning. 2012. “Binding of the Amphetamine-like 1-Phenyl-Piperazine to Monoamine Transporters.” *ACS Chemical Neuroscience* 3 (9). American Chemical Society: 693–705. doi:10.1021/cn300040f.

Shen, Bo, Xiaojuan Zhao, Kelly A O’Brien, Aleksandra Stojanovic-Terpo, M Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C-T Lam, and Xiaoping Du. 2013. “A Directional Switch of Integrin Signalling and a New Anti-Thrombotic Strategy.” *Nature* 503 (7474): 131–35. doi:10.1038/nature12613.

Shen, Kuo-Ping, Li-Wen Chu, Su-Ling Hsieh, Li-Mei An, Ing-Jun Chen, and Bin-Nan

Wu. 2008. “Hypotensive and Antiaggregative Effects of Eugenosedin-B with Serotonin and Alpha/beta-Adrenoceptor Antagonistic Activities in Rats and Human Platelets.” *Journal of Cardiovascular Pharmacology* 51 (2): 154–61. doi:10.1097/FJC.0b013e31815e852c.

Sherwin, R. P., J. C. Shih, J. D. Lee, and R. Ransom. 1986. “Serotonin Content of the Lungs, Brains, and Blood of Mice Exposed to 0.45 Ppm Nitrogen Dioxide.” *International Journal of Toxicology* 5 (6): 583–88. doi:10.3109/10915818609141027.

Shulman, N R, R H Aster, A Leitner, and M C Hiller. 1961. “Immunoractions involving platelets.” *The Journal of Clinical Investigation* 40 (9). American Society for Clinical Investigation: 1597–1620. doi:10.1172/JCI104383.

Siekhaus, Daria E., and David G. Drubin. 2003. “Spontaneous Receptor-Independent Heterotrimeric G-Protein Signalling in an RGS Mutant.” *Nature Cell Biology* 5 (3). Nature Publishing Group: 231–35. doi:10.1038/ncb941.

Simon, Melvin I., Stefan Offermanns, Christopher F. Toombs, and Yi-Hui Hu. 1997. “Defective Platelet Activation in G|[alpha]|q-Deficient Mice.” *Nature* 389 (6647). Nature Publishing Group: 183–86. doi:10.1038/38284.

Singh, Preeti, Terry W Fletcher, Yicong Li, Nancy J Rusch, and Fusun Kilic. 2013. “Serotonin Uptake Rates in Platelets from Angiotensin II-Induced Hypertensive Mice.” *Health* 5 (4A): 31–39. doi:10.4236/health.2013.54A005.

Smyth, S S, E D Reis, H Väänänen, W Zhang, B S Coller, AI. Schafer, MH. Kroll, et al. 2001. “Variable Protection of Beta 3-Integrin--Deficient Mice from Thrombosis Initiated by Different Mechanisms.” *Blood* 98 (4). American Society of Hematology: 1055–62. doi:10.1182/blood.v98.4.1055.

Sondek, J, A Bohm, D G Lambright, H E Hamm, and P B Sigler. 1996. “Crystal Structure of a G-Protein Beta Gamma Dimer at 2.1A Resolution.” *Nature* 379 (6563): 369–74. doi:10.1038/379369a0.

Soulet, C, B Hechler, M-P Gratacap, M Plantavid, S Offermanns, C Gachet, and B Payrastre. 2005. “A Differential Role of the Platelet ADP Receptors P2Y1 and P2Y12 in Rac Activation.” *Journal of Thrombosis and Haemostasis : JTH* 3 (10): 2296–2306. doi:10.1111/j.1538-7836.2005.01588.x.

Stahl, Edward L, Lei Zhou, Frederick J Ehlert, and Laura M Bohn. 2015. “A Novel Method for Analyzing Extremely Biased Agonism at G Protein – Coupled Receptors S,” 866–77.

Stahl, Stephen M, Clara Lee-Zimmerman, Sylvia Cartwright, and Debbi Ann Morrissette. 2013. “Serotonergic Drugs for Depression and Beyond.” *Current Drug Targets* 14 (5). Netherlands: 578–85.

Stalker, Timothy J, Elizabeth a Traxler, Jie Wu, Kenneth M Wannemacher, Samantha L Cermignano, Roman Voronov, Scott L Diamond, and Lawrence F Brass. 2013. "Hierarchical Organization in the Hemostatic Response and Its Relationship to the Platelet-Signaling Network." *Blood* 121 (10): 1875–85. doi:10.1182/blood-2012-09-457739.

Steiner, Jennifer a, Ana Marin D Carneiro, and Randy D Blakely. 2008. "Going with the Flow: Trafficking-Dependent and -Independent Regulation of Serotonin Transport." *Traffic (Copenhagen, Denmark)* 9 (9): 1393–1402. doi:10.1111/j.1600-0854.2008.00757.x.

Stritt, Simon, Karen Wolf, Viola Lorenz, Timo Vögtle, Shuchi Gupta, Michael R Bösl, and Bernhard Nieswandt. 2015. "Rap1-GTP-Interacting Adaptor Molecule (RIAM) Is Dispensable for Platelet Integrin Activation and Function in Mice." *Blood* 125 (2): 219–22. doi:10.1182/blood-2014-08-597542.

Stutz, Christopher M, Lynda D O'Rear, Kevin R O'Neill, Maria E Tamborski, Colin G Crosby, Clinton J Devin, and Jonathan G Schoenecker. 2013. "Coagulopathies in Orthopaedics: Links to Inflammation and the Potential of Individualizing Treatment Strategies." *Journal of Orthopaedic Trauma* 27 (4): 236–41. doi:10.1097/BOT.0b013e318269b782.

Suzuki, Hidenori, Kagari Murasaki, Kumi Kodama, and Hiroshi Takayama. 2003. "Intracellular Localization of Glycoprotein VI in Human Platelets and Its Surface Expression upon Activation." *British Journal of Haematology* 121 (6): 904–12. http://www.ncbi.nlm.nih.gov/pubmed/12786802.

Szasz, Robert, and George L Dale. 2002. "Thrombospondin and Fibrinogen Bind Serotonin-Derivatized Proteins on COAT-Platelets." *Blood* 100 (8): 2827–31. doi:10.1182/blood-2002-02-0354.

Tabu, A, and A Yo. 2001. "Small G TPase Rho Regula T Es Thrombin- I Nduced Pla T Ele T Aggrega T Ion" 975: 970–75.

Tadokoro, Seiji, Tsuyoshi Nakazawa, Tsuyoshi Kamae, Kazunobu Kiyomizu, Hirokazu Kashiwagi, Shigenori Honda, Yuzuru Kanakura, and Yoshiaki Tomiyama. 2011. "A Potential Role for α-Actinin in inside-out αIIbβ3 Signaling." *Blood* 117 (1): 250–58. doi:10.1182/blood-2009-10-246751.

Taub, R, R J Gould, V M Garsky, T M Ciccarone, J Hoxie, P A Friedman, and S J Shattil. 1989. "A Monoclonal Antibody against the Platelet Fibrinogen Receptor Contains a Sequence That Mimics a Receptor Recognition Domain in Fibrinogen." *The Journal of Biological Chemistry* 264 (1): 259–65. http://www.ncbi.nlm.nih.gov/pubmed/2909518.

Thakker, D R, F Natt, D Hüsken, H van der Putten, R Maier, D Hoyer, and J F Cryan. 2005. "siRNA-Mediated Knockdown of the Serotonin Transporter in the Adult Mouse Brain." *Molecular Psychiatry* 10 (8): 782–89, 714.

doi:10.1038/sj.mp.4001687.

Thase, M E, A R Entsuah, and R L Rudolph. 2001. “Remission Rates during Treatment with Venlafaxine or Selective Serotonin Reuptake Inhibitors.” *The British Journal of Psychiatry : The Journal of Mental Science* 178 (March): 234–41. http://www.ncbi.nlm.nih.gov/pubmed/11230034.

Thompson NT, Scrutton MC, Wallis RB. 1986. “Synergistic Responses in Human Platelets. Comparison between Aggregation, Secretion and Cytosolic Ca2+ Concentration.” *Eur J Biochem.*

Thompson, Brent J, Tammy Jessen, L K Henry, Julie R Field, Karen L Gamble, Paul J Gresch, Ana M Carneiro, et al. 2011. “Transgenic Elimination of High-Affinity Antidepressant and Cocaine Sensitivity in the Presynaptic Serotonin Transporter.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (9). National Academy of Sciences: 3785–90. doi:10.1073/pnas.1011920108.

Thompson, N T, M C Scrutton, and R B Wallis. 1986. “Synergistic Responses in Human Platelets. Comparison between Aggregation, Secretion and Cytosolic Ca2+ Concentration.” *European Journal of Biochemistry / FEBS* 161 (2): 399–408. http://www.ncbi.nlm.nih.gov/pubmed/3490977.

Thurlow, P. J., D. A. Kenneally, and J. M. Connellan. 1990. “The Role of Fibronectin in Platelet Aggregation.” *British Journal of Haematology* 75 (4). Blackwell Publishing Ltd: 549–56. doi:10.1111/j.1365-2141.1990.tb07797.x.

Toh, C. C. 1956. “Release of 5-Hydroxytryptamine (Serotonin) and Histamine from Platelets by Tissue Extracts.” *The Journal of Physiology* 133 (2): 402–11. doi:10.1113/jphysiol.1956.sp005595.

Tollefson, G D, and S L Holman. 1994. “How Long to Onset of Antidepressant Action: A Meta-Analysis of Patients Treated with Fluoxetine or Placebo.” *International Clinical Psychopharmacology* 9 (4): 245–50. http://www.ncbi.nlm.nih.gov/pubmed/7868846.

Torres, Gonzalo E, Wei-Dong Yao, Amy R Mohn, Hui Quan, Kyeong-Man Kim, Allan I Levey, Jeff Staudinger, and Marc G Caron. 2001. “Functional Interaction between Monoamine Plasma Membrane Transporters and the Synaptic PDZ Domain–Containing Protein PICK1.” *Neuron* 30 (1): 121–34. doi:10.1016/S0896-6273(01)00267-7.

Touhara, K, J Inglese, J a Pitcher, G Shaw, and R J Lefkowitz. 1994. “Binding of G Protein Beta Gamma-Subunits to Pleckstrin Homology Domains.” *The Journal of Biological Chemistry* 269 (14): 10217–20. http://www.ncbi.nlm.nih.gov/pubmed/8144601.

Tsakiris, D A, L Scudder, K Hodivala-Dilke, R O Hynes, and B S Coller. 1999. “Hemostasis in the Mouse (Mus Musculus): A Review.” *Thrombosis and*

Haemostasis 81 (2): 177–88. http://www.ncbi.nlm.nih.gov/pubmed/10063988.

Tseng, Yu-Lun, Meng-Ling Chiang, Tur-Fu Huang, Kuan-Pin Su, Hsien-Yuan Lane, and Yu-Chen Lai. 2010. "A Selective Serotonin Reuptake Inhibitor, Citalopram, Inhibits Collagen-Induced Platelet Aggregation and Activation." *Thrombosis Research* 126 (6). Elsevier Ltd: 517–23. doi:10.1016/j.thromres.2010.09.017.

Tseng, Yu-Lun, Meng-Ling Chiang, Hsien-Yuan Lane, Kuan-Pin Su, and Yu-Chen Lai. 2013. "Selective Serotonin Reuptake Inhibitors Reduce P2Y12 Receptor-Mediated Amplification of Platelet Aggregation." *Thrombosis Research* 131 (4): 325–32. doi:10.1016/j.thromres.2013.02.007.

Ueno, Masafumi, Murali Kodali, Antonio Tello-Montoliu, and Dominick Joseph Angiolillo. 2011. "Role of Platelets and Antiplatelet Therapy in Cardiovascular Disease." *Journal of Atherosclerosis and Thrombosis* 18 (6): 431–42. http://www.ncbi.nlm.nih.gov/pubmed/21427504.

van EE, R, and J C van Oene. 1991. "Effect of Ketanserin on Intraoperative Blood Loss during Total Hip Arthroplasty in Elderly Patients under General Anaesthesia." *British Journal of Anaesthesia* 66 (4): 496–99. http://www.ncbi.nlm.nih.gov/pubmed/2025478.

Vandendries, Erik R, Justin R Hamilton, Shaun R Coughlin, Bruce Furie, and Barbara C Furie. 2007. "Par4 Is Required for Platelet Thrombus Propagation but Not Fibrin Generation in a Mouse Model of Thrombosis." *Proceedings of the National Academy of Sciences of the United States of America* 104 (1): 288–92. doi:10.1073/pnas.0610188104.

Vanhoutte, P M. 1987. "Cardiovascular Effects of Serotonin." *Journal of Cardiovascular Pharmacology* 10 Suppl 3. UNITED STATES: S8–11.

Vanhoutte, Paul M, Gabor M Rubanyi, Virginia M Miller, and Donald S Houston. 1986. "Modulation of Vascular sooth muscle contraction by the endothelium." *Ann. Rev. Physiol* 48: 307–20.

Vasiliou, Vasilis, Konstandinos Vasiliou, and Daniel W Nebert. 2009. "Human ATP-Binding Cassette (ABC) Transporter Family." *Human Genomics* 3 (3): 281–90. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2752038&tool=pmcentrez&rendertype=abstract.

Versteeg, Henri H, Johan W M Heemskerk, Marcel Levi, Pieter H Reitsma, J. Ahamed, W. Ruf, J. Ahamed, et al. 2013. "New Fundamentals in Hemostasis." *Physiological Reviews* 93 (1). American Physiological Society: 327–58. doi:10.1152/physrev.00016.2011.

Virmani, R., F. D. Kolodgie, A. P. Burke, A. Farb, and S. M. Schwartz. 2000. "Lessons From Sudden Coronary Death : A Comprehensive Morphological Classification Scheme for Atherosclerotic Lesions." *Arteriosclerosis, Thrombosis, and Vascular*

Biology 20 (5): 1262–75. doi:10.1161/01.ATV.20.5.1262.

Visvikis, Orane, Madhavi P Maddugoda, and Emmanuel Lemichez. 2010. "Direct Modifications of Rho Proteins: Deconstructing GTPase Regulation." *Biology of the Cell / under the Auspices of the European Cell Biology Organization* 102 (7): 377–89. doi:10.1042/BC20090151.

Vogel, Carola, Rainald Mössner, Manfred Gerlach, Thoralf Heinemann, Dennis L Murphy, Peter Riederer, Klaus-Peter Lesch, and Claudia Sommer. 2003. "Absence of Thermal Hyperalgesia in Serotonin Transporter-Deficient Mice." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 23 (2): 708–15. http://www.ncbi.nlm.nih.gov/pubmed/12533631.

Vu, Thien-Khai H., David T. Hung, Virginia I. Wheaton, and Shaun R. Coughlin. 1991. "Molecular Cloning of a Functional Thrombin Receptor Reveals a Novel Proteolytic Mechanism of Receptor Activation." *Cell* 64 (6). Cell Press: 1057–68. doi:10.1016/0092-8674(91)90261-V.

Vuorenpää, Anne, Ina Ammendrup-Johnsen, Trine N. Jørgensen, and Ulrik Gether. 2016. "A Kinome Wide Screen Identifies Novel Kinases Involved in Regulation of Monoamine Transporter Function." *Neurochemistry International*. doi:10.1016/j.neuint.2016.03.013.

Walter, Dirk H, Volker Schächinger, Mathias Elsner, Stefanie Dimmeler, and Andreas M Zeiher. 1997. "Platelet Glycoprotein IIIa Polymorphisms and Risk of Coronary Stent Thrombosis." *The Lancet* 350 (9086): 1217–19. doi:10.1016/S0140-6736(97)05399-3.

Walther, D. J. 2003. "Synthesis of Serotonin by a Second Tryptophan Hydroxylase Isoform." *Science* 299 (5603). American Association for the Advancement of Science: 76–76. doi:10.1126/science.1078197.

Walther, Diego J, Jens-Uwe Peter, Sandra Winter, Markus Höltje, Nils Paulmann, Maik Grohmann, Jakob Vowinckel, et al. 2003. "Serotonylation of Small GTPases Is a Signal Transduction Pathway That Triggers Platelet Alpha-Granule Release." *Cell* 115 (7): 851–62. http://www.ncbi.nlm.nih.gov/pubmed/14697203.

Ware, Jerry. 2004. "Dysfunctional Platelet Membrane Receptors: From Humans to Mice." *Thrombosis and Haemostasis* 92 (3): 478–85. doi:10.1160/TH04-05-0308.

Watson, Steve P. 2009. "Platelet Activation by Extracellular Matrix Proteins in Haemostasis and Thrombosis." *Current Pharmaceutical Design* 15 (12): 1358–72. http://www.ncbi.nlm.nih.gov/pubmed/19355974.

Watts, Stephanie W, Jessica R C Priestley, and Janice M Thompson. 2009a. "Serotonylation of Vascular Proteins Important to Contraction." *PloS One* 4 (5). Public Library of Science: e5682. doi:10.1371/journal.pone.0005682.

Weiss, Ethan J, Justin R Hamilton, Katy E Lease, and Shaun R Coughlin. 2002. "Protection against Thrombosis in Mice Lacking PAR3." *Blood* 100 (9): 3240–44. doi:10.1182/blood-2002-05-1470.

Weiss, Ethan J., Paul F. Bray, Matthew Tayback, Steven P. Schulman, Thomas S. Kickler, Lewis C. Becker, James L. Weiss, Gary Gerstenblith, and Pascal J. Goldschmidt-Clermont. 2009. "A Polymorphism of a Platelet Glycoprotein Receptor as an Inherited Risk Factor for Coronary Thrombosis." *http://dx.doi.org/10.1056/NEJM199604253341703*. Massachusetts Medical Society.

White, J G, and W J Conard. 1973. "The Fine Structure of Freeze-Fractured Blood Platelets." *The American Journal of Pathology* 70 (1). American Society for Investigative Pathology: 45–56. http://www.ncbi.nlm.nih.gov/pubmed/4566474.

Wihlborg, A.-K., Lingwei Wang, Oscar Östberg Braun, Atli Eyjolfsson, Ronny Gustafsson, Tomas Gudbjartsson, and David Erlinge. 2004. "ADP Receptor P2Y12 Is Expressed in Vascular Smooth Muscle Cells and Stimulates Contraction in Human Blood Vessels." *Arteriosclerosis, Thrombosis, and Vascular Biology* 24 (10): 1810–15. doi:10.1161/01.ATV.0000142376.30582.ed.

Wildling, Linda, Christian Rankl, Thomas Haselgrübler, Hermann J Gruber, Marion Holy, Amy Hauck Newman, Mu-Fa Zou, et al. 2012. "Probing Binding Pocket of Serotonin Transporter by Single Molecular Force Spectroscopy on Living Cells." *The Journal of Biological Chemistry* 287 (1): 105–13. doi:10.1074/jbc.M111.304873.

Wong, Albert, Yuan-Wei Zhang, Grace R Jeschke, Benjamin E Turk, and Gary Rudnick. 2012. "Cyclic GMP-Dependent Stimulation of Serotonin Transport Does Not Involve Direct Transporter Phosphorylation by cGMP-Dependent Protein Kinase." *The Journal of Biological Chemistry* 287 (43): 36051–58. doi:10.1074/jbc.M112.394726.

Worm, K., C. Dragsholt, K. Simonsen, and B. Kringsholm. 1998. "Citalopram Concentrations in Samples from Autopsies and Living Persons." *International Journal of Legal Medicine* 111 (4). Springer-Verlag: 188–90. doi:10.1007/s004140050147.

Woulfe, Donna, Hong Jiang, Alicia Morgans, Robert Monks, Morris Birnbaum, Lawrence F. Brass, C Trumel, et al. 2004. "Defects in Secretion, Aggregation, and Thrombus Formation in Platelets from Mice Lacking Akt2." *Journal of Clinical Investigation* 113 (3). American Society for Clinical Investigation: 441–50. doi:10.1172/JCI20267.

Woulfe, Donna, Hong Jiang, Richard Mortensen, Jing Yang, and Lawrence F Brass. 2002. "Activation of Rap1B by G(i) Family Members in Platelets." *The Journal of Biological Chemistry* 277 (26): 23382–90. doi:10.1074/jbc.M202212200.

Wozniak, Greta, Aikaterini Toska, Maria Saridi, and Odysseas Mouzas. 2011. "Serotonin

Reuptake Inhibitor Antidepressants (SSRIs) against Atherosclerosis." *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research* 17 (9): RA205–14.

Xiang, Binggang, Guoying Zhang, Lucia Stefanini, Wolfgang Bergmeier, T Kent Gartner, Sidney W Whiteheart, and Zhenyu Li. 2012. "The Src Family Kinases and Protein Kinase C Synergize to Mediate Gq-Dependent Platelet Activation." *The Journal of Biological Chemistry* 287 (49): 41277–87. doi:10.1074/jbc.M112.393124.

Yamauchi, Miki, Takako Miyara, Tetsuya Matsushima, and Taiichiro Imanishi. 2006. "Desensitization of 5-HT2A Receptor Function by Chronic Administration of Selective Serotonin Reuptake Inhibitors." *Brain Research* 1067 (1): 164–69. doi:10.1016/j.brainres.2005.10.075.

Yeagle, Philip L., and Arlene D. Albert. 2003. "A Conformational Trigger for Activation of a G Protein by a G Protein-Coupled Receptor†." American Chemical Society .

Yim, Yun Young, Katherine Betke, and Heidi Hamm. 2015. "Using Peptide Arrays Created by the SPOT Method for Defining Protein-Protein Interactions." *Methods in Molecular Biology (Clifton, N.J.)* 1278: 307–20. doi:10.1007/978-1-4939-2425-7_18.

Yoda, Emiko, Kohmi Rai, Mai Ogawa, Yuki Takakura, Hiroshi Kuwata, Hidenori Suzuki, Yoshihito Nakatani, Makoto Murakami, and Shuntaro Hara. 2014. "Group VIB Calcium-Independent Phospholipase A2 (iPLA2γ) Regulates Platelet Activation, Hemostasis and Thrombosis in Mice." *PloS One* 9 (10): e109409. doi:10.1371/journal.pone.0109409.

Yoshinaga, I G, S K Dekker, M C Mihm, and H R Byers. 1994. "Differential Effect of Magnesium and Calcium on Integrin-Mediated Melanoma Cell Migration on Type IV Collagen and Fibronectin." *Melanoma Research* 4 (6): 371–78. http://www.ncbi.nlm.nih.gov/pubmed/7703716.

Zahn, F. Wilh. 1874. "Untersuchungen Über Thrombose." *Archiv Für Pathologische Anatomie Und Physiologie Und Für Klinische Medicin* 62 (1). Springer-Verlag: 81–124. doi:10.1007/BF01927438.

Zarbock, Alexander, Renata K Polanowska-Grabowska, and Klaus Ley. 2007. "Platelet-Neutrophil-Interactions: Linking Hemostasis and Inflammation." *Blood Reviews* 21 (2): 99–111. doi:10.1016/j.blre.2006.06.001.

Zeinali, Fatemeh, Reidar Fossmark, Oyvind Hauso, Rune Wiseth, Oyvind Hjertner, and Helge L Waldum. 2013. "Serotonin in Blood: Assessment of Its Origin by Concomitant Determination of β-Thromboglobulin (Platelets) and Chromogranin A (Enterochromaffin Cells)." *Scandinavian Journal of Clinical and Laboratory Investigation* 73 (2): 148–53. doi:10.3109/00365513.2012.756121.

Zhou, Feng C, Klaus-Peter Lesch, and Dennis L Murphy. 2002. “Serotonin Uptake into Dopamine Neurons via Dopamine Transporters: A Compensatory Alternative.” *Brain Research* 942 (1-2): 109–19. http://www.ncbi.nlm.nih.gov/pubmed/12031859.

Zhou, Zheng, Juan Zhen, Nathan K Karpowich, Christopher J Law, Maarten E A Reith, and Da-Neng Wang. 2009. “Antidepressant Specificity of Serotonin Transporter Suggested by Three LeuT-SSRI Structures.” *Nature Structural & Molecular Biology* 16 (6). NIH Public Access: 652–57. doi:10.1038/nsmb.1602.

Zhu, Chong-Bin, Ana M Carneiro, Wolfgang R Dostmann, William a Hewlett, and Randy D Blakely. 2005. “p38 MAPK Activation Elevates Serotonin Transport Activity via a Trafficking-Independent, Protein Phosphatase 2A-Dependent Process.” *The Journal of Biological Chemistry* 280 (16): 15649–58. doi:10.1074/jbc.M410858200.

Ziu, Endrit, Coedy Hadden, Yicong Li, Curtis Lee Lowery, Preeti Singh, Serra S Ucer, Charles P Mercado, Howard H Gu, and Fusun Kilic. 2014. “Effect of Serotonin on Platelet Function in Cocaine Exposed Blood.” *Scientific Reports* 4: 5945. doi:10.1038/srep05945.

Ziu, Endrit, Charles P Mercado, Yicong Li, Preeti Singh, Billow a Ahmed, Samuel Freyaldenhoven, Shelly Lensing, Jerry Ware, and Fusun Kilic. 2012. “Down-Regulation of the Serotonin Transporter in Hyperreactive Platelets Counteracts the pro-Thrombotic Effect of Serotonin.” *Journal of Molecular and Cellular Cardiology* 52 (5). Elsevier B.V.: 1112–21. doi:10.1016/j.yjmcc.2012.02.004.

Zotz, Rainer B., Michael Klein, Hans Peter Dauben, Christiane Moser, Emmeran Gams, and Rüdiger E. Scharf. 2000. “Prospective Analysis after Coronary-Artery Bypass Grafting: Platelet GP IIIa Polymorphism (HPA-1b/Pl A2) Is a Risk Factor for Bypass Occlusion, Myocardial Infarction, and Death.” *Thrombosis and Haemostasis* 83 (3). Schattauer Publishers: 404–7.

Zurawski, Zack, Shelagh Rodriguez, Karren Hyde, Simon Alford, and Heidi E Hamm. 2016. “Gβγ Binds to the Extreme C Terminus of SNAP25 to Mediate the Action of Gi/o-Coupled G Protein-Coupled Receptors.” *Molecular Pharmacology* 89 (1): 75–83. doi:10.1124/mol.115.101600.