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COMMUNICATION BETWEEN 5-HT AND SMALL GTPases

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Abstract

Advances over the past decade have improved our understanding of the serotonin (5-HT) biology outside the central nervous system specifically the molecular mechanisms of serotonergic signaling in association with small GTPases. It is now recognized that the communication between 5-HT and GTPases plays important roles in peripheral tissues, vascular cells and are involved in coagulation, hypertension, inflammation, healing and protection. Furthermore, 5-HT receptors as heterotrimeric GTP-binding protein-coupled receptors act as effector protein on the small GTPases. Therefore, the antagonists or agonists of the effector proteins of small GTPases could be useful therapeutic agents for the treatment of several diseases and disorders.

INTRODUCTION

Serotonin (5-hydroxytryptamine (5-HT)) was first noted by Dr. V. Ersparmer in 1935 in enterochromaffin cells and labeled as *enteramine* for its ability to contract the intestines. A decade later, the term “serotonin” was used by Rapport and colleagues when it was discovered in the blood serum and characterized as an arterial constrictor [1]. A detailed history on the discovery and characterization of 5-HT is reviewed by Sjoerdsma and Palfreyman in 1990 [2]. Currently, it is accepted that 5-HT acts differently at different locations (organ-systems) in a concentration-dependent manner. For instance, as a neurotransmitter, 5-HT is related to a variety of mood disorders and psychiatric diseases whereas in the periphery, it can act as a mitogen where it influences the rate of cell division, as a developmental signal early in rodent embryogenesis, and as a vasoconstrictor in the circulation system. Genetic and pharmacological disruption of 5-HT-signaling has been attributed as a cause of maternal and prenatal morbidity and mortality via mediating high blood pressure and neuroanatomical abnormalities, respectively [3–5]. The actions of 5-HT are mediated by different types of receptors and terminated by a single 5-HT transporter (SERT) [6–9].

SERT is a member of the Na⁺/Cl[−]-dependent solute carrier 6 (SLC6) family, which includes transporters of norepinephrine, dopamine, γ -aminobutyric acid, glycine, proline, creatine, and betaine [10]. The detailed mechanism by which SERT activity depends upon transmembrane ion gradients is still not understood; however, the X-ray crystal structure of LeuT [11], a prokaryotic amino acid transporter and homolog of mammalian

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neurotransmitter transporters, has significantly elevated our understanding on SERT and its interaction with 5-HT.

5-HT is a derivative of the amino acid, L-tryptophan. Initially, tryptophan hydroxylase (TPH), using tetrahydrobiopterine as cofactor, adds a hydroxyl group into the lipophilic ring of tryptophan. This irreversible reaction in 5-HT synthesis is followed by the removal of the carboxyl group by L-amino acid decarboxylase and leaving the amine group on 5-hydroxytryptophan, (5-HT). Previous studies comprehensively described the importance of amine group in the covalent interaction between 5-HT and the proteins in transamidation reactions [12]. 5-HT is metabolized in the liver and eventually excreted as 5-hydroxyindoleacetic acid (5-HIAA) following the decarboxylation of amine group.

In a series of elegant studies, Walther and colleagues discovered a gene encoding the second isoform of TPH enzyme, TPH2, which is localized in the raphe nuclei and axonal projections of brain and synthesized 5-HT in brain and the central nervous system [13]. Although, TPH2 is also expressed by 5-HT neurons in the gut, TPH1 is responsible for the production of 5-HT outside the central nervous system such as the intestinal enterochromaffin cells [13]. Spatial and temporal expressions differentiate the two isoforms of the enzyme and their differences are further emphasized by their distinct kinetic properties.

Peripheral 5-HT produced in the enterochromaffin cells is secreted in the bloodstream and taken up by the platelet plasma membrane SERT. 5-HT is then stored in the dense granules through a second membrane transporter, the vesicular monoamine transporter (VMAT2) [14]. The dense granules act as storage pool of 5-HT in the circulation, which is critical in hemostasis and thrombosis.

5-HT modulates a myriad of physiologic processes in the brain and peripheral organs exhibiting a pleiotropic behavior. This behavior reflects the presence of multiple receptors, the heterogeneity of their distribution, the signaling pathway activated, as well as the opposing actions that can be elicited, i.e. ability to mediate both excitatory and inhibitory transmission in certain target organs. The spatial distribution of the 5-HT receptors reflects a multitude of effects that is triggered by 5-HT stimulation [15]. 5-HT has the capacity to regulate physiologic processes at various levels at multiple steps through different opposing mechanisms [16].

5-HT receptors are post-transcriptionally modified to yield different isoforms, and hence differences in properties and activities [17]. Activation of 5-HT receptors initiates signaling through different cascades. One of these is the alteration of cGMP content with 5-HT N-acetyltransferase activity in pineal glands [18]. Later on, studies of axon growth cones and wound healing have demonstrated that signals created by 5-HT result in rearrangements of the myosin-actin cytoskeleton and other actin-binding protein bundles [19–21]. Pharmacologic intervention utilizing 5-HT receptors has widened understanding of neurogenesis and neural survival in adults, axonal growth and regulation of neuronal morphology [18–21].

However, small GTP-binding proteins (GTPases) have long been implicated in the initiation of neuronal outgrowth, polarity and axonal debranching, and playing pivotal role on neuronal morphogenesis via directing the cytoskeletal organization and membrane trafficking [22,23]. Recent studies brought 5-HT and GTPases in neuronal cells to understand the involvement of 5-HT in these processes via small GTPases [24–27]. A good example for these studies was by Fricker et al. [24] who suggested that Gai-Rap1-Src-STAT-3 pathway plays a major role in 5-HT_{1A} receptor-induced neurite outgrowth, in which the activation of the receptor leads to profound morphological changes that increases

survival and neurite outgrowth [24]. The coupling of 5-HT₇ receptor with Rho GTPase leads to changes in morphology and motility of neural cells, specifically induction of stress fiber formation leading to growth cone collapse and neurite retraction [25].

While 5-HT is well studied as a neurotransmitter, it is not surprising that most of the 5-HT receptors are expressed outside of CNS [16]. In muscle cells, it was shown that 5-HT-mediated signal activates p21 activating kinase (PAK), which in turn phosphorylates vimentin on the serine residue at position 56 [28]. Following phosphorylation, the curved filamentous structure of vimentin undergoes reorganization and straightens [29]. Vimentin is the major structural component of the intermediate filaments in cells of mesenchymal origin including platelets [30]. In exploring the impact of plasma level 5-HT on platelets, we tested if SERT utilizes the vimentin network in trafficking between intracellular locations and the plasma membrane [31]. We reported that plasma 5-HT mediates phosphorylation of vimentin, which uncurls the filamentous structure of vimentin, enhances vimentin-SERT association and promotes the internalization of SERT on a “paralyzed” vimentin network [31]. One other signaling cascade activated by 5-HT-receptor coupling was reported as facilitating the covalent modification of cytosolic small GTPases with 5-HT via phospholipase C which involved in the production of diacylglycerol (DAG) and inositol 3-phosphate (IP3) and increases intracellular Ca⁺⁺ concentration and downstream pathway [12]. The physical interaction between 5-HT and GTPases is highlighted by transglutaminase (TGase), now referred to as serotonylation [12], which is a downstream effect of a G-protein coupled signal transduction.

5-HT-receptor coupling elevates the intracellular Ca⁺⁺ concentration that activates a cytoplasmic enzyme, TGase, which belongs to an important family of enzymes including factor XIII-A (FXIII-A), in coagulation cascade [32]. TGases are expressed in several tissues including blood and platelets and play important functions such as blood clotting, tissue remodeling in cancer and wound repair, skin maintenance, and phagocytosis to name a few. Main functions of this group of enzymes are to catalyze three types of post translational modifications - hydrolysis, transamidation and esterification. These functions are essential for skin barrier maintenance and extracellular matrix remodeling. TGases have been involved in many diseases such as gluten sensitivity and inflammatory bowel syndrome. The review article by Lismaa et al. [33] included TGases and knock out animal models extensively. Basically, TGases catalyze the formation of a covalent cross link between glutamine and lysine residues of two proteins. This covalent linkage is strong enough to bring the TGase-linked proteins in an aggregated form inside cells during proteolysis. Each TGase has different affinity and specificity thus allowing them to be identified from different kinetic parameters even though they might recognize the same substrate. Although TGases are involved in many different processes we will focus on particular reaction where a primary amine like 5-HT is covalently attached to proteins, specifically small GTPases. Covalent linkage of 5-HT in return changes the functional state of those proteins allowing signaling pathway to be conveyed downstream.

Dale and colleagues pioneered in showing 5-HT as a substrate in the transamidation of von Willebrand factor (vWF) and other proaggregatory molecules [34]. Termed as COAT-platelets upon dual stimulation with collagen and thrombin, the role of this subpopulation of platelets has been investigated in stroke [35] and other bleeding diathesis [36]. These studies branched 5-HT out as a mere neurotransmitter in the CNS. Exerting its effect through TGase, and in concert with small GTPases, 5-HT has gained significance in the physiology and pathophysiology of many relevant diseases.

Human cells contain more than 100 members of small GTPase superfamily, with a molecular mass between 20–30 kDa, and members of this large family are localized to

distinct membrane-bound compartments [37]. The structural basis of the small GTPases is unique to the GDP and GTP-bound conformations which is called “GTP/GDP hydrolysis region.” These proteins are classified into five families: Ras, Rho, Rab, ADP-ribosylation factor (ARF), and Ran family of proteins in which the cycle of their GTP binding and hydrolysis have been postulated to ensure directionally in the gene expression [38], cytoskeletal reorganization [39,40], vesicle trafficking [41], microtubule organization, the spatial and temporal organization of the eukaryotic cell [42], and nucleocytoplasmic transport [43], respectively. Small GTPases have highly conserved domains required for GTP-binding, GTP/GDP exchange, and GTP hydrolysis that are essential for their proper functions [41]. Therefore they could be thought of as molecular clocks or biotimers (temporal expression) which regulate critical biological processes such as initiation and termination of cell signaling and at the same time as a spatial determinant for cellular functions. One other common feature of small GTPases is their membrane association by using the covalently attached carboxyl terminal lipids such as palmitoyl, farnesyl, geranylgeranyl group(s) [38]. Despite all these efforts, the factors modulate the GTP-binding, hydrolysis of small GTPases or even factors involved in their interactions with their effectors have not been fully identified yet. However, serotonylation of small GTPases via elevated 5-HT level have been of recent medical interest.

Studies in the 1990's by Karniguian et al., identified the involvement and role of small GTP-binding proteins in human platelets [44]. A decade ago, Sirakawa *et al.*, [45] had reported the involvement of Rab proteins in Ca⁺⁺ induced α -granules secretion in platelets. However, recently the requirement for intracellular 5-HT together with Ca²⁺ for the release of α -granules were nicely demonstrated by the studies using isolated platelets from mice lacking the gene for TPH [12]. Later on, we developed a novel mouse model system in which blood 5-HT was chronically elevated by an osmotic mini-pump [46]. Platelets from 5-HT-infused mice aggregated more readily and their aggregation rate were significantly altered by the inhibitors of SERT, dense granules-specific VMAT or platelet-specific 5-HT receptor [46]. These studies indicate that 5-HT-stimulation accelerated the exocytosis of α -granules, which secrete their ingredients, the procoagulant molecules, into the plasma [47]. Therefore, these findings confirm an important regulatory role for elevated extracellular 5-HT in the exocytosis of α -granules.

In the pancreas, Rab3 and Rab27a have been recently shown to facilitate the secretion of insulin granules from beta-islet cells of the pancreas [48]. Constitutive expression of these GTPases promotes the secretion of insulin, whereas inhibition of serotonylation via GTPases or enhanced proteasomal degradation impedes insulin secretion. While its clinical relevance is yet to be elucidated, this mechanism highlights a new aspect in the pathophysiology of diabetes mellitus. In a similar manner, TGase-dependent RhoA serotonylation and consequent activation of Rho kinase in pulmonary vascular smooth muscle cells is pivotal in the etiology of pulmonary hypertension [49,50]. Elevated 5-HT renders RhoA constitutively active, whereas the depletion of GTP-RhoA activates downstream Akt signaling which leads to decreased smooth muscle cell contraction. As such, the accumulation of hypertrophied cells induced by high 5-HT contributes to pulmonary hypertension as exhibited by rats under hypoxic conditions [50] and in vascular smooth muscle [51].

The action of 5-HT through coupling to its receptors produces the serotonergic signal which is correlated with 5-HT level of extracellular surface. However, the 5-HT uptake capacity of cells depends on the number of SERT molecules on the plasma membrane. Our earlier studies established that surface SERT expression in platelets shows a biphasic relationship to extracellular 5-HT concentrations [52]. Specifically, in platelets, plasma membrane SERT levels and 5-HT uptake by SERT initially rise as plasma 5-HT levels are increased, but then fall below normal as the plasma 5-HT level continues to rise. To understand the biphasic

relationship between 5-HT-SERT and the mechanism by which elevated plasma 5-HT accomplishes this task further, we studied recycling of SERT in platelets under high level of plasma 5-HT [31,46,47,52,53]. Indeed, our *in vivo*, 5-HT-infused mice model [46,47] and *in vitro*, isolated platelets [31,52] studies confirm a dynamic relationship between extracellular 5-HT elevation, loss of surface SERT, and depletion of platelet 5-HT. It appears that elevated plasma 5-HT alters the trafficking pathways in platelets to down-regulate the density of SERT molecules on the plasma membrane. When exposed to high level of 5-HT, platelet Rab4, is first transamidated by 5-HT and then forms Rab4-GTP, which binds and blocks the translocation of SERT to the plasma membrane [50]. Concurrently, elevated 5-HT mediates phosphorylation of vimentin to permit the phosphovimentin-SERT association that accelerates SERT internalization [31]. These findings point to a novel role for extracellular 5-HT on the C-terminus of SERT in regulating its auto-expression in the plasma membrane. Interestingly, the C-terminus appears to contain the domain that associates with Rab4 [53] and vimentin in response to elevated 5-HT level [49].

CONCLUSION

In summary, 5-HT was linked to the functional characterization of small GTPases more specifically Rab4-GTP accelerates the exocytosis of α -granules but also associates with SERT and tethers the translocation of SERT to the plasma membrane (Fig. 1). Considering the role of 5-HT in platelet aggregation, it is possible that a loss of platelet SERT coupled with elevated plasma 5-HT may play a significant role in the cluster of cardiovascular diseases including diabetes, metabolic syndrome, atherosclerosis, and peripheral arterial disease that are thought to reflect a prothrombotic state susceptible to coagulation and thrombotic events. The development of possible antithrombotic therapies for patients with cardiovascular disease has focused on reducing these risk factors rather than on promoting endogenous mechanisms of anti-thrombosis. In this regard, therapies designed to promote the expression of SERT on the platelet surface may represent a novel approach to alleviating thrombotic events by taking advantage of an endogenous protective mechanism already in place to reduce plasma 5-HT. Other circumstances, such as in the CNS where the level of 5-HT is altered and serotonylates the small GTPases, in addition to platelet aggregation, are eagerly awaited.

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Fig. 1.

5-HT in platelet physiology. The endogenously expressed Rab4 is located in CHO cells expressing YFP-SERT via labeling the cells with Rab4 antibody followed by staining with Texas Red conjugated rabbit IgG. To contrast the localization of SERT and Rab4, the overlaid images are presented with Rab4 signal pseudocolored in red. (A) In the absence of 5-HT, a strong signal of YFP-SERT on the plasma membrane and a less pronounced intracellular signal are seen as shown in the image. Rab4 localizes primarily to intracellular structures, consistent with its known endosomal localization. The merged images emphasize the separate distributions of YFP-SERT and Rab4 [53]. The schematic diagram illustrates the circulating platelets, SERT (dark yellow) within the plasma membrane (black line) transports 5-HT from the blood plasma into the platelet cytoplasm. 5-HT is stored within the platelet in dense granules (green circles); α -granules (light blue circles) store procoagulant molecules. Both types of granules can release their contents into the blood plasma under appropriate platelet stimulation such as elevated 5-HT concentration in blood plasma [46,47,52,53]. The internalization and recycling of SERT through platelet trafficking machinery provides a means of regulating 5-HT uptake. B. In contrast, when 5-HT concentration was increased to 100 mM, extensive co-localization emerges between YFP-SERT and endogenous Rab4. These results suggest that 5-HT pretreatment facilitates the co-localization of Rab4 with SERT at intracellular locations. However, our studies support the biphasic relationship between extracellular 5-HT concentration and the density of SERT on the plasma membrane [31,46,47,52,53]. It is reported that upon elevation of 5-HT concentration in the plasma, the uptake rate of 5-HT increases, as small vesicles deliver SERT to the platelet surface [52]. These findings are illustrated in schematic diagram as the rise in cytoplasmic 5-HT promotes the transamination of Rab4 (schematized in pink), thereby activating Rab4 to Rab4-GTP and promoting its association with SERT; the SERT-Rab4-GTP interaction prevents the trafficking of SERT to the plasma membrane [53]. The rise in cytoplasmic 5-HT also promotes exocytosis of dense granules [46,47] and α -granules [12,45]. The internalization of SERT is also promoted as 5-HT activates PAK, which phosphorylates vimentin [31], causing its association with SERT. The indicated roles of Rab4-GTP and vimentin culminate in the reduced surface expression of SERT, which delimits further intake of 5-HT, restoring cytoplasmic levels to their initial values.