Loss of Serotonin Transporter Function Alters ADP-mediated Glycoprotein αIIbβ3 Activation through Dysregulation of the 5-HT2A Receptor*

Received for publication, May 6, 2016, and in revised form, July 14, 2016 Published, JBC Papers in Press, July 15, 2016, DOI 10.1074/jbc.M116.736983

Kendra H. Oliver, Matthew T. Duvernay, Heidi E. Hamm1, and Ana M. D. Carneiro2

From the Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

Reduced platelet aggregation and a mild bleeding phenotype have been observed in patients chronically taking selective serotonin reuptake inhibitors (SSRIs). However, it remains unclear how SSRIs, which inhibit the plasma membrane serotonin transporter (SERT), modulate hemostasis. Here, we examine how sustained inhibition of SERT activity alters serotonergic signaling and influences platelet activation and hemostasis. Pharmaceutical blockade (citalopram dosing) or genetic ablation (SERT−/−) of SERT function in vivo led to reduced serotonin (5-hydroxytryptamine (5-HT)) blood levels that paralleled a mild bleeding phenotype in mice. Transfusion of wild-type platelets to SERT−/− mice normalized bleeding times to wild-type levels, suggesting that loss of SERTs causes a deficiency in platelet activation. Although SERT−/− platelets displayed no difference in P-selectin or αIIbβ3 activation upon stimulation with thrombin, ADP-mediated αIIbβ3 activation is reduced in SERT−/− platelets. Additionally, synergistic potentiation of αIIbβ3 activation by ADP and 5-HT is lost in SERT−/− platelets. Acute treatment of wild-type platelets with 5-HT2A receptor (5-HT2AR) antagonists or SSRIs revealed that functional 5-HT2ARs, not SERTs, are necessary for the synergistic activation of αIIbβ3 by dual 5-HT/ADP stimulation. Pharmacological studies using radiolabeled guanosine 5′-3-O-([35S]thio)triphosphate and [3H]ketanserin revealed that platelets isolated from SERT−/− or citalopram-treated mice have reduced activation of G-proteins coupled to 5-HT2ARs and receptor surface expression. Taken together, these data demonstrate that sustained SERT loss of function reduces 5-HT2AR surface expression that is critical for the synergistic activation of αIIbβ3 by 5-HT and ADP. These results highlight an antiplatelet strategy centered on blocking or desensitizing 5-HT2AR to attenuate ADP-mediated αIIbβ3 activation.

In the periphery, serotonin (5-HT)3 is produced by enterochromaffin cells in the gastrointestinal tract, released into the plasma, and quickly taken up by platelets via the plasma membrane serotonin transporter (SERT). Following uptake, 5-HT is stored in dense granules by the actions of the vesicular monoamine transporter 2 (1–4). Chronic inhibition of SERT through selective serotonin reuptake inhibitors (SSRIs) (e.g. citalopram and paroxetine) leads to dramatically reduced platelet 5-HT granule content (5, 6), altering peripheral 5-HT homeostasis and potentially modifying multiple physiological processes including hemostasis (7–10). Clinically, increased bleeding risk has been observed in patients taking SSRIs, and platelet aggregation is disrupted (5, 11). Here, we have characterized a similar effect in two distinct mouse models of lost SERT function, suggesting that sustained loss of SERT function influences hemostasis.

Platelet dense granules contain 5-HT along with other platelet agonists including adenosine diphosphate (ADP), thromboxane (TXA2), and histamine. Appropriate platelet activation depends on the timely release of these factors (4, 5, 12). Platelet aggregation is crucial early in thrombus formation (4, 5, 13). Aggregation, which is the bridging of platelet-platelet contacts, requires a conformational alteration in the glycoprotein αIIbβ3, leading to its activation and fibrinogen binding. 5-HT has been shown to enhance aggregation in a 5-HT2A receptor (5-HT2AR)-dependent manner (4, 14–17). The 5-HT2AR is the only serotonergic receptor found on platelets and potentiates platelet responses to weak agonists like ADP (18). Subthreshold concentrations of two different platelet agonists can exert a synergistic effect on platelet activation. One example includes dual ADP and 5-HT activation leading to increases in cytosolic [Ca2+] (13). However, the role of 5-HT during in vivo hemostasis remains unclear, particularly in the context of chronic SERT inhibition.

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. Acute and chronic blockage of SERT function results in distinct scenarios regarding the effects on 5-HT homeostasis. Acute inhibition of SERT blocks the amount of

*This work was supported by National Institutes of Health Grant MH090256 (to A. M. D. C.); Clinical and Translational Science Awards UL1TR000445, KL2TR000446, and TL1TR000447 (to Vanderbilt); and Molecular Libraries Probe Production Centers Network Grant U54MH084659 (to H. E. H.) and by American Heart Association Predoctoral Fellowship Award 14PRE19640007 (to K. H. O.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence may be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, 442 Robinson Research Bldg., 23rd Ave. South at Pierce, Nashville, TN 37232-6600. E-mail: heidihamm@vanderbilt.edu.

2 To whom correspondence may be addressed: Dept. of Pharmacology, Vanderbilt University Medical Center, 461 Preston Research Bldg., 23rd Ave. South at Pierce, Nashville, TN 37232-6600. E-mail: ana.carneiro@vanderbilt.edu.

The abbreviations used are: 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; Cit, citalopram; 5-HT2AR, 5-HT2AR receptor; SERT−/−, wild-type mice; SERT−/−, serotonin transporter knock-out mice; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminoethylindole; GTPγS, guanosine 5′-3-O-([35S]thio)triphosphate; ANOVA, analysis of variance.
5-HT carried into the cytosol during platelet activation, whereas chronic blockade of SERT slowly depletes granule 5-HT, resulting in loss of 5-HT secretion and 5-HT signaling during platelet activation. Some have shown that acute SERT-mediated 5-HT uptake modulates platelet function (20, 21) in part through the transamination of small GTPase proteins important for platelet α-granule exocytosis. However, the effects of chronic loss of SERT function on platelet activation remain unresolved.

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 hypothesized that the bleeding effects noted with the disruption of SERT function are due to altered 5-HT2A signaling during platelet activation. Indeed, we found that mice 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 5-HT2A signaling and surface expression.

Results

Decreased 5-HT Content in Platelets Isolated from SERT−− and Citalopram-treated Mice Parallels Bleeding Phenotype

Mice Treated with Citalopram for 6 Days Have Reduced Whole Blood 5-HT Levels and Increased Tail Bleed Time—To determine whether SSRIs alter blood 5-HT homeostasis, mice were exposed to citalopram-treated water for 6 days, 1 day beyond the lifetime of a circulating platelet in a mouse (22). We used high performance liquid chromatography (HPLC) to measure whole blood levels of 5-HT and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA). Whole blood from wild-type mice undergoing citalopram (Cit) treatment showed reduced 5-HT levels as compared with water-treated controls (Fig. 1A). No significant difference in 5-HIAA levels was found between water- and Cit-treated samples (Fig. 1B). To determine whether citalopram treatment alters hemostasis, we performed a tail bleed assay. We found that citalopram-treated mice exhibited a significantly longer tail bleed time as compared with water-treated controls (Fig. 1C). These findings indicate a phenotypic association between reduced 5-HT levels and increased bleeding time in mice treated with SSRIs.

Reduced 5-HT Whole Blood Levels and Increased Tail Bleed Times in SERT−−—We observed very little measurable 5-HT in SERT−− platelets (Fig. 1D). Similar to whole blood samples isolated from citalopram-treated mice, there was no significant difference in 5-HIAA between SERT+/+ and SERT−− mice (Fig. 1E). We found that bleeding time was significantly increased in SERT−− mice as compared with SERT+/+ (Fig. 1F). These data show that SERT function modulates whole blood 5-HT levels and that SERT inhibition is associated with increased bleeding time.

Bleeding in SERT−− Mice Is Rescued by Transfusion of SERT+/+ Platelets

As 5-HT potentiates platelet activation and previous studies have shown that SERT function alters platelet aggregation (5), 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 (Fig. 2A). To determine whether addition of SERT\(^{+/+}\) platelets rescued the SERT\(^{-/-}\) bleeding phenotype, mice were transfused with gel-filtered platelets resuspended in saline via jugular vein injection. A tail bleed was performed 3 min after 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). Tail bleed time in saline-transfused SERT\(^{-/-}\) was significantly increased compared with SERT\(^{+/+}\) (p = 0.0039 (***) and was rescued to SERT\(^{+/+}\) bleeding time following platelet transfusion (saline-infused SERT\(^{-/-}\) versus platelet-infused SERT\(^{-/-}\), \(p = 0.002\) (#); saline-infused SERT\(^{+/+}\) versus platelet-infused SERT\(^{-/-}\), \(p = 0.9893\); saline-infused SERT\(^{-/-}\) versus platelet-infused SERT\(^{-/-}\), \(p = 0.9837\); Tukey’s multiple comparison test following two-way ANOVA). Data are shown with median, range, and individual points. Error bars represent the range of the data set.

**5-HT\(_{2A}\)R Surface Expression Reduced with Lost SERT Function**

5-HT\(_{2A}\)R stimulation enhances ADP-mediated αIibβ3 activation

To examine the role of 5-HT in ADP-mediated platelet activation, we distinguished the role of two plasma membrane proteins that are responsive to 5-HT 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 5-[\(^{3}\)H]HT platelet uptake with an IC\(_{50}\) of 1.5 \(\times 10^{-11}\) and 1.3 \(\times 10^{-11}\), respectively (Fig. 4A). Platelet activation with 1 and 10 \(\mu M\) ADP was examined with the addition of either 5-HT\(_{2A}\)R antagonists (ketanserin and methysergide) or SERT inhibitors (citalopram and paroxetine) 5 min before ADP activation. Both ketanserin and methysergide treatment significantly reduced ADP-mediated activation at 1 \(\mu M\) (Fig. 4B). Acute inhibition of 5-HT uptake by SERT with citalopram (10 \(\mu M\)) or paroxetine (10 \(\mu M\)) had no effect on ADP-mediated αIibβ3 activation despite effectively blocking SERT-mediated 5-HT uptake. These data reveal a role for 5-HT\(_{2A}\)R activation, but not 5-HT 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 5-HT and ADP receptor stimulation. 5-HT alone was unable to elicit activation of the αIibβ3 integrin at either 1 or 10 \(\mu M\) concentration in either SERT\(^{+/+}\) or SERT\(^{-/-}\) platelets (Fig. 4C). However, using a submaximal concentration of ADP (1 \(\mu M\)), we observed that 10 \(\mu M\) 5-HT potentiated the ADP-mediated activation of αIibβ3 in SERT\(^{+/+}\) platelets. 5-HT was unable to potentiate ADP-mediated platelet activation in SERT\(^{-/-}\) platelets (Fig. 4D). In a separate experiment, we found that incubation with ketanserin prevented the 5-HT-dependent potentiation of αIibβ3 activation in SERT\(^{+/+}\) platelets (Fig. 4E). 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$^{-/-}$ platelets.

**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 percentage of total [³H]ADP binding blocked in the presence of 2,2'-pyridilisotobienyl, a P2Y antagonist (Fig. 5A). Competitive inhibition with MRS2179 (P2Y$_1$-specific antagonist) and ticagrelor (P2Y$_{12}$-specific antagonist) was used to determine relative receptor binding in SERT$^{+/+}$ and SERT$^{-/-}$ platelet membrane preparations (Fig. 5, C and D). P2Y$_1$ accounted for ~46.42% of [³H]ADP binding, whereas P2Y$_{12}$ accounted for ~55.89% of total [³H]ADP binding. Inhibition at 1 μM for both MRS2179 and ticagrelor was used to determine percentage of [³H]ADP binding to P2Y$_1$ in platelet membranes. We found no statistically significant difference in either P2Y$_{12}$ or P2Y$_1$ binding between SERT$^{+/+}$ and SERT$^{-/-}$ membrane preparations. Additionally, we measured whole blood ADP levels using an ELISA and observed no difference between SERT$^{+/+}$ and SERT$^{-/-}$ preparations (Fig. 5E). 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]GTPγS incorporation to 5-HT$_{2A}$R following activation with 1-[2,5-dimethoxy-4-iodophenyl]-2-amino propane (DOI), a 5-HT$_{2A}$R-specific agonist. We observed a significant reduction in [³⁵S]GTPγS incorporation to SERT$^{-/-}$ membrane preparations as compared with SERT$^{+/+}$ (Fig. 5F; n = 8; p = 0.0002, Mann-Whitney test). To determine whether 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 ($B_{max}$) of 5-HT$_{2A}$R (Fig. 5G). SERT$^{-/-}$ platelets showed significantly reduced $B_{max}$ for 5-HT$_{2A}$R surface levels as compared with those observed in SERT$^{+/+}$ platelets (Fig. 5H). There was no significant difference in $K_d$ between
SERT\textsuperscript{+/+} and SERT\textsuperscript{−/−} \textsuperscript{3}H]ketanserin binding (SERT\textsuperscript{+/+} median, 7.32 nM; SERT\textsuperscript{−/−} median, 6.71 nM; \(p = 0.134\), Mann-Whitney test). These results show reduced 5-HT\textsubscript{2A}R signaling in SERT\textsuperscript{−/−} platelets, likely resulting from reduced 5-HT\textsubscript{2A}R surface expression.

**Citalopram-treated Platelets Recapitulate the SERT\textsuperscript{−/−} Platelet Phenotype: Citalopram-treated Platelets Have Reduced ADP-mediated \(\alpha_{IIb}\beta_{3}\) Activation and Reduced 5-HT\textsubscript{2A}R Surface Expression**

To determine whether we could recapitulate the loss of 5-HT\textsubscript{2A}R surface expression using an alternative model of lost SERT function, we treated mice chronically with citalopram for 6 days. Similar to SERT\textsuperscript{−/−} platelets, citalopram-treated platelets showed reduced ADP-dependent \(\alpha_{IIb}\beta_{3}\) activation compared with water-treated controls (Fig. 6A). In control water-treated mouse platelets, \(\alpha_{IIb}\beta_{3}\) activation by ADP was enhanced by 5-HT, whereas 5-HT potentiation of ADP signaling was absent in citalopram-treated mouse platelets (Fig. 6B). Furthermore, 5-HT\textsubscript{2A}R levels were reduced in platelets isolated from citalopram-treated mice (Fig. 6C). These findings establish that 6-day treatment with SSRI is sufficient to reduce platelet expression of 5-HT\textsubscript{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\textsubscript{2A}R surface expression and platelet function in SERT\textsuperscript{−/−} mice is not due to loss of the SERT protein but rather results from sustained loss of SERT function. Finally, to test whether premature exposure to 5-HT could alter ADP-mediated \(\alpha_{IIb}\beta_{3}\) activation, wild-type platelets were incubated with serotonin (10 \mu M) for 15 min, washed, and resuspended. They were then activated with 10 \mu M ADP, and JON/A binding was measured (Fig. 6D). We found that platelets pretreated with 5-HT for 15 min lost ADP-mediated \(\alpha_{IIb}\beta_{3}\) activation similarly to both SERT\textsuperscript{−/−} and citalopram-treated platelets. These results suggest that the pre-activation or possible desensitization of the 5-HT\textsubscript{2A}R reduces platelet ADP-mediated \(\alpha_{IIb}\beta_{3}\) activation.

**Discussion**

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 (4, 23). 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\textsubscript{2A}R surface expression, leading to reduced ADP-mediated \(\alpha_{IIb}\beta_{3}\) activation. Therefore, our findings support a novel mechanism for the reduced aggregation in SSRI-treated patients:

\[
5-HT_{2A}R \text{ Surface Expression Reduced with Lost SERT Function}
\]

**FIGURE 4.** ADP requires 5-HT\textsubscript{2A} Receptor activation for ADP/5-HT-mediated \(\alpha_{IIb}\beta_{3}\) synergy, which is disrupted in SERT\textsuperscript{−/−} platelets. A, isolated platelet uptake of 5-[\textsuperscript{3}H]HT blocked with either citalopram or paroxetine (log[inhibition]) versus normalized response-variable slope: citalopram robust sum of squares, 20.58; paroxetine robust sum of squares, 22.92. B, JON/A binding following 15-min ADP activation was measured using geometric mean fluorescence intensity (gMFI) following a 5-min preincubation with either 5-HT\textsubscript{2A} receptor antagonist ketanserin or methysergide or selective serotonin reuptake inhibitor citalopram or paroxetine on the same platelet preparation (n = 6). Both ketanserin and methysergide treatment significantly reduced ADP \(\alpha_{IIb}\beta_{3}\) activation at both 1 \mu M (ketanserin, \(p = 0.0409\) (**); methysergide, \(p = 0.0041\) (**)) and 10 \mu M (ketanserin, \(p = 0.0187\) (*); methysergide, \(p < 0.0001\) (****)); Dunnett’s test with two-way repeated measures 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 5-HT alone (C), ADP 1 \mu M + 5-HT (\(p < 0.0001\) (****)) (D), or ADP/5-HT following a 5-min incubation with ketanserin (Sidak’s test following two-way ANOVA) (E). Data are shown with median, range, and individual points. Error bars represent the range of the data set.
5-HT_{2A}R Surface Expression Reduced with Lost SERT Function

FIGURE 5. SERT^+/− platelets have reduced 5-HT_{2A}R receptor surface expression but no change in P_{Y_{12}} and P_{Y_{10}}. A and B, competitive inhibition with 2,2′-pyridylisatogen tosylate (PIT) (dual P_{Y_{1}} and P_{Y_{3}} antagonist; IC_{50} = 7.80 × 10^{-8}, 83.71%), MRS2179 (P_{Y_{1}}-specific antagonist; IC_{50} = 2.08 × 10^{-9}, 46.42%), and ticagrelor (Ticag.) (P_{Y_{12}}-specific antagonist; IC_{50} = 1.25 × 10^{-7}, 55.89%) was used with [3H]ADP to determine relative binding of receptors. Single point inhibition was used to determine percentage [3H]ADP binding for P_{Y_{12}} (n = 9 SERT^+/−; n = 7 SERT^−/−; p = 0.4592, Mann-Whitney test) (C) and P_{Y_{1}} receptors (n = 9 SERT^+/−; n = 7 SERT^−/−; p = 0.9646, Mann-Whitney test) (D) in SERT^+/− and SERT^−/− platelets. Data are shown with median, range, and individual points. E, ADP whole blood levels were determined by ELISA (n = 6; p = 0.675, Mann-Whitney test). F, [35S]GTPγS incorporation was used to measure 5-HT_{2A}R receptor-specific G-protein activation in purified platelet membranes (n = 8; p = 0.0002 (**), Mann-Whitney test). G, 5-HT_{2A}R specific saturation curve with [3H]ketanserin is shown for both SERT^+/− and SERT^−/− platelets. H, calculated B_{max} values are significantly reduced in SERT^−/− platelets (n = 5 SERT^+/−; n = 6 SERT^−/−; p = 0.0173 (*), Mann-Whitney test). There was no significant difference in K_{D} between SERT^+/− and SERT^−/− [3H]ketanserin binding (SERT^+/− median, 7.32 nM; SERT^−/− median, 6.71 nM; p = 0.134, Mann-Whitney test). Error bars represent the range of the data set.

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 blocking of SERT function and 5-HT uptake, 2) depletion of granule 5-HT and loss of 5-HT secretion during activation, and 3) increased extracellular 5-HT levels. Many studies concluding that acute uptake of 5-HT contributes to platelet function used SSRIs as tool compounds to acutely inhibit 5-HT uptake. However, these studies utilized SSRIs at high concentrations (>10 μM) that are known to exert off-target effects (e.g. the σ1 receptor) (11, 24). 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 (10 nM; Fig. 4). Here, we observed no change in ADP-mediated αIIbβ3 activation following acute SSRi treatment, suggesting that acute 5-HT 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 5-HT by SERT leads to a receptor-independent signaling pathway via the posttranslational modification of intracellular proteins (20). This process, known as serotonylation, involves the covalent attachment of 5-HT molecules to proteins mediated by the enzyme transglutaminase, resulting in transamination of small GTPases important for platelet activation (20–25). In our experiments, we demonstrated no difference in P-selectin antibody as a measure for α-granule exocytosis in SERT^+/− platelets following thrombin activation (Fig. 3B). 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 5-HT levels, one would expect that chronic SERT inhibition would increase plasma concentrations of 5-HT as suggested by the effects of SSRIs in the CNS (26, 27). However, because 5-HT_{2A}R stimulation by 5-HT alone does not lead to platelet activation, loss of SERT function likely leads to local increases in plasma 5-HT levels within the portal vein and indirectly triggers internalization of the 5-HT_{2A}R. It has been demonstrated that 5-HT induces internalization of 5-HT_{2A}R in a β-arrestin-dependent manner within 30 min of initial exposure (28, 29). 5-HT_{2A}R
Desensitization has been shown in the brain following prolonged exposure to elevated levels of serotonin in citalopram-treated mice (30, 31). However, it has yet to be validated whether extracellular plasma 5-HT levels increase following SSRI treatment. Evaluating changes in plasma (i.e., extracellular) 5-HT levels as compared with platelet (i.e., intracellular) 5-HT levels following SSRI treatment has been challenging. Measuring plasma 5-HT levels has proven difficult due to very low concentration by extraordinarily efficient liver metabolism as well as the free diffusion of 5-HT out of circulation (32–34). Furthermore, levels of plasma 5-HT can vary greatly, possibly due to contamination by platelet granule release during plasma purification (1, 2, 34, 35). Reported plasma 5-HT levels fluctuate between investigators and preparations, but our data clearly demonstrate that 5-HT homeostasis is altered by loss of SERT function (36–38). Furthermore, our data demonstrate that improper exposure of platelets to 5-HT likely desensitizes the receptor, leading to reduced ADP-mediated αIIbβ3 activation (Fig. 6D). Our findings indicate that chronic loss of SERT function reduces 5-HT2A surface expression and signaling in each of two models of altered 5-HT homeostasis.

It has previously been established that 5-HT plays a synergistic role with ADP activation during platelet aggregation (12, 13, 39, 40). Here, we demonstrate that 5-HT2A stimulation potentiates ADP-dependent signaling to activate αIIbβ3 (i.e. JON/A binding), leading to aggregation. Although 5-HT2A stimulation alone did not influence αIIbβ3-activation, dual stimulation of 5-HT2A with a submaximal concentration of ADP enhanced JON/A binding. 5-HT2A stimulation may be required to enhance P2Y1 Gq-mediated signaling and Gi-mediated P2Y12 activation converging on αIIbβ3 inside-out activation. It is well known that the 5-HT2AR can signal through multiple non-G-protein pathways including β-arrestins (28, 41, 42). Therefore, although canonical 5-HT2AR signaling would be redundant relative to ADP-mediated P2Y1 activation, alternative signaling pathways downstream of β-arrestins, such as ERK1/2 and c-Src, could culminate in αIIbβ3 activation (28, 41–43). Additionally, 5-HT2AR signaling through arachidonic acid, 2-arachidonoylglycerol, calmodulin, or AKT could also synergize with ADP...
signaling (44–47). Further experiments are required to determine what signaling pathways are necessary and sufficient for maximal αIIbβ3 activation via ADP/5-HT synergy. Clinically, targeted inhibition of the 5-HT2AR has been efficacious (17). APD791, an inverse agonist of 5-HT2AR, is currently in clinical trials and attenuated recurrent thrombosis irrespective of the time of treatment (16, 17). No increase in bleeding time in the presence of APD791 was observed as compared with other antiplatelet therapies. Additionally, APD791 was able to block 5-HT-dependent platelet activation over a short time scale (2 h) (16, 17). Our data provide mechanistic insight into the reduced aggregation previously observed in SSRI-treated patients and support explorations of peripherally restricted, well tolerated SSRIs or 5-HT2AR antagonists as an antiplatelet therapeutic approach. The therapeutic implications of this work suggest a novel approach targeting the 5-HT signaling systems via SERT inhibition, leading to reduced 5-HT2AR-mediated platelet aggregation.

**Experimental Procedures**

**Materials—**Thrombin was purchased from Chronolog (Haventown, PA). Sterile saline, ADP, fibrinogen, ketanserin, DOIs, and citalopram were purchased from Sigma-Aldrich. Flow cytometry antibodies anti-activated αIIbβ3 (JON/A-phycocerythrin) and anti-P-selectin-fluorescein isothiocyanate (FITC) antibodies were purchased from EMFRET Analytics & Co. KG (Würzburg, Germany). Radioactive compounds [3H]Ketanserin, 5-[3H]HT, [3H]ADP, and [35S]GTPγS were purchased through PerkinElmer Life Sciences.

**Animals and Housing—**All mice were group-housed (two to five mice per cage) in temperature- and humidity-controlled conditions under a 12-h 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 (M/12/121). Age- and sex-matched mice were used in all experiments (8–20 weeks of age). All experiments were run with either SERT+/+ (wild-type) or SERT−/− (serotonin transporter knock-out) homozygous mice of both sexes in a C57BL6 background.

**Administration of Citalopram—**Citalopram was administered acutely (addition of drug during platelet activation) or chronically (via drinking water treatment). Citalopram-treated drinking water was prepared based on average weight of the mice and an average consumption of ~7 ml/day/mouse for a dosing of 15 mg/kg/day. Citalopram-prepared water was added to a water dispenser, and mice were given full access to either non-treated control or citalopram-treated water. Mice were exposed to citalopram-treated water for 6 days, 1 day beyond the lifetime of a circulating platelet in a mouse (22), to model chronic loss of SERT function in the periphery but minimize central nervous systems effects. After 6 days, mice were euthanized, and experiments were performed as indicated.

**Tail Bleed—**Experiments were carried out as described previously (48). Briefly, mice were maintained under anesthesia (2% isoflurane and 1 ml/min oxygen; JD Medical Distributing Co., Inc., Phoenix, AZ), and a transverse incision was made over a lateral tail 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 was recorded as the bleeding time. Maximum time allowed for cessation of bleeding was 12 min before manually stopping bleeding.

**Blood Collection and Platelet Purification—**We used isoflurane because the effect is considered negligible on platelets in most species (19). Cardiopuncture was performed in euthanized mice using a 25-gauge needle/1-ml syringe containing anticoagulant. For platelet isolation, whole blood was diluted 1:1 in Tyrroe’s buffer (10 mM HEPES, 11.9 mM NaHCO3, 127.2 mM NaCl, 5 mM KCl, 0.4 mM NaH2PO4, 1 mM MgCl2, 5 mM glucose, pH 7.4), layered onto 2 ml of Fico/Lite platelets (Atlanta Biologicals, Inc., Lawrenceville, GA), and spun for 15 min at 350 × g. Three antiplatelet activation agents used during our purification include acid-citrate-dextrose, 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. Platelets were washed in Tyrode’s buffer containing acid-citrate-dextrose, 0.2 unit/ml apyrase, and 5 μg/ml PGE1 (10 min at room temperature) and spun at 1000 × g for 10 min to obtain a washed platelet pellet. Platelets were resuspended in Tyrode’s buffer and allowed to equilibrate for 30 min before experimentation. Platelets were counted on a Coulter counter and diluted in Tyrode’s buffer with 0.1% BSA to the indicated concentrations.

**Platelet Transfusion—**Platelet transfusions were performed by jugular vein injection of 200 μl of 1 × 10^8/ml gel-filtered platelets. A 3-min incubation time was allowed before tail bleed time was determined. Tail bleed was performed as described above.

**ADP, 5-HT, and 5-HIAA Levels—**Whole blood was collected in 3.2% sodium citrate and stored at −80 °C until analyzed. 5-HT and 5-HIAA levels were measured by HPLC by the Vanderbilt Neurochemistry core. Briefly, 5-HT was determined by a specific HPLC assay utilizing an Antec Decade (oxidation, 0.4) electrochemical detector. Samples were injected using a Waters 2707 autosampler onto a Phenomenex Kintex (2.6-μm, 100-Å) C18 HPLC column (100 × 4.60 mm). 5-HT eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10 −3 M sodium acetate, 10 −4 M EDTA, and 10.5% methanol, pH 3.8. HPLC control and data acquisition were managed by Millennium 32 software. Daily calibration curves were generated by supplementing whole blood with stock 5-HT solutions to yield final concentrations of 1, 3, 6, 12, 25, 50, 75, and 100 ng/ml using 3,4-dihydroxybenzylamine as an internal standard. An ADP ELISA kit was purchased from Abcam (ab83359). ELISA was performed as described by the manufacturer.

**Gel-filtered Platelet Flow Cytometry—**After purifying platelets, platelet counts were determined and normalized to 1 × 10^7/ml. 25 μl of platelets were added and mixed with 5 μl each of both anti-mouse P-selectin and anti-JON/A that binds a conformation of αIIbβ3 (EMFRET Analytics & Co. KG). Platelets were allowed to incubate with the antibodies for 15 min before activation. Antagonists were allowed to incubate with the platelet antibody mixture for 5 min before activation. Activation was stopped by addition of 100 μl of 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.138 M NaCl, 0.0027 M KCl, pH 7.4) 15 min after activation followed by addition of 300 μl of...
PBS after 15 min. Samples were analyzed at the Nashville Veterans Affairs Medical Center Flow Cytometry Resource Center (17, 48) using geometric mean fluorescence intensity, which gives the mean fluorescence intensity of each fluorescence channel for the sample (17, 48).

**Radioligand Binding**—Platelet counts were normalized before analysis using a Coulter Counter at 5–3 × 10^9 cells/ml. A saturation curve was done with increasing concentrations of [3H]ketanserin (0.625–20 nm) incubated with 50 μl of platelets in suspension and methysergide at 20 μM to determine nonspecific binding. B_{max} 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 [3H]ADP at 20 nM was added to platelet membrane preparations. 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 min. 1 μM DOI and 0.2 μM [35S]GTPγS were added to samples and incubated for 60 min at room temperature. Counts were standardized by protein concentration of membrane preparation. For nonspecific [35S]GTPγS incorporation, excess unlabeled GTPγS (100 μM) was added. 5-HT_{2A} receptor-specific [35S]GTPγS was measured following normalization 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 of [3H]ADP to platelet membrane preparations was used to determine relative percentages of P_{2Y_1} and P_{2Y_{12}} receptors. [3H]ADP at 20 nm was added to platelet membrane preparations (5–20 μg total). Platelets were then incubated with the indicated concentration of either P_{2Y_{12}} (ticagrelor), P_{2Y_1} (MRS2179), or dual P_{2Y_1} and P_{2Y_{12}} antagonist (2,2’-pyridylisotosylate) to determine receptor-specific binding.

**Statistics**—All data were analyzed in Prism 4.0c (GraphPad Software, Inc.). Outliers were determined using ROUT methods with Q = 1%. A non-parametric Mann-Whitney test was performed to account for non-determination of equal variance (included in results). All figures display the median and the range. Median values are reported in the text. Student’s t tests or two-way analysis of variance (ANOVA) followed by Tukey’s post-tests were performed as appropriate (indicated in results).

**Author Contributions**—K. H. O. designed, performed, analyzed, and interpreted results and wrote the manuscript. H. E. H. and A. M. D. C. both equally designed, interpreted results, and wrote the manuscript. Scientific interpretation, experiment guidance, and manuscript editing was performed by M. T. D.

**Acknowledgments**—We acknowledge Drs. Matthew Mazalouskas and Michael Dohn for assistance with editing of the manuscript. We also thank Jae Maeng for animal husbandry. Additionally, we thank the undergraduate researchers who participated, including Cecilia Li and Cynthia Mairink.

**References**

1. Brenner, B., Harney, J. T., Ahmed, B. A., Jefferis, B. C., Unal, R., Mehta, J. L., and Kilic, F. (2007) Plasma serotonin levels and the platelet serotonin transporter. J. Neurochem. 102, 206–215
2. Matondo, R. B., Punt, C., Homberg, J., Toussaint, M. J., Kisjes, R., Korporaal, S. J., Akkerman, J. W., Cuppen, E., and de Bruin, A. (2009) Deletion of the serotonin transporter in rats disturbs serotonin homeostasis without impairing liver regeneration. Am. J. Physiol. Gastrointest. Liver Physiol. 296, G963–G968
3. Beikmann, B. S., Tomlinson, I. D., Rosenthal, S. J., and Andrews, A. M. (2013) Serotonin uptake is largely mediated by platelets versus lymphocytes in peripheral blood cells. ACS Chem. Neurosci. 4, 161–170
4. Bismuth-Evenzal, Y., Gonołopsky, Y., Gurwitz, D., Iancu, I., Weizman, A., and Rehavi, M. (2012) Decreased serotonin content and reduced agonist-induced aggregation in platelets of patients chronically medicated with SSRI drugs. J. Affect. Disord. 136, 99–103
5. Carneiro, A. M., Cook, E. H., Murphy, D. L., and Blakely, R. D. (2008) Interactions between integrin αIIbβ3 and the serotonin transporter regulate serotonin transport and platelet aggregation in mice and humans. J. Clin. Investig. 118, 1544–1552
6. Abdelmalik, N., Ruhé, H. G., Barwari, K., van den Dool, E.-J., Meijers, J. C., Middeldorp, S., Bühler, H. R., Schene, A. H., and Kamphuisen, P. W. (2008) Effect of the selective serotonin reuptake inhibitor paroxetine on platelet function is modified by a SLC6A4 serotonin transporter polymorphism. J. Thromb. Haemost. 6, 2168–74
7. Namkung, J., Kim, H., and Park, S. (2015) Peripheral serotonin: a new player in systemic energy homeostasis. Mol. Cells 38, 1023–1028
8. Hranilovic, D., Blazevic, S., Ivica, N., Cicin-Sain, L., and Oreskovic, D. (2011) The effects of the perinatal treatment with 5-hydroxytryptophan or tryptophan on the peripheral and central serotonin homeostasis in adult rats. Neurochem. Int. 59, 202–207
9. Blazevic, S., Erjavac, I., Brizic, M., Vukicevic, S., and Hranilovic, D. (2015) Molecular background and physiological consequences of altered peripheral serotonin homeostasis in adult rats perinatally treated with tryptophan. J. Physiol. Pharmacol. 66, 529–537
10. Amireault, P., Sibon, D., and Côté, F. (2013) Life without peripheral serotonin: insights from tryptophan hydroxylase 1 knockout mice reveal the existence of paracrine/autocrine serotonergic networks. ACS Chem. Neurosci. 4, 64–71
11. Tseng, Y.-L., Chiang, M.-L., Huang, T.-F., Su, K.-P., Lane, H.-Y., and Lai, Y.-C. (2010) A selective serotonin reuptake inhibitor, citalopram, inhibits collagen-induced platelet aggregation and activation. J. Clin. Investig. 127, 3–17
12. Cerrito, F., Lazzaro, M. P., Gaudio, E., Arminio, P., and Aloisi, G. (1993) 5-HT_{2A} receptor antagonists. Mol. Cells 4, 209–215
13. Thompson, N. T., Scrutton, M. C., and Wallis, R. B. (1986) Synergistic responses in human platelets. Comparison between aggregation, secretion, and cytosolic Ca^{2+} concentration. Eur. J. Biochem. 161, 399–408
14. Mercado, C. P., Quintero, M. V., Li, Y., Singh, P., Byrd, A. K., Talabnin, K., Ono, S., Azadi, P., Rutsch, N. J., Khasawneh, F. T. (2014) The antidepressant 5-HT_{2A} receptor antagonists pizotifen and cyproheptadine inhibit serotonin-enhanced platelet function. PLoS One 9, e87026
15. Adams, J. W., Ramirez, J., Shi, Y., Thomsen, W., Frazer, J., Morgan, M., Edwards, J. E., Chen, W., Teegarden, B. R., Xiong, Y., Al-Shamma, H., Behan, D. P., and Connolly, D. T. (2009) APD791, 3-methoxy-N-(3-(1-methyl-1H-pyrazol-5-yl)-4-(2-morpholinooethoxy)phenyl)benzamide, a novel 5-hydroxytryptamine 2A receptor antagonist: pharmacological profile, pharmacokinetics, platelet activity and vascular biology. J. Pharmacol. Exp. Ther. 331, 96–103
16. Przyklenk, K., Frelinger, A. L., 3rd, Linden, M. D., Whittaker, P., Li, Y., Barnard, M. R., Adams, J., Morgan, M., Al-Shamma, H., and Michelson, A. D. (2010) Targeted inhibition of the serotonin 5HT2A receptor im-
proves coronary patency in an in vivo model of recurrent thrombosis. J. Thromb Haemost. 8, 331–340
18. Li, N., Wallén, N. H., Ladjevardi, M., and Hjemdahl, P. (1997) Effects of serotonin on platelet activation in whole blood. Blood Coagul. Fibrinolysis 8, 517–523
19. Jiroušková, M., Shet, A. S., and Johnson, G. J. (2007) A guide to murine platelet structure, function, assays, and genetic alterations. J. Thromb Haemost. 5, 661–669
20. Walther, D. J., Peter, J.-U., Winter, S., Höltje, M., Paulmann, N., Grohm, M., Yowinckel, J., Alamo-Bethencourt, V., Wilhelm, C. S., Ahnert-Hilger, G., and Bader, M. (2003) Serotonin binding of small GTPases is a signal transduction pathway that triggers platelet α-granule release. Cell 115, 851–862
21. Hummerich, R., and Schloss, P. (2010) Serotonin—more than a neurotransmitter: transglutaminase-mediated serotoninization of C6 glia cells and fibroblasts. Neurochem. Int. 57, 67–75
22. Malszko, J., Urano, T., Knoller, R., Taminato, A., Yoshimi, T., Yakuda, Y., and Takada, A. (1994) Daily variations of platelet aggregation in relation to blood and plasma serotonin in diabetes. Thromb. Res. 75, 569–576
23. Andrade, C., Sandarsh, S., Chethan, K. B., and Nagesh, K. S. (2010) Serotonin reuptake inhibitor antidepressants and abnormal bleeding: a review for clinicians and a reconsideration of mechanisms. J. Clin. Psychiatry 71, 1565–1575
24. Maurice, T., and Su, T.-P. (2009) The pharmacology of α-1 receptors. Pharmacol. Ther. 124, 195–206
25. Ahmed, B. A., Jeffus, B. C., Bukhari, S. I., Harney, J. T., Unal, R., Lupashin, V. V., van der Sluijs, P., and Kilic, F. (2008) Serotonin transmades Rab4 and facilitates its binding to the C terminus of serotonin transporter. J. Biol. Chem. 283, 9388–9398
26. Gartsde, S. E., Umers, B., Hajoš, M., and Sharp, T. (1995) Interaction between a selective 5-HT1A receptor antagonist and an SSRI signaling in vivo: effects on 5-HT cell firing and extracellular 5-HT. Br. J. Pharmacol. 115, 1064–1070
27. Artigas, F., Romero, L., de Montigny, C., and Blier, P. (1996) Acceleration of the effect of selective antidepressant drugs in major depression by 5-HT1A antagonists. Trends Neurosci. 19, 378–383
28. Schmid, C. L., Raehal, K. M., and Bohn, L. M. (2008) Agonist-directed signaling of the serotonin 2A receptor depends on β-arrestin-2 interactions in vivo. Proc. Natl. Acad. Sci. USA. 105, 1079–1084
29. Bohn, L. M., and Schmid, C. L. (2010) Serotonin receptor signaling and regulation via β-arrestins. Crit. Rev. Biochem. Mol. Biol. 45, 555–566
30. Schaff, M., Receveur, N., Bourdon, C., Ohlmann, P., Lanza, F., Gachet, C., and Mangin, P. H. (2012) β-Arrestin-1 participates in thrombosis and regulates integrin αIIbβ3 signalling without affecting P2Y receptors desensitisation and function. Thromb. Haemost. 107, 735–748
31. Yamachuchi, M., Miyara, T., Matsushima, T., and Imanishi, T. (2006) Desensitization of 5-HT2A receptor function by chronic administration of selective serotonin reuptake inhibitors. Brain Res. 1067, 164–169
32. Moore, T. C., and Eiseman, B. (1966) Serotonin metabolism in the isolated perfused canine liver. Surgery 59, 765–769
33. Anderson, G. M., Barr, C. S., Lindell, S., Durham, A. C., Shifrovich, I., and Higley, J. D. (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, 339–346
34. Anderson, G. M., and Cook, E. H. (2016) Commentary on “platelet studies in autism spectrum disorder patients and first-degree relatives.” Mol. Autism 7, 20
35. Lau, W. K., Chan-Yeung, M. M., Yip, B. H., Cheung, A. H., Ip, M. S., Mak, J. C., and COPD Study Group of the Hong Kong Thoracic Society (2012) The role of circulating serotonin in the development of chronic obstructive pulmonary disease. PloS One 7, e31617
36. Jerme, B., Vlaďíč, A., Cinc-Sain, L., Hranilović, D., Banović, M., Balija, M., Bilić, E., Sucić, Z., Vukadin, S., and Grigicević, D. (2002) Platelet serotonin measures in migraine. Headache 42, 588–595
37. Singh, P., Fletcher, T. W., Li, Y., Ruch, N. J., and Kilic, F. (2013) Serotonin uptake rates in platelets from angiotensin II-induced hypertensive mice. Health 5, 31–39
38. Walther, D. J., Peter, J. U., Bashammakh, S., Hörtgand, H., Voits, M., Fink, H., and Bader, M. (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science 299, 76–76
39. Berger, J. S., Becker, R. C., Kuhn, C., Helms, M. J., Ortel, T. L., and Williams, R. (2013) Hyperreceptive platelet phenotypes: relationship to altered serotonin transporter number, transport kinetics and intrinsic response to adrenergic co-stimulation. Thromb. Haemost. 109, 85–92
40. Hirsh-Rokach, B., Spectre, G., Shai, E., Lotan, A., Ritter, A., Al-Aieshy, F., Malmström, R. E., Varon, D., and Alcalai, R. (2015) Differential impact of selective serotonin reuptake inhibitors on platelet response to clopidogrel: a randomized, double-blind, crossover trial. Pharmacotherapy 35, 140–147
41. Stahl, E. L., Zhou, L., Ehler, F. J., and Bohn, L. M. (2015) A novel method for analyzing extremely biased agonism at G protein-coupled receptors. Mol. Pharmacol. 87, 866–877
42. Schmid, C. L., and Bohn, L. M. (2010) Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a β-arrestin2/Src/Akt signaling complex in vivo. J. Neurosci. 30, 13513–13524
43. Li, Z., Delaney, M. K., O’Brien, K. A., and Du, X. (2010) Signaling during platelet adhesion and activation. Arterioscler. Thromb. Vasc. Biol. 30, 2341–2349
44. Quinn, J. C., Johnson-Farley, N. N., Yoon, J., and Cowen, D. S. (2002) Activation of extracellular-regulated kinase by 5-hydroxytryptamine, receptors in PC12 cells is protein kinase C-independent and requires calmodulin and tyrosine kinases. J. Pharmacol. Exp. Ther. 303, 746–752
45. Johnson-Farley, N. N., Kertesy, S. B., Dubyak, G. R., and Cowen, D. S. (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. J. Neurochem. 92, 72–82
46. Gööö, M., Gööö, P., Lutfret, L. M., and Raymond, J. R. (2006) 5-HT2A receptor induces ERK phosphorylation and proliferation through ADAM-17 tumor necrosis factor-α-converting enzyme (TACE) activation and heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding in mesangial cells. J. Biol. Chem. 281, 21004–21012
47. Miller, K. J., Mariano, C. L., and Cruz, W. R. (1997) Serotonin 5HT2A receptor activation inhibits inductive nitric oxide synthase activity in C6 glia cells. Life Sci. 61, 1819–1827
48. Oliver, K. R., Jessen, T., Crawford, E. L., Chung, C. Y., Sutcliffe, J. S., and Carneiro, A. M. (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. Mol. Pharmacol. 85, 921–931