The essential contribution of the antidepressant-sensitive serotonin (5-HT) transporter SERT (which is encoded by the SLC6A4 gene) to platelet 5-HT stores suggests an important role of this transporter in platelet function. Here, using SERT-deficient mice, we have established a role for constitutive SERT expression in efficient ADP- and thrombin-triggered platelet aggregation. Additionally, using pharmacological blockers of SERT and the vesicular monoamine transporter (VMAT), we have identified a role for ongoing 5-HT release and SERT activity in efficient human platelet aggregation. We have also demonstrated that fibrinogen, an activator of integrin αIIbβ3, enhances SERT activity in human platelets and that integrin αIIbβ3 interacts directly with the C terminus of SERT. Consistent with these findings, knockout mice lacking integrin β3 displayed diminished platelet SERT activity. Conversely, HEK293 cells engineered to express human SERT and an activated form of integrin β3 exhibited enhanced SERT function that coincided with elevated SERT surface expression. Our results support an unsuspected role of αIIbβ3/SERT associations as well as αIIbβ3 activation in control of SERT activity in vivo that may have broad implications for hyperserotonemia, cardiovascular disorders, and autism.

Introduction

Before gaining attention as a neurotransmitter, serotonin (5-hydroxytryptamine [5-HT]) was first detected in the blood and initially studied for its ability to trigger vasoconstriction (1). Although platelets store large quantities of 5-HT, the amine is actually synthesized in the gut, where it is released after the activation of presynaptic neurons or the stimulation of enterochromaffin cells (2). Once 5-HT enters the circulation, it is sequestered inside platelets by antidepressant-sensitive 5-HT transporters (SERTs, SLC6A4) and then into secretory granules by the vesicular monoamine transporter (VMAT). Chronic use of 5-HT selective reuptake inhibitors (SSRIs) decreases platelet 5-HT content and can exacerbate bleeding (3, 4). Secreted 5-HT, acting on platelet 5-HT2A receptors, has been proposed to augment the effects of other prothrombotic agents that act through integral membrane receptor proteins, including the αIIbβ3 complex (also known as glycoprotein GPIIb/IIIa) (5). These actions of 5-HT may also represent a pathway for platelet hyperactivity and spontaneous aggregation that is observed in patients with atherosclerotic disease (6). Whether continuous SERT activity is needed to sustain platelet aggregation is unknown, though the recognition that serotoninota of intracellular Rac and Rho proteins supports platelet granule secretion suggests a role for SERT in sustaining platelet aggregation independent of 5-HT2A receptors (7). Together these studies provide indirect support for SERT as a key player in platelet 5-HT homeostasis and sustaining thrombosis, conclusions reinforced by association of SERT polymorphisms with cardiovascular disease (8).

SERTs in the brain, platelets, and transfected cells are increasingly recognized to be regulated by complex signaling mechanisms that involve the actions of multiple Ser/Thr kinases and phosphatases (9, 10) as well as scaffolding/adaptor proteins (11). These interactions can rapidly redistribute the transporter among distinct membrane subdomains, alter transporter cell surface expression, and/or control SERT catalytic activity (11–13). Additionally, SERT proteins are phosphorylated at several different sites, where the balance between phosphorylation by PKC and PKG and dephosphorylation by protein phosphatase 2A (PP2A) influence different aspects of SERT catalytic activity and trafficking (12, 14, 15). Tonic activation p38 MAPK signaling pathways, without direct phosphorylation of the transporter, controls basal surface SERT expression levels, whereas acute p38 MAPK activation induces trafficking-independent stimulation of SERT transport activity (12, 16). SERT is now recognized as a downstream effector of multiple cell-surface receptors (17, 18). In platelets, the activation of thrombin and ADP receptors initiates “inside-out” signaling, leading to integrin αIIbβ3 serine and tyrosine phosphorylation and integrin receptor activation and clustering (19). Consequently, binding of αIIbβ3 ligand (e.g., fibrinogen) triggers “outside-in” signaling that promotes further cytoskeletal (CS) reorganization and granule secretion (20). The propagation of outside-in signaling leads to the formation of focal adhesion structures, continued granule secretion, and thrombus formation (21). The current studies, which

Nonstandard abbreviations used: CS, cytoskeleton/cytoskeletal; GST, glutathione-transferase; 5-HT, serotonin; ITGB3, integrin β3; MS, membrane skeleton/skeletal; PP2A, protein phosphatase 2A; SERT, 5-HT transporter; SRSI, 5-HT selective reuptake inhibitor; TS, Triton X-100–soluble; VMAT, vesicular monoamine transporter.

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establish physical associations between SERT and αIIbβ3, reveal the regulation of SERT trafficking and 5-HT as additional facets of integrin-mediated platelet activation.

Genetic variation leading to altered αIIbβ3 activity is associated with platelet dysfunction, exemplified by the association of constitutively active forms of αIIbβ3 with elevated blood 5-HT and arterial thrombosis and myocardial infarction (22, 23). Here we report that a constitutively active allele of integrin β3, encoded by the human gene ITGB3, modulates SERT surface and subcellular localization via a PP2A- and p38 MAPK–dependent signaling pathway. Our findings provide a mechanistic basis for established genetic interactions of ITGB3 and SLC6A4 polymorphisms in setting 5-HT blood levels (24). Additionally, they reveal an unsuspected coordination between αIIbβ3 signaling and SERT activity that can sustain platelet aggregation and may contribute to the recognized comorbidities between cardiovascular and brain disorders.

**Results**

Platelet SERT expression and function are involved in platelet aggregation. To examine the role for SERT in thrombus formation, we isolated platelets from wild-type mice or mice lacking SERT, which have significantly reduced blood 5-HT levels (25, 26). Platelet aggregation assays conducted with wild-type mouse platelets using 20 μM ADP or 0.05 U/ml thrombin demonstrated approximately 50% aggregation within approximately 10 minutes. In contrast, SERT–/– mice showed an 80% reduction in ADP-induced aggregation over the same time period and a significantly slower rate of platelet aggregation (t = 10 minutes, P = 0.0179, n = 4; rate: P = 0.0108; Figure 1A). In response to thrombin, platelets lacking SERT had an approximately 50% reduction in aggregation over the same time period and a significantly slower rate of platelet aggregation (t = 10 minutes, P = 0.0171, n = 4; Figure 1B). In contrast to ADP, thrombin did not induce a decrease in aggregation rates in SERT–/– platelets, suggesting a more prominent role of the transporter in maintaining
aggregation. These data reveal agonist-dependent roles for SERT in the rate and extent of platelet aggregation.

To examine whether loss of sensitivity to ADP in SERT−/− platelets arises from loss of SERT protein per se versus loss of intracellular 5-HT, we treated normal platelets with the VMAT inhibitor reserpine (27). Acute (30-minute) reserpine treatments led to a loss of granule stores but overall retention of 5-HT in the cytoplasm, and reserpine-treated platelets remained competent for attachment and spreading on fibrinogen-coated plates (Figure 1E). However, reserpine-treated platelets exhibited a marked reduction in aggregation rate and extent that could not be reversed by acute incubation in 10 μM external 5-HT (t = 10 minutes, P < 0.0001, n = 4; rate analysis: P = 0.0169, n = 4; Figure 1C). Platelets with normal intracellular stores of 5-HT that were treated acutely with the SSRIs citalopram or fluoxetine at concentrations required to fully block platelet SERT attenuated ADP-induced platelet aggregation, reducing the initial rate of aggregation (Figure 1, D and E; t = 10 minutes, P = 0.006, n = 6; rate analysis: P = 0.0354, n = 8) and also diminishing spreading of platelets onto fibrinogen-coated plates (Supplemental Figure 1; supplemental material available online changes in levels of platelet 5-HT, as HPLC analysis of these levels showed no difference between Itgb3−/− and Itgb3+/− animals (data not shown). These findings reveal that cTnIβ3 expression or signaling is required for normal platelet SERT function.

The activation of inside-out pathways by platelet aggregation agonists triggered widespread signaling cascades within platelets, leading to different effects on SERT function (thrombin, ADP, Ca2+, and PKC activation; shown in Supplemental Figure 2, A and B). To examine whether cTnIβ3 signaling imparts changes in SERT activity, we took advantage of the ability of immobilized fibrinogen to induce cTnIβ3 clustering and localized outside-in signaling in nonactivated platelets (28). Whereas the α2B1 ligand collagen failed to stimulate SERT activity to a greater extent than immobilized poly-D-lysine, platelets seeded on cTnIβ3 ligand fibrinogen showed an enhanced level of 5-HT uptake as measured 30 minutes after plating (P = 0.0403, n = 4; Figure 2D). Soluble fibrinogen also enhanced SERT activity, though higher concentrations were required (Supplemental Figure 2C). The impact of cTnIβ3 gene dosage on SERT activity was not an indirect result of with this article; doi:10.1172/JCI33374DS1). These findings indicate that 5-HT recycling via SERT is required to sustain stable rates of platelet aggregation. Although we did not observe a significant enhancement of platelet aggregation in samples preincubated with 10 μM 5-HT in vitro (data not shown), the treatment did promote the formation of platelet aggregates (Figure 1E). Taken together, our findings indicate that SERT function plays a role not only in the acquisition of 5-HT to load granule stores but also in 5-HT recycling that supports platelet aggregation.

**Figure 2**

Integrin regulation of SERT function. (A and B) SERT expression levels are conserved regardless of the Itgb3 genotype. (A) Western blot of native SERT from platelet lysates from Itgb3+/+, Itgb3−/−, and Itgb3+/− mice. (B) Platelets were isolated from Itgb3+/+, Itgb3−/−, and Itgb3+/− mice and incubated with [3H]citalopram (20 nM) for 20 minutes at 4°C. (C) Integrin cTnIβ3 loss of function dramatically reduces SERT uptake activity. Platelets were isolated from Itgb3+/+, Itgb3−/−, and Itgb3+/− mice and [3H]5-HT (50 nM) transport assayed. For B and C, data are shown as mean ± SEM. One-way ANOVA with Dunnett’s post-test, *P < 0.05. (D) Immobilized fibrinogen, but not collagen, enhances human platelet SERT uptake activity. Human platelets were seeded on plates coated with either poly-D-lysine, collagen, or fibrinogen and [3H]5-HT uptake measured. One-way ANOVA with Bonferroni’s post-test, *P < 0.05. (E) The competing peptide GRGDSP (1 mM) reverses the fibrinogen-mediated enhancement of SERT activity in seeded human platelets. n = 4. (F) The fibrinogen-mediated enhancement of SERT uptake activity can be partially reversed by the p38 MAPK inhibitor SB203580. Platelets attached to fibrinogen-coated plates were incubated for 10 minutes with p38 MAPK inhibitor SB203580 (20 μM), PD98059 (10 μM), H8 (0.1 μM), or genistein (20 μM) before measurement of [3H]5-HT uptake. n = 8. The data for D–F are presented as mean ± SEM with 1-way ANOVA and Dunnnett’s post-test, *P < 0.05 and **P < 0.005.
confirmed by blocking available binding sites with the αIIbβ3-binding peptide GRGDSP, which reduced platelet 5-HT uptake to poly-L-lysine levels (P < 0.0001, n = 4; Figure 2E). The control peptide GRADSP had no effect on fibrinogen-induced stimulation of SERT activity. To explore αIIbβ3-signaling pathways that support fibrinogen-induced SERT activation, we treated platelets for 10 minutes with inhibitors of tyrosine kinase (genistein, 20 μM), or focal adhesion kinase (FAK) [H-89, 10 μM], or p38 MAPK (SB203580, 20 μM), or PP2A (fasoracetin, 1nM) prior to fibrinogen incubation. Only the p38 MAPK inhibitor SB203580 affected a significant attenuation of fibrinogen stimulation (P = 0.0244, n = 8; Figure 2F). These data suggest that signaling arising from fibrinogen-bound αIIbβ3 can enhance SERT surface expression and/or catalytic function through pathways linked to p38 MAPK.

SERT participates in the αIIbβ3 complex through direct protein-protein interactions. Our evidence of a functional relationship between αIIbβ3 and SERT led us to ask whether a physical complex exists that could support coordinated receptor-transporter signaling. Indeed, proteomic analysis of SERT fusion protein complexes following incubation with human platelet extracts revealed multiple peptides for components of the αIIbβ3 complex (Supplemental Table 1), including both αIIb and β3 subunits. To validate these findings, we immunoprecipitated full-length SERT from detergent extracts of platelets and also recovered both αIIb and β3 integrins, as well as the integrin β3-associated proteins talin and adaptor protein Hic-5 (Figure 3A), suggesting recovery of a larger protein complex. To assess the specificity of this complex, we lysed human platelets with a high-stringency buffer (RIPA, containing 1% SDS) and isolated SERT using 2 different SERT antibodies (nos. 48 and 50). Using both SERT antisera, we recovered significant quantities of αIIbβ3 protein, whereas no αIIbβ3 signal was detected when using normal rabbit serum (Figure 3B). Notably, under these more stringent conditions, talin was not retained in coimmunoprecipitations, suggesting that it associates indirectly or is more detergent-sensitive. To localize a domain in SERT that might support these associations, we performed pull-down assays from platelet detergent extracts using glutathione-S-transferase (GST) or GST fused to the N or C terminus of αIIbβ3 (Enzyme Research) demonstrate that the β3 subunit interacts with the C terminus of SERT. (E) The fibrinogen-mediated enhancement of SERT uptake activity is not mediated by increased interactions with the focal adhesion complex. No significant differences were found between collagen and fibrinogen samples. For A–E, representative blots from at least 3 independent experiments are shown.

Figure 3
Physical interactions between SERT and integrin αIIbβ3. Western blots showing the association of SERT with members of the focal adhesion complex. Human platelet lysates were prepared under low-stringency (A, 1% Triton X-100, n = 2 shown) and high-stringency (B, RIPA buffer) conditions, and SERT immunocomplexes were isolated (normal rabbit serum [lane 1, NRS] or 2 different anti-SERT polyclonal sera [nos. 48 and 50; see ref. 49 for details] were used). The focal adhesion proteins integrin αIIbβ3, vinculin, talin, and actin were identified by immunoblotting with specific antibodies. Only integrin αIIbβ3 interacts with SERT under high-stringency conditions. (C) GST pulldowns performed in human platelets under high-stringency conditions (RIPA buffer) demonstrate that the C terminus of SERT (CSERT) mediates the SERT/αIIbβ3 interaction. (D) GST pulldowns performed using purified αIIbβ3 (Enzyme Research) demonstrate that the β3 subunit directly interacts with the C terminus of SERT. (E) The fibrinogen-mediated enhancement of SERT uptake activity is not mediated by increased interactions with the focal adhesion complex. No significant differences were found between collagen and fibrinogen samples. For A–E, representative blots from at least 3 independent experiments are shown.

The ITGB3 P33I (Pro33) variant enhances SERT uptake activity via enhanced transport surface expression. The ITGB3 P33I and P33L polymorphisms correspond to Leu33 and Pro33 coding variants, respectively. Platelets carrying the Pro33 isoform show increased...
the membrane skeleton (MS). We also identified a transient compart-
ments (11), with a quantitatively similar distribution evident
alters the distribution of SERT protein between platelet subcellular
Previously, we found that modification of intracellular pathways
was correlated with elevated SERT plasma membrane expression
V
This increase was supported by an approximately 2-fold increase in
pared with the Leu33 cells (2-way ANOVA,
P
isoforms of integrin
ficking, and subcellular localization. (4)
Figure 4C).
α
ITGB3
V
V
P
n
= 3 per group.

Figure 4
Genetic variation in ITGB3 alters SERT transport activity, surface traf-
cking, and subcellular localization. (A) Saturation analyses of HEK293
cells expressing SERT, αIIbβ3, and either the Leu33 or the Pro33
forms of integrin β3. Data are presented as mean ± SEM. Two-way
ANOVA, P < 0.0001, n = 10. (B) Pro33/β3 expression increases SERT
V
max
Unpaired Student’s t test, ***P < 0.0001, n = 10. (C) The increase in
SERT V
max
by Pro33/β3 is reflected by enhancement of SERT plasma
membrane expression. Intact HEK293 cells were exposed to bio-
tin, and surface proteins were captured by streptavidin beads. Surface
SERT was detected by Western blot analysis. No significant differenc-
est were found in total SERT or αIIbβ3 expression. Representative blot
from 3 independent experiments. (D) SERT subcellular distribution in
platelets from Leu33 or Pro33 homozygous subjects. Human platelets
were fractionated, and CS and MS fractions were collected. SERT is
evenly distributed among the CS, MS, and TS compartments in Pro33
subjects. Data are presented as mean ± SEM. Two-way ANOVA,
P < 0.05, n = 3 per group.

αIIbβ3 activation, fibrinogen binding, and platelet reactivity upon
ADP stimulation (29). Given our evidence of a physical and func-
tional interaction between SERT and αIIbβ3, we asked whether these
2 ITGB3 alleles differentially impact SERT function. HEK293
cells stably expressing either the αIIbLeu33 or αIIbPro33 were
transfected with human SERT and evaluated for 5-HT transport
activity. Saturation analyses revealed that the Pro33 allele con-
ferred a significant (260%) increase in 5-HT transport activity, com-
pared with the Leu33 cells (2-way ANOVA,
P
β
= 10. (C)
Saturation analyses of HEK293
expressed SERT/PP2A complexes (or possibly other pools of PP2A)
inhibition of PP2A increases SERT phosphorylation and internaliza-
tion. Since coimmunoprecipitation studies did not reveal a differ-
ence between SERT/Pro33 and SERT/Leu33 cells in the relative levels of
SERT/PP2A complexes, it is possible that Pro33 signaling targets
preformed SERT/PP2A complexes (or possibly other pools of PP2A)
to support SERT trafficking (Figure 5C).

Enhanced cell adhesion and migration attributed to integrin
β3 Pro33 can be abolished by MAPK inhibition (31), suggesting that
activated MAPKs could play a role in the Pro33 regulation of
SERT. Therefore, we investigated the role of MAPK signaling in the
enhancement of SERT activity by incubating SERT/Leu33 and
SERT/Pro33 cells for 10 minutes with either a specific ERK2
inhibitor (PD98059, 10 μM) or the p38 MAPK inhibitor SB203580
(20 μM). The ERK inhibitor PD98059 failed to reduce Pro33-
stimulated 5-HT transport (Supplemental Figure 4C). In contrast,
SERT activity was reduced upon treatment with the p38 MAPK
inhibitor SB203580 specifically in SERT/Pro33 cells (Figure 5D,
P = 0.0365, n = 6; Supplemental Figure 4F). These studies support
the idea that p38 MAPK is upregulated or activated in Pro33 cells.
Indeed, whereas basal and phosphorylated ERK levels did not differ
between Pro33 and Leu33 cells (Supplemental Figure 4D), Pro33
cells demonstrated a consistent elevation of p38 MAPK phos-
phorylation, relative to Leu33 cells (Figure 5E; P = 0.0238, n = 3).
Treatments with either SB203580 (Figure 5E; P = 0.0045, paired
Student’s t test, n = 3) or okadaic acid (Figure 5F; P = 0.0068, paired
Student’s t test, n = 3) eliminated this increase. In contrast to the
Pro33-supported stimulation of 5-HT uptake, p38 MAPK phos-
phorylation was insensitive to foscirenic (Supplemental Figure 4G),
suggesting a role for PP1 in sustaining p38 MAPK phosphorylation
(32). Taken together, our findings indicate that the Pro33-triggered
elevation of 5-HT uptake arises from enhanced p38 MAPK signal-
ing supported by both PP1 and PP2A.
Figure 5

**ITGB3** Pro33 regulates SERT transport activity via serine/threonine protein phosphatases and p38 MAPK signaling. (A) Coimmunoprecipitation experiment showing no changes in the SERT/αIIb3 complexes between SERT/Leu33 and SERT/Pro33 cells. (B) Reduction of SERT uptake activity in Pro33, but not Leu33, cells by inhibition of serine/threonine phosphatases. HEK293 cells expressing SERT and either Leu33 or Pro33 were incubated for 10 minutes with vehicle, okadaic acid (250 nM), or fostriecin (1 nM) before measurement of [³H]5-HT uptake. Data are presented as mean ± SEM. Paired Student’s t-test: *P < 0.05, 1-way ANOVA with Dunnett’s post-test: ***P < 0.0005, n = 6. (C) Coimmunoprecipitation experiment showing no changes in the SERT/PP2A complexes between Leu33 and Pro33 cells. (D) Reduction of SERT uptake activity in Pro33 by inhibition of p38 MAPK. HEK293 cells expressing SERT and either Leu33 or Pro33 were incubated for 10 minutes with SB203580 (20 μM) before measurement of [³H]5-HT uptake. Data are presented as mean ± SEM. Paired Student’s t-test: *P < 0.05; 1-way ANOVA with Dunnett’s post-test: **P < 0.01; n = 6. (E and F) Enhanced p38 MAPK phosphorylation levels in Pro33 cells are sensitive to SB203580 and okadaic acid (250 nM). Data were normalized to total p38 MAPK levels and are presented as mean ± SEM. One-way ANOVA with Dunnett’s post-test: *P < 0.01; paired Student’s t-test: ***P < 0.005; n = 3.

Discussion

In the present study, we demonstrate that SERT expression supports efficient platelet aggregation; we identify the integrin receptor αIIb3 as a novel component of SERT regulatory protein complexes and demonstrate that SERT trafficking and transport activity are influenced by functional ITGB3 signaling variants. Genetic ablation of platelet 5-HT uptake in SERT−/− mice, SSRI blockade of human SERT, and depletion of platelet 5-HT granules with reserpine significantly reduced the rate and extent of platelet aggregation, findings best explained by an ongoing need for platelets to recycle releasable stores of 5-HT (Figure 6, A and B). The effects of genetic SERT elimination on aggregation were more pronounced than those observed with ex vivo SSRI treatments, consistent with the preservation of intracellular 5-HT stores during acute SSRI treatments and the more profound aggregation impact observed with reserpine-induced granule depletion. The prothrombotic human integrin β3 polymorphism Pro33 (P33) induced elevated SERT surface expression and enhanced 5-HT uptake activity (Figure 6C), effects that plausibly contribute to elevated 5-HT blood levels. The recognition of an important role for SERT in platelet aggregation and identification of αIIb3/SERT interactions that can impact transporter trafficking and 5-HT uptake capacity provides a mechanistic basis for established interactions between SLC6A4 and ITGB3 genetic variation in hyperserotonemia and cardiovascular disease.

In addition to associated proteins, SERT is responsive to multiple kinase-mediated signaling pathways including those linked to PKC, PKG, and p38 MAPK activation (10, 12, 14), as well as dephosphorylation events mediated by PP2A (9). Although extensive work has been done regarding the identification and characterization of signaling pathways that regulate SERT, only PKC and PKG have been shown to directly phosphorylate the transporter (14, 15), suggesting that multiple pathways may interact to affect transporter function and trafficking. Our implication of p38 MAPK pathways in both rapid, fibrinogen-modulated SERT activity and the constitutive effects of ITGB3 Pro33 elevation of SERT surface expression is consistent with recent findings that p38 MAPK controls both basal SERT catalytic function (18) and trafficking (16). Despite the role for p38 MAPK in fibrinogen stimulation of SERT, as implicated by attenuation of stimulation by SB203580, fibrinogen stimulation does not elevate total p38 MAPK phosphorylation levels (data not shown). This finding is not surprising if only a limited pool of p38 MAPK, perhaps associated with the transporter, is responsible for SERT catalytic modulation. Since a stable complex is evident between αIIb3 and SERT, it seems altogether possible that fibrinogen binding can impact SERT conformations directly, stabilizing a state that is more susceptible to existing modulators (such as p38 MAPK).

Although we could not detect acute activation of p38 MAPK by fibrinogen, basal p38 MAPK phosphorylation in Pro33 cells was clearly elevated above levels found in Leu33 cells. That this activation was related to increased SERT trafficking and activity became evident with the ability of SB203580 to significantly reduce 5-HT uptake in the SERT/Pro33 cells but not the SERT/Leu33 cells. A constitutive elevation in p38 MAPK phosphorylation that drives elevated SERT surface expression and increased 5-HT uptake has been previously demonstrated by Samuvel and coworkers (16) using overexpression of the upstream p38 MAPK activator MKK3b. p38 MAPK is known to activate PP2A (32, 33), and the specific PP2A inhibitor fostriecin reduced the stimulation of SERT evident in SERT/Pro33 cells. Interestingly, fostriecin had no effect on the elevation of p38 MAPK phosphorylation found in Pro33 cells, though the PP1/PP2A inhibitor okadaic acid was effective. Thus, we suspect that PP1 activation downstream of Pro33 may be responsible for p38 MAPK activation, whereas transmission of kinase activation to SERT upregulation involves PP2A (Figure 6C) (34).
The decrease in platelet aggregation in SERT-null mice is reminiscent of the diminished 5-HT content found in subjects chronically exposed to SSRIs (35) and the reduced platelet activation observed in depressed patients after chronic SSRI treatment (36). Additionally, short-term, open-label treatment of depressed patients with the SSRI paroxetine leads to a significant reduction in the number of functional αIIbβ3 isoforms and plasma concentrations of proaggregatory factors (37). Whereas acute blockade of SERT did not compromise αIIbβ3 activation per se, as assessed by activated αIIbβ3 antibody binding (data not shown), acute SSRI treatments reduced platelet aggregation in vitro. It is possible that SERT activity is necessary not only for maintaining the granular 5-HT pools, but also to resupply cytoplasmic pools utilized for the amplification of signaling pathways that facilitate platelet aggregation. Indeed, high cytoplasmic 5-HT concentrations are necessary for rapid exocytosis of α-granules and controlled serotonylation of small G proteins during irreversible platelet aggregation (7, 38, 39). This idea is consistent with a differential effect of reserpine and citalopram on platelet spreading on fibrinogen-coated plates. A more detailed study of how various platelet agonists trigger platelet aggregation will provide a deeper understanding of how SERT activity collaborates with the multiple pathways that drive platelet aggregation. These ideas may also contribute to the negative correlation between cardiovascular disease and SSRI-induced remission of depression (40, 41).

Our studies have focused on platelet SERT and integrin β3 interactions due to the genetic interactions evident between ITGB3 and SLc6A4 for hyperserotonemia and also due to the tractability of platelets as a model system. Although the association of ITGB3 P3A2 polymorphisms with acute thrombosis and stroke remains controversial, with contradictory findings in the general population, it may enhance the risk in populations already susceptible to coronary disease, such as in the elderly (42–44). It has not escaped our attention that similar mechanisms may also play a role in the regulation of SERT in other tissues, such as the gut or the brain. Integrin β3 is expressed in the developing and mature CNS and acts as a modulator of neurite formation and synaptic plasticity (45). In this regard, the enhanced SERT activity supported by αIIbβ3 polymorphisms may generate behavioral phenotypes similar to those of hSERT polymorphisms associated with altered SERT gene and protein expression (46, 47). In this regard, recent studies indicate a genetic interaction between ITGB3 and SLc6A4 in mediating autism susceptibility (30, 48). The finding that SERT and integrin β3 interact genetically and physically reveals an entirely unsuspected function for adhesion proteins in 5-HT homeostasis and represents a mechanism worthy of further investigation as a component of comorbidities between cardiovascular and mental illness. Studies of mice expressing human coding variants of ITGB3 and SLc6A4 should be useful in further analysis of this possibility.

**Methods**

Reagents and Antibodies. Most reagents, including thrombin from bovine plasma, adenosine 5'-diphosphate (ADP), collagen type I from rat tail, β-actin, vinculin, and talin mouse monoclonal antibodies were purchased from Sigma-Aldrich. Fibrinogen from human plasma, αIIbβ3-binding peptide GRGDSP, and the control peptide GRADSP were purchased from Calbiochem (EMD Biosciences). Mouse monoclonal antibodies against integrin αIIb, integrin β3, Hic-5, and focal adhesion kinase (FAK) were obtained from BD Transduction Laboratories (BD Biosciences). Mouse monoclonal antibodies against p44/42 MAPK (ERK1/2), phospho-p44/42 (phospho-ERK1/2), p38 MAPK, and phospho-p38 MAPK were purchased from Cell Signaling Technology Inc. SERT antisera (nos. 48 and 50) have been previously characterized elsewhere (49). Preparation of platelets. Outdated human platelet-rich plasma (PRP) concentrates were obtained from the American Red Cross. Platelets were used within 72 hours and tested initially for aggregation by thrombin (0.01–0.5 U/ml), to ensure that platelets were viable and competent for aggregation (50). Studies with mouse platelets were performed in accordance with humane guidelines established by the Vanderbilt IACUC under an approved protocol (M/04/376). For these studies, we used littermates as controls, although both Itgb3 and Slc6a4 mice have already been backcrossed 10 generations onto a C57BL/6 background. Mouse platelets were collected in a tube containing acid citrate dextrose (39 mM citric acid, 75 mM sodium citrate, and 135 mM glucose, pH 7.4) from the superior vena cava after euthanasia. Whole blood was centrifuged immediately at 190 g for 15 minutes at room temperature to obtain PRP. The PRP was centrifuged at 2,500 × g for 15 minutes at 22 °C, and the resulting platelet pellet was gently resuspended in PBS buffer, pH 7.4, and used immediately.

Platelet aggregation. Platelet aggregations were measured using washed platelets. The pellet platelets were resuspended in Tyrode buffer and adjusted to a concentration of 3 × 10^8 platelets/ml using a Coulter counter (Beckman Coulter). Platelets were tested for aggregation in a thrombin concentration curve and compared with fresh samples activated with...
Experiments were performed in duplicate and replicated at least 4 times. Most of the data analysis was performed using 1-way ANOVA and data are presented as mean ± SEM of 3 or more independent experiments. The authors gratefully acknowledge Vinod Vijayan for provision of HEK293 cells stably transfected with the αIIBLeu33β3 or αIIBPro33β3 integrins. We also acknowledge Bryan Voss for help with the platelet aggregation assays, Jane Wright for animal husbandry and genotyping, and Tammy Jessen, Angela Steele, and Qiao Han for general laboratory support. We thank Mary Zutter and Sam Santoro for reagent provision and advice on experiments and early stages of our analyses and Jeremy Veenstra-Vander Weele for critical reading of the final manuscript. The authors have been supported as follows: NARSAD Young Investigator Award and NIDA DA007390 to A.M.D. Carneiro; the NIMH Intramural Program for D.L. Murphy; NICHD/CPEA U19 HD35482 to E.H. Cook; and NIMH P50 MH078028 to R.D. Blakely. Received for publication July 23, 2007, and accepted in revised form January 9, 2008.

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