Effect of serotonin on platelet function in cocaine exposed blood

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5-hydroxytryptamine (5-HT) reuptake inhibitors counteract the pro-thrombotic effect of elevated plasma 5-HT by down-regulating the 5-HT uptake rates of platelets. Cocaine also down-regulates the platelet 5-HT uptake rates but in contrast, the platelets of cocaine-injected mice show a much higher aggregation rate than the platelets of control mice. To examine the involvement of plasma 5-HT in cocaine-mediated platelet aggregation, we studied the function of platelets isolated from wild-type and transgenic, peripheral 5-HT knock-out (TPH1-KO) mice, and cocaine-insensitive dopamine transporter knock in (DAT-KI) mice. In cocaine-injected mice compared to the control mice, the plasma 5-HT level as well as the surface level of P-selectin was elevated; in vitro platelet aggregation in the presence of type I fibrillar collagen was enhanced. However, cocaine injection lowered the 5-HT uptake rates of platelets and increased the plasma 5-HT levels of the DAT-KI mice but did not change their platelets aggregation rates further which are already hyper-reactive. Furthermore, the in vitro studies supporting these in vivo findings suggest that cocaine mimics the effect of elevated plasma 5-HT level on platelets and in 5-HT receptor- and transporter-dependent pathways in a two-step process propagates platelet aggregation by an additive effect of 5-HT and nonserotonergic catecholamine.

Cocaine use is associated with complications in the cardiovascular (CV) system directly and indirectly via stimulating the sympathetic nervous system. In peripheral tissue, cocaine produces a sympathomimetic response by inhibiting the reuptake of serotonin (5-HT) and catecholamine leading to a transient bradycardia followed by tachycardia, hypertension and acute thrombosis in coronary arteries. However, it is unclear if the hyper-reactivity of platelets originating from catecholamine or different pathogenic origins differentially predisposes platelets to thrombosis.

Studies agree that the development of cocaine-related CV problems does not require a preexisting vascular disease. Even in the absence of systemic platelet activation, endothelial dysfunction, or CV complications, cocaine is associated with acute thrombosis of coronary arteries; however, the mechanism by which cocaine predisposes to platelet-dependent thrombus formation is poorly understood.

Cocaine is a potent antagonist of 5-HT transporter, SERT and catecholamine transporters, norepinephrine (NE) transporter (NET) and dopamine (DA) transporter (DAT). Specifically, DAT is the primary target for cocaine1–6. Based on the similarities of these transporters and their receptors in between platelets and central nervous system, the inhibitory effect of cocaine on platelet is expected to be strong enough and elevates their plasma levels. Platelet membrane has SERT and residual level of NET but DA uptake of platelet is still controversial; specifically the α-granules of platelet contain DA, but there is no indication for DAT in platelet. On the other hand, DATs are expressed in the stomach, pancreas, as well as in lymphocytes. Cocaine inhibits DAT at these sites, and the secretion of α-granules from activated platelets elevate the plasma DA level. While some studies accept the involvements of plasma catecholamines in platelet activation and aggregation, there are some controversial reports not accepting this.

Cocaine acts as a ligand on SERT and reduces the 5-HT reuptake rates of the cells. 5-HT is synthesized and secreted into blood by enterochromaffin cells of the intestine but its plasma concentration is primarily regulated by SERT on the plasma membrane of platelet via a saturable reuptake mechanism. Once in the platelet cytoplasm, 5-HT molecules are sequestered by the vesicular monoamine transporter type 2 (VMAT2) into intracellular dense granules. Notably, the 5-HT concentration in blood plasma is in the low nanomolar range, but the dense granules of resting platelets store millimolar concentrations of 5-HT10–11. The actions of 5-HT are mediated by different types of receptors but terminated by a single transporter, SERT12. Therefore, plasma vs. platelet 5-HT ratio, primarily regulated by SERT, plays an important role in CV system.
Notably, 5-HT is an independent risk factor for platelet aggregation and for thrombus formation in animals and humans. The involvement of plasma 5-HT in platelet aggregation process may also occur in 5-HT$_{2A}$-independent, SERT-dependent signaling pathways. Interestingly, the pharmacological block of the 5-HT$_{2A}$ receptor elevates the 5-HT uptake rates and inhibits 5-HT-induced platelet activation in animal models of hypertension, as well as ex vivo platelet aggregation. Selective 5-HT reuptake inhibitors (SSRI) lowers the 5-HT uptake rates of platelets and predispose to platelet dysfunction and bleeding. In an earlier study, we showed that when 5-HT-infused mice were treated with paroxetine, an SSRI, the 5-HT uptake rates of platelets as well as the platelet content of 5-HT were reduced but in contrast the markers of platelet activation associated with a pro-aggregation phenotype were reduced. These studies strongly infer the involvement of 5-HT in platelet aggregation by acting as ligand for the 5-HT$_{2A}$ receptor and SERT situated on the platelet plasma membrane.

In the current study, we are exploring the involvement of 5-HT in cocaine-mediated platelet activation, we studied the platelets of mice knocked in with cocaine-insensitive dopamine transporter, DAT-KI. The injection of cocaine on DAT-KI mice did not change the aggregation rates of platelets further but reduced their 5-HT uptake rates significantly which caused an elevation in plasma 5-HT levels. Following the cocaine-injection, the plasma 5-HT levels and the response of platelet to collagen were elevated in wild-type, peripheral 5-HT KO (TPH1-KO). Based on our results, we hypothesize that cocaine mimics the combined effect that SERT and 5-HT$_{2A}$ play together in a two-step process to propagate the platelet aggregation by additive effect of 5-HT and the nonserotonergic catecholamine. Findings from these studies will impact the field in terms of demonstrating the mechanism by which serotonergic and nonserotonergic catecholamines play a role in the cocaine-mediated platelet aggregation process.

**Results**

**Cocaine and 5-HT in circulation system.** Our earlier studies demonstrated that 5-HT concentration in platelet cytoplasm is an important factor in promoting the aggregation ability of platelets. Here, we are investigating the involvement of 5-HT on cocaine-mediated platelet aggregation rates in detail.

Mice were injected with cocaine at different doses (ranging from 10 to 30 mg/kg, intraperitoneally) over 30 minutes; their locomotor activities were measured and found to be elevated by 2-fold relative to control (saline-injected) mice (Fig. 1A).

At the end of 30 min of cocaine injection, platelets were isolated from the blood samples of each group of mice and their 5-HT uptake rates were measured (P < 0.01) (Fig. 1B and Table 1). The 5-HT uptake rate of the platelets reduced in parallel to the elevated concentration of the cocaine injected to the mice. However, the highest reduction in the uptake rates was found in the platelets of 30 mg/kg cocaine-injected mice which was 86% lower than the control groups (n = 5). Therefore, in the following studies, the mice were injected with 30 mg/kg cocaine and the assays were performed 30 min following injections.

**Comparing the platelets isolated from cocaine-injected mice with the platelets of Paroxetine-injected mice.** Blood samples were analyzed using an ELISA assay to measure the 5-HT levels (Fig. 1A). As anticipated, the 5-HT concentrations in the plasma of cocaine-injected mice (3.50 ± 0.18 ng/µl blood) were 4-fold higher than the levels in the plasma of control mice (0.88 ± 0.07 ng/µl blood). Similarly, plasma 5-HT concentrations in PAR-injected mice also showed a 3.4-fold higher level compared to the control mice plasma level (5 mice per group) (Fig. 2A and Table 1). The circulating platelets counts were similar between control and the cocaine-injected mice. However, the 5-HT uptake rates of platelets of PAR and cocaine-injected mice were much lower than the control platelets’ 5-HT uptake rates (Fig. 2B).

Next, in stirred platelet aggregometer the rates of percent aggregation of the platelets from control-, PAR- or cocaine-injected mice were determined as their response to stimulation with collagen (3 µg/ml). The representative aggregation tracings are shown (Fig. 3A) revealing an opposite response in the percent aggregation in response to collagen. While the PAR-injection reduces the extent of aggregation by 46%, platelets of cocaine-injected mice showed 34% elevation (Fig. 3A and Table 1).

The level of P-selectin on the platelet surface is a good marker for their shift from the resting stage. Therefore, the surface expression of P-selectin on platelets isolated from cocaine-injected mice compared to control and PAR-injected mice platelets were analyzed by flow cytometry. Thus, in vivo exposure to cocaine coincides with a heightened platelet response as measured by stirred platelet aggregations and increased markers of platelet activation. In contrast, platelets of PAR-injected mice appeared to have lower aggregation rates and lower levels of surface P-selectin compared to control mice platelets (Fig. 3B. Table 1).

The significance of 5-HT plasma/platelet ratio in platelet aggregation mechanism. In an effort to understand the impact of the
environment that cocaine-injection created on platelet behavior, a mouse model was created to mimic the platelets of cocaine-injected mice. The involvement of SERT and 5-HT receptor in 5-HT-mediated platelet aggregation was analyzed by injecting the mice with SSRI or 5-HT receptor blocker before or after the 5-HT infusion through osmotic mini pumps.

Our earlier studies showed that the plasma 5-HT concentration was 0.85 ± 0.04 ng/μl blood for saline-infused animals (n=15) and 2.74 ± 0.37 ng/μl blood for 5-HT-infused animals (n=15); at the end of 24 hours infusion, platelet count did not show a difference between blood samples from saline and 5-HT–infused mice. Mice implanted with osmotic mini-pumps were injected with PAR (3 mg/kg) as a SERT inhibitor or Sarpogrelate (Sarpo, 30 mg/kg) as a 5-HT2A antagonist, either before the infusion of 5-HT started (pre-infusion), or at the end of 24-hr 5-HT infusion (post-infusion) (5 mice per group). The half-life of PAR is 24-hr but Sarpo is only 12-hr. Therefore, for the pre-infusion, Sarpo injection was repeated as two doses in 12-hr periods.

The 5-HT uptake rates of the platelets from 5-HT-infused or only saline-infused mice were measured by a competitive ELISA technique (0.88 ± 0.07 ng/μl blood in saline-infused, and 2.95 ± 0.05 ng/μl blood in PAR-injected, and 3.5 ± 0.18 ng/μl blood in cocaine-injected) 1. Both, cocaine and PAR-injection increased the plasma 5-HT concentration by 3.5-fold. (B) 5-HT uptake rates of platelets. Platelets of saline- and PAR- or cocaine-injected mice were isolated and the 5-HT uptake rates of platelets measured as described previously. Both, PAR and cocaine-injection were associated with a 61% and 85% decrease, respectively in 5-HT uptake rates of platelets compared to the saline-infused mice. All assays were performed in triplicate (n = 5 group). Asterisks indicate statistical difference between saline- and cocaine-injected (*). mice.

**Figure 2** | (A). Blood 5-HT concentration. The 5-HT concentrations in blood samples of the saline–, PAR (3 mg/kg)- or cocaine (30 mg/kg)-injected mice were measured by a competitive ELISA technique (0.88 ± 0.07 ng/μl blood in saline-infused, and 2.95 ± 0.05 ng/μl blood in PAR-injected, and 3.5 ± 0.18 ng/μl blood in cocaine-injected) 1. Both, cocaine and PAR-injection increased the plasma 5-HT concentration by 3.5-fold. (B) 5-HT uptake rates of platelets. Platelets of saline- and PAR- or cocaine-injected mice were isolated and the 5-HT uptake rates of platelets measured as described previously. Both, PAR and cocaine-injection were associated with a 61% and 85% decrease, respectively in 5-HT uptake rates of platelets compared to the saline-infused mice. All assays were performed in triplicate (n = 5 group). Asterisks indicate statistical difference between saline- and cocaine-injected (*). mice.

| Table 1 | The summary of our findings in 8 groups of mice model system |

| WT | WT + PAR | WT + Sarpo | WT + Coc. | TPH1-KO | TPH1-KO + Coc. | DAT-KI | DAT-KI + Coc. |
|-----|----------|------------|----------|---------|----------------|--------|-------------|
| [5-HT] in plasma (ng/μl blood) | 0.88 ± 0.07 | 2.95 ± 0.05 | 0.68 ± 0.07 | 3.50 ± 0.18 | u.n.d. | u.n.d. | 0.68 ± 0.09 | 4.1 ± 0.17 |
| 5-HT uptake rates (pmol/min/mg protein) | 0.76 ± 0.04 | 0.30 ± 0.02 | 0.92 ± 0.05 | 0.11 ± 0.02 | 0.89 ± 0.05 | 0.19 ± 0.01 | 1.01 ± 0.1 | 0.17 ± 0.02 |
| Platelet aggregation | 43 ± 5.5 | 23 ± 4.1 | 38 ± 5.7 | 64.33 ± 4.04 | 30 ± 2.83 | 40 ± 4.62 | 60.67 ± 2.31 | 63.33 ± 1.15 |
| Flow cytometry analysis of P-selectin | 181.7 ± 8.00 | 110.32 ± 9.35 | 145.3 ± 7.35 | 325.21 ± 11.2 | 93.13 ± 7.77 | 101.09 ± 2.67 | 297.11 ± 8.31 | 312.89 ± 9.44 |

u.n.d. = undetectable; defined as [5-HT] < 0.1 ng/μl or 5-HT uptake rate less than the background accumulation of 3H-5HT.
However, while the cocaine effect on the 5-HT uptake rates of platelets appeared as down-regulating in both, in vivo and in vitro systems, its effect on the aggregation rates of platelets was not same. In an in vitro system, cocaine-treatment did not elevate the aggregation rates of platelets, as was seen in the aggregation rates of the platelets from cocaine-injected mice (Figs 3 and 5).

Impact of cocaine stimulation on the platelets of cocaine insensitive transgenic mice. To determine the participation of plasma 5-HT in the function of platelets in cocaine-exposed blood, here we tested the source for the cocaine-mediated platelet aggregation on transgenic mice: specifically mice lack of 5-HT in blood (TPH1-KO) and the mice knocked in with cocaine-insensitive dopamine transporter, DAT-KI. The biochemical and functional analysis of the platelets of transgenic mice were performed with or without cocaine-injection and compared the ones in control mice.

As described previously, DAT in DAT-KI mice are 70-fold less sensitive to cocaine but fully functional for DA uptake. Tryptophan hydroxylase 1 (TPH1)-KO mice lack the TPH1 gene that encodes the rate-limiting enzyme in peripheral 5-HT synthesis.

First the locomotor activities of WT, TPH1-KO and DAT-KI mice were monitored in the presence and absence of cocaine (n = 5). Cocaine (30 mg/kg) was injected intraperitoneally in mice and at the end of 30 min of injection, the locomotor activities of all, cocaine- or saline-injected mice were measured (Fig. 6A). Interestingly DAT-KI mice did not show any change on their locomotor activities following cocaine injection. But the locomotor activity of saline-injected DAT-KI mice appeared 2-fold higher than the TPH1-KO and WT. Cocaine injection can be significantly seen on TPH-KO and WT mice (Fig. 6A).

Next, platelets were isolated from each group of mice and the 5-HT uptake rates were measured (P < 0.01) (Fig. 6B and Table 1). Regardless of the study model, cocaine-injection reduced the 5-HT uptake rates of their platelets (n = 5). While, the 5-HT uptake rates of platelets isolated from DAT-KI mice were 25% and 12% higher than the uptake rates of platelets in WT or in TPH1 KO mice, respectively.

In correlating the 5-HT uptake rates with the plasma 5-HT levels, the cocaine- or control mice plasma 5-HT levels were measured (Table 1). At the end of 30 min of cocaine injection, platelets were counted for all models. Circulating platelets counts were unchanged as a result of 30 min cocaine-injection. Then, the 5-HT concentrations in platelet and in plasma were determined by ELISA. The plasma 5-HT levels of control and cocaine-injected DAT-KI mice (n = 4) were determined as 0.68 ± 0.09 ng/µl blood and 4.1 ± 0.17 ng/µl blood, respectively (Fig. 7A). As anticipated, cocaine-injected DAT-KI mice showed a 6.0-fold higher plasma 5-HT concentration than the 5-HT level in control DAT-KI mice blood plasma. The plasma 5-HT levels for TPH1-KO or cocaine injected counterparts appeared undetectably low.

Platelets from control- or cocaine-injected WT, DAT-KI and TPH1-KO mice were prepared in plasma and stimulated with col-
lager (3 μg/ml) and tested in their stirred platelet aggregation assays. The representative aggregation tracings are shown (Fig. 7B) revealing an interesting response in the percent aggregation in response to collagen. The platelets of control or cocaine-injected DAT-KI did not show any difference. The cocaine-injection elevated the extent of aggregation by 30%, platelets of cocaine-injected TPH1-KO mice (Fig. 7B and Table 1).

The surface expression of P-selectin on platelets isolated from cocaine-injected mice compared to the control mouse platelets were analyzed by flow cytometry. Thus, in vivo exposure to cocaine coincides with a heightened platelet response as measured by stirred platelet aggregations and increased markers of platelet activation only for WT and TPH1-KO but not DAT-KI mice (Table 1).

**Discussion**

Cocaine is the second most commonly used illicit drug. Moreover, 5–10% of emergency department visits in the United States are believed to be secondary to cocaine usage and 40% of those admitted with chest pain, the most common cocaine-related medical problem. Through a variety of mechanisms, cocaine increases the risk of CV complications specifically thrombosis. Autopsy studies demonstrated the presence of coronary atherosclerosis in young cocaine users along with associated thrombus formation; thus, cocaine use is associated with premature coronary atherosclerosis and thrombosis. Platelets are derived from the fragmented cytoplasm of megakaryocytes and enter the circulation in an inactive form. The initial activation of platelets stabilizes them in hemostasis. Further platelet activation enlists more platelets at a fibrin-stabilized hemostatic area to form a thrombus after associating with the endothelium or each other. Platelets isolated from cocaine-injected mice appear more hyper-reactive and form thrombus as a result of elevated platelet α-granule release, platelet count and level of plasminogen-activator inhibitor and decreased level of antithrombin-III. Previously published studies agree that the development of cocaine-related CV problems does not require preexisting vascular disease. Even in the absence of systemic platelet activation, endothelial dysfunction, or CV complications, cocaine is associated with acute thrombosis of coronary arteries; however, the mechanism by which cocaine predisposes to platelet-dependent thrombus formation is poorly understood.

The role of circulating free 5-HT in platelet adhesion, aggregation and thrombus formation has been studied by various laboratories including ours, and clinical and biochemical observations infer a complex involvement. For example, increased thrombus incidence including hypertension exhibit elevated plasma 5-HT and enhanced aggregation responses are a feature of isolated human platelets exposed to 5-HT1A. The 5-HT signaling pathway in platelet aggregation was defined in 5-HT2A - or SERT-dependent pathways. The receptor-dependent pathway is related to plasma 5-HT signaling which is initiated by an interaction between plasma 5-HT and 5-HT2A, a G-protein coupled receptor on the platelet surface. 5-HT signaling transduced by 5-HT2A mobilizes calcium from intracellular stores to trigger the vesicular release of pro-coagulant molecules from α-granules. Studies demonstrated that 5-HT2A antagonist elevates the 5-HT uptake rates of platelets but the blocked 5-HT signaling can neither mobilize the intracellular calcium nor lead to the secretion of granules. Therefore in blood exposed to 5-HT2A antagonist aggregation rates of platelets appear even lower than the physiological level.

![Figure 4](https://www.nature.com/scientificreports)

**Figure 4** | Time-dependent evaluation of PAR and Sarpo on platelets. The osmotic mini-pumps were filled with 90 ± 10 μl of saline or 5-HT (dissolved in saline to provide a dose of 0.1 mg/ml). One group of mice was only injected with PAR or Sarpo (30 mg/kg). The effect of PAR and Sarpo was tested on 5-HT-infused mice either by injecting the mice before the 5-HT-infusion started (Pre-) or at the end of 24 hour infusion (n=5 group). At the end of 24-hr infusion, platelets from 8 groups of mice were isolated and assayed. (A) 5-HT uptake rates. 5-HT uptake assay was performed as described in the Method section. As reported previously, elevated plasma 5-HT was associated with a 37% decrease in 5-HT uptake rates of platelets compared to the saline-infused mice. 5-HT uptake rates of platelets were assayed in triplicate. While PAR-injection lowered the 5-HT uptake rates of platelets 60%, Sarpo elevated their rates by 21%. (B) Effect of PAR and Sarpo on platelet aggregation. The effect of PAR or Sarpo on platelet aggregation was monitored. Platelets were isolated from 8 study models of mice and stimulated with collagen and their aggregation profile was monitored in an aggregometer. Approximately 35% of the platelets from PAR-injected saline-infused mice, 54% of platelets from PAR-injected on pre-5-HT-infused mice and 75% of platelets from PAR-injected on post-5-HT-infused mice were aggregated at the end of 4 min, whereas in the absence of PAR, saline-infused mice platelets showed ~42% and the 5-HT-infused mice platelets showed ~75% aggregation. Similarly 42% of the platelets from Sarpo-injected saline-infused mice, 51% of platelets from Sarpo-injected on pre-5-HT-infused mice and 75% of platelets from PAR-injected on post-5-HT-infused mice were aggregated at the end of 4 min. The average of 5 measurements is presented in the bar graph. PAR- or Sarpo-injection on pre-5-HT-infused mice lowered the 5-HT uptake rates of platelets to the level found in cocaine-injected mice plasma. Their rate of aggregations was also 30% higher than the aggregation rates of platelets from control group. Asterisks indicate statistical difference between saline- and PAR- or Sarpo-injected or 5-HT-infused (*)/5-HT- and PAR- or Sarpo-injected on 5-HT-infused (†) mice. All assays were performed in triplicate (n = 5 group).
The SERT-dependent pathway is related with free levels of 5-HT present in platelet cytoplasm which binds to small GTPases, such as Rab4 and regulates the membrane trafficking of granules as well as SERT. Thus, selective 5-HT reuptake inhibitors (SSRI) downregulate the platelet 5-HT uptake rates which elevates the plasma 5-HT concentration yet blunts the aggregation rates of platelets via blocking the 5-HT signaling in platelets. These studies emphasize the importance of plasma vs. platelet 5-HT ratio in platelet physiology.

One of our major findings was that platelets from cocaine-injected mice showed blunted 5-HT uptake rates by SERT resulting in a loss of the primary mechanism for regulating plasma levels of 5-HT and depletion of 5-HT signaling in the platelet cytosol. However, oppos-

Figure 5 | Isolated platelets of WT mice were pretreated with SSRI or 5-HT antagonists or cocaine or 5-HT. (A) 300K platelets were incubated with various concentrations of citalopram, PAR, MDL, Sarpo, cocaine or 5-HT for 30 min at RT. Then, the 5-HT uptake rates were measured as described in the Method section. The in vitro treatments of all these reagents showed similar effect on the 5-HT rates as they did in in vivo treatments. (B) Percent aggregation in response to collagen (3 μg/ml) of isolated platelets pre-incubated in drug-free solution (control) or the listed drugs for 30 min. The aggregation response to collagen was significantly less in platelets incubated in PAR or Sarpo cocaine did not show a significant increase in their aggregation rates as it did in intraperitoneal injection. These assays were performed in triplicate (n = 5 group). Asterisks indicate statistical difference between control- and drug treated (*) platelets.

Figure 6 | (A) Locomotor Activity. WT, TPH1-KO and DAT-KI mice were injected either with 30 mg/kg cocaine or with saline. The basal locomotor activity of DAT-KI appeared 2-fold higher than the others however after cocaine-injection that was not changed. Cocaine-injection elevated the locomotor activity of WT mice by 3-fold and TPH1-KO by 2-fold. (B) 5-HT uptake rates of platelets. 5-HT uptake rates of the platelets isolated from DAT-KI was 33% higher than the uptake rates of platelets isolated from WT mice. However, 5-HT uptake rates of platelets from all models showed ~80% decrease following cocaine-injection. These assays were performed in triplicate (n = 5 group). Asterisks indicate statistical difference between WT and DAT-KI (*); cocaine-injected WT and cocaine-injected transgenic (**) mice.
ite to the SSRI, cocaine elevates the aggregation rates of platelets. Experiments using the platelets of cocaine-insensitive DAT knock-
transgenic mice. (DAT-CI) tested the involvement of sympathetic nervous
system on platelet aggregation due to the fact that the effects of cocaine on CV system could be acting directly or via sympathetic
nervous system. DAT-KI mice appeared to have higher 5-HT uptake rates, elevated plasma 5-HT level and higher platelet aggregation rate
than the WT mice. Cocaine administration on DAT-KI mice reduced their 5-HT uptake rates significantly which caused an elevation in plasma 5-HT levels as found in cocaine-injected WT and peripheral
5-HT KO (TPH1-KO). However cocaine did not change the aggregation rates of the platelets of DAT-KI mice suggesting the participa-
tion of cocaine on DAT-KI plasma 5-HT levels were elevated by 4- and 6-fold following cocaine-injection. All assays were performed in triplicate. * = statistical difference between saline- and cocaine-injected mice (n = 5 for each group). (B) The effect of cocaine on platelet aggregation was monitored. Platelets were isolated
from saline- or cocaine-injected WT, TPH1-KO or DAT-KI mice and stimulated with collagen and their aggregation profile was monitored in an
aggregometer. Approximately 30% of the platelets from saline-injected TPH1-KO mice and 43% of platelets from WT mice and 63% of the platelets from
DAT-KI mice were aggregated at the end of 10 min, whereas in the presence of cocaine, WT mice platelets showed ~65% and the TPH1-KO mice platelets showed ~40% and interestingly the DAT-KI mice platelets showed no elevation in aggregation. The average of 5 measurements is presented in the bar
graph. Asterisks indicate statistical difference between control and cocaine-injected (*) WT and transgenic (**) mice (n = 5 group).

Methods

Animals. Adult male C57BL/6J wild-type (WT) mice, or TPH1-KO21 or DAT-KI24 mice on a C57BL6 genetic background were anesthetized with isoflurane for
subcutaneous implantation of osmotic mini-pumps. Pumps were filled with saline (SAL) or 0.05 mg/ml 5-HT dissolved in saline to provide an infusion rate of 1.66 µg/kg/hr.13. Procedures involving animals were approved by the Institutional Animal
Care and Use Committee at the University of Arkansas for Medical Sciences and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory
Animals.

Blood sampling and platelet preparation. After 24 hours of continuous infusion with either saline or 5-HT, blood was withdrawn into a syringe containing 3.8%
sodium citrate solution by cardiac puncture from each animal. Samples of platelet and plasma were prepared from the whole blood.13. In biochemical studies, each assay was
performed using the same number 300,000/µL of platelets. The concentrations of 5-HT in platelets and plasma were quantified by competitive enzyme-linked
immunosorbent assay (ELISA). The platelet 5-HT uptake assay was performed as described.

Quantitative measurement of 5HT levels by ELISA. The 5-HT levels in plasma
which were prepared from the blood samples of each animal model was measured by
competitive ELISA technique by following the manufacturer’s instructions (IBL
Imuno-Biological Laboratories, Hamburg, Germany) as described previously13.

Platelet 5HT uptake assay. Platelet (300,000/µL of platelets) pellet was quickly
washed with phosphate-buffered saline (PBS) containing 0.1 mM CaCl2 and 1 mM
MgCl2 (PBSCM) then resuspended in PBSCM with 14.6 nM 3H-5-HT at room

Figure 7 | (A) Blood 5-HT concentration. The 5-HT concentrations in blood plasma samples of the saline- and cocaine-injected WT, TPH1-KO and
DAT-KI mice were measured by a competitive ELISA technique. The plasma 5-HT level was too low to be detected in TPH1-KO mice. But in WT and
DAT-KI plasma 5-HT were elevated by 4- and 6-fold following cocaine-injection. All assays were performed in triplicate. * = statistical difference
between saline- and cocaine-injected mice (n = 5 for each group). (B) The effect of cocaine on platelet aggregation was monitored. Platelets were isolated
from saline- or cocaine-injected WT, TPH1-KO or DAT-KI mice and stimulated with collagen and their aggregation profile was monitored in an
aggregometer. Approximately 30% of the platelets from saline-injected TPH1-KO mice and 43% of platelets from WT mice and 63% of the platelets from
DAT-KI mice were aggregated at the end of 10 min, whereas in the presence of cocaine, WT mice platelets showed ~65% and the TPH1-KO mice platelets showed ~40% and interestingly the DAT-KI mice platelets showed no elevation in aggregation. The average of 5 measurements is presented in the bar
graph. Asterisks indicate statistical difference between control and cocaine-injected (*) WT and transgenic (**) mice (n = 5 group).
temperature (RT) for 10 min, to include only the initial linear phase of transport in human platelets. Platelets were collected by rapid filtration through Whatman GF/B filters and were washed twice with 5 ml of ice-cold PBS. Filters were placed in scintillation vials containing 5 ml scintillation cocktail and immediately counted. Background accumulation of 3H-5-HT that occurred independent of SERT was measured in the same experiment treating platelets with the high-affinity cocaine analog, 1 µmol/L 2β-carboxybenzoyl-3-tropane (B-CIT) (Chemical Synthesis Service, NIMH, Bethesda, MD) and subtracted from each experimental value. In parallel, the protein concentration for 0.2 X 106 (0.015 mg cellular protein) platelets was determined using the Micro BCA protein assay kit. The 5-HT uptake rates of transporters were calculated as means of standard deviation values from three independent experiments.

**Stirred platelet aggregation.** For aggregation assays, platelets in plasma were prepared and platelet counts were normalized (300,000/µL) using a Hemavet 950 (Drew Scientific, Waterbury, CT). Collagen (3 µg/ml) was monitored by light transmittance (Chrono-log Corp., Haverton, PA).

**Flow cytometry.** All flow cytometry experiments were performed at the UAMS Flow Cytometry Core Facility. The impact of stimulants such as 5-HT, cocaine or other drugs on platelet activation was assessed using anti-P-selectin (Em忻ntics, Cat M130-1). Platelets in plasma (300,000/µL) were incubated with the Ab and followed the procedure reported previously. The samples were gated for single platelets based on forward and side scatter profiles and 20,000 events were recorded and read at the UAMS Flow Cytometry Core Facility.

**Data analysis.** Nonlinear regression fits of experimental and calculated data were performed with Origin, which uses the Marquart-Levenberg non-linear least squares curve fitting algorithm. Each figure shows a representative experiment that was performed with Origin. The analysis of variance (ANOVA) was performed with Origin, which uses the Marquardt-Levenberg non-linear least squares curve fitting algorithm. Each figure shows a representative experiment that was performed with Origin. The analysis of variance (ANOVA) was performed with Origin, which uses the Marquardt-Levenberg non-linear least squares curve fitting algorithm. Each figure shows a representative experiment that was performed with Origin.

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